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Penicillium chrysogenum Pex5p mediates differential sorting of PTS1 proteins to microbodies of the methylotrophic yeast *Hansenula polymorpha*

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Abstract

We have isolated the *Penicillium chrysogenum pex5* gene encoding the receptor for microbody matrix proteins containing a type 1 peroxisomal targeting signal (PTS1). *Pc-pex5* contains 2 introns and encodes a protein of approximately 75 kDa. *P. chrysogenum pex5* disruptants appear to be highly unstable, show poor growth, and are unable to sporulate asexually. Furthermore, *pex5* cells mislocalize a fluorescent PTS1 reporter protein to the cytosol. *Pc-pex5* was expressed in a *PEX5* null mutant of the yeast *Hansenula polymorpha*. Detailed analysis demonstrated that the PTS1 proteins dihydroxyacetone synthase and catalase were almost fully imported into microbodies. Surprisingly, alcohol oxidase, which also depends on Pex5p for import into microbodies, remained mainly in the cytosol. Thus, *P. chrysogenum* Pex5p has a different specificity of cargo recognition than its *H. polymorpha* counterpart. This was also suggested by the observation that Pc-Pex5p sorted a reporter protein fused to various functional PTS1 signals with different efficiencies.
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Keywords: Filamentous fungus; Methylotrophic yeast; Microbodies; Penicillin; *PEX* gene

1. Introduction

Microbodies (peroxisomes, glyoxysomes, and glycosomes) are vital cellular organelles of a simple architecture, namely a single membrane that encloses a proteinaceous matrix. Despite this simplicity, these organelles control many important metabolic processes (van den Bosch et al., 1992; Veenhuis and Harder, 1991). Microbodies are essential organelles in plants and *Trypanosomes* (Guerra-Giraldez et al., 2002; Schumann et al., 2003). Moreover, their importance is demonstrated by the discovery of inherited human metabolic disorders—prototype the Zellweger Syndrome—that are associated with defects in microbody biogenesis or function (Gould and Valle, 2000). Microbodies obtain their enzyme contents via post-translational import of proteins synthesized in the cytosol. To enable sorting into the organelle, these proteins contain specific peroxisomal targeting signals (PTS), that fall into at least two categories. The most abundant signal (PTS1) is located at the carboxy terminus of enzymes and has a consensus sequence related to the canonical -S-K-L-*COOH* sequence observed in firefly luciferase [reviewed by Purdue and Lazarow (2001)]. The PTS1 receptor, Pex5p, contains six tetratricopeptide repeat motifs (TPR) in its C-terminus. Analysis of the crystal structure of the TPR domain of human PEX5 indicates that this forms a negatively charged pocket that binds the PTS1 signal (Gatto et al., 2000). PTS1 protein import into microbodies is rather unusual in that the PTS1 receptor accompanies its cargo into the lumen of the organelle, where dissociation takes place (Dammai and Subramani, 2001). Subsequently, the receptor may shuttle back to the cytosol for a new import cycle.

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So far, studies of microbody biogenesis/matrix protein import have concentrated on yeast and mammalian systems (see Purdue and Lazarow, 2001). However, microbodies also play a vital role in filamentous fungi. In addition to a role in β-oxidation of fatty acids, microbodies in filamentous fungi are required for the production of β-lactam antibiotics like penicillin, notably in the final stages of their synthesis [reviewed by Martín and Gutiérrez (1995)]. Moreover, a correlation seems to exist between the amount of penicillin produced by certain *Penicillium chrysogenum* and *Aspergillus nidulans* strains and the number of microbodies per cell (Müller et al., 1991; Valenciano et al., 1998). Furthermore, two enzymes of penicillin biosynthesis, isopenicillin N:acyl CoA acyltransferase (IAT) and phenylacetyl-CoA ligase (PACL), contain putative PTS1 signals (-A-R-L-*COOH* and -S-K-I-*COOH*, respectively; Barredo et al., 1989; Gledhill et al., 1998). Indeed, IAT has been shown to be located in the microbody matrix (Müller et al., 1991) and a mutant strain of *P. chrysogenum* in which the PTS1 of IAT was removed was unable to synthesize penicillin (Müller et al., 1992). Another important function for microbodies in filamentous fungi is exemplified by the discovery that Woronin bodies of *Neurospora crassa* represent a special class of microbodies, that are required to plug the septal pore upon hyphal damage in order to avoid cytoplasmic leakage. Woronin bodies contain major amounts of a PTS1-containing protein, designated Hex1p, essential for the plug function of the organelles (Jedd and Chua, 2000).

Two *pex* genes involved in microbody biogenesis in filamentous fungi have been identified by serendipity. Thus, *Podospora anserina pex2* was isolated by functional complementation of mutants affected in caryogamy, a process necessary for sexual sporulation (Berteaux-Lecellier et al., 1995). Additionally, Kimura et al. (2001) identified *pex6* of the plant pathogenic fungus *Colletotrichum lagenarium* as the gene disrupted in a mutant that could no longer infect host plants. In both cases, the respective *pex* mutants lacked normal microbodies, were unable to metabolize oleate, and mislocalized microbody matrix proteins to the cytosol.

Our aim is to analyse the precise function of microbodies in penicillin biosynthesis in *P. chrysogenum* at the molecular level. Recently, we isolated the *P. chrysogenum pex1* and *pex6* genes (Kiel et al., 2000). However, we were unable to isolate disruption mutants for either of these genes, thereby precluding any investigation of the effect of loss of functional microbodies. In order to affect microbody biogenesis to a lesser extent, we isolated the *P. chrysogenum pex5* gene encoding the PTS1 receptor. Our data show that a *pex5* disruption mutant is genetically unstable. Nevertheless, *pex5* disrupted cells are deficient in asexual sporulation, have low viability and mislocalize a fluorescent PTS1 reporter protein to the cytosol. Expression of *Pc-pex5* in the heterologous host *Hansenula polymorpha* demonstrated that this gene encodes a bona fide PTS1 receptor that has a different specificity of cargo recognition than the PTS1 receptor of the host *H. polymorpha*.

2. Materials and methods

2.1. Strains

Escherichia coli DH5α (Sambrook et al., 1989) and XL1-Blue (Stratagene, La Jolla, CA), used for plasmid constructions and bacteriophage propagations, respectively, were cultivated according to Sambrook et al. (1989). *P. chrysogenum* strains used in this study (listed in Table 1) were cultured as described by Gouka et al. (1991). *P. chrysogenum* transformants were selected on media with acetamide as sole nitrogen source as described by Cantoral et al. (1987). *H. polymorpha* strains used in this study (listed in Table 1) were grown in batch cultures on mineral medium (MM) (van Dijken et al., 1976) using glucose (0.5% w/v), methanol (0.5% v/v) or glycerol + methanol (0.1 + 0.5% v/v, respectively) as carbon sources. Leucine was added as required (final concentration 30 mg/L). Leucine prototrophic *H. polymorpha* transformants were selected on YND plates containing 0.67% (w/v) Yeast Nitrogen Base without amino acids (Difco), 1% (w/v) glucose, and 1.5% (w/v) agar. Zeocin-resistant transformants were selected on YPD plates containing 1% (w/v) yeast extract, 1% (w/v) peptone, 1% (w/v) glucose, and 100 mg/L zeocin (Invitrogen).

2.2. DNA procedures

Standard recombinant DNA manipulations according to Sambrook et al. (1989) were used throughout this study. Preparation and transformation of *P. chrysogenum* protoplasts was performed as described by Cantoral et al. (1987). Electro-transformation of *H. polymorpha* cells was performed as described previously (Faber et al., 1994).

Penicillium chrysogenum DS04825 genomic DNA was isolated essentially as described by Kolar et al. (1988). Southern blot analysis was performed using the ECL direct nucleic acid labelling and detection system according to the instructions of the supplier (Amersham, Arlington Heights, IL). The *P. chrysogenum* DS04825 genomic DNA library in λZAPII and the *P. chrysogenum* cDNA libraries in pCMV-SPORT4 have been described previously (Kiel et al., 2000). Plaque hybridization was performed according to Sambrook et al. (1989). The helper phage M13 k07 was used for in vivo excision of phagemids from λZAPII. Polymerase chain reaction (PCR)-mediated DNA amplification using degenerate primers (obtained from Eurogentec, Seraing, Belgium) was performed on a GeneAmp 2400 DNA amplifier (Perkin–Elmer, Norwalk, CT) with Gold Star RED *Taq*

Table 1
Fungal strains used in this study

Strain	Genotype/characteristics	Reference
Penicillium chrysogenum		
Wis54-1255 (ATCC28089)	Wild type	American Type Culture Collection
DS04825	Strain with improved penicillin production	Gouka et al. (1991)
Wis54-1255::pPC-mb-eYFP	Wis54-1255 expressing eYFP-SKL ($AmdS^+$)	van der Lende et al. (2002)
Wis54-1255::pPC-cyt-eYFP	Wis54-1255 expressing eYFP ($AmdS^+$)	van der Lende et al. (2002)
Wis54-1255::pPC-mb-eYFP ($AmdS^-$)	Wis54-1255::pPC-mb-eYFP, with *amdS* marker removed by fluoroacetamide treatment	van der Lende, unpublished
pex5	Wis54-1255 disrupted in *pex5* gene; $AmdS^+$; genetically unstable	This study
pex5::pPC-mb-eYFP	Wis54-1255::pPC-mb-eYFP ($AmdS^-$) disrupted in *pex5* gene; $AmdS^+$; genetically unstable	This study
Hansenula polymorpha		
NCYC495 (*leu1.1*)	Wild type	Gleeson and Sudbery (1988)
HF246 (*leu1.1*)	NCYC495::(P_{AOX} · *GFP-SKL*)^{1c}	van Dijk et al. (2001)
Δ*pex5* (*leu1.1*)	NCYC495 with deletion in *PEX5*	Salomons et al. (2001)
Δ*pex5*::(P_{AMO} · *GFP-SKL*)^{1c}	Δ*pex5* expressing *eGFP-SKL*	K. Gunkel, unpublished
Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc}	Δ*pex5* expressing *Pc-pex5*	This study
Pc-pex5::(P_{AOX} · *GFP-SKL*)	Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} expressing *eGFP-SKL*	This study
Pc-pex5::(P_{AOX} · *GFP-SKI*)	Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} expressing *eGFP-SKI*	This study
Pc-pex5::(P_{AOX} · *GFP-LARF*)	Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} expressing *eGFP-LARF*	This study

polymerase (Eurogentec) using an annealing temperature of 60 °C. DNA fragments were isolated from cDNA libraries using the Expand high fidelity PCR system (Roche, Mannheim, Germany) and gene-specific oligonucleotides (Gibco, Life Technologies, Breda, The Netherlands). DNA sequencing of genomic *P. chrysogenum* DNA fragments was carried out on a ABI 373A DNA sequencer using the BigDye Terminator system (Perkin–Elmer/Applied Biosystems). cDNA fragments were cloned in pBluescript II SK+ (Stratagene) and sequenced using both vector-based and gene-specific primers.

2.3. Cloning and sequencing of the *P. chrysogenum pex5* gene

To enable isolation of the *P. chrysogenum pex5* gene, two homologous regions in the primary sequences of four yeast Pex5p's—amino acid sequences SEA(A/G) LAFEAA and WNRLGA(S/A/T)LAN, respectively—were used to design the degenerate PCR primers PcPEX5-F and PcPEX5-R (see Table 2). Using these primers a specific 520 bp *P. chrysogenum pex5* fragment was obtained with *P. chrysogenum* genomic DNA, which was cloned into *Sma*I-digested pBluescript SK+. Sequence analysis demonstrated that the cloned fragment indeed contained part of a putative *pex5* gene. To isolate the genomic region comprising the entire *P. chrysogenum pex5* gene, the PCR probe was used to screen a *P. chrysogenum* gene library in λZAPII by plaque hybridization. Four positive plaques were obtained and the corresponding phagemids isolated. Subsequently, fragments containing the hybridizing regions were subcloned and sequenced.

To localize the intron positions in *Pc-pex5* and be able to deduce the primary sequence of Pc-Pex5p, we designed gene-specific PCR primers (Table 2) and isolated DNA fragments from *P. chrysogenum* cDNA

Table 2
Primers used in this study

PcPEX5-F	5′-TCC GAG GCY GSY CTS GCY TTY GAR GCN GC-3′
PcPEX5-R	5′-TTG GCG AGR GHR GCR CCS AGN CKR TTC CA-3′
PcPEX5-1	5′-AGA AAG CTT ATG TCG TTC CTC GGT GGC GCA G-3′
PcPEX5-2	5′-GCA GCG TGT CAT ACA GAT TCG TGC-3′
PcPEX5-3	5′-GCA GTC TCC ATC TCG GCA AAC TGC-3′
PcPEX5-4	5′-GCC ATC GAA GCC TAC GAG CAA GC-3′
PcPEX5-STOP	5′-CAA TGT ACG CAA CCA ACA GAT C-3′
PC1	5′-ACA CAG GAT AAG GGT AAA GG-3′
PC2	5′-TAC CCT TAT CCT GTG TCT GC-3′
PC3	5′-GGA AGT ATC CGG TGA CGC AG-3′
PC4	5′-CTG CGT CAC CGG ATA CTT CC-3′
Universal M13/pUC	5′-GTA AAA CGA CGG CCA GT-3′
Reverse M13/pUC	5′-CAG GAA ACA GCT ATG AC-3′
AMDS-P	5′-GCC AGA AAG AGT CAC CGG TCA CTG-3′
AMDS-T	5′-AGG TGA CTC TGG ATG GCC CCA TAC-3′

libraries, which were sequenced. Additionally, we isolated the entire *pex5* coding region by PCR from a *P. chrysogenum* cDNA library using primers PcPEX5-1 and PcPEX5-stop. The resulting 1.9 kb fragment was digested with *Hin*dIII and cloned into *Hin*dIII + *Sma*I-digested pBluescript, resulting in plasmid pB-PcPEX5. Two independently isolated clones were sequenced. For analysis of the *P. chrysogenum* DNA sequences and deduced amino acid sequences the PC-GENE program (release 6.80, Intelligenetics, Mountain View, CA) was used. Amino acid sequences were aligned using the Clustal_X program (Thompson et al., 1997). A 2488 bp region comprising the nucleotide sequence of the genomic *P. chrysogenum pex5* gene as well as its cDNA sequence has been deposited at GenBank and was assigned the Accession No. AY366189.

2.4. Disruption of pex5 in P. chrysogenum

To enable disruption of the *Pc-pex5* gene, we constructed plasmid pGB-PEX5-del2, which consists of the pBluescript vector that harbours between its *Asp*718I and *Bam*HI sites the following three fragments: a 0.77 kb *Asp*718I–*Eco*RI fragment (comprising the *Asp*718I–*Xho*I region of *Pc-pex5*: nt 771–1541 in AY366189), a 3.1 kb *Eco*RI–*Asp*718I (blunted by Klenow treatment) fragment from plasmid pNiGANi (a gift from DSM, Delft, The Netherlands) containing the *A. nidulans amdS* gene under the control of the *A. nidulans* P_{gpdA} promoter and a 0.63 kb *Sma*I–*Bam*HI fragment (nt 587–1213) of *Pc-pex5*. Digestion of plasmid pGB-PEX5-del2 with *Mlu*I (nt 1062 in *Pc-pex5*) allows the isolation of a 4.1 kb disruption cassette containing exclusively an internal portion of *Pc-pex5* (nt 587–1541) and the P_{gpdA}-*amdS* cassette as selectable marker, but lacks all vector sequences (Fig. 1). The disruption cassette was transformed into protoplasts of *P. chrysogenum* Wis54-1255 as well as protoplasts of a

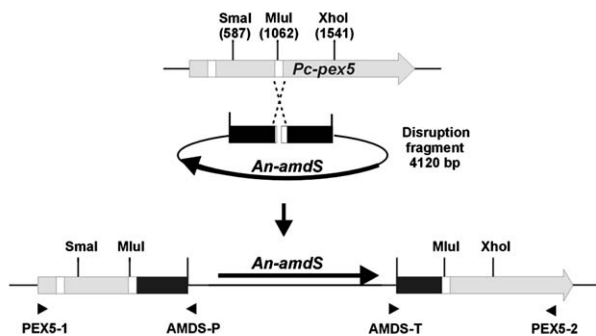

Fig. 1. Schematic representation of the genomic *P. chrysogenum pex5* gene and the *pex5* disruption strategy. The white areas in *Pc-pex5* represent the location of the two introns. The arrowheads indicate the location of the primers that were used to identify *pex5* disruptants among AmdS$^+$ transformants (for sequences see Table 2). Only relevant restriction sites are indicated. *An*, *A. nidulans*; *Pc*, *P. chrysogenum*.

derivative of strain Wis54-1255::pPC-mb-eYFP (van der Lende et al., 2002) that had lost its *amdS* cassette as a result of fluoroacetamide selection (cf. Royer et al., 1999). Transformants able to grow on plates with acetamide as sole nitrogen source were selected and analysed by PCR using primers specific for the *Pc-pex5* gene and the P_{gpdA}-*amdS* cassette (data not shown). Transformants showing the expected PCR fragments were chosen for further study.

2.5. Heterologous expression of Pc-pex5 in a H. polymorpha Δpex5 strain

To enable expression of the *P. chrysogenum pex5* gene in *H. polymorpha* we placed the *Pc-pex5* cDNA behind the strong, inducible *H. polymorpha AOX* promoter. The *Pc-pex5* cDNA was isolated as a 1.9 kb *Hin*dIII–*Not*I (blunted by Klenow treatment) fragment from pB-PcPEX5 and cloned between the *Hin*dIII and *Sma*I sites of vector pHIPX4 (Gietl et al., 1994). Subsequently, the resulting plasmid pHIPX4-PcPEX5 was linearized with *Stu*I in the P_{AOX} region and integrated into the *H. polymorpha* Δ*pex5* genome. Proper integration of the plasmid was tested by Southern blot analysis (data not shown). A strain that harboured multiple copies of the integrated plasmid was chosen for further study. To enable further investigation of the functionality of *Pc-pex5* in *H. polymorpha*, we also constructed derivatives of this strain that produced the fluorescent model protein eGFP carrying different PTS1 signals. Thus, plasmids pHIPZ4-GFP.LARF, pHIPZ4-GFP.SKI (Salomons et al., 2000), and pANL29 (producing eGFP-SKL; Leão-Helder et al., 2003) were linearized with *Asp*718I and randomly integrated into the *H. polymorpha* genome. Strains with bright fluorescence were chosen for further analysis.

2.6. Biochemical techniques

Crude extracts of *H. polymorpha* cells were prepared using the TCA method (Baerends et al., 2000). *H. polymorpha* protoplasts were generated and lysed according to van der Klei et al. (1998). Differential centrifugation was performed as described previously (van der Klei et al., 1998). Post-nuclear supernatants were loaded onto discontinuous sucrose gradients as described by Douma et al. (1985). Fractions of gradients were analysed for sucrose concentrations and alcohol oxidase (AO; Verduyn et al., 1984) and cytochrome *c* oxidase (COX; Douma et al., 1985) activities. Protein concentrations were determined using the Bio-Rad Protein Assay system using bovine serum albumin as a standard. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting were performed by established procedures. Western blots were decorated with polyclonal antibodies against

Saccharomyces cerevisiae Pex5p and selected *H. polymorpha* proteins.

2.7. Ultrastructural techniques

Cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Waterham et al., 1994). Immunolabeling was performed on ultrathin sections of Unicryl-embedded *H. polymorpha* cells using specific antibodies against selected proteins. Fluorescence microscopy studies were performed using a Zeiss Axioskop microscope (Carl Zeiss, Göttingen, Germany).

3. Results

3.1. Isolation of P. chrysogenum pex5

PTS1 receptors (Pex5ps) contain in their C-termini a conserved region consisting of two sets of 3 tetratricopeptide repeat (TPR) motifs. We have used two highly conserved amino acid sequences of TPR1 and TPR5 present in PTS1 receptors of the yeast species *S. cerevisiae*, *H. polymorpha*, *Pichia pastoris*, and *Yarrowia lipolytica* to design degenerate oligonucleotides that enabled to isolate a portion of the *P. chrysogenum pex5* gene by PCR. Southern blot analyses of *P. chrysogenum* genomic DNA using the isolated fragment as a probe demonstrated that the fragment truly specified part of the *P. chrysogenum* genome. Furthermore, since single hybridization signals were observed, *pex5* is apparently present in a single copy in the *P. chrysogenum* genome (data not shown). Subsequently, using the PCR fragment as a probe, we isolated the entire *pex5* gene from a gene library containing genomic *P. chrysogenum* DNA. Additionally, *pex5* cDNAs were isolated by PCR from a *P. chrysogenum* cDNA library.

Sequence analysis indicated that the *pex5* cDNA sequence differs at one position (C1761T) from the genomic *pex5* sequence, resulting in a Ala467Val replacement in TPR4 of *P. chrysogenum* Pex5p. This substitution has been observed in two independently isolated cDNAs and is presumably caused by the use of different *P. chrysogenum* strains in the construction of the gene libraries (Kiel et al., 2000). Comparison of the genomic *pex5* sequence with that of the cDNA shows that two introns of 81 and 69 nt, respectively, are present in the 5′ end of the *P. chrysogenum pex5* gene (GenBank Accession No. AY366189, see also Fig. 1). The size of these introns as well as the intron sequences themselves (splice donor and acceptor sites and the lariat) are similar to those observed for introns in many other *P. chrysogenum* genes (size 50–139 nt; consensus sequence: GT(A/g) AGT-$(N)_{26-118}$-CT(A/g)A(C/t)-$(N)_{5-18}$-(C/T) AG; Kiel et al., 2000).

The *P. chrysogenum pex5* gene encodes a protein of 632 amino acids that is highly similar to PTS1 receptors from other fungi (Fig. 2). Like other PTS1 receptors, *P. chrysogenum* Pex5p contains a highly conserved N-terminal region (amino acids 1–36) of unknown function, which is followed by a weakly conserved region that contains two WxxxY/F motifs (amino acids 101 to 105—WAKEF—and 116 to 120—WEAQF). For human PEX5, these have been demonstrated to bind the receptor docking protein PEX14 on the microbody membrane (Saidowsky et al., 2001). Finally, the C-terminus of Pc-Pex5p contains the typical two sets of 3 TPR motifs (indicated in Fig. 2) that constitute the binding site for the PTS1 tripeptide. Notably, in its C-terminus Pc-Pex5p contains additional stretches of amino acids between TPRs 4 and 5 and after TPR6, which are not observed in yeast and human PTS1 receptors. These may constitute additional small loops in the structure of the protein, the function of which is currently unknown. Similar loops are also present in a putative *N. crassa* Pex5p (GenBank Accession No. EAA36111), suggesting a feature specific to PTS1 receptors from filamentous fungi (data not shown).

3.2. Disruption of P. chrysogenum pex5

To enable investigation of the role of *P. chrysogenum pex5* in microbody biogenesis and penicillin production, we attempted to disrupt the gene. To this end, a plasmid was constructed that upon *Mlu*I digestion will provide a disruption cassette containing an internal fragment of *P. chrysogenum pex5* (nt 587–1541) and the *A. nidulans amdS* marker (Fig. 1). Initially, the disruption cassette was transformed into protoplasts of strain Wis54-1255. Among 106 transformants, we obtained 6 clones in which the disruption cassette had integrated at the right location on the *P. chrysogenum* genome as deduced by colony PCR, using *pex5*- and *amdS*-specific primers (data not shown). Typically, these colonies showed very poor growth on acetamide selection plates, did not turn green like other transformants after prolonged incubation on these plates and displayed a very poor viability. Surprisingly, it was observed that, in addition to the *pex5* disruption-specific fragments, colony PCR of these transformants also produced a DNA fragment indicative of the presence of the wild type *pex5* gene. Therefore, we aimed to isolate a true *pex5* disruptant by screening colonies generated from single spores. We observed that, when placed on sporulation media, the putative *pex5* disruptants produced segmented colonies with white and green sectors. The green sectors of these colonies contained many spores that were, however, unable to grow on selective acetamide plates suggesting that they represented non-transformed wild type cells or cells that had lost the disruption cassette. By contrast, hyphae from the white sectors grew slowly on acetamide

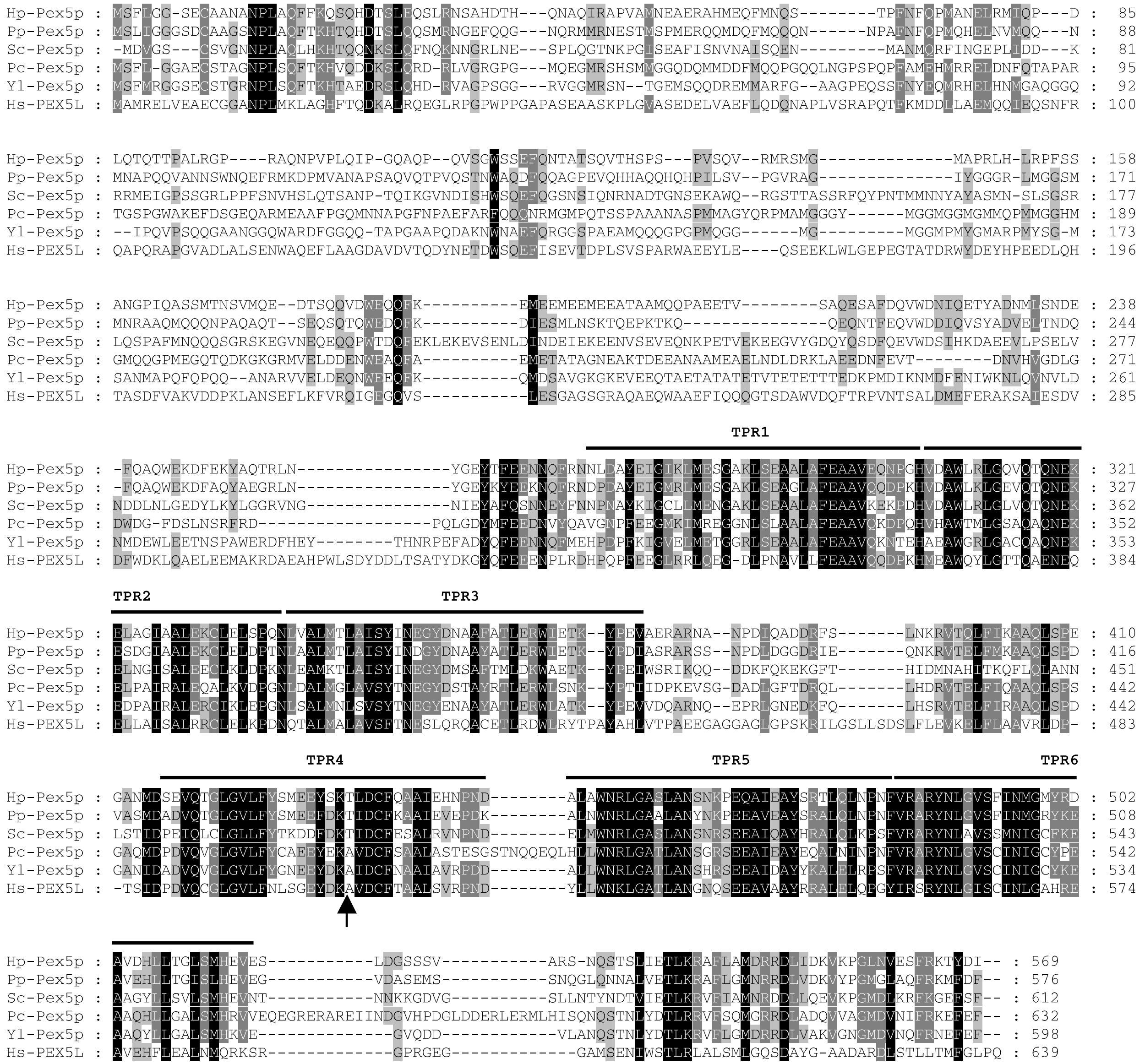

Fig. 2. Sequence alignment of PTS1 receptors. The deduced primary sequence of *P. chrysogenum* (Pc) Pex5p was aligned with those of PTS1 receptors from *H. polymorpha* (Hp; GenBank Accession No. AAC49040), *P. pastoris* (Pp; GenBank Accession No. P33292), *S. cerevisiae* (Sc; GenBank Accession No. P35056), *Y. lipolytica* (Yl; GenBank Accession No. Q99144), and human PEX5-L (Hs; GenBank Accession No. A56126) using the Clustal_X program. The one-letter code is shown. Gaps were introduced to maximize the similarity. Identical and conserved residues are shaded. The six TPR motifs in the C-terminus of the PTS1 receptors are indicated by the bars above the sequences. The arrow points at alanine 467 of Pc-Pex5p that was changed to valine in the protein encoded by the *Pc-pex5* cDNA.

plates, but failed to produce any spores on sporulation media. Thus, disruption of *P. chrysogenum pex5* resulted in colonies with poor viability that are disturbed in asexual sporulation. We have attempted to determine the location of the PTS1 matrix protein IAT in such clones by immunoelectronmicroscopy after culturing them on selective penicillin production media. Unfortunately, these studies were inconclusive (data not shown).

To overcome this problem, an alternative strategy was used that allowed direct visualization of the effect of *pex5* disruption, even in unstable transformants. To this end we used an AmdS$^-$ derivative of strain *P. chrysogenum* Wis54-1255::pPC-mb-eYFP (van der Lende et al., 2002). This strain produces a fluorescent protein (YFP) that is targeted to microbodies by a C-terminal PTS1 signal (-S-K-L-*COOH*). Disruption of the *pex5* gene in this strain is expected to result in mislocalization of the YFP-SKL protein to the cytosol, which should be visible in a significant number of cells even in unstable transformants with low viability. Fig. 3A demonstrates that YFP fluorescence was present in a punctate pattern in hyphae of Wis54-1255::pPC-mb-eYFP colonies grown on selective acetamide plates, indicative of its location in

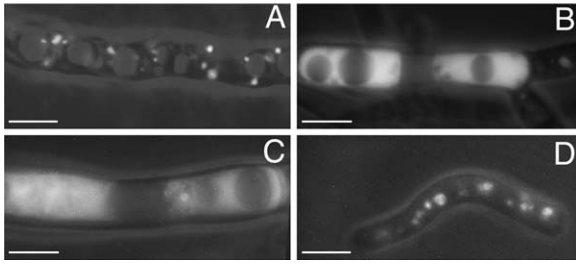

Fig. 3. *Penicillium chrysogenum* cells disrupted in pex5 mislocalize PTS1 proteins. Colonies of Wis54-1255::pPC-mb-eYFP (A), Wis54-1255::pPC-cyt-eYFP (B) and *pex5*::pPC-mb-eYFP, an AmdS$^+$ *pex5* disruptant of Wis54-1255::pPC-mb-eYFP (AmdS$^-$) (C,D) were grown on selective acetamide plates for 3 days at 25 °C. The location of YFP-SKL was analysed by fluorescence microscopy. The non-fluorescent regions in (B,C) represent vacuoles of the cells that do not import the YFP proteins. (D) A cell with a wild type phenotype thought to result from genetic instability of the *pex5* disruptant. Such cells no longer have the *An-amdS* cassette, and are unable to grow on selective acetamide plates, which may explain the smaller and strange shape of the cell. The scale bar represents 5 μm.

microbodies. In contrast, hyphae of the control strain Wis54-1255::pPC-cyt-eYFP (van der Lende et al., 2002) producing YFP lacking a PTS1 signal, showed an exclusive cytosolic fluorescence (Fig. 3B). Subsequently, we transformed Wis54-1255::pPC-mb-eYFP (AmdS$^-$) protoplasts with the *pex5* disruption cassette and obtained two positive clones out of 99 transformants tested, which again displayed the sporulation-deficient/poor growth/low viability phenotype. Analysis of hyphae of these colonies by fluorescence microscopy showed a heterogenous cell population. In many cells the YFP-SKL protein was mislocalized to the cytosol (Fig. 3C), while in other cells the presence of punctate fluorescence suggested localization of the YFP-SKL protein in microbodies (Fig. 3D). Again, these data are indicative of unstable transformants. Nevertheless, since disruption of *pex5* in *P. chrysogenum* results in mislocalization of PTS1 proteins, we concluded that this gene is involved in matrix protein import into microbodies.

3.3. Heterologous expression of *P. chrysogenum pex5* in the methylotrophic yeast *H. polymorpha*

To further establish the function of Pc-Pex5p in PTS1 matrix protein import, we expressed the corresponding cDNA in a *PEX5* null mutant of the methylotrophic yeast *H. polymorpha*. In this yeast, proliferation of microbodies can be easily manipulated by adaptation of the growth conditions. *H. polymorpha* cells require functional microbodies to allow growth on methanol as sole carbon and energy source, as they contain the key enzymes of the metabolism of this compound, alcohol oxidase (AO), catalase (CAT), and dihydroxyacetone synthase (DHAS), sorting of which is mediated by *H. polymorpha* Pex5p (van der Klei et al., 1995; Veenhuis et al., 1978). Nevertheless, microbody deficient mutants are viable and can be propagated in rich media that do not require functional microbodies.

Expression of *Pc-pex5* in the *H. polymorpha PEX5* null mutant resulted in the production of a protein of approximately 75 kDa, that cross-reacted with antibodies against *S. cerevisiae* Pex5p (Fig. 4A). Despite the synthesis of significant amounts of Pc-Pex5p, *H. polymorpha* $\Delta pex5$::($P_{AOX} \cdot Pc\text{-}pex5$)^{mc} was unable to grow on methanol as sole carbon and energy source, indicating that expression of the heterologous gene could not complement the microbody biogenesis defect of the mutant. To investigate whether Pc-Pex5p could at least partly import PTS1 proteins into *H. polymorpha* microbodies, we analysed PTS1 protein import in $\Delta pex5$::($P_{AOX} \cdot Pc\text{-}pex5$)^{mc} biochemically and ultrastructurally.

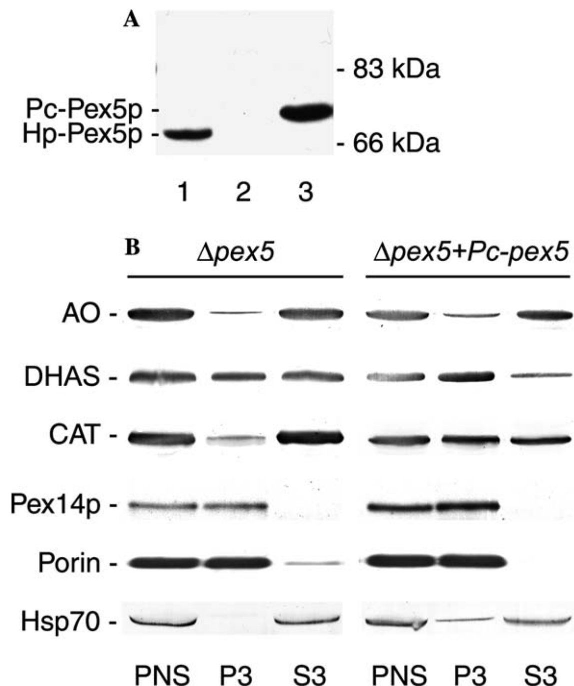

Fig. 4. Specific PTS1 proteins co-localize with organelles upon expression of Pc-pex5 in a *H. polymorpha* PEX5 null mutant. (A) Western blot analysis of crude extracts prepared from methanol/glycerol grown cells of *H. polymorpha* wild type (lane 1), $\Delta pex5$ (lane 2), and $\Delta pex5$::($P_{AOX} \cdot Pc\text{-}pex5$)^{mc} (lane 3) to demonstrate the production of Pc-Pex5p. Equal amounts of protein were loaded per lane. The Western blots were decorated with specific antibodies against *S. cerevisiae* Pex5p. (B) Post-nuclear supernatants (PNS) were prepared from lysed protoplasts of *H. polymorpha* $\Delta pex5$ and $\Delta pex5$::($P_{AOX} \cdot Pc\text{-}pex5$)^{mc} cells, cultured for 18 h on glycerol/methanol. Centrifugation of each PNS for 30 min at 30,000*g* resulted in an organellar pellet fraction (P3) and the corresponding supernatant fraction (S3)—containing soluble proteins. Western blots were prepared of equal volumes loaded per lane. The blots were decorated with specific antibodies against the indicated *H. polymopha* proteins. It should be noted that the sedimented DHAS protein in the P3 fraction of the $\Delta pex5$ samples is believed to be due to aspecific binding to membranes, a feature observed before for this protein (our unpublished data).

First, we analysed by differential centrifugation a post-nuclear supernatant (PNS) obtained from lysed protoplasts of glycerol/methanol-grown *H. polymorpha* Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} cells to address whether PTS1 matrix proteins were pelletable, using identically treated *H. polymorpha* Δ*pex5* cells as control. Fig. 4B demonstrates that in both the Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} strain and the control, AO remained soluble. By contrast, significant amounts of DHAS and CAT were pelletable in the Pc-Pex5p producing strain, but not in the Δ*pex5* control, suggesting Pc-Pex5p-dependent import of these proteins into organelles. Other control proteins, the microbody membrane protein Pex14p, mitochondrial porin and cytosolic Hsp70, were at their expected locations.

Sucrose density gradients of PNS fractions prepared from glycerol/methanol-grown *H. polymorpha* Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} and Δ*pex5* cells were analysed to discern co-localization of PTS1 matrix proteins with microbodies. When wild type *H. polymorpha* cells are used in such experiments, microbodies (and their PTS1 proteins) sediment at approximately 53% sucrose (cf. Kiel et al., 1999; see also arrow in Fig. 5). By contrast, using a PNS from Δ*pex5* cells, all PTS1 matrix proteins are located in the cytosol, and microbody membrane proteins sediment at approximately 33% sucrose (Fig. 5A). Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} cells do not contain high density microbodies (Fig. 5B). Instead, significant amounts of the PTS1 protein CAT sediment together with the microbody membrane proteins Pex14p and Pex3p as well as Pc-Pex5p at 43% sucrose. The PTS1 protein AO remained present in the cytosolic fractions.

These biochemical data were supported by ultrastructural analysis. Glycerol/methanol-grown *H. polymorpha* Δ*pex5* cells do not contain normal microbodies and their PTS1 proteins are located in the cytosol (Fig. 6B; van der Klei et al., 1995). Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} cells have microbodies that are smaller but more abundant relative to the organelles found in *H. polymorpha* wild type cells (compare Fig. 6A and C). Immunocytochemistry demonstrated that these organelles contained DHAS (Fig. 6E) and CAT protein (Fig. 6F), while the α-AO dependent labeling was predominantly on the cytosol with low numbers of gold particles on peroxisomal profiles (Fig. 6D). Clearly, Pc-Pex5p production results in a partial restoration of PTS1 protein import into microbodies. Therefore, we conclude that Pc-Pex5p is a true PTS1 receptor that apparently poorly recognizes AO as a cargo protein.

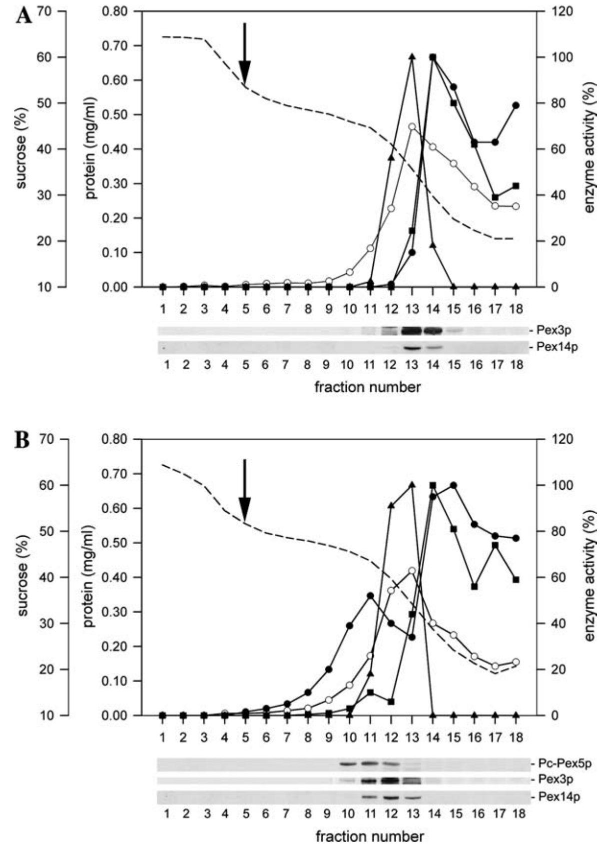

Fig. 5. Pc-pex5 expression in *H. polymorpha* Δ*pex5* cells results in the formation of low density microbodies. Sucrose density gradient centrifugation of a PNS of *H. polymorpha* Δ*pex5* (A) and Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} (B) cells cultured for 18 h on glycerol/methanol. Sucrose (- - - - - -), protein concentration (○) and the distribution of the activities of the microbody matrix proteins AO (■) and CAT (●) and the mitochondrial marker COX (▲) are indicated. Enzyme activities are expressed as percentages of the specific activities of the peak fractions, which were arbitrarily set to 100%. The Western blots show the distribution of the microbody membrane proteins Pex3p and Pex14p as well as Pc-Pex5p in the fractions of the gradients. Equal portions of each fraction were loaded per lane. The arrows indicate the expected location of normal wild type high density microbodies. In both gradients mitochondria sediment at approximately 37% sucrose. Peroxisomal membrane proteins of the PNS of the *PEX5* null mutant peak at 33% sucrose, while PTS1 proteins (AO and CAT) are present on top of the gradient. In the sucrose gradient prepared with the PNS of Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} the peroxisomal membrane proteins Pex3p and Pex14p and a major portion of the PTS1 protein CAT as well as PcPex5p sediment at approximately 43% sucrose, while almost all AO is present on top of the gradient.

3.4. *The P. chrysogenum PTS1 receptor imports an eGFP-LARF fusion protein less efficiently than eGFP-SKL*

To understand more about why production of Pc-Pex5p in *H. polymorpha* Δ*pex5* resulted in a differentiated import between the major microbody proteins AO, DHAS, and CAT, we constructed derivatives of Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} producing eGFP proteins with three different PTS1 signals. *H. polymorpha Pc-pex5*::(P_{AOX} · *eGFP-SKL*), a strain producing eGFP with the canonical PTS1 sequence (-S-K-L-*COOH*), a bona fide signal for import into *P. chrysogenum* microbodies (van der Lende et al., 2002; Fig. 3), showed an exclusive punctate fluorescence pattern reminiscent of import of the protein into microbodies (Figs. 7E and F). Similarly, *Pc-pex5*::(P_{AOX} · *eGFP-SKI*), producing eGFP provided

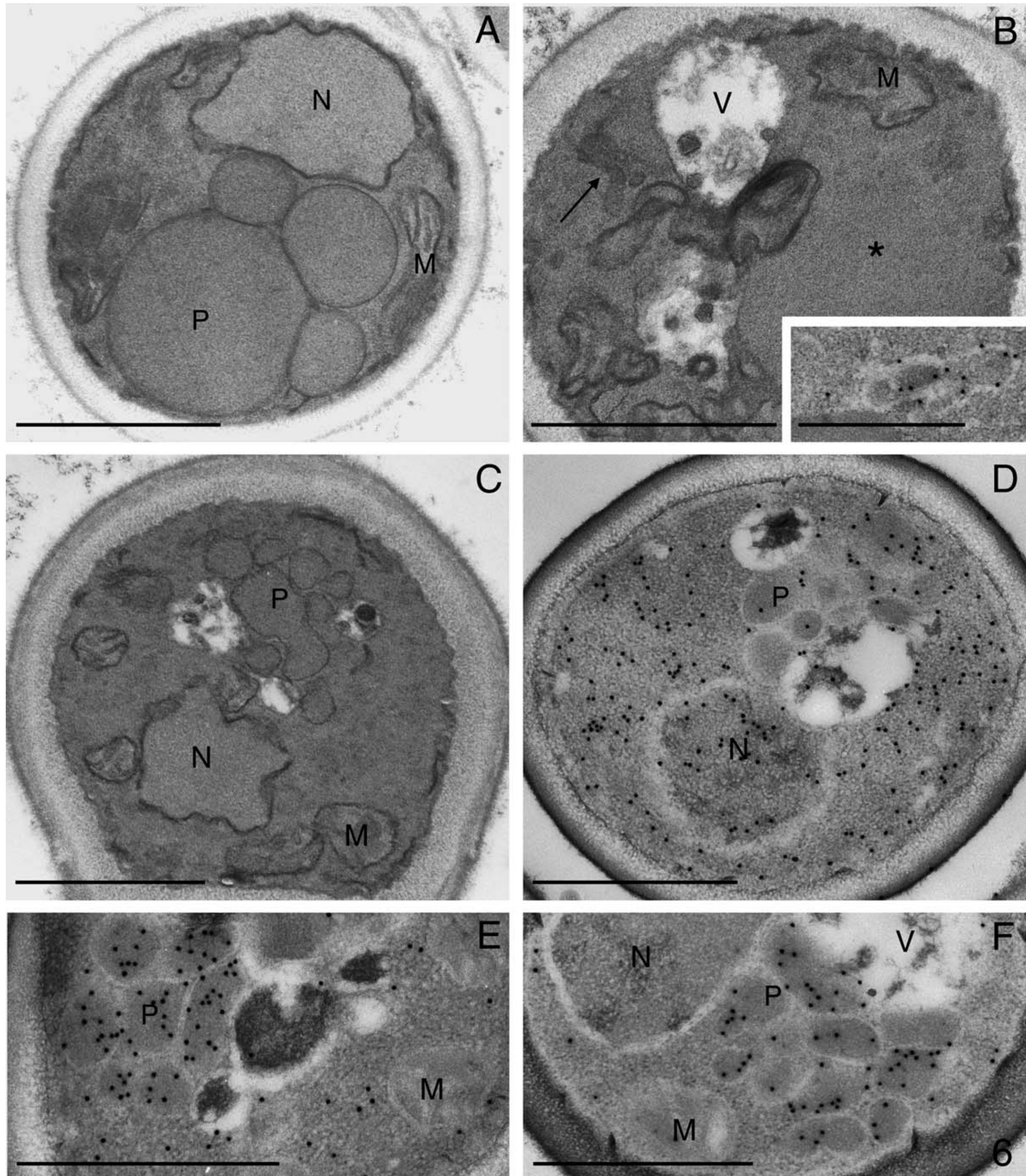

Fig. 6. *Hansenula polymorpha* Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} cells contain microbodies that harbour DHAS and CAT in conjunction with low AO amounts. (A–C) ($KMnO_4$-fixed cells): Morphology of a methanol-grown *H. polymorpha* wild type cell containing many microbodies (A), a glycerol/methanol-grown Δ*pex5* cell, that contains several small microbody profiles (arrow) and a cytosolic AO crystalloid (asterisk) (B). The microbodies are labeled with specific antibodies against the *H. polymorpha* microbody membrane protein Pex3p (inset, B). (C) A section of a glycerol/methanol-grown cell of Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} demonstrating the presence of many relatively small rounded microbodies in these cells. (D–F) Immunocytochemistry (aldehyde, primary antibody/GAR-gold, and uranylacetate). Sections of Δ*pex5*::(P_{AOX} · *Pc-pex5*)^{mc} cells were labeled with specific antibodies against *H. polymorpha* AO (D), DHAS (E), and CAT (F). AO labeling is dominant on the cytosol and very low on peroxisomal profiles. Conversely, most of the DHAS and CAT specific labeling is present on microbody profiles. *Abbreviations*: M, mitochondrion; N, nucleus; P, microbody (peroxisome); and V, vacuole. An asterisk indicates the location of a cytosolic AO crystalloid. The scale bar represents 1 μm except in the inset of (B) where it represents 0.5 μm.

with the PTS1 signal of *H. polymorpha* CAT (Didion and Roggenkamp, 1992) and *P. chrysogenum* PACL (Gledhill et al., 1998), showed an almost completely punctate fluorescence pattern, with only little fluorescent signal in the cytosol (Figs. 7G and H). By contrast, *H. polymorpha Pc-pex5*::(P_{AOX} · *eGFP-LARF*), producing eGFP with the 4 C-terminal amino acids of *H. polymorpha* AO, showed a dual location, with punctate

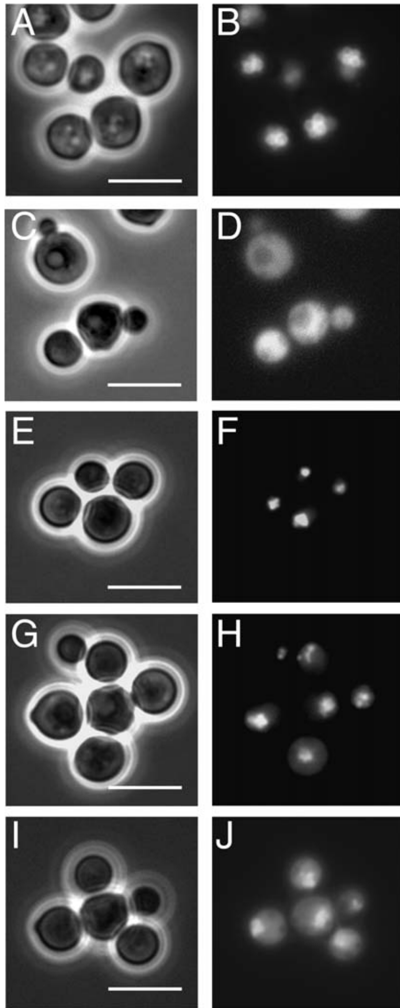

Fig. 7. *The P. chrysogenum PTS1 receptor shows a differential import of the reporter protein eGFP fused to various PTS1 signals*. Bright field (A, C, E, G, and I) and fluorescence pictures (B, D, F, H, and J) are shown of methanol/glycerol-grown cells that synthesize eGFP derivatives with different PTS1 signals. (A,B) *H. polymorpha* wild type cells expressing *eGFP-SKL* (strain HF246). All fluorescence is present in the large clusters of peroxisomes. Similar results were obtained when *eGFP-SKI* or *eGFP-LARF* are expressed in wild type *H. polymorpha* (data not shown). (C,D) *H. polymorpha* Δ*pex5* cells expressing *eGFP-SKL*. Fluorescence is located in the cytosol. (E,F) *Pc-pex5*::(P_{AOX} · *GFP-SKL*) cells; fluorescence is confined to the organelles. Essentially similar results were obtained for *Pc-pex5*::(P_{AOX} · *GFP-SKI*) cells producing eGFP with the PTS1 of CAT. However, occasionally a faint cytosolic fluorescence is also visible (G,H). (I,J) *Pc-pex5*::(P_{AOX} · *GFP-LARF*) cells producing eGFP fused to the C-terminal 4 amino acids of AO. Although punctate fluorescence is visible, significant fluorescence is present in the cytosol, indicative of a less efficient import of eGFP-LARF. The scale bar represents 5 μm.

fluorescent structures in conjunction with significant cytosolic fluorescence (Figs. 7I and J).

4. Discussion

We described the isolation of the *P. chrysogenum pex5* gene that contains 2 introns and encodes a protein of approximately 75 kDa with all the sequence characteristics of a PTS1 receptor. All attempts to disrupt *pex5* in *P. chrysogenum* resulted in unstable transformants; *pex5* disrupted cells appeared to be unable to sporulate, showed poor growth and low viability. Furthermore, they mislocalized the PTS1 reporter protein eYFP-SKL to the cytosol. *Pc-pex5* expression in a *PEX5* null mutant of *H. polymorpha* did not complement the microbody development defect of this strain. Nevertheless, biochemical and ultrastructural analysis demonstrated that significant amounts of the PTS1 proteins DHAS and CAT had been imported into microbodies, while AO, another protein that requires Pex5p for import into microbodies, was present in these organelles at very low amounts. A comparison between the import efficiency of the reporter protein eGFP fused to either the canonical PTS1 signal (-S-K-L-*COOH*) or two degenerate PTS1 signals (-S-K-I-*COOH* and -L-A-R-F-*COOH*), demonstrated that Pc-Pex5p was able to import eGFP-SKL and eGFP-SKI with high efficiency in *H. polymorpha* microbodies, but that eGFP-LARF import was much less efficient.

We envisage two possible explanations for the observed instability of *Pc-pex5*-disrupted colonies. One option is that transformation of the disruption cassette resulted in the formation of *pex5* disruptants in which occasionally the cassette is removed from the *P. chrysogenum* genome by perfect DNA excision. In this scenario, the phenotype of the transformants, i.e., sporulation-deficiency, poor growth, low viability, and mislocalization of PTS1 proteins, is the direct consequence of the *pex5* disruption, a phenotype that is apparently lost upon excision of the disruption cassette. An alternative explanation is that multi-nucleated protoplasts have been transformed. In this scenario, each cell of a positive clone contains at least one copy of the disrupted gene, together with one or more copies of the wild type gene, with the disrupted copy having a dominant negative effect. It must be noted that our *pex5* disruption strategy results in a gene that could theoretically still produce a truncated protein of 393 amino acids (consisting of the N-terminus plus TPR regions 1 and 2, see Fig. 2), which might negatively influence import of PTS1 proteins by the wild type PTS1 receptor. Growth of such multi-nucleated cells will occassionally produce wild type cells that have lost the transformed nucleus, while cells containing exclusively the nucleus with the disrupted *pex5* gene may be inviable. Unfortunately, our data did not allow to discriminate between these possibilities.

Expression of *Pc-pex5* in *H. polymorpha* has led to a number of interesting observations. First, *Pc-pex5* does not fully complement the PTS1 protein import defect of the *H. polymorpha PEX5* null mutant. Our biochemical and ultrastructural studies clearly demonstrated that only two of the key enzymes of methanol metabolism,

DHAS and CAT, were significantly imported by Pc-Pex5p into the microbody matrix, suggesting that *Pc-pex5* indeed encodes a bona fide PTS1 receptor. By contrast, AO, catalyzing the first step of methanol metabolism, remained predominantly mislocalized to the cytosol. Since methanol metabolism requires an exclusive microbody localization of AO (van der Klei et al., 1991), the lack of AO import explains the inability of the *Pc-pex5* expression strain to grow on this compound as sole carbon and energy source. This lack of complementation does not seem to be the result of the sequence difference between the genomic and the cDNA clone of *Pc-pex5* (Ala467Val). We have analysed in parallel strains expressing a *Pc-pex5* cDNA with Ala467, and obtained identical results (data not shown). Previously, van der Klei et al. (1995) demonstrated that even the highly related *P. pastoris PEX5* gene was not able to fully complement the *H. polymorpha pex5* mutant, suggesting that the *H. polymorpha* PTS1 receptor has certain features that have not been (completely) conserved in other species.

Our data suggest that like other PTS1 receptors, *P. chrysogenum* Pex5p has a preference for certain PTS1 signals (see Neuberger et al., 2003). Apparently, the canonical PTS1 (-S-K-L-*COOH*) and the signals of *P. chrysogenum* IAT (-A-R-L-*COOH*) and *H. polymorpha* DHAS (-N-K-L-*COOH*) and CAT (-S-K-I-*COOH*) are efficiently recognized, while the C-terminal 4 amino acids of *H. polymorpha* AO (-L-A-R-F-*COOH*) are not. Previously, we demonstrated that in *H. polymorpha*, fusion proteins between eGFP and these PTS1 signals were normally imported into microbodies, suggesting that they all can function as efficient sorting signals in this host (Salomons et al., 2000; van Dijk et al., 2001). It must be noted that recent data indicate that the C-terminus of AO (-L-A-R-F-*COOH*) is not the PTS of this protein at all (Gunkel et al., 2004). These authors showed that deletion of the C-terminal 16 amino acids of AO did not significantly affect import of this protein into microbodies in *H. polymorpha*. In addition, efficient import of AO required only the N-terminal part of *H. polymorpha* Pex5p and not the C-terminal TPR domains, that were supposed to recognize the -L-A-R-F-*COOH* sequence. This implies that AO contains an specific internal PTS that has not been identified yet. The low levels of import of AO in a *H. polymorpha pex5* strain expressing *Pc-pex5* clearly implies that the *P. chrysogenum* PTS1 receptor does not recognize this novel motif significantly. Recently, a gene encoding an AO from *P. chrysogenum* was isolated (Holzmann et al., 2002). Although no data are available concerning a possible microbody location of this protein, inspection of the C-terminus of Pc-AO shows a sequence (-S-R-L-*COOH*) highly similar to the canonical PTS1 signal (see Fig. 8), making it tempting to assume that this protein is sorted to microbodies via this signal.

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Pp-AO1    : EDLGYSGEALDMTVPQFKLGTYEKTGLARF : 663
Pp-AO2    : EDLGYTGEALDMTVPQFKLGTYEKTGLARF : 663
Hp-AO     : EDLGYSGSDLDMTIPNFRLGTYEETGLARF : 664
Pm-AO2    : EDLGYSGAELDMTIPGFKLGTYESTGLGRF : 663
Pm-AO1    : EDLGYSGDALKMTVPNFKLGTYEEAGLARF : 664
Cb-AO     : EDLGYSGSELDMEVPQHKLKTYEQTGAARY : 663
Pc-AO     : EDLGYSGADLDMKVPTYHAPA-EFSNLSRL : 666
Cv-AO-p68 : EDLGYSGSALDMKVPNYHAPR-EIAGLSRL : 665
Nc-ORF    : EDLGYSGEALQMKVPDYHAPG-ESRLSSRL : 666
Cf-AO     : EDLGYTGRALDMRVPDYQANR-EITGLARL : 665
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Fig. 8. Sequence alignment of C-termini of fungal AO proteins. The C-terminus of *P. chrysogenum* alcohol oxidase (Pc-AO, GenBank Accession No. AAL56054) was aligned with the corresponding regions from the AO proteins from the methylotrophic yeasts *P. pastoris* (Pp-AO1, GenBank Accession No. AAB57849 and Pp-AO2, GenBank Accession No. AAB57850), *H. polymorpha* (Hp-AO, GenBank Accession No. P04841), *Pichia methanolica* (Pm-AO1, GenBank Accession No. AAF02494; Pm-AO2, GenBank Accession No. AAF02495), and *Candida boidinii* (Cb-AO, GenBank Accession No. Q00922). Also included in the alignment are the C-termini of AO proteins from the fungi *Cochliobolus victoriae* (Cv-AO-p68, GenBank Accession No. AAK14990) and *Cladosporium fulvum* (Cf-AO, GenBank Accession No. AAF82788) as well as a putative AO from *N. crassa* (Nc-ORF, translation of Genbank Accession No. AABX01000068). The one-letter code is shown. Gaps were introduced to maximize the similarity. Identical and conserved residues are shaded. Note that all proteins show putative PTS1 signals.

Initially, we set out to analyse the effect of disruption of *pex5* on β-lactam production by *P. chrysogenum.* Unfortunately, the genetic instability of *pex5* disruptants, their poor growth and inability to sporulate have precluded such analyses. We sought to completely delete *Pc-pex5* from the *P. chrysogenum* genome via a double cross-over event. However, so far, all attempts to obtain a *pex5* null mutant have been unsuccesful. Previously, we were also unable to isolate null mutants of *P. chrysogenum pex1* and *pex6* (Kiel et al., 2000). Combined, this may point towards possible inviability of *P. chrysogenum* mutants completely devoid of microbodies. Filamentous fungi like *P. chrysogenum* contain in addition to microbodies required for fatty acid β-oxidation and penicillin production, a microbody-related structure known as the Woronin body. This organelle is essential for plugging the septal pore upon hyphal damage and contains a proteinaceous core consisting of Hex1p, a protein with a PTS1, which is essential for this function (Jedd and Chua, 2000). *Hex1* mutants are viable, but have a bleeding phenotype, i.e., upon physical damage cytoplasm leaks out of the hyphae via the septal pore into the environment, a phenotype that can also be expected for a null mutant of any *pex* gene involved in PTS1 matrix protein import. Therefore, we propose that the presumed inviability of *pex* null mutants in filamentous fungi like *P. chrysogenum* may be the result of an accumulation of the negative effects of phenotypes like sporulation-deficiency, hyphal bleeding, etc. on growth and survival. Clearly, the isolation of temperature sensitive *pex* mutants or the identification of a tightly controlled promoter to enable

regulatable *pex* gene expression is required to analyse this hypothesis in detail. Such strains should also be instrumental in determining the effect of inhibition of microbody biogenesis on penicillin production in *P. chrysogenum*.

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