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Heterologous complementation of peroxisome function in yeast: the *Saccharomyces cerevisiae PAS3* gene restores peroxisome biogenesis in a *Hansenula polymorpha per9* disruption mutant

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Abstract ***PER*** **genes are essential for the biogenesis of peroxisomes in the yeast *Hansenula polymorpha*. Here we describe the functional complementation of a *H. polymorpha per9* disruption strain (*Δper9*) by a heterologous gene. The *Saccharomyces cerevisiae* Pas3p, a homologue of Per9p, restored peroxisome biogenesis and peroxisomal protein import in the *Δper9* mutant, allowing it to grow again on methanol as sole carbon and energy source. This result shows that heterologous complementation of peroxisome function in yeast is indeed feasible and furthermore suggests that *H. polymorpha Δper9* may be the candidate of choice to attempt the isolation of Per9p homologues from higher eukaryotes by functional complementation.**

Key words: Functional complementation; Inducible promoter; Matrix protein; Microbody; Shuttle vector; Yeast

1. Introduction

Peroxisomes are subcellular organelles surrounded by a single membrane, which are characterized by the presence of H_2O_2-producing oxidases as well as the H_2O_2-scavenger catalase. Dependent on the organism in which they occur, peroxisomes may vary in size, number and enzyme repertoires. Peroxisomes do not contain DNA. As a consequence, peroxisomal matrix proteins, synthesized in the cytosol, have to be imported into the growing organelle (reviewed in [1,2]).

Peroxisomes fulfil essential functions in cellular metabolism. Defects in the biogenesis/function of these organelles have severe consequences, exemplified by the lethal genetic disorders such as the classical Zellweger syndrome in humans [3]. Yeasts are a major exception in that they remain completely viable and can grow normally on rich media in the absence of peroxisomes. Hence, various mutants involved in peroxisome biogenesis have been isolated from several yeast species (reviewed in [4]). The isolation of the corresponding yeast genes has rapidly expanded our knowledge on the molecular mechanisms underlying peroxisome biogenesis and also had a distinct spin-off in the understanding of human peroxisomal disorders [5–9].

There is a firm belief that the molecular basis of peroxisome biogenesis, like for other organelles, is conserved from lower to higher eukaryotes. However, so far functional complementation of peroxisome-deficient yeast mutants by heterologous genes has not been achieved [8,9]. This contrasts to the situation observed for other organelles like mitochondria and endoplasmic reticulum, where yeast mutants have been complemented by genes from higher eukaryotes [10,11].

In our laboratory we use the methylotrophic yeast *Hansenula polymorpha* as a model organism for studies on peroxisome biogenesis and function. Recently, we have isolated and characterized the *PER9* gene [12]. The *H. polymorpha* Per9p is presumed to be the functional homologue of the *S. cerevisiae* peroxisomal membrane protein Pas3p [13]. In the present study we show that the *PAS3* gene could functionally completed a *H. polymorpha per9* disruption mutant and thus restore peroxisome assembly. The data suggest that in particular the *Δper9* mutant may be suited for the isolation of heterologous genes by functional complementation, using cDNA or genome banks.

2. Materials and methods

2.1. Micro-organisms and growth conditions

Hansenula polymorpha NCYC495 (*leu1-1 ura3*) and transformants of the peroxisome-deficient *H. polymorpha* strain *Δper9* (*leu1-1 ura3 Δper9::URA3*) [12] were grown in batch cultures on mineral medium [14] using either glucose (0.5%), glycerol (0.5%) or methanol (0.5%) as carbon source. *Escherichia coli* DH5α (*supE44 ΔlacU169* (*φ80lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) [15] was used for plasmid constructions and was grown on LB-medium supplemented with the appropriate antibiotics.

2.2. DNA procedures

H. polymorpha was transformed using the electroporation method [16]. Recombinant DNA manipulations were as described [15]. Polymerase chain reaction-mediated DNA amplification (PCR) was performed with *Vent* polymerase (New England Biolabs, Beverly, MA) according to [17]. Oligonucleotides were obtained from Eurosequence bv, Groningen, The Netherlands. Biochemicals were obtained from Boehringer, Mannheim, Germany.

2.3. Construction of plasmids

The *E. coli/H. polymorpha* shuttle vectors pHIPX5 and pHIPX6, shown in Fig. 1, were constructed as follows: for pHIPX5, a 1.0 kb fragment containing the promoter of the *H. polymorpha* amine oxidase gene (P_{AMO}; nt 1–989 of EMBL accession number X15111) was isolated by PCR with the P_{AMO} primer (5′ GGG GCA TGC CCA TGG ATC CTG TAC GAT ATG AAG GAC 3′) and the M13/pUC sequencing primer. After subcloning in a pBluescript vector (Stratagene, LaJolla, CA), P_{AMO} was cloned as an *Eco*RI (blunted)–*Xma*I fragment in *Not*I (blunted) + *Xma*I digested pHIPX4 [18], thus replacing the alcohol oxidase promoter in this plasmid. Similarly, for pHIPX6 a 1.2 kb fragment containing the promoter of the *H. polymorpha PER9* gene (nt 2–1196 in Genbank accession number U37763) was obtained by PCR with the P_{PER9} primer (5′ TGG ATC CAC CAA GTA TCA CAG AG 3′) and the M13/pUC reverse sequencing primer. The amplified frag-

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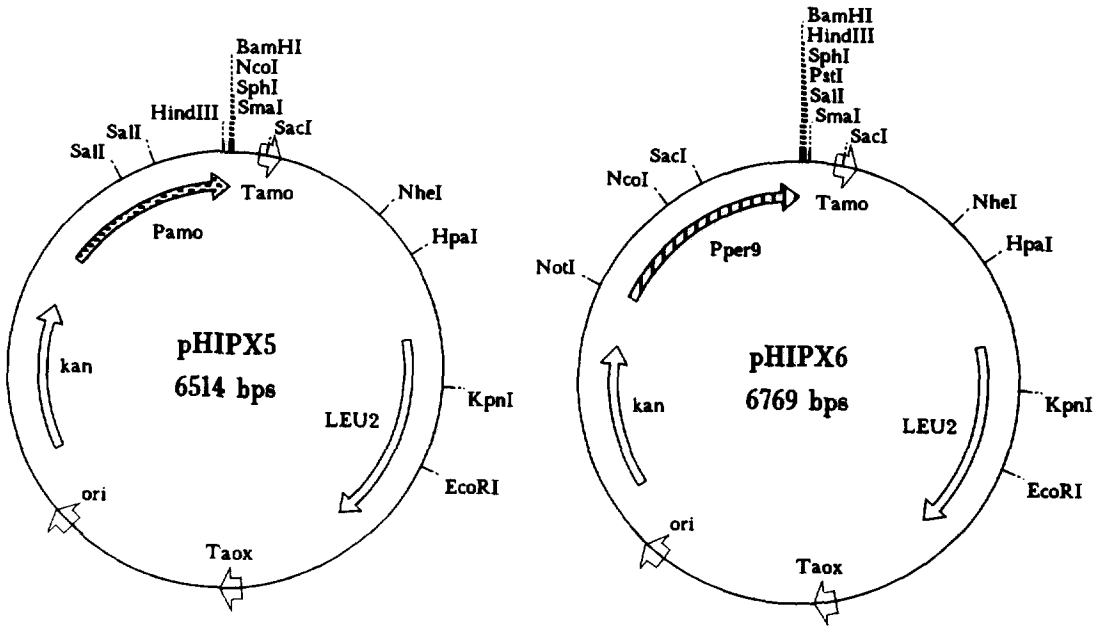

Fig. 1. Physical map of the *E. coli*/*H. polymorpha* shuttle vectors pHIPX5 and pHIPX6. Abbreviations: kan, kanamycin resistance marker; ori, origin for replication in *E. coli*; P_{AMO} and P_{PER9}, promoters of the *H. polymorpha* amine oxidase and *PER9* genes, respectively. T_{AMO} and T_{AOX}, terminators of the *H. polymorpha* amine oxidase and alcohol oxidase genes, respectively.

ment was digested with *Not*I and cloned in *Not*I + *Hin*dIII (blunted) pHIPX4.

For plasmids expressing the *H. polymorpha PER9* gene (pHIPX4-PER9, pHIPX5-PER9 and pHIPX6-PER9), the gene was amplified by PCR, using a PER9ATG primer (5′ GTT CTC TGT GAT ACG GAT CCA TGT TCC AAT ATT GTA G 3′) and the M13/pUC reverse sequencing primer. After subcloning, the *PER9*-containing fragment was subsequently cloned as a *Bam*HI (blunted)–*Sal*I fragment in *Hin*dIII (blunted) + *Sal*I digested pHIPX4, as a *Bam*HI–*Sal*I (blunted) fragment in *Bam*HI + *Sma*I digested pHIPX5, or as a *Bam*HI–*Sal*I fragment in *Bam*HI + *Sal*I digested pHIPX6.

For plasmids expressing the *S. cerevisiae PAS3* gene (pHIPX4-PAS3, pHIPX5-PAS3 and pHIPX6-PAS3), a 1.6 kb *Bam*HI–*Sal*I fragment from plasmid pTK9 (T. Krause and W.H. Kunau, unpublished) was inserted in *Bam*HI + *Sal*I-digested pHIPX4-B (a slightly modified pHIPX4 derivative; M. Komori, unpublished) and *Bam*HI + *Sal*I-digested pHIPX6. In addition, the *PAS3* gene was cloned as a *Bam*HI–*Sal*I (blunted) fragment in *Bam*HI + *Sma*I digested pHIPX5.

2.4. Biochemical methods

Lysis of *H. polymorpha* cells [19], protein determination [20], SDS-PAGE [21] and Western blotting [22] were performed by established methods. Blots were decorated with specific antibodies against *S. cerevisiae* Pas3p or *H. polymorpha* Per9p.

2.5. Electron microscopy

Cells were prepared for electron microscopy as described previously [23]. Immunocytochemical experiments were performed on ultrathin sections of Unicryl-embedded cells using polyclonal antibodies raised against *H. polymorpha* catalase and *S. cerevisiae* Pas3p and gold-conjugated goat-anti-rabbit antibodies.

3. Results and discussion

To determine whether the *S. cerevisiae PAS3* gene could functionally complement the peroxisome-deficient *H. polymorpha Δper9* mutant, we utilized plasmids that allowed synthesis of Pas3p at various levels, prescribed by the growth conditions. During growth on methanol/ammoniumsulphate, the strong alcohol oxidase promoter (P_{AOX}), present on pHIPX4, and the weaker *PER9* promoter (P_{PER9}), present on pHIPX6, are induced [12]. The amine oxidase promoter (P_{AMO}), present on pHIPX5, is repressed when *H. polymorpha* cells are grown on ammoniumsulphate as nitrogen source, and is normally induced in the presence of primary amines [24]. However, during growth on ammoniumsulphate partial derepression of P_{AMO} occurs when the cells reach the early stationary phase (J.A.K.W. Kiel, unpublished). *H. polymorpha Δper9* was transformed with the different plasmids carrying the *PAS3* gene

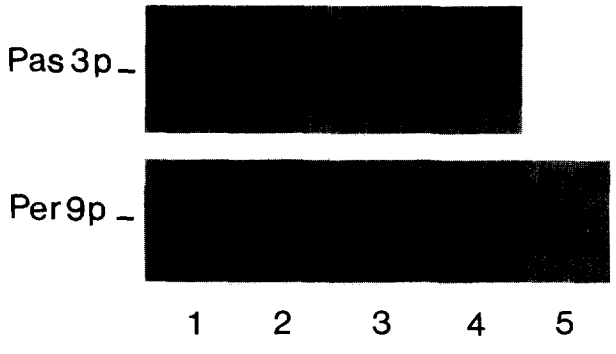

Fig. 2. Western blots of *H. polymorpha Δper9* transformants. Cells were grown on methanol/ammoniumsulphate as described in Table 1 and harvested after 24 h of cultivation. Panel A: *Δper9* cells carrying plasmids expressing *S. cerevisiae PAS3*: pHIPX4-PAS3 (lane 2), pHIPX5-PAS3 (lane 3) and pHIPX6-PAS3 (lane 4). Panel B: *Δper9* cells carrying plasmids expressing *H. polymorpha PER9*: pHIPX4-PER9 (lane 2), pHIPX5-PER9 (lane 3), pHIPX6-PER9 (lane 4) and wild-type *H. polymorpha* NCYC495 (*leu1-1 ura3*) (lane 5). In both panels *Δper9* cells carrying vector pHIPX4 induced on methanol/ammoniumsulphate were used as control (lanes 1). Equal amounts of protein were loaded in each lane. Blots were decorated with specific antibodies against *S. cerevisiae* Pas3p (panel A) or *H. polymorpha* Per9p (panel B).

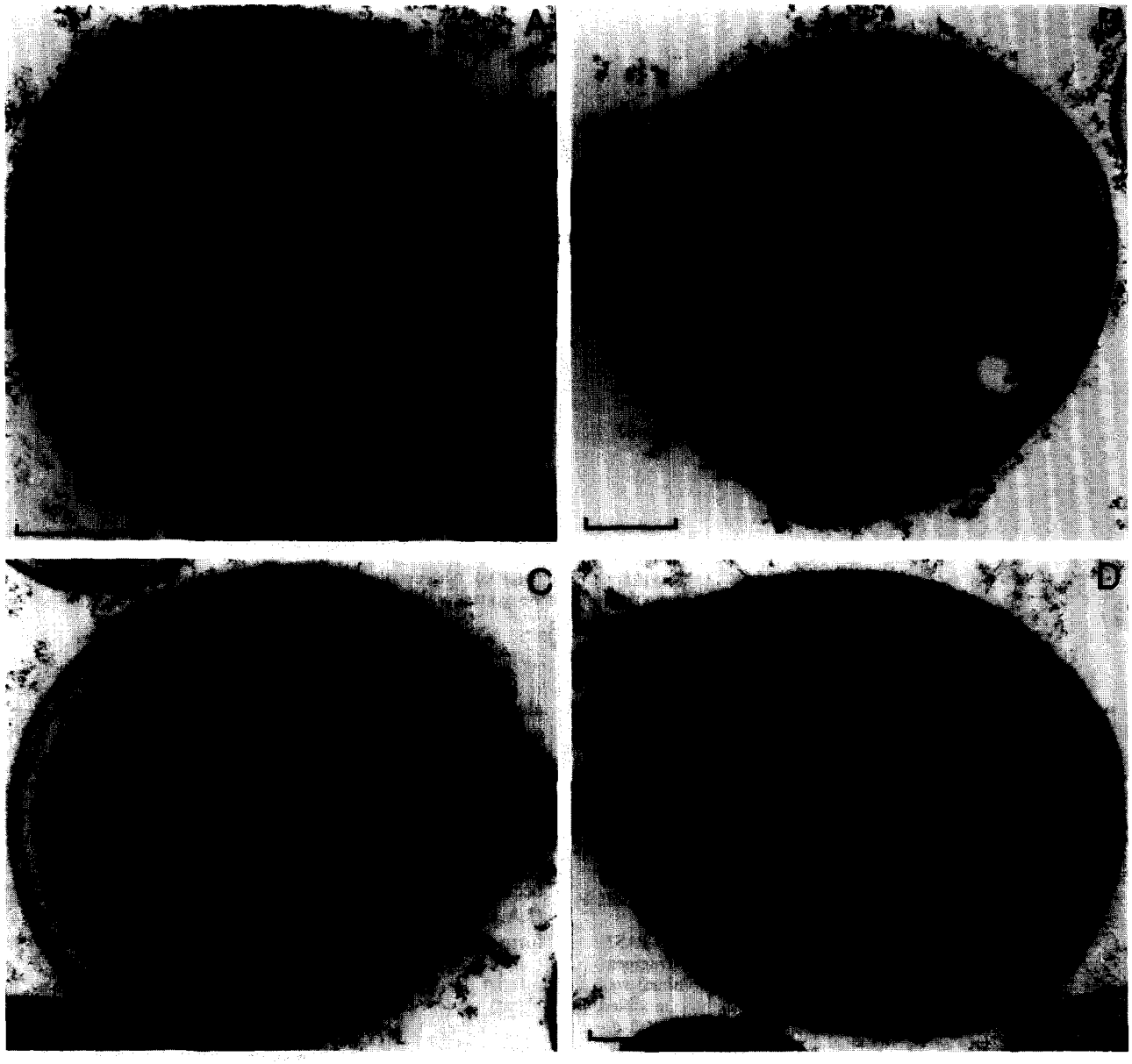

Fig. 3. Ultrastructural analysis of transformed *H. polymorpha Δper9* cells, incubated on methanol/ammoniumsulphate-containing media. Cell of *Δper9* carrying vector pHIPX4, showing the absence of peroxisomes; instead a cytosolic alcohol oxidase crystalloid (*) was present. The architecture of this crystalloid is not well preserved due to the $KMnO_4$ fixation method (A). Cell of *Δper9* carrying pHIPX4-PER9 (B) and cell of *Δper9* carrying pHIPX4-PAS3 (C) showing numerous peroxisomes. Cell of *Δper9* carrying pHIPX5-PAS3 containing relatively few peroxisomes, which in part are enlarged in size (D). Abbreviations: M, mitochondrion; N, nucleus; P, peroxisome. The bar represents 0.5 μm.

(pHIPX4-PAS3, pHIPX5-PAS3 and pHIPX6-PAS3). Plasmid pHIPX4 and plasmids carrying the *PER9* gene (pHIPX4-PER9, pHIPX5-PER9 and pHIPX6-PER9) were used as controls. Growth experiments, summerized in Table 1, showed that all constructs carrying *H. polymorpha PER9*, as well as pHIPX4-PAS3 and pHIPX6-PAS3 fully complemented the methanol-utilizing deficient (Mut^-) phenotype of the *H. polymorpha Δper9* mutant strain. However, pHIPX5-PAS3 transformants grew only slowly on methanol/ammoniumsulphate-containing media. Even after 48 h these cells did not reach the final density obtained with the other transformants. pHIPX4-transformants, used as control, were fully impaired to grow on methanol, confirming the Mut^- phenotype of the *Δper9* mutant [12].

The *S. cerevisiae* Pas3p and *H. polymorpha* Per9p protein levels in the various transformants of *H. polymorpha Δper9* were determined by Western blotting (Fig. 2). The data indicated that both Pas3p and Per9p were predominantly present in the pellet fractions which were separated from the crude cell homogenates by a short centrifugation step (not shown). The presence of Pas3p in these fractions suggests that this protein is either strongly bound to organellar membranes, as is the case for Per9p [12], or alternatively may be present in proteinaceous aggregates. Nevertheless, the relative amounts of both *S. cerevisiae* Pas3p and *H. polymorpha* Per9p observed in the pellet fractions corresponded to the expected levels of the proteins in that cells carrying a P_{AOX}-driven *PAS3* or *PER9* gene contained

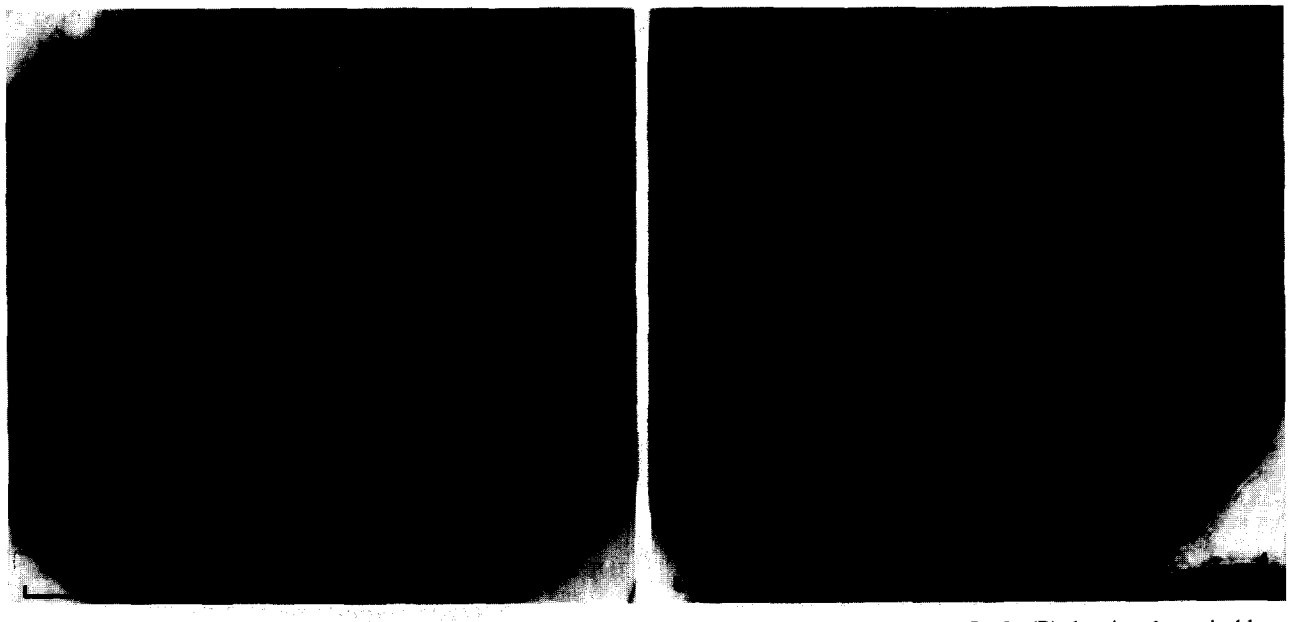

Fig. 4. Immunocytochemistry, using specific antibodies against *H. polymorpha* catalase (A) and *S. cerevisiae* Pas3p (B) showing the typical location of catalase at the edge of intact peroxisomes (A) and localization of *S. cerevisiae* Pas3p on the peroxisomal membrane (B) in methanol/ammoniumsulphate-grown *H. polymorpha Δper9* cells, carrying pHIPX4-PAS3. Abbreviations: M, mitochondrion, N, nucleus; P, peroxisome. The bar represents 0.5 μm.

the highest amounts of Pas3p or Per9p. In cells carrying a P_{AMO}-driven *PAS3* gene Pas3p was hardly detectable. However, even this low expression level allowed *Δper9* cells to grow on methanol, albeit slowly (Table 1).

The overall morphology of the various transformants of *Δper9* was studied by electron microscopy. Analyses of ultrathin sections of $KMnO_4$-fixed cells revealed that in *Δper9* [pHIPX4] no peroxisomes were discernible, as expected (Fig. 3 panel A). However, *Δper9* cells synthesizing either *S. cerevisiae* Pas3p or *H. polymorpha* Per9p clearly contained normal intact peroxisomes (Fig. 3, panels B–D). On average, cells containing a P_{AOX}-driven *PAS3* or *PER9* gene contained increased numbers of peroxisomes, compared to *Δper9* cells containing a P_{AMO}-driven *PAS3* or *PER9* gene (compare in Fig. 3, panels B–D). This is in line with the finding that Per9p plays a role in peroxisome multiplication [12].

Immunocytochemistry confirmed that in *H. polymorpha Δper9* cells complemented with *S. cerevisiae* Pas3p the peroxisomal matrix enzymes alcohol oxidase, dihydroxyacetone synthase and catalase were normally incorporated in peroxisomes (Fig. 4, panel A, only shown for catalase).

Using antibodies against *S. cerevisiae* Pas3p, the subcellular location of this protein in *H. polymorpha Δper9* carrying pHIPX4-PAS3 was determined by immunocytochemistry. Using this method, labelling appeared to be confined to the peroxisomal membrane (Fig. 4, panel B), indicating that Pas3p was located in this membrane. Proteinaceous aggregates containing Pas3p were never observed. This location of *S. cerevisiae* Pas3p in *H. polymorpha* is in line with the location of the protein in *S. cerevisiae*, in which Pas3p was characterized as an integral peroxisomal membrane protein [13]. Similarly, the *H. polymorpha PER9* gene product was recently identified in the peroxisomal membrane [12].

4. Concluding remarks

Summarizing, our data led us to conclude that in *H. polymorpha* the heterologous *S. cerevisiae* Pas3p is not only normally synthesized and incorporated in the peroxisomal membrane, but is also able to functionally complement for the Per9p function. A partial complementation of the *Δper9* mutant by the *PAS3* gene, resulting in import of only a portion of the key enzymes of methanol metabolism – alcohol oxidase, dihydroxyacetone synthase and catalase – into the peroxisomal matrix, would undoubtedly lead to cells exhibiting a Mut^- phenotype. As shown before, the presence of even low quantities of alcohol oxidase activity in the cytosol prevents growth of the cells in liquid methanol-containing cultures [25,26], and is also the most likely explanation for the failure of the *Pichia pastoris* Pas8p, the putative homologue of *H. polymorpha* Per3p, to

Table 1
Growth properties of *Hansenula polymorpha Δper9* transformants in methanol

Plasmid	A_{663} after 24 h	A_{663} after 48 h
pHIPX4	0.2	0.3
pHIPX4-PAS3	3.1	2.8
pHIPX5-PAS3	1.3	2.4
pHIPX6-PAS3	3.0	3.0
pHIPX4-PER9	3.1	3.1
pHIPX5-PER9	3.4	3.1
pHIPX6-PER9	3.2	3.1

Transformed *H. polymorpha Δper9* cells were precultured on glucose/ammoniumsulphate-containing media (twice), subsequently on glycerol/ammoniumsulphate-containing media (twice), and finally diluted at an absorption at 663 nm (A_{663}) of 0.1 in methanol/ammoniumsulphate-containing media. Growth is expressed as A_{663} after incubation of cells for 24 and 48 h at 37°C.

functionally complement a *H. polymorpha per3* disruption mutant [8]. Partial complementation was observed in a *P. pastoris pas8* mutant synthesizing a fusion protein between *P. pastoris* Pas8p and its human homologue, Pxrlp. The transformed cells contained peroxisomes, and could grow on oleate, which also requires functional peroxisomes. Surprisingly, methylotrophic growth was not restored [9]. The reason for this is not yet known.

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