A comparative study of peroxisomal structures in *Hansenula polymorpha* pex mutants

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**Abstract**

In a recent study, we performed a systematic genome analysis for the conservation of genes involved in peroxisome biogenesis (PEX genes) in various fungi. We have now performed a systematic study of the morphology of peroxisome remnants (‘ghosts’) in *Hansenula polymorpha* pex mutants (pex1–pex20) and the level of peroxins and matrix proteins in these strains. To this end, all available *H. polymorpha* pex mutants could be categorized into four distinct groups, namely pex mutants containing: (1) virtually normal peroxisomal structures (pex7, pex17, pex20); (2) small peroxisomal membrane structures with a distinct lumen (pex2, pex4, pex5, pex10, pex12, pex14); (3) multilayered membrane structures lacking apparent matrix protein content (pex1, pex6, pex8, pex13); and (4) no peroxisomal structures (pex3, pex19).

The morphologic phenotype of yeast PEX deletion cells (pex) may provide important clues on the function of the deleted gene. For careful comparison of the effects of deletion of a given PEX gene, it is essential to analyze cells that have been grown identically and are in an optimal physiologic state. Hence, the use of batch cultures and peroxisome-inducing cultivation media (oleic acid, methanol) may have major disadvantages, as the pex mutant cells are generally unable to grow on these compounds. This may at least in part explain the wide range of peroxisomal phenotypes that have been described and that have often seemed contradictory (Hohfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996).

In this study, we analyzed PEX1–PEX20 deletion mutants of the methylotrophic yeast *Hansenula polymorpha*, which are affected in peroxisome biogenesis and growth on methanol. To ensure reproducible, optimal cultivation and maximal peroxisome induction, pex mutant cells were grown in glucose-limited chemostat cultures, using choline as sole nitrogen source (van der Klei et al., 1991). Oxidation of choline results in the release of formaldehyde, which subsequently induces the major peroxisomal enzymes involved in methanol metabolism, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS), and catalase (CAT). The use of

**Introduction**

In recent years, much progress has been made in identifying and characterizing proteins involved in peroxisome biogenesis. These proteins are called peroxins, and are encoded by PEX genes (Distel et al., 1996). Up to now, 32 peroxins have been identified (Kiel et al., 2006). With the exception of PEX11, the first 22 described PEX genes (PEX1–PEX22) all function in matrix protein import or in the formation of peroxisomal membranes. Yeast mutants in which a PEX gene has been deleted that plays a role in peroxisomal matrix protein import still maintain peroxisomal membrane structures (also termed peroxisomal ghosts) to which peroxisomal membrane proteins are normally sorted, whereas the bulk of the matrix proteins are mislocalized to the cytosol. Mutants defective in membrane formation (PEX3 and PEX19) lack peroxisomal membranes and mislocalize both peroxisomal membrane and matrix proteins.

The most recently identified PEX genes, PEX23–PEX32, together with PEX11, are involved in the regulation of peroxisome abundance, size and distribution. Deletion of one of these genes generally does not have major effects on normal sorting of peroxisomal membrane and matrix proteins (Yan et al., 2005).
steady-state chemostat cells has the advantage of highly controlled growth conditions (e.g. constant growth rate, pH, and temperature), allowing accurate comparisons between different strains. The data revealed that four distinct mutant peroxisomal phenotypes could be discriminated among the available *H. polymorpha* pex mutants.

**Materials and methods**

**Organisms and growth**

The *H. polymorpha* strains used in this study are listed in Table 1. The cells were grown in glucose-limited chemostat cultures at 37 °C in mineral medium (van Dijken et al., 1976) containing 0.25% glucose as sole carbon source and 0.2% choline as nitrogen source. The dilution rate (*D*) was set at 0.1 h⁻¹. When needed, leucine was added to a final concentration of 30 μg mL⁻¹.

*Escherichia coli* strain DH5α was cultivated as previously described (Sambrook et al., 1989).

**Molecular techniques**

Standard recombinant DNA techniques (Sambrook et al., 1989) and genetic manipulations of *H. polymorpha* (Faber et al., 1994) were performed as detailed previously. Restriction and DNA-modifying enzymes were obtained from Roche Molecular Biochemicals (Almere, the Netherlands). PCRs were performed with Expand High Fidelity PCR enzyme (Roche Molecular Biochemicals, Almere, the Netherlands) as described by the supplier, using a Perkin-Elmer GeneAmp PCR 2400 thermocycler. Oligonucleotides were synthesized by Baseclear (Leiden, the Netherlands) using a LiCore automated DNA sequencer and dye primer chemistry (LiCore, Lincoln, NE). For DNA and amino acid sequence analysis, the CLONE MANAGER 5 program (Scientific and Educational software, Durham) 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 CLUSTALX program (Thompson et al., 1997).

Southern blot analysis was performed using the ECL direct nucleic acid labeling and detection system, as described by the manufacturer (GE Healthcare).

**Isolation of the *H. polymorpha* PEX2 gene**

The *H. polymorpha* PEX2 gene was isolated from the *H. polymorpha* genomic library pYT3 (Tan et al., 1995) using degenerate primers (reverse primer, 5’-CACACG TAACARTANAYRTGNCRCRA-3’; forward primer, 5’-GAGTWCCAGAAGRCRACYTBSTNNGG-3’). Transformants were selected that carried vector pYT3 with an 8-kb insert. By subcloning, a 3-kb SalI–NdeI (blunt-ended) fragment was isolated. This fragment was ligated between SalI and Smal into pBluescript II KS⁺ (Stratagene, San Diego, CA) and sequenced. The nucleotide sequence of the PEX2 gene was deposited at GenBank and assigned accession numberAY688949.

**Deletion of PEX2**

For the deletion of PEX2, a plasmid with a 3-kb subclone containing PEX2 was amplified by PCR using primer DelPEX2-1 (5’-AGATGATCAGCTACTAAATGATTAGGCTATAG-3’), which introduces a BstI site upstream of the ATG start codon, and primer DelPEX2-2 (5’-AGAATCGATGGTCTATAATTAAACAAAATATGCTGGCACGAAG-3’), which introduces

| Table 1. *Hansenula polymorpha* strains used in this study |
|---------------------------------|---------------------------------|---------------------------------|
| **Strain** | **Relevant properties** | **Source or reference** |
| Wild-type leu1.1 ura3 | NCYC495 leu1.1 ura3 | Gleson & Sudbery (1988) |
| Wild-type leu1.1 | NCYC495 leu1.1 URA3 | Gleson & Sudbery (1988) |
| pex1 leu1.1 | PEX1 disruption strain, leu1.1 | Kiel et al. (1999) |
| pex2 leu1.1 | pex2 disruption strain, leu1.1 | This study |
| pex3 leu1.1 | pex3 disruption strain, leu1.1 | Baereuds et al. (1996) |
| pex4 leu1.1 | pex4 disruption strain, leu1.1 | van der Klei et al. (1998) |
| pex5 leu1.1 | pex5 disruption strain, leu1.1 | van der Klei et al. (1995) |
| pex5 ura3 | Pex5 disruption strain, ura3 | van der Klei et al. (1995) |
| pex6 leu1.1 | pex6 disruption strain, leu1.1 | Kiel et al. (1999) |
| pex7 leu1.1 | pex7 disruption strain, leu1.1 | This study |
| pex8 leu1.1 | pex8 disruption strain, leu1.1 | Haan et al. (2002) |
| pex10 | pex10 disruption strain | Tan et al. (1995) |
| pex12 leu 1.1 | pex12 disruption strain, leu1.1 | This study |
| pex13 leu 1.1 | pex13 disruption strain, leu1.1 | Komori, Osaka, Japan, laboratory collection |
| pex14 leu 1.1 | pex14 disruption strain, leu1.1 | Komori et al. (1997) |
| pex17 leu 1.1 | pex17 disruption strain, leu1.1 | Komori, Osaka, Japan, laboratory collection |
| pex20 leu 1.1 | pex20 disruption strain, leu1.1 | Otzen et al. (2005) |
a ClaI site downstream of the stop codon. The resulting 4.7-kb PCR product was digested with NotI and Asp718I. The 1.3-kb NotI-blunt fragment and the 0.5-kb Asp718I-blunt fragment were, in two subsequent steps, ligated into pBluescript KS\(^+\), using NotI–SmaI in the first step, and Asp718I–EcoRV in the second step. The plasmid was cut with BclI and ClaI and ligated with the \(H.\) polymorpha \textit{URA3} gene (Merckelbach et al., 1993), which was isolated as a BamHI–ClaI fragment, resulting in plasmid pHp\_pex2. The 4.1-kb deletion fragment was isolated from pHp\_pex2 by digestion with BamHI and partial digestion with Sall, and used to transform \(H.\) polymorpha \textit{NCYC495} leu1.1 ura3. Methanol utilization-defective strains (Mut\(^-\)) were selected, and correct integration was confirmed by Southern blot analysis.

\section*{Isolation of the \textit{PEX7} gene and construction of the deletion strain}

The \(H.\) polymorpha \textit{PEX7} gene was isolated from the \(H.\) polymorpha genomic library pYT3 using degenerate primers \([5'\text{ primer}, 5'\text{-AACTACGG(A/C)(C/T)TTGTNGG NAA(T/C)GG-3'}; \text{ and } 3'\text{ primer}, 5'\text{-CCACA(G/A)TTT(G/A)A(T/C)(A/C)GANCC(G/A)TCCCA)-3'}]\) based on conserved regions in \textit{PEX7} genes of \textit{Pichia pastoris}, \textit{Saccharomyces cerevisiae}, \textit{Homo sapiens} and \textit{Kluyveromyces lactis}.

Transformants were selected that carried vector pYT3 with a 7-kb insert. By subcloning, a 2.7-kb XbaI–NruI fragment was isolated. This fragment was cloned into the Smal site of pUC19, and analyzed by sequencing. The nucleotide sequence of \(H.\) polymorpha \textit{PEX7} was deposited at GenBank and assigned accession number DQ217754.

A disruption mutant was created by cloning a 1.8-kb BamHI (filled in by Klenow treatment), XbaI \textit{URA3} fragment of \(H.\) polymorpha (Merckelbach et al., 1993) between the MluI (filled in by Klenow treatment) and SpeI sites of the 2.7-kb \textit{PEX7} fragment in pUC19. The disruption cassette was isolated by digestion with NsiI and NheI, and transformed to \textit{NCYC495} leu1.1 ura3. Transformants were selected and tested for correct integration using PCR and Southern blot analysis (data not shown).

\section*{Disruption of PEX12}

An \(H.\) polymorpha \textit{pex12} strain was isolated in a screen of random integration of linear DNA fragments (RALF) mutants that were unable to grow on methanol (van Dijk et al., 2001). Sequence analysis revealed that, in this mutant, the linear p-REMI fragment was inserted between base pairs 55 and 56 of the \textit{PEX12} gene, and hence represented a \textit{PEX12} disruption mutant. The \textit{PEX12} gene was cloned by functional complementation of the original RALF mutant and deposited at GenBank (accession number AF333026).

\section*{Biochemical methods}

Crude extracts were prepared as described previously (Baerends et al., 2000). Protein concentrations were determined by the Bradford assay using the Bio–Rad protein assay kit (Bio–Rad GmbH, Munich, Germany), using bovine serum albumin as standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (Laemmli, 1970). Gels were subjected to Western blotting (Kyhse-Andersen, 1984), and nitrocellulose blots were decorated using the chromogenic (NBT-BCIP) (NBT, nitro-blue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3’-indolylphosphate p-toluene salt) or chemiluminescent (peroxidase) Western blotting kit (Roche) and specific polyclonal antisera against various \(H.\) polymorpha proteins.

\section*{Microscopy}

Cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Waterham et al., 1994). Immunocytochemistry was performed on ultrathin sections of Unicryl-embedded cells, using specific polyclonal antisera against various \(H.\) polymorpha peroxisomal proteins and gold-conjugated goat anti-rabbit serum (Waterham et al., 1994).

\section*{Results}

\textit{Hansenula polymorpha} \textit{pex} cells contain four distinct groups of peroxisomal structures

We restricted our study to \textit{PEX} genes that play a role in peroxisome biogenesis (i.e. matrix protein import and membrane formation), namely \textit{PEXI}–\textit{PEX20}, excluding \textit{PEX11}, which is involved in controlling peroxisome numbers (Erdmann & Blobel, 1995; Marshall et al., 1995). \textit{Hansenula polymorpha} Pex1p–Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, Pex17p, Pex19p and Pex20p have been identified before in the laboratory (Veenhuis et al., 2003). The two peroxins Pex9p and Pex15p that also have been implicated in peroxisomal matrix protein import could not be identified in \(H.\) polymorpha (Kiel et al., 2006). \textit{Hansenula polymorpha} also does not contain the \textit{S. cerevisiae} Pex7p auxiliary proteins Pex18p and Pex21p, but contains Pex20p instead (Purdue et al., 1998; Otzen et al., 2005).

From the group of \textit{PEX} genes that are thought to play a role in peroxisomal membrane formation, \textit{PEX3}, \textit{PEX16} and \textit{PEX19}, \textit{PEX16} has so far only been identified in mammals, plants and the yeast \textit{Yarrowia lipolytica}, but is absent in \(H.\) polymorpha (Kiel et al., 2006).

Cells of the selected \(H.\) polymorpha \textit{PEX} deletion strains were grown in glucose-limited chemostat cultures using choline as sole nitrogen source. Samples were taken from
steady-state cultures for morphologic and biochemical analysis.

Electron microscopy revealed that all *H. polymorpha* pex mutants studied could be placed in four morphologically distinct groups (Fig. 1). The first group is characterized by the presence of almost normal peroxisomes (Figs 1a and 2a). This group includes *H. polymorpha* pex7 and pex20 cells, which are defective in PTS2 (PTS, peroxisomal targeting signal) protein import, as well as pex17 (Otzen et al., 2005). However, almost normal peroxisomal structures were also observed in *H. polymorpha* pex17 cells (Fig. 2a). These organelles were reduced in size relative to those observed in cells of wild-type *H. polymorpha*, but still harbored significant amounts of AO and DHAS proteins (Fig. 2c and d). This was an unexpected observation, as *S. cerevisiae* and *P. pastoris* pex17 mutants contain only very small peroxisomal structures or membrane remnants (ghosts) (Snyder et al., 1999; Harper et al., 2002).

The cells in the second group of mutants contained very small remaining peroxisomal structures, which harbored a matrix space. These included *H. polymorpha* pex2, pex4, pex5, pex10, pex12 and pex14 (shown for pex2 in Fig. 1d). Apparently, these structures contain very limited amounts of matrix proteins (Komori et al., 1997).

The third group of pex mutants harbors multilamellar membrane sheets that lack an apparent organellar matrix. Members of this group are pex1, pex6, pex8 and pex13 (shown for pex13 in Fig. 1c). This suggests that matrix protein import is fully blocked in these mutants.

The final group comprised *H. polymorpha* pex3 (Fig. 1b) and pex19 cells, which lacked recognizable peroxisomal membranes.

**Peroxin levels in *H. polymorpha* pex mutants**

We subsequently analyzed the various pex mutants for peroxin contents. Crude extracts, prepared from chemostat-grown...
cells, were analyzed for levels of Pex3p, Pex10p and Pex14p by Western blotting. In addition, we studied the levels of the peroxisomal matrix proteins AO, CAT, DHAS, and amine oxidase (AMO).

The levels of these major matrix proteins were comparable in all pex mutants analyzed (shown for CAT in Fig. 3; AMO, AO and DHAS not shown), indicating that the defects in peroxisome biogenesis do not significantly affect the levels of these proteins. This is in line with earlier observations that these enzymes are normally assembled and stable in the cytosol of pex mutants (van der Klei et al., 1998).

Except for Pex3p in pex3 cells, Pex10p in pex10 cells, and Pex14p in pex14 cells, these three membrane-bound peroxins were detected in cells of all strains (Fig. 3a and c). Variations in protein levels were observed for Pex14p, but not for Pex3p and Pex10p. We did not observe any direct relationship between enhanced Pex14p levels and pex cells that contained matrix space. Moreover, normal levels of Pex3p have been observed before in H. polymorpha pex19 cells that lack peroxisomal membranes (Otzen et al., 2004).

Discussion

This article describes the characterization of peroxisomal structures that remain in H. polymorpha pex mutants and their classification into four distinct groups. Among the pex mutant cells that contained normal peroxisomal structures, pex7, pex17 and pex20, the presence of pex17 is remarkable (Fig. 2). Pex7 and pex20 were expected to be in this group, as they are affected in PTS2 protein import only. Hence, in these cells, import of the major proteins of methanol metabolism, AO, DHAS and CAT, is not abolished, resulting in the formation of almost normal peroxisomes. However, Pex17p is proposed to represent a component of the PTS1/PTS2 receptor docking machinery. The fact that, in pex17, significant amounts of AO, DHAS and CAT are imported.

Fig. 2. Hansenula polymorpha pex17 form peroxisomal structures that harbor matrix proteins. Ultrathin sections of Hansenula polymorpha pex17 cells grown at steady-state levels in chemostat culture (a, c, d) on glucose/choline, or in batch culture on methanol (b). In chemostat-grown pex17 cells, several peroxisomes are observed, whereas in batch culture, one small peroxisome could typically be observed. Immunocytochemistry revealed that the organelles contain AO (c) and DHAS (d) protein, and that these proteins are also present in the cytosol, where they are frequently organized in crystalloids (*). (a, b) KMnO₄-fixed cells, and (c, d) glutaraldehyde-fixed cells. P, peroxisome; V, vacuole; N, nucleus; M, mitochondrion. The bar represents 0.5 μm. (b) is from Kuravi VK, PhD Thesis, University of Groningen, 2007.

Bar 0.5 μm

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Harper tubular peroxisomal structures were observed. Later studies of P. pastoris pex17 cells. Instead, very small vesicular and induced some-like structures observed in batch-cultured, methanol-containing peroxisomal structures, similar to the few peroxisomal structures that lack luminal space were observed only in pex13 cells (Fig. 1). These findings stress the importance of using controlled cultivation schemes when analyzing yeast pex mutants in comparative studies (e.g. in genomics, transcriptomics, and proteomics).

Hansenula polymorpha pex2, pex10, pex12 and pex4 belong to the same morphologic class as pex14 (Fig. 1). The luminal space observed in H. polymorpha pex4 and pex14 cells is in line with previous observations that these structures contain limited amounts of matrix proteins (Purdue et al., 1998; van der Klei et al., 1998).

Pex2p, Pex10p and Pex12p are ring finger proteins that have been implicated in peroxisomal matrix protein import. The presence of a luminal space in these mutants suggests that some residual peroxisomal matrix protein import is possible in the absence of either one of these ring finger proteins. Hence, in these mutants, the defect in the import process most likely occurs after docking and translocation, as in pex4 and pex14 mutants. Possibly, in these mutants, Pex5p is defective in that it cannot reach the receptor recycling machinery. The latter suggestion is based on previous findings that H. polymorpha Pex5p accumulates at peroxisomal ghosts in pex2, pex10 and pex12 cells (Fig. 1d). Also, in human cell lines obtained from patients suffering from a peroxisome biogenesis disorder due to a mutation in PEX10 (complementation group 7) (Warren et al., 1998), HsPex5p was shown to accumulate inside peroxisomes (Dodt & Gould, 1996).

The last morphologic group of H. polymorpha pex strains includes pex1, pex6, pex8 and pex13, which have ghosts lacking a distinct lumen (Fig. 1). These findings suggest that import of matrix proteins in these mutants is strongly affected. This is in agreement with the proposed important function of Pex13p in receptor docking. The intraperoxisomal peroxin Pex8p plays a key role in peroxisomal matrix protein import in H. polymorpha (Waterham et al., 1994), where it is proposed to mediate dissociation of the PTS1 cargo from Pex5p (Wang et al., 2003), but is also important for associating the two core complexes for matrix protein import [the Pex13p–Pex14p–Pex17p docking complex and the Pex2p–Pex10p–Pex12p ring finger complex (Agne et al., 2003)]. The latter most likely explains why, in the absence of Pex8p, peroxisomal matrix protein import is severely blocked.

Pex1p and Pex6p are AAA-ATPases (AAA, ATPases associated with various cellular activities) that form a complex at the peroxisomal membrane (Kiel et al., 1999). Data from epistasis analysis indicated that P. pastoris Pex1p and Pex6p function at a very late stage of the Pex5p import cycle (Collins et al., 2000). This possibility was recently experimentally tested and confirmed (Platta et al., 2004). Also, Pex4p is important at a very late stage of the matrix protein import process. Interestingly, the morphologic peroxisome phenotype of H. polymorpha pex1 and pex6 cells differs from
that of pex4 cells, which could be explained by the fact that these peroxins are not equally important in receptor recycling. Alternatively, the pex1 and pex6 phenotypes may also be due to additional functions of Pex1p and Pex6p, e.g. in the formation of peroxisomes from the endoplasmic reticulum (ER). Studies in Y. lipolytica revealed that Pex1p and Pex6p are important for the formation and fusion of ER-derived vesicles, processes that are required to form new peroxisomes from the ER (Titorenko & Rachubinski, 2000). Hence, the peroxisomal membrane remnants that were detected by electron microscopy in H. polymorpha pex1 and pex6 cells may also include early intermediates of peroxisome formation from the ER.

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