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The *Hansenula polymorpha per6* mutant is affected in two adjacent genes which encode dihydroxyacetone kinase and a novel protein, Pak1p, involved in peroxisome integrity

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**Abstract**  The *Hansenula polymorpha per6-210* mutant is impaired in respect of growth on methanol (Mut−) and is characterized by aberrant peroxisome formation. The functionally complementing DNA fragment contains two open reading frames. The first encodes dihydroxyacetone kinase (DAK), a cytosolic enzyme essential for formaldehyde assimilation; the second ORF codes for a novel protein (Pak1p). We have demonstrated that *per6-210* cells lack DAK activity, causing the Mut− phenotype, and have strongly reduced levels of Pak1p, resulting in peroxisomal defects. Sequence analysis revealed that *per6-210* contains a mutation in the 3′ end of the *DAK* coding region, which overlaps with the promoter region of *PAK1*. Possibly this mutation also negatively affects *PAK1* expression.

**Key words** Peroxisome biogenesis · Methanol metabolism · Yeast

**Introduction**

Peroxisomes are essential for growth of methylotrophic yeasts on methanol as the sole source of carbon and energy. These organelles harbour the key enzymes of methanol metabolism: alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT). Other enzymes of the dissimilatory (formaldehyde and formate dehydrogenase) and assimilatory (the xylulose-5-phosphate) pathways are located in the cytosol (reviewed by Veenhuis and Harder 1988).

In order to identify the genes essential for the growth of methylotrophic yeasts on methanol, various methanol-utilization-deficient (Mut−) mutants of the yeast *Hansenula polymorpha* have been isolated (De Koning et al. 1987; Cregg et al. 1990). Within these collections different classes of Mut− strains have been discriminated, namely: (1) strains mutated in structural genes encoding peroxisomal or cytosolic enzymes involved in methanol metabolism (Verduyn et al. 1984; de Koning et al. 1987), and (2) *pex* mutants, which are defective in peroxisome biogenesis and/or function (Cregg et al. 1990). In these mutants the Mut− phenotype is due to the mislocation of enzymatically active peroxisomal enzymes to the cytosol (van der Klei et al. 1991).

Here, we describe the *H. polymorpha per6-210* mutant, which has been identified within a previously described collection of *H. polymorpha* Mut− mutants (Titorenko et al. 1993). We found that this mutant is deficient in an enzyme required for methanol metabolism, but is also disturbed in peroxisome formation. We show that a single mutation in *per6-210* affects the protein products of two adjacent genes. The first encodes the enzyme dihydroxyacetone kinase (DAK), a cytosolic enzyme essential for the assimilation of formaldehyde produced from methanol oxidation. The second ORF, designated *PAK1*, encodes a novel protein. The peroxisomal defect in *per6-210* is most probably due to strongly reduced levels of Pak1p.

**Materials and methods**

Organisms and growth conditions. *H. polymorpha* wild-type (CBS 4732), the *per6-210* mutant (Titorenko et al. 1993), NCYC 495 *leu1* (Waterham et al. 1994), NCYC 495 *leu1 ura1* (Waterham et al. 1994), and the constructed disruption strains (see below) were grown
Materials and methods section). Only relevant restriction sites are indicated.

**Biochemical methods.** Crude extracts were prepared as described (Waterham et al. 1994). For the generation of protoplasts whole cells were pre-incubated in a solution containing 100 mM Tris pH 8.0,
50 mM EDTA, 140 mM β-mercaptoethanol and 1.2 M sorbitol for 15 min at 37°C. The cells were harvested by centrifugation, washed once in a 50 mM potassium phosphate buffer pH 7.2, containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mg ml⁻¹ of zymolyase 20 T for 15 min at 37°C. The cells were harvested by centrifugation, washed once in a 50 mM potassium phosphate buffer pH 7.2, containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mglm l⁻¹ of zymolyase 20 T at 37°C for 30–120 min. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation, washed in 5 mM MES pH 5.5 containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mglm l⁻¹ of zymolyase 20 T at 37°C for 30–120 min. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation, washed in 5 mM MES pH 5.5 containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mglm l⁻¹ of zymolyase 20 T at 37°C for 30–120 min. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation, washed in 5 mM MES pH 5.5 containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mglm l⁻¹ of zymolyase 20 T at 37°C for 30–120 min. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation, washed in 5 mM MES pH 5.5 containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mglm l⁻¹ of zymolyase 20 T at 37°C for 30–120 min. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation, washed in 5 mM MES pH 5.5 containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mglm l⁻¹ of zymolyase 20 T at 37°C for 30–120 min. All subsequent steps were performed at 4°C. 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tion by the mechanisms detailed before for the turnover of redundant intact peroxisomes in wild-type *H. polymorpha* (Veenhuis et al. 1983; data not shown, compare Fig. 7). Alternatively, AO crystalloids, lacking a surrounding membrane, remained in the cytosol where they often fragmented (data not shown; compare Fig. 7).

Cloning of *per6-210*-complementing DNA

In order to clone the complementing DNA fragment, mutant *per6-210* was transformed with a genomic *H. polymorpha* library. Among approximately 10 000 transformants obtained, four grew well on methanol-containing agar plates (Mut+ phenotype). These clones appeared to carry overlapping genomic inserts. One clone was selected which contained plasmid DNA with a genomic insert of 5.2 kb. The nucleotide sequence of the complementing portion of this fragment was determined and was deposited at GenBank (accession number AF061946). A BLASTN database search revealed that this fragment corresponds to a region in a large *H. polymorpha* DL1 genomic DNA fragment which was not further characterized, but was cloned by functional complementation of a dihydroxyacetone kinase (DAK)-deficient mutant (Tikhomirova et al. 1988; EMBL accession number X58862).

Analysis of the *per6-210*-complementing sequence revealed two ORFs (Fig. 1). ORF1 encodes a protein of 609 amino acids with a predicted mass of 65 kDa. The protein product was highly similar (34% identity) to the translation product of the *Citrobacter freundii dhaK* gene, encoding the enzyme dihydroxyacetone kinase (DAK; Daniel et al. 1995). In addition two *Saccharomyces cerevisiae* ORFs were found to display strong similarity to ORF1 (51% and 46% identity; SwissProt accession number P43550 and PIR accession number S48327). An alignment of these sequences is given in Fig. 2.

The second ORF encodes a protein of 592 amino acids with a calculated molecular weight of 66.1 kDa (Fig. 3). It showed sequence homology (32% identity) over the entire

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length with the Neurospora crassa THI4 gene, which is involved in thiamine biosynthesis (Akiyama and Nakashima 1996). Also the putative protein products of three S. cerevisiae ORFs [YOL055 C (PIR S66740), YPR121 W (PIR S69014) and YPL258 C (PIR S65289)] are homologous over their entire length (31–37% identity). Their functions are, however, as yet unknown. The N-terminal half of the ORF2 protein product showed similarity to a bacterial protein involved in thiamine biosynthesis (33% identity), namely Salmonella typhimurium 4 amino-5-hydroxymethyl-2-methyl pyrimidine phosphate kinase (HMPP-kinase, Swiss Prot P55882; Petersen and Downs 1997). In addition, the C-terminal domain of the ORF2 protein was similar to Bacillus subtilis tenA, a putative regulatory component of protein secretion (20% identity; Swiss Prot P25052; Pang et al. 1991).

A hydrophobicity plot of the gene product of ORF2 predicts one possible membrane-spanning region (amino acids 262–292). At the N-terminus a degenerate form of the N-terminal peroxisomal targeting signal PTS2 (RV-X5-QL) is present. This sequence is identical to the putative PTS2 of Trypanosoma brucei glycosomal aldolase (Clayton 1985). We designated ORF2 as PAK1 and its translation product as Pak1p.

ORF1 encodes the cytosolic enzyme dihydroxyacetone kinase

To test whether ORF1 indeed represented the H. polymorpha DAK gene, as suggested by sequence analysis, a strain was constructed in which ORF1 was disrupted (see Fig. 1). Cells of this strain grew normally on mineral media containing glucose, ethanol or glycerol, but were unable to grow on methanol. In addition, cells of this strain were unable to grow on dihydroxyacetone, a compound which requires DAK activity for its metabolism (de Koning et al. 1987). Control cultures of wild-type (WT) or a peroxisome-deficient mutant (Δpex8; Waterham et al. 1994) grew well on DHA. The absence of DAK activity in this strain was confirmed biochemically by enzyme activity measurements which revealed that in crude extracts, prepared from cells of the ORF1 disruption strain grown on glucose or glycerol or incubated for 24 h in methanol-containing media, DAK activity was invariably absent. Based on these findings we conclude that ORF1 encodes the H. polymorpha DAK enzyme, and designated it as DAK.

The morphology of peroxisomes in cells of the strain in which ORF1 was disrupted (Δdak) was similar to those of normal intact peroxisomes present in WT cells (data not shown). Immunocytochemistry revealed that peroxisomal matrix proteins were, as in WT controls, confined to the organelar matrix (data not shown). Peroxisome disassem-
bly or increased organelar turnover, typical for per6-210
cells, was never observed in
Ddak
cells. The subcellular location of DAK was determined by
cell-fractionation experiments (Fig. 4). Sucrose-density
centrifugation of a post-nuclear supernatant obtained from
homogenates of methanol-grown WT
H. polymorpha
resulted in a clear separation of peroxisomes (marker pro-
tein AO, fraction 6), mitochondria (marker protein cyto-
chrome c oxidase, fraction 16) and soluble proteins (frac-
tions 23–25). As shown in Fig. 4, DAK activity was con-
fined to the upper part of the gradient, which indicates that
it is a cytosolic protein.

Characterization of a
P AK1
disruption mutant

In order to test whether the peroxisomal aberrations ob-
served in per6-210 cells were related to a deficiency in the
protein product of the second ORF (PAKI), a PAK1 disrup-
tion mutant was constructed (Fig. 1). Physiological stud-
ies revealed that the resulting strain (∆pak1) grew normally
on methanol and peroxisomes were formed in methanol-
grown ∆pak1 cells. Occasionally, peroxisomal abnormal-
ities like those observed in per6-210 cells were encoun-
tered, especially in cells from batch cultures in the late ex-
ponential and stationary growth phase. Under these condi-
tions immunocytochemical experiments revealed that the
peroxisomal matrix proteins were predominantly peroxi-
somal but were also present in the cytosol (Fig. 5A. AO;
DHAS not shown). In WT control cells labelling was in-
variably confined to the peroxisomal matrix when the same
antisera were used (data not shown; see Douma et al. 1985).

Pak1p is a low abundant, methanol-inducible protein

In order to obtain insight into the function of the PAK1 gene
product, the levels of Pak1p were determined in cells
grown under various conditions. For this purpose, Pak1p
antibodies were raised using a Pak1p-MBP fusion protein
synthesized in
E. coli.
With these antibodies, a single pro-
tein band of approximately 68 kDa was observed in West-
ern blots prepared from crude extracts of methanol-grown
H. polymorpha
WT cells (Fig. 6A). The apparent molecu-
lar weight of the protein was in good agreement with the
calculated mass of the protein (66.1 kDa). The intensity of
the band was very low and only detectable using chemilu-
minescence techniques. In extracts prepared from ∆pak1
cells the 68 kDa-band was invariably absent. To increase
the Pak1p protein level, a strain was constructed which
contained an additional copy of PAK1 under the control
of the strong AO promoter. In Western blots of crude extracts
prepared from methanol-induced cells of this strain, the in-
tensity of the 68-kDa band had significantly increased. We
concluded from these data that the 68-kDa band repre-
sented Pak1p and that the antiserum specifically recog-
nized Pak1p, but no other
H. polymorpha
proteins.

The induction of Pak1p was subsequently analyzed in
crude extracts of variously grown H. polymorpha WT cells
by Western blotting using α-Pak1p antibodies. The results,
presented in Fig. 6B, indicate that synthesis of Pak1p is en-
hanced in methanol-grown cells compared to glucose-
grown cells. In cells grown on glucose in the presence of
thiamine, Pak1p was not detected, but a faint band became

Fig. 5 A shows the characteristic labelling pattern after immuno-
cytochemical experiments on methanol-induced ∆pak1 cells, using
α-AO antibodies. The bulk of the labelling is localized on the per-
oxisomes, while a minor labelling is detectable in the cytosol. Using
methanol-grown WT cells (B) and α-Pak1p antibodies, labelling
is localized on the peroxisomal membrane. In identical experiments
on WT::P
AOX
::PAK1
peroxisomal labelling is increased,
while labelling is also observed in the cytosol. Aldehyde fixation.
Abbreviations: M mitochondrion, N nucleus, P peroxisome. The
marker represents 0.5 μm
visible when crude extracts were used prepared from cells grown on glucose under thiamine limitation. An increase in Pak1p levels due to thiamine limitation was more evident when cells grown on methanol under conditions of thiamine excess were compared with cells grown on methanol under thiamine-limitation conditions (Fig. 6B).

Subcellular location of Pak1p

Immunocytochemical experiments on ultrathin sections of WT H. polymorpha cells, using α-Pak1p antibodies, resulted in a specific labelling of the peroxisomal membrane. However, the labelling intensities were very low (generally 2–3 gold particles/organelle; Fig. 5B), in agreement with the very low levels of Pak1p in WT cells. The ultrastructural data, which suggested that Pak1p is associated with the peroxisomal membrane, could not be confirmed biochemically using cell-fractionation experiments. In sucrose gradients, prepared from a post-nuclear supernatant of methanol-grown WT cells, a very minor portion of Pak1p co-sedimented with the peroxisomes (fraction 6) while the bulk of the protein was found on top of the gradient (Fig. 4). Upon extraction of an enriched peroxisomal fraction (30 000 g pellet) by high salt, all sedimentable Pak1p became solubilized. This suggests that the organellar Pak1p is loosely associated with the outer surface of the organellar membrane (Fig. 6C). After over-production of Pak1p, α-Pak1p-specific labelling was predominantly localized at the peroxisomal membrane, but was also present in the cytosol (Fig. 5C). The latter results should however be interpreted with care, because over-production of Pak1p may alter its subcellular location. Taken together these data suggest that Pak1p may have a dual location and is present both at the peroxisomal membrane and in the cytosol.

Per6-210 is affected in both DAK and Pak1p

The phenotypes of the single Δdak and Δapk1 mutants differed from that of the original per6-210 mutant. Analysis of the original mutant per6-210 revealed that this strain is unable to grow on DHA and lacked DAK activity, indicating that the DAK gene is inactivated. In addition, methanol-induced per6-210 cells contain strongly reduced Pak1p levels (Fig. 6D), which suggests that expression of PAK1 may be disturbed as well. Additional evidence that per6-210 is defective in both gene products came from
studies on a constructed double mutant in which major parts of both the DAK and PAK1 genes were deleted (Δdak Δpak1; Fig. 1). As expected, Δdak Δpak1 cells lacked DAK activity and were unable to grow on methanol or DHA. Also Pak1p was absent in these cells (Fig. 6D). Ultrastructural analysis revealed that the peroxisomal morphology in methanol-induced cells of the double mutant was similar to that of the original per6-210 mutant (Fig. 7).

A single mutation in per6-210 may affect both DAK activity and PAK1 expression.

The smallest DNA fragment still able to functionally complement the per6-210 mutation is a 3.2-kb PvuII fragment (nt 1357–4422) containing the 3' half of ORF1 (encoding the C-terminal 310 amino acids of DAK) and the complete ORF2 (see Fig. 1). Most likely, functional complementation was due to integration/recombination events. Sequence analysis of a cloned 1.1-kb fragment which included the 3' half of DAK and the 5' end of PAK1, revealed that in per6-210 a G-to-A transition had occurred in nucleotide 1658. This mutation resulted in the substitution of the conserved glycine 401 of DAK into glutamic acid (Fig. 2). This mutation is present only 670 bp upstream of the start codon of PAK1 and hence could affect the regulatory elements of the PAK1 promoter.

Taken together, these data support the view that possibly both genes may be inactivated in per6-210 due to a single mutation.
Physiological studies

To gain further insight in the role of DAK and Pak1p in methanol metabolism, physiological studies were carried out using glucose-limited chemostat cultures of Δpak1, Δdak or Δdak Δpak1 cells. In WT *H. polymorpha* the addition of methanol as a second carbon source to glucose-limited chemostat cultures results in an increase in yield due to the simultaneous utilization of glucose and methanol under these conditions (van der Klei et al. 1991). In experiments, in which 0.2% methanol was added to a glucose-limited chemostat culture of the Δdak Δpak1 double mutant the yield of the culture increased only slightly (0.4 D_{660} units). In similar experiments, carried out with the single mutant Δpak1, the increase in biomass was considerably higher (1.9 D_{660} units) and comparable to the values observed in cultures of WT cells (van der Klei et al. 1991). In case of the Δdak mutant an intermediate increase was observed (0.8 D_{660} units). Hence, the low increase observed in Δdak Δpak1 cultures most likely reflects the combined defects of both DAK and Pak1p. In vivo 31P NMR studies on methanol-induced cells demonstrated that the reduction in yields of Δdak Δpak1 cultures were not due to specific energetic disadvantages caused by damaged or non-functional peroxisomes, as have been observed in various *pex* strains (van der Klei et al. 1991). In both cultures normal ATP levels were observed and the inorganic phosphate peaks, indicating the acidic nature of the peroxisomal lumen (pH approximately 5.8–6.0), were normally found (data not shown, see Nicolay et al. 1987). These results therefore confirm the morphological data which indicated that in Δdak Δpak1 cells at least a substantial portion of the peroxisomal population is intact and physiologically active.

An alternative explanation could be that, due to the absence of DAK activity, the cells start to produce dihydroxyacetone (DHA). Indeed, after the addition of methanol up to 12 mM, DHA was detectable in the growth medium. Hence, part of the formaldehyde produced from methanol oxidation is assimilated by DHAS, resulting in the formation of DHA in methanol cultures of these strains.

The effects of the *pak1* disruption on the efficiency of methanol metabolism and peroxisome integrity was particularly prominent in a Δdak Δpak1 double mutant during the growth of cells in a chemostat on glucose/methanol mixtures. Essentially, the phenotype of methanol-induced *per6-210* cells (Mut’, peroxisomal defects, no DAK activity) was akin to the constructed Δdak Δpak1 cells and thus most likely represents the combined effects of the disruption of either of the DAK or the *Pak1* genes alone. This view is supported by the observed reduced Pak1p levels in *per6-210* cells which might be explained by a point mutation in the coding region of DAK, which supposedly overlaps with the promoter region of *Pak1*.

In contrast to DAK, the function of Pak1p is less clear. The sequence analyses revealed that the N-terminal half of Pak1p and the protein product of *N. crassa* *THH4*, essential for thiamine biosynthesis in this organism, are homologous to *Salmonella typhimurium* hydroxymethyl pyrimidine kinase, also involved in thiamine biosynthesis. Thiamine is the precursor of thiamine pyrophosphate (TPP), which is the co-factor of α-keto acid decarboxylase, α-keto acid oxidase and several transketolases (Begley 1996), including *H. polymorpha* DHAS (Bystryk et al. 1990), which may explain the increased level of Pak1p in methanol-grown *H. polymorpha* cells compared to glucose-
grown cells. Also, PAK1 expression is partially repressed by thiamine, a phenomenon which has been reported for several, but not all, genes involved in thiamine biosynthesis (Begley 1996).

Although the above findings suggest a role of Pak1p in thiamine biosynthesis, this function is not immediately clear in the case of Hansenula polymorpha because this organism is not able to synthesize thiamine, but is dependent on the addition of this vitamin to the cultivation medium for growth. A clue to the function of Pak1p may be deduced from studies on thiamine biosynthesis in Saccharomyces cerevisiae and S. pombe. This view is based on the fact that the subcellular locations of the known yeast enzymes involved in thiamine biosynthesis are not yet known. However, TPP-dependent enzymes are present in various cell compartments (e.g. mitochondria, peroxisomes). This therefore implies that TPP (or precursor forms) are either transported across intracellular membranes or else synthesized in different subcellular compartments. Interestingly, in S. cerevisiae three Pak1p homologues have been found which may be located at different sites in the cell. If so, it can be envisaged that, due to transport barriers, defects in the synthesis at one site can not be (fully) restored by thiamine added to the growth medium or synthesized in another organelle.

This may serve as an explanation as to why in Hansenula polymorpha a defect in a gene required for thiamine biosynthesis still shows a phenotype even when thiamine is supplemented in the growth medium. Decreased TPP levels in PAK1 disruption strains may explain the mislocation of a portion of the peroxisomal enzymes in the cytosol. The assumption that limitation of a co-factor of a peroxisomal enzyme may affect its subcellular location is not without precedent. Studies on a riboflavin (Rf)-auxotrophic mutant of Hansenula polymorpha showed that the limitation of Rf (and thus FAD, the AO co-factor) resulted in the mislocation of a portion of the AO protein and other peroxisomal matrix enzymes in the cytosol (Evers et al. 1994, 1996). A similar phenomenon may explain the observed cytosolic portion of peroxisomal enzymes in a Pak1p-deficient environment, namely an imbalance in the amount of newly synthesized DHAS protein and its co-factor TPP. Taken these data together, we propose that Pak1p plays a role in thiamine biosynthesis and is possibly involved in the generation of adequate amounts of the co-factor TPP for the peroxisomal enzyme DHAS.

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