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# The *Pichia pastoris* Dihydroxyacetone Kinase is a PTS1-containing, but Cytosolic, Protein that is Essential for Growth on Methanol

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Dihydroxyacetone kinase (DAK) is essential for methanol assimilation in methylotrophic yeasts. We have cloned the *DAK* gene from *Pichia pastoris* by functional complementation of a mutant that was unable to grow on methanol. An open reading frame of 1824 bp was identified that encodes a 65·3 kDa protein with high homology to DAK from *Saccharomyces cerevisiae*. Although DAK from *P. pastoris* contained a C-terminal tripeptide, TKL, which we showed can act as a peroxisomal targeting signal when fused to the green fluorescent protein, the enzyme was primarily cytosolic. The TKL tripeptide was not required for the biochemical function of DAK because a deletion construct lacking the DNA encoding this tripeptide was able to complement the *P. pastoris dak*Δ mutant. Peroxisomes, which are essential for growth of *P. pastoris* on methanol, were present in the *dak*Δ mutant and the import of peroxisomal proteins was not disturbed. The *dak*Δ mutant grew at normal rates on glycerol and oleate media. However, unlike the wild-type cells, the *dak*Δ mutant was unable to grow on methanol as the sole carbon source but was able to grow on dihydroxyacetone at a much slower rate. The metabolic pathway explaining the reduced growth rate of the *dak*Δ mutant on dihydroxyacetone is discussed. The nucleotide sequence reported in this paper has been submitted to GenBank with Accession Number AF019198. © 1998 John Wiley & Sons, Ltd.

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KEY WORDS — *Pichia pastoris*; methylotrophic yeasts; dihydroxyacetone kinase; DNA sequencing

## INTRODUCTION

Methylotrophic yeasts are widely used as organisms for the production of recombinant heterologous proteins (Gellisen *et al*., 1995; Faber *et al*., 1995; Cregg *et al*., 1993). As unicellular eukaryotic organisms they combine the advantages of simplicity of molecular genetic manipulation with the ability to introduce post-translational eukaryotic modifications into protein products or foreign proteins (Cregg *et al*., 1993). The use of strong and tightly regulated methanol-inducible promoters allows for controlled induction of recombinant gene expression and the generation of high yields of foreign proteins. For *Pichia pastoris* and *Hansenula polymorpha (P. angusta)*, the most commonly used yeasts for heterologous gene expression, yields of the recombinant proteins of up to 30% of the total cellular protein have been achieved (Gellisen *et al*., 1995; Faber *et al*., 1995; Cregg *et al*., 1993).

Methylotrophic yeasts are capable of utilizing methanol as the sole carbon and energy source. In order to grow on methanol, these yeasts have to generate energy from methanol oxidation and to assemble multicarbon molecules from C1 units. The key enzymes for methanol metabolism,

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The abbreviations used are: DHA, dihydroxyacetone; DAK, dihydroxyacetone kinase; PCR, polymerase chain reaction; GFP, green fluorescent protein; Pi, inorganic phosphate; PNS, post-nuclear supernatant; PTS, peroxisomal targeting signal.
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alcohol oxidase, catalase and dihydroxyacetone synthase, are localized in peroxisomes (Fukui *et al.*, 1975; Douma *et al.*, 1985; Goodman, 1985). The subsequent reactions in methanol assimilation and dissimilation are localized in the cytosol (Veenhuis and Harder, 1987).

Methanol metabolism starts with the FAD-dependent oxidation of methanol to formaldehyde and hydrogen peroxide, a reaction catalysed by alcohol oxidase (methanol oxidase). Dissimilation of formaldehyde proceeds, in the cytoplasm, to formate and further to $CO_2$, and is catalysed by formaldehyde dehydrogenase and by formate dehydrogenase, respectively, resulting in the generation of two molecules of NADH (Veenhuis and Harder, 1987).

Assimilation of formaldehyde occurs via the xylulose monophosphate pathway, which catalyses the net conversion of three molecules of formaldehyde and three of ATP into dihydroxyacetone phosphate, three ADPs and two inorganic phosphates (van Dijken *et al.*, 1978). The first enzyme for formaldehyde assimilation, dihydroxyacetone synthase, is localized in the peroxisomal compartment and catalyses a transketolase reaction between xylulose-5-phosphate and formaldehyde to generate dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate. DHA is phosphorylated by dihydroxyacetone kinase (DAK) in the cytosol and the resulting dihydroxyacetone phosphate reacts with glyceraldehyde-3-phosphate to form fructose-1,6-bisphosphate, which is converted to fructose-6-phosphate by the actions of fructose-1,6-bisphosphate aldolase and fructose-1,6-bisphosphatase, respectively. Two molecules of fructose-6-phosphate and one molecule of dihydroxyacetone phosphate are rearranged by a series of reactions to generate three molecules of xylulose-5-phosphate.

In order to understand fully the pathways of methanol metabolism in methylotrophic yeasts, the genes encoding the enzymes involved need to be cloned and the proteins have to be characterized. Two alcohol oxidase genes (*AOX1* and *AOX2*) have been cloned and characterized from *P. pastoris* (Ellis *et al.*, 1985; Cregg *et al.*, 1989). In *H. polymorpha*, besides a single alcohol oxidase gene (*MOX1*), the genes for dihydroxyacetone synthase (*DAS*) and catalase (*CAT*) have been cloned (Ledeboer *et al.*, 1985; Janowicz *et al.*, 1985; Didion and Roggenkamp, 1992). Dihydroxyacetone kinase has been purified from various methylotrophic yeasts and characterized enzymatically (Hoffmann and Babel, 1980, 1981; Kato *et al.*, 1988; Bystrykh *et al.*, 1990) but its subcellular localization has not been firmly established. Isolation of a clone that complements a DAK-deficient strain of *H. polymorpha* has been reported (Tikhomirova *et al.*, 1988), but the full-length sequence has not been published.

In this study we report the cloning, sequencing and disruption of the *DAK* gene from the methylotrophic yeast *P. pastoris* as well as the characterization of the *dak*Δ mutant and the analysis of the subcellular localization of PpDAK. This is the first detailed characterization of a eukaryotic DAK.

## MATERIALS AND METHODS

### *Strains and growth conditions*

The yeast strains used in this study are summarized in Table 1. Strain STW211 is the original DAK-deficient mutant strain (Wenzel *et al.*, in preparation). Strain SGL1 is the *dak*Δ null mutant and was generated by integration of the knockout targeting construct (see below) into the *DAK* locus of strain PPY12. Strains SGL2 to SGL6 and SGL7 to SGL10 were generated by integration of the indicated *Stu*I-linearized, *HIS4*-based, expression constructs (see plasmids) into the *his4* loci of strains SGL1, PPY4 or PPY12.

Yeast strains were grown at 30°C either in YPD (1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 2% (w/v) dextrose), YPM (1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 0·5% (v/v) methanol), YPOT (1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 0·2% (v/v) oleate, 0·02% (v/v) Tween40) or in synthetic medium consisting of 0·67% (w/v) yeast nitrogen base, supplemented with 50 µg/ml of the appropriate amino acids and with the following carbon sources: 2% (w/v) dextrose (SD), 0·5% (v/v) methanol (SM), 0·25% (v/v) dihydroxyacetone (SDHA) or 1% (v/v) glycerol (SG). Bacto agar (2% w/v) was added for solid media.

### *Recombinant DNA techniques*

*Escherichia coli* strain JM109 (Invitrogen, Carlsbad, CA) was used in all cloning procedures involving plasmid propagation. Polymerase chain reactions (PCR), plasmid isolation, restriction analysis, cloning techniques, transformation of *E. coli* and isolation of genomic DNA were performed according to standard protocols

 

Table 1. *Pichia pastoris* strains

| Strain | Genotype (expression construct) |
|---|---|
| PPY4 | *his4* |
| PPY12 | *his4, arg4* |
| STW211 | *his4, arg4, dak* |
| SGL1 | *his4, arg4, dak*Δ :: *ARG4* (*dak* null) |
| SGL2 | *arg4, dak*Δ :: *ARG4, his4* :: pTW74 ($P_{GAP}$GFP-PTS1, HIS4) |
| SGL3 | *arg4, dak*Δ:: *ARG4, his4* :: pTW66 ($P_{GAP}$PTS2-GFP, HIS4) |
| SGL4 | *arg4, dak*Δ :: *ARG4, his4* :: pGHL63 ($P_{DAK}$DAK, HIS4) |
| SGL5 | *arg4, dak*Δ :: *ARG4, his4* :: pGHL64 ($P_{DAK}$DAKΔTKL, HIS4) |
| SGL6 | *arg4, dak*Δ :: *ARG4, his4* :: pGHL44 ($P_{GAP}$GFP-DAK, HIS4) |
| SGL7 | *his4* :: pTW74 ($P_{GAP}$GFP-PTS1, HIS4) |
| SGL8 | *his4* :: pTW66 ($P_{GAP}$PTS2-GFP, HIS4) |
| SGL9 | *his4* :: pGHL44 ($P_{GAP}$GFP-DAK, HIS4) |
| SGL10 | *arg4, his4* :: pGHL69 ($P_{GAP}$GFP-TKL, HIS4) |

Strains PPY4 and PPY12 have been described earlier (Gould *et al.*, 1992). All other strains are generated and described in this study.

(Sambrook *et al.*, 1989). *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used for all PCR reactions. Yeast cells were transformed by electroporation (Becker and Guarente, 1991). The ECL direct nucleic acid labelling and detection system (Amersham, Bucks, UK) was used for labelling of the probes and visualization of the labelled probes after hybridization on Southern blots. The probes used were a 1·1 kb genomic *Bgl*II fragment consisting of a 5′ untranslated region including the first 34 nucleotides of the *DAK* open reading frame and the entire *DAK* open reading frame amplified by PCR. DNA sequencing was performed on an Applied Biosystems/Perkin Elmer 373A automated DNA sequencer according to the manufacturer's specifications using 500 ng DNA and 3·2 pmoles of primer per sequencing reaction.

### *Cloning of PpDAK and generation of the dakΔ mutant*

Mutant strain STW211 was isolated after chemical mutagenesis of strain PPY12 (T. Wenzel *et al.*, in preparation) because of its inability to grow on methanol as the sole carbon source. Strain STW211 was transformed with a genomic *P. pastoris* library based on vector pYM8 described earlier (Liu *et al.*, 1995) and transformants were selected for restoration of growth on SM medium. Three plasmids (pRAJ1,2,3) were isolated from those transformants and physical maps, made by restriction analysis, revealed overlapping inserts in all three plasmids. The *PpDAK* open reading frame was identified by sequencing of pRAJ2 in both directions. A 3·9 kb genomic fragment containing the entire *PpDAK* was generated by partial *Bam*HI digest of the complementing plasmid, pRAJ3, and cloned into pBluescript leading to plasmid pGHL41. A *Sty*I fragment of pGHL41 was replaced by a 2·1 kb *Hin*dIII/*Pst*I fragment (Figure 2) containing the *PpARG4* gene resulting in pGHL42. (The *Sty*I, *Hin*dIII and *Pst*I sites were filled in using Klenow DNA polymerase.) Strain PPY12 was transformed with a 4·8 kb *Cla*I/partial *Bam*HI fragment from pGHL42 containing the *PpARG4* and *PpDAK* flanking regions. Transformants were selected for arginine prototrophy and clones that were unable to grow on methanol were analysed by Southern blotting to confirm targeted integration of the *PpARG4* into the *PpDAK* locus (Figure 2). The resulting *dak*Δ mutant was named SGL1.

### *Plasmids*

Construction of plasmid pTW66 has been described previously (Wiemer *et al.*, 1996). Plasmid pTW74 was cloned by replacing the peroxisomal targeting signal (PTS)2–green fluorescent protein (GFP) fragment of pTW66 with a *Bgl*II/*Eco*R1 fragment containing a GFP–PTS1 fusion from pTW51 (Wiemer *et al.*, 1996), thus placing the GFP–PTS1 under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter ($P_{GAP}$, Waterham *et al.*, 1997). To generate a GFP–DAK fusion construct, the *PpDAK* open

 

reading frame without the start codon was amplified by PCR from pRAJ2 using oligonucleotides GLO73 and GLO74. The PCR product was cloned in-frame, downstream of the GFP, into plasmid pTW114 which contains the GFP without its stop codon under control of the GAP promoter resulting in plasmid pGHL44. The sequence of the in-frame fusion and of the C-terminus was confirmed by sequence analysis.

The full-length *DAK* with 360 bp of its own promoter region ($P_{DAK}$) and a truncated *DAK* lacking the last three amino acids were amplified using oligos GLO51/GLO74 and GLO51/GLO75, respectively, with pRAJ2 as the template. PCR fragments were cloned into the *Nde*I/*Eco*RI-digested and blunted pHIL-D2 (Invitrogen, Carlsbad, CA) resulting in pGHL63 for the full-length DAK and pGHL64 for the truncated DAK (DAKΔTKL) respectively. The correct C-terminus was confirmed for both plasmids (pGHL63 and pGHL64) by sequence analysis. A fusion of GFP with the tripeptide TKL (GFP–TKL) was generated by PCR using oligos GLO93 and GLO94 with pTW74 as the template. The PCR product was cloned as a *Bam*HI/*Eco*RI fragment into *Bgl*II/*Eco*RI-digested pTW66 (Wiemer *et al.*, 1996), leading to a construct (pGHL69) expressing the GFP–TKL fusion under control of the GAP promoter. The oligonucleotides used in this study were:

GLO51-GGGCCATGCAACAATAGGC;
GLO73-CCATATGTCTAGTAAACATTGG;
GLO74-TATACCGGTCTACAACTTGGTTTC AGATTTG;
GLO75-TATACCGGTCATTCAGATTTGAAG TATGC;
GLO93-GAATTCTACAACTTGGTTTTGTAT AGTTCATCCATG;
GLO94-GGATCCATGAGTAAAGGAGAACT TTTC.

*Preparation of crude yeast lysates*

Yeast cells were washed with sterile ice-cold water and harvested by centrifugation. The cell pellets were resuspended in an equal volume of 50 mM-Tris–HCl, pH 7 with 0·2 mM-PMSF; 5 μg/ml leupeptin and 5 μg/ml aprotinin as protease inhibitors. 1 ml of the suspension was mixed with 500 μl of acid-washed glass beads and vortexed for 5 min. Cell debris was pelleted for 20 s at maximal speed in an Eppendorf centrifuge and the clear supernatant representing the crude cell lysate was used for biochemical analysis.

*Subcellular fractionations*

Strain PPY12 was used for cell fractionation experiments. Cells were cultured in 500 ml YPD medium to near saturation, pelleted, resuspended in 2 l of SM medium and incubated for 16 h. Preparation of spheroplasts and cell homogenates, as well as the subsequent differential centrifugation were performed essentially as described previously (Monosov *et al.*, 1996). The postnuclear supernatant (PNS) was subjected to two sequential centrifugations at 27,000 *g* and at 100,000 *g* resulting in two pellet fractions 27P and 100P, respectively, as well as the cytosolic fraction (100S).

*Analytical methods*

DAK activity was analysed essentially according to Kato *et al.* (1988). Glass bead lysates and subfractions from cells induced on SM medium were dialysed for 2 × 1 h against 50 mM-Tris–HCl, pH 7 containing 0·001% (v/v) Triton X100 before enzyme activity was measured. The reaction mixture for the enzyme assay contained 0·1 mM-DHA, 0·15 mM-NADH, 5 mM-ATP, 5 mM-$MgCl_2$, 0·001% (v/v) Triton X100 and 50 mM-Tris–HCl, pH 7. In control reactions without sample, ATP or DHA, no activity could be detected. Protein content was analysed essentially according to Bradford (1976) using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

*Fluorescence microscopy*

Living cells were applied onto poly-L lysine-coated coverslips and embedded in 14% (w/v) Mowiol 4–88 (Calbiochem, La Jolla, CA) and 28% (v/v) glycerol to achieve sufficient immobilization. Samples with living cells were analysed immediately after embedding using a Nikon microphot-SA microscope and a Plan 100/125 oil DIC objective. GFP fluorescence was detected using the FITC channel.

## RESULTS

*Cloning, sequencing and disruption of the P. pastoris dihydroxyacetone kinase (PpDAK)*

The *P. pastoris* mutant strain STW211 was unable to grow on methanol as the sole carbon source. The methanol-utilization defect was complemented by crossing mutant STW211 with alcohol oxidase-, formaldehyde dehydrogenase-, formate dehydrogenase- and catalase- deficient

 

mutants, indicating that none of these genes involved in methanol metabolism was defective in mutant STW211. The mutant strain was transformed with a genomic *P. pastoris* library in pYM8 (Liu *et al.*, 1995) and three plasmids, including pRAJ2, with overlapping inserts were isolated from transformants that were able to grow on methanol. The insert of pRAJ2 was sequenced and an open reading frame of 1824 bp encoding a protein of 608 amino acids with a calculated molecular weight of 65·3 kDa and an estimated pI of 5·2 was identified (GenBank accession number AF019198). The amino acid sequence of PpDAK was homologous (33% identity) to the known bacterial DAK from *Citrobacter freundii* (Figure 1). Sequence comparison revealed also high homology (Figure 1) with two putative dihydroxyacetone kinases from *S. cerevisiae* (about 50% and 45% identity with YFL053w and SC9745 2, respectively). The PpDAK and one of the putative kinases from *S. cerevisiae* (encoded by SC9745 2) contained a C-terminal tripeptide (TKL and SAL, respectively) with the consensus sequence for a peroxisomal targeting signal (PTS1).

The *PpDAK* gene was disrupted by integrating the *PpARG4* into the *PpDAK* locus (Figure 2A). The resulting *dak*Δ mutant (strain SGL1) should only produce a truncated protein resulting in an enzymatically inactive peptide of 64 amino acids of the DAK. Targeted integration of the *PpARG4* into the *PpDAK* locus was confirmed by Southern blot analysis (Figure 2B).

### *The dakΔ mutant strain SGL1 was unable to grow on methanol and lacked DAK activity*

The *dak*Δ mutant strain SGL1 grew on glucose but was unable to grow on methanol as the sole carbon source (Figure 3). Strain SGL1 was able to grow on oleic acid and glycerol like the wild-type strain PPY4 (not shown), and on dihydroxyacetone but at a much slower rate than the wild type. In batch cultures containing SDHA medium, the generation times at mid-log phase were 4 h and 14·5 h for the wild-type strain PPY4 and the knockout strain SGL1, respectively. The generation times were identical for both strains grown on SD medium (Figure 4).

Wild-type and mutant strains were analysed for DAK enzyme activity as described in Materials and Methods. No DAK activity could be detected in the null mutant strain (Table 2). Furthermore, using the *PpDAK* open reading frame as probe, no homologous isoform could be detected by Southern blot analysis of genomic *P. pastoris* DNA (not shown).

### *The dakΔ mutant has peroxisomes and import of PTS1- and PTS2-containing peroxisomal proteins is unaffected*

Since functional peroxisomes are essential for growth of *P. pastoris* on methanol as the sole carbon source, the null mutant was analysed for the presence of peroxisomes. Wild-type strains SGL7 and SGL8 and *dak*Δ mutant strains SGL2 and SGL3, producing GFP–PTS1 or PTS2–GFP under control of the GAP promoter respectively, were grown on SD, SM, YPOT and SDHA medium; cells were analysed by fluorescence microscopy for the localization of those reporter molecules (Figure 5). After induction of wild-type strain SGL7 on SM medium, GFP–PTS1 was localized into large clustered peroxisomes (Figure 5A) as expected. Although the null mutant strain SGL2 was unable to grow on methanol, GFP–PTS1 was clearly imported into large peroxisomes after induction on methanol (Figure 5B). PTS2–GFP was imported into peroxisomes of wild-type strain SGL8 and null mutant strain SGL3 upon growth on oleic acid (data not shown). During growth on glucose, peroxisomes were not induced, but GFP–PTS1 was imported into a small peroxisome which was present in every cell (Figure 5C). Upon growth of cells on DHA, GFP–PTS1 was located in small peroxisomes (Figure 5E) of similar size to those observed after growth of the wild-type and the null mutant cells on glucose (not shown). Cells with several (two to four) of these small peroxisomes were observed more frequently after growth on DHA rather than on glucose.

### *PpDAK is a cytosolic protein*

PpDAK has a C-terminal TKL tripeptide which has been shown to function as a PTS in *S. cerevisiae* (Elgersma *et al.*, 1996). We have analysed the significance of this tripeptide for the biochemical and physiological function of PpDAK. The full-length DAK, as well as a truncated version lacking the C-terminal tripeptide (DAKΔTKL), both expressed under the endogenous promoter, were integrated into the *his4* locus of the null mutant leading to strains SGL4 and SGL5, respectively. Strains SGL4 and SGL5 are both complemented for the growth defect of

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PpDHAK          MSSKHWDYKKDLVLSHLAGLCQSNP--HVRLIESE---RVVISAENQED-----KITLIS
YFL053w         MSHKQFKSDGNIVTPYLLGLARSNP--GLTVIKHD---RVVFRTASAPNSGNPPKVSLVS
SC9745_2        MSAKSFEVT-DPVNSSLKGFALANP--SITLVPEE---KILFRKTDSDK------IALIS
CfDHAK          MSQFFFNQRTHLVSDVIDGAIIASPWNNLARLESDPAIRIVVRRDLNKN-----NVAVIS
                **   :.   . *   : *   :.*   :  :  :   :::.      .      ::::*

PpDHAK          GGGSGHEPLHAGFVTKDGLLDAAVAGFIFASPSTKQIFSAIKAK-PSKKGTLIIVKNYTG
YFL053w         GGGSGHEPTHAGFVG-EGALDAIAAGAIFASPSTKQIYSAIKAV-ESPKGTLIIVKNYTG
SC9745_2        GGGSGHEPTHAGFIG-KGMLSGAVVGEIFASPSTKQILNAIRLVNENASGVLLIVKNYTG
CfDHAK          GGGSGHEPAHVGFIG-KGMLTAAVCGDVFASPSVDAVLTAIQAV-TGEAGCLLIVKNYTG
                ******** *.**:  .* * . . * :*****.. : .**:    .  * *:*******

PpDHAK          DILHFGLAAEKAKAEGLNAELLIVQDDVSVGKAKNGLVGRRGLAGTSLVHKILGAKAYLQ
YFL053w         DIIHFGLAAERAKAAGMKVELVAVGDDVSVGKKKGSLVGRRGLGATVLVHKIAGAAASHG
SC9745_2        DVLHFGLSAERARALGINCRVAVIGDDVAVGREKGGMVGRRALAGTVLVHKIVGAFAEEY
CfDHAK          DRLNFGLAAEKARRLGYNVEMLIVGDDISLPDNK----HPRGIAGTILVHKIAGYFAERG
                * ::***:**:*:  * : .:  : **:::   *      *.:.* ***** *  *

PpDHAK          KDNLELHQLVTFGEKVVANLVTIGASLDHVTIPARANKQEEDDSDDEHGYEVLKHDEFEI
YFL053w         ---LELAEVAEVAQSVVDNSVTIAASLDHCTVPG--HKPE---A-------ILGENEYEI
SC9745_2        SSKYGLDGTAKVAKIINDNLVTIGSSLDHCKVPG--RKFES----------ELNEKQMEL
CfDHAK          ---YNLATVLREAQYAASNTFSLGVALSSCHLPQETDAAP-----------RHHPGHAEL
                     *      .:    * .::. :*.   :*                       . *:

PpDHAK          GMGIHNEPGIKKSSPIPTVDELVAE-LLEYLLSTTDKDRNYVQFDKNDEVVLLINNLGGT
YFL053w         GMGIHNESGTYKSSPLPSISELVSQ-MLPLLLDE-DEDRSYVKFEPKEDVVLMVNNMGGM
SC9745_2        GMGIHNEPGVKVLDPIPSTEDLISKYMLPKLLDPNDKDRAFVKFDEDDEVVLLVNNLGGV
CfDHAK          GMGIHGEPGASVID-TQNSAQVVNL-MVDKLLAA---------LPETGRLAVMINNLGGV
                *****.*.*    .   .  :::   ::  **          :     :.:::**:**

PpDHAK          SVLELYAIQ-NIVVDQLASKYSIKPVRIFTGTFTTSLDGPGFSITLLNATKTGDKDILKF
YFL053w         SNLELGYAA-EVISEQLIDKYQIVPKRTITGAFITALNGPGFGITLMNASKAGG-DILKY
SC9745_2        SNFVISSIT-SKTTDFLKENYNITPVQTIAGTLMTSFNGNGFSITLLNATKATKALQSDF
CfDHAK          SVAEMAIITRELASSPLHSRIDWLIG---PASLVTALDMKGFSLTAIVLEESIE--KALL
                *   :     .   . * .. .       ..:: *:::  **.:* :   ::

PpDHAK          LDHKTSAPGWNSNIS---DWSGRVDNFIVAAPEIDEG-----DSSSKVSVDAKLYADLLE
YFL053w         FDYPTTASGWNQMYHSAKDWEVLAKGQVPTAPSLKTLRN---EKGSGVKADYDTFAKILL
SC9745_2        EEIKSVLDLLNAFTN-APGWPIADFEKTSAPSVNDDLLHNEVTAKAVGTYDFDKFAEWMK
CfDHAK          TEVETS------------NWPTPVPPR-----EITCVV----SSHASARVEFQPSANALV
                 :  :             .*                          :    : .  *. :

PpDHAK          SGVKKVISK-----EPKITLYDTVAGDGDCGETLANGSNAILKALAEGKLDLKDGVKSLV
YFL053w         AGIAKINEV-----EPKVTWYDTIAGDGDCGTTLVSGGEALEEAIKNHTLRLEDAALGIE
SC9745_2        SGAEQVIKS-----EPHITELDNQVGDGDCGYTLVAGVKGITENLD--KLSKDSLSQAVA
CfDHAK          AGIVELVTATLSDLETHLNALDAKVGDGDTGSTFAAAAREIASLLHRQQLPLNNLATLFA
                :*  ::        *.::.  *  .**** * *:. . . : . :    *  :.    .

PpDHAK          QITDIVETAMGGTSGGLYSIFISALAKSLKEKELSEGAYTLTLETISGSLQAALQSLFKY
YFL053w         DIAYMVEDSMGGTSGGLYSIYLSALAQGVRD----SGDKELTAETFKKASNVALDALYKY
SC9745_2        QISDFIEGSMGGTSGGLYSILLSGFSHGLIQVCK-SKDEPVTKEIVAKSLGIALDTLYKY
CfDHAK          LIGERLTVVMGGSSGVLMSIFFTAAGQKLEQ-----GAN------VVEALNTGLAQMKFY
                 *   :   ***:** * ** ::. .: : :              .  :   .*  :  *

PpDHAK          TRARTGDRTLIDALEPFVKEFAKS-KDLKLANKAAHDGAEAT-RKLEAKFGRASYVAEEE
YFL053w         TRARPGYRTLIDALQPFVEALKAG-KGPRAAAQAAYDGAEKT-RKMDALVGRASYVAKEE
SC9745_2        TKARKGSSTMIDALEPFVKEFTAS-KDFNKAVKAAEEGAKST-ATFEAKFGRASYVGDSS
CfDHAK          GGADEGDRTMIDALQPALTSLLAQPKNLQAAFDAAQAGAERTCLSSKANAGRASYLSSES
                  *  *  *:****:* :  :    *. . * .**  **: *  . .*  *****:....

PpDHAK          FKQFESEGGLPDPGAIGLAALISGITDAYFKSETKL
YFL053w         LRKLDSEGGLPDPGAVGLAALLDGFVTAAGY-----
SC9745_2        --QVE------DPGAVGLCEFLKGVQSAL-------
CfDHAK          --LLGN----MDPGAQRLAMVFKALAESELG-----
                   .       ****  *. .:...  :
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the null mutant on methanol (Figure 3). The specific DAK activity in lysates of methanol-induced cells of both strains was identical and interestingly about twice as high as in the wild-type strain (Table 2). Thus, the C-terminal tripeptide of the wild-type protein is not essential for its enzymatic activity nor its physiological function.

The subcellular localization of PpDAK was analysed by expressing a fusion protein of GFP and DAK under control of the GAP promoter in the null mutant (strain SGL6) and the wild type (strain SGL9). This fusion protein expressed in strain SGL6 was enzymatically active (Table 2) and complemented the null mutant for the growth defect on methanol (Figure 3).

Cells of strain SGL9 were grown on SD or SM medium and analysed by fluorescence microscopy. In strain SGL9 grown on SM medium (Figure 5F) and on SD medium (not shown) the fluorescent label was clearly cytosolic and no punctate staining could be observed. The C-terminal tripeptide TKL therefore does not function as a PTS1 in the context of the DAK protein in *P. pastoris*.

A fusion protein of GFP and the tripeptide TKL (GFP-TKL) expressed in the wild-type strain SGL10 was targeted to large punctate structures after induction of cells on SM medium (Figure 5D). Since these structures are inducible upon shift of the SGL10 strain from glucose to methanol medium, we conclude that they are peroxisomes. These results show that the tripeptide TKL can function as a PTS1 in *P. pastoris*. Such context dependence of the ability of PTS1 to function in peroxisomal targeting has been documented earlier (Gould *et al.*, 1989).

The biochemical activity of the wild-type PpDAK was further localized in subcellular fractions after differential centrifugation. A PNS from wild-type cells (PPY12) grown on SM medium was fractionated into two organelle pellets (27,000 *g* and 100,000 *g*) and a cytosolic fraction (100,000 *g* supernatant) as described in Materials and Methods. About 95% of the total DAK activity present in the PNS was localized in the cytosolic subfraction (Table 3). With only about 54% of the total protein, the highest specific DAK activity was in the cytosolic fraction, confirming the cytosolic location of the PpDAK.

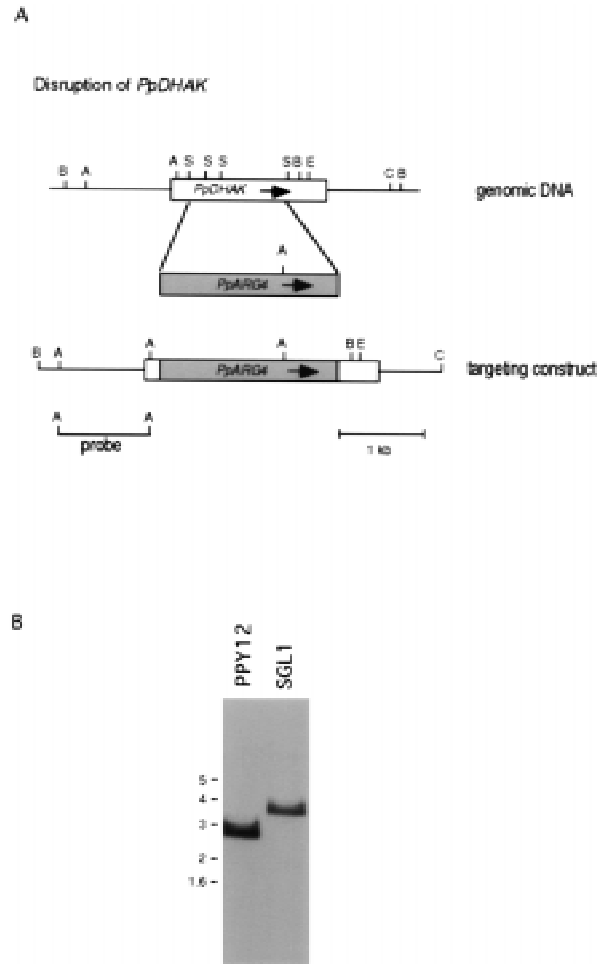


Figure 2. Disruption of the *PpDAK* gene. (A) Schematic representation of the *dak* disruption construct. The white bar represents the *DAK* open reading frame and the grey bar the *PpARG4* gene used to disrupt the *PpDAK*. Arrows indicate the direction of transcription. Restriction sites: A, *Bgl*II; B, *Bam*HI; C, *Cla*I; E, *Eco*RV; S, *Sty*I. (B) Southern blot of genomic DNA from wild-type (PPY12) and the *dak*Δ mutant (SGL1) strains after digestion with *Bam*HI. A 1·1 kb *Bgl*II fragment indicated in (A) was used as the probe. Targeted integration of the *PpARG4* into the *PpDAK* locus results in an increase of the *Bam*HI fragment from 2·7 kb (PPY12) to 3·5 kb (SGL1).

Figure 1. Sequence comparison of PpDAK with two putative dihydroxyacetone kinases from *S. cerevisiae* (YFL053w and SC9745 2) and with the bacterial DAK from *C. freundii*. The amino acid sequences were aligned using the 'Clustal W 1.7' program with the gap opening penalty of 10·0 and the gap extension penalty of 0·05. Identical residues are indicated by an asterisk. Two dots represent highly conserved residues whereas weakly conserved residues are indicated by a single dot. Note the distinct C-terminus with a TKL motif for the PpDAK.

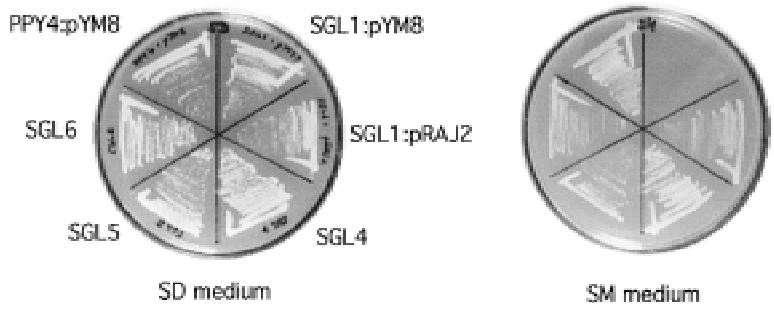


Figure 3. Growth characteristics of the wild-type and *dak*Δ mutant strains on solid SD and SM medium. Wild-type strain PPY4 and the null mutant strain SGL1 are carrying the pYM8 plasmid for histidine auxotrophy. Strains SGL4, 5 and 6 have different DAK expression constructs integrated into the *his4* locus of the null mutant (see Materials and Methods). Note that strain SGL1 is unable to grow on methanol and that plasmid pRAJ2 is able to rescue its methanol growth defect.

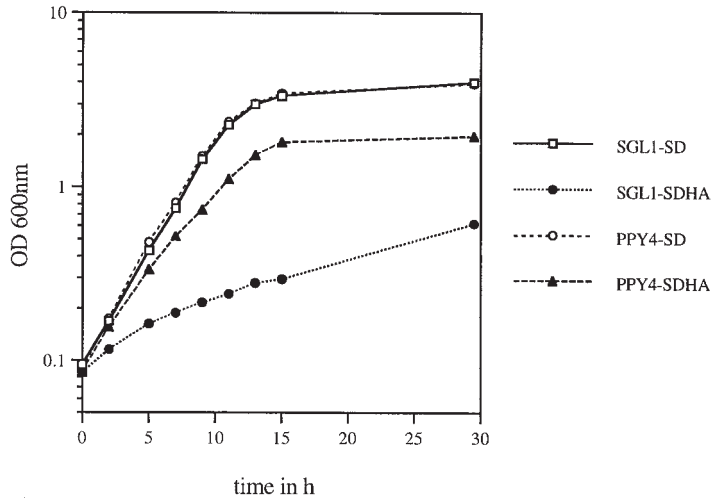


Figure 4. Growth characteristics of the wild-type (PPY4) and the *dak*Δ null mutant (SGL1) strains on liquid SD and SDHA medium. Cells growing in the indicated medium were diluted at time 0 to an $OD_{600nm}$ of about 0·1 and changes in optical density were followed over time.

Table 2.

| Strain | PPY4 | SGL1 | SGL4 | SGL5 | SGL6 |
|---|---|---|---|---|---|
| Enzyme activity | 1·79 | 0 | 3·79 | 3·79 | 2·20 |

Specific DAK activity (μmol/min per mg protein) in lysates after induction of indicated strains on SM medium.

## DISCUSSION

The PpDAK is essential for growth of *P. pastoris* on methanol as the sole carbon source. We have cloned and sequenced the *PpDAK* and analysed the *dak*Δ mutant strain SGL1 which is unable to grow on methanol and lacks DAK activity. Reintroduction of the *PpDAK* gene into the null mutant restores enzyme activity and the ability to grow on methanol. The growth defect of the null mutant on methanol emphasizes the essential role of DAK in the assimilation of methanol via the xylulose monophosphate cycle (van Dijken *et al.*, 1978) in this methylotrophic yeast. In glucose- and glycerol-containing medium, both wild-type and null mutant strains grow at identical rates. The null mutant is also able to grow on DHA as sole carbon source but at a much

 

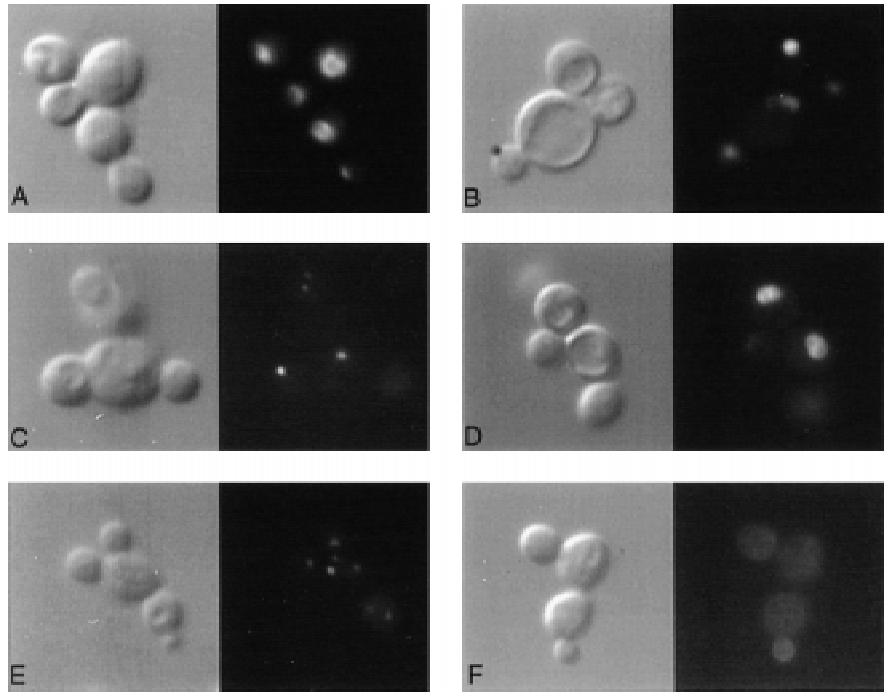


Figure 5. Subcellular localization of GFP fusion proteins in the wild-type and the *dak*Δ mutant. Panels show the corresponding Nomarski (left) and fluorescence images (right). All strains were induced on the indicated carbon source for 16 h. (A, C, E) Wild-type strain SGL7 expressing GFP-PTS1 after growth on SM, SD and SDHA medium, respectively. (B) *dak*Δ mutant strain SGL2 expressing GFP-PTS1 after induction on SM medium. (D, F) Wild-type strains SGL10 and SGL9 expressing GFP–TKL and GFP–DAK, respectively, after growth on SM medium.

Table 3. Subcellular distribution of DAK activity and protein content.

| | Protein (%) | DAK (%) | DAK (U/mg) |
|---|---|---|---|
| PNS | 100 | 100 | 0·387 |
| 27P | 21·7 | 2 | 0·039 |
| 100P | 23·9 | 2·8 | 0·034 |
| 100S | 54·4 | 95·2 | 0·509 |

Wild-type (PPY12) cells were grown on YPM medium and subcellular fractions were obtained as described in Materials and Methods. Note that 95% of the DAK activity is in the cytosolic fraction (100S) whereas only 54% of the protein content is localized in this fraction.

slower rate than the wild-type strain (Figure 4). This is not due to the presence of an isoform of DAK because no homologous gene could be identified by Southern blot analysis and no enzymatic activity was present in the *dak*Δ mutant strain, implicating an alternative but less efficient pathway for DHA metabolism.

A similar phenotype has been observed for a *H. polymorpha* mutant (17B) lacking DAK activity (de Koning *et al.*, 1987a). Using a double mutant lacking DAK and dihydroxyacetone synthase activities, it was shown (de Koning *et al.*, 1987a) that the growth rate of the *Hpdak*-deficient strain 17B on DHA was not due to the reverse dihydroxyacetone synthase reaction. The slow growth rate was abolished in double mutants lacking DAK activity and either glycerol kinase or glycerol-3-phosphate dehydrogenase activity. The slow growth rate of the *dak*-deficient strain on DHA was therefore attributed to the phosphorylative pathway of glycerol metabolism after NADH-dependent reduction of DHA to glycerol by DHA reductase (de Koning *et al.*, 1987b). The biochemical activities of enzymes required for the phosphorylative pathway of glycerol metabolism have been shown to be present in *P. pastoris* (de Koning *et al.*, 1987b).

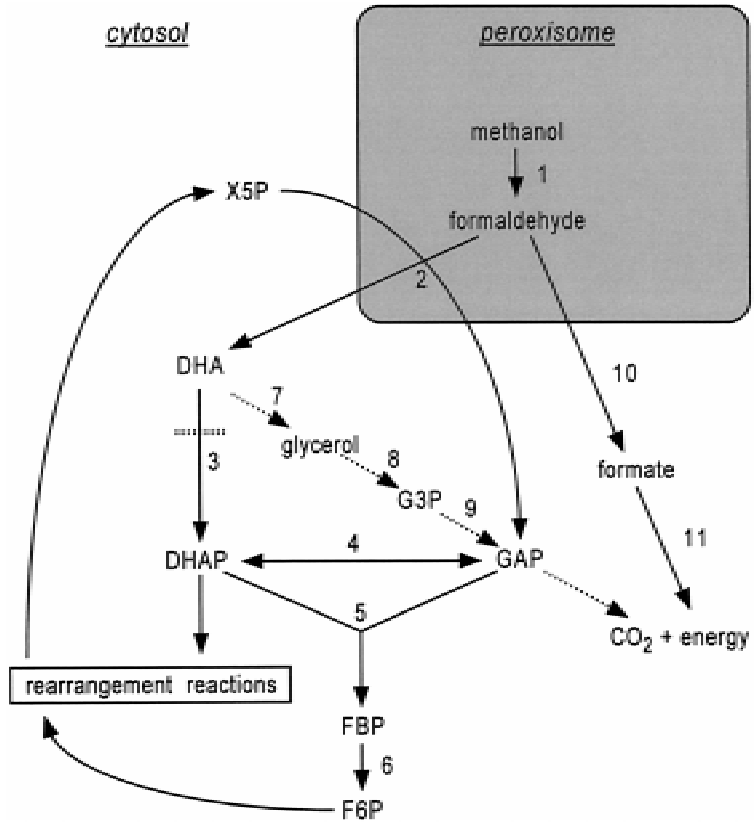


Figure 6. Schematic representation of the pathways of methanol and DHA metabolism in *P. pastoris*. The solid arrows indicate the pathways of methanol and DHA metabolism in the wild type. The dotted arrows indicate the alternative but less efficient pathway of DHA metabolism in the *dak*Δ mutant. 1, Alcohol oxidase; 2, dihydroxyacetone synthase; 3, dihydroxyacetone kinase; 4, triosephosphate isomerase; 5, fructose-1,6-bisphosphate aldolase; 6, fructose-1,6-bisphosphatase; 7, dihydroxyacetone reductase; 8, glycerol kinase; 9, glycerol-3-phosphate dehydrogenase; 10, formaldehyde dehydrogenase; 11, formate dehydrogenase; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; X5P, xylulose-5-phosphate; G3P, glycerol-3-phosphate; GAP, glyceraldehyde-3-phosphate.

Based on the observation that DAK activity was partly inhibited by the presence of glycerol in the reaction mixture when cells were grown on glycerol but not after cells were grown on methanol, it was suggested that glycerol kinase also catalyses the conversion of DHA to DHA phosphate in *P. pastoris* (de Koning *et al.*, 1987b). We analysed the PpDAK activity in lysates of wild-type cells grown in SM, SG and SDHA medium in the presence and absence of 5 mM-glycerol in the reaction mixture. Glycerol did not affect the DAK activity in our enzyme assays (not shown). Furthermore no DAK activity could be detected in lysates from *dak*Δ mutant cells grown on glycerol. We therefore propose that, according to the model in Figure 6, in the *dak*Δ mutant DHA is reduced to glycerol before entering the phosphorylative pathway of glycerol metabolism. Because the *dak*Δ mutant (SGL1) and the wild-type strains (PPY4) both grew at the same rate on glycerol and glucose media, the slower growth rate of the null mutant on DHA medium is likely to be due to the rate-limiting reduction of DHA to glycerol by DHA reductase. The affinity of the DHA reductase for DHA has indeed been reported to be much lower than that of DAK for DHA (Bystrykh *et al.*, 1990).

 

The inability of the *dak*Δ mutant to grow on methanol is probably due to insufficient regeneration of the C1-acceptor molecule xylulose-5-phosphate. For the *H. polymorpha* mutant (17B) lacking DAK activity it has been shown that, after growth on methanol and xylose, which is metabolized via xylulose-5-phosphate, in spite of the DHA accumulation, the xylose became rate limiting with increasing methanol concentrations in the culture (de Koning *et al*., 1990).

PpDAK has a C-terminal tripeptide TKL with the consensus sequence (Elgersma *et al*., 1996; Gould *et al*., 1989) for a peroxisomal targeting signal (PTS1). Because alcohol oxidase and dihydroxyacetone synthase, the enzymes catalysing the two preceding reactions in methanol assimilation, are both located in peroxisomes, we analysed the subcellular localization of PpDAK and the function of its C-terminal tripeptide. We fused the tripeptide TKL to GFP which has previously been shown to be a reliable reporter molecule with respect to peroxisomal protein import (Monosov *et al*., 1996). The GFP–TKL is, like GFP–SKL, imported into peroxisomes and TKL is thus sufficient as a targeting signal in *P. pastoris*. PpDAK, however, is a cytosolic protein. Biochemical analysis shows that DAK activity is localized in the cytosolic subfraction, and a GFP–DAK fusion protein that is able to complement the methanol growth defect of the null mutant is localized in the cytosol (Table 3 and Figure 5). Furthermore, deletion of the last three amino acids does not affect the biochemical activity of the DAK nor its ability to functionally complement the null mutant. The C-terminal PTS1 has to specifically interact with the PTS1 receptor (PpPEX5p) (McCollum *et al*., 1993; Terlecky *et al*., 1995) prior to import into the peroxisomes. We assume that PpDAK is folded to a conformation that makes its C-terminus inaccessible to the PTS1 receptor.

There are two putative DAKs (encoded by the genes SC9745 2 and YFL053w) in *S. cerevisiae*, which are 46% identical to each other (Norbeck and Blomberg, 1997). Both are approximately 37% identical (56% similar) to the bacterial *C. freundii* DAK which has been purified and characterized (Daniel *et al*., 1995). The *P. pastoris* DAK is homologous to these three proteins, as shown in Figure 1. Neither the subcellular locations nor the precise metabolic functions of the *S. cerevisiae* DAKs are known at present. One of the two *S. cerevisiae* DAK homologs (SC9745 2) ends in the sequence SAL, which is known to function as a PTS in *S. cerevisiae* when fused to a reporter protein (Elgersma *et al*., 1996). Since *S. cerevisiae* is not a methylotrophic yeast, these *S. cerevisiae* proteins, unlike the *P. pastoris* DAK, cannot be involved in methanol utilization. The *S. cerevisiae* DAK homolog encoded by SC9745 2 is induced by high salt and osmotic stress. This protein has therefore been proposed to be involved in the metabolism of the excess glycerol that is produced by cells to counteract the effects of growth on saline media. In this model, the excess glycerol produced is converted, by glycerol dehydrogenase, another salt-inducible protein encoded by the *GCY1* gene, to dihydroxyacetone, which is then converted to dihydroxyacetone phosphate by DAK (Norbeck and Blomberg, 1997). If this mechanism is correct, it is quite likely that, like the *P. pastoris* DAK that has been characterized in this study, the product of the SC9745 2 gene is cytosolic, even though it has a putative PTS1 at its C-terminus.

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## REFERENCES

Becker, D. M. and Guarente, L. (1991). High-efficiency transformation of yeast by electroporation. *Methods. Enzymol.* **194,** 182–187.

Bystrykh, L. V., de Koning, W. and Harder, W. (1990). Triokinase from *Candida bodinii* KD1. *Methods. Enzymol.* **188,** 445–451.

Cregg, J. A., Madden, K. R., Barringer, K. J., Thill, G. P. and Stillman, C. A. (1989). Functional characterization of two alcohol oxidase genes from the yeast *Pichia pastoris. Mol. Cell. Biol.* **9,** 1316–1323.

Cregg, J. M., Vedvick, T. S. and Raschke, W. C. (1993). Recent advances in the expression of foreign genes in *Pichia pastoris. Bio/Technology* **11,** 905–910.

Daniel, R., Stuertz, K. and Gottschalk, G. (1995). Biochemical and molecular characterization of the oxidative branch of glycerol utilization by *Citrobacter freundii. J. Bacteriol.* **177,** 4392–4401.

 

de Koning, W., Gleeson, M. A. G., Harder, W. and Dijkhuizen, L. (1987a). Regulation of methanol metabolism in the yeast *Hansenula polymorpha*. *Arch. Microbiol.* **147,** 375–382.

de Koning, W., Harder, W. and Dijkhuizen, L. (1987b). Glycerol metabolism in the methylotrophic yeast *Hansenula polymorpha*: phosphorylation as the initial step. *Arch. Microbiol.* **148,** 314–320.

de Koning, W., Weusthuis, R. A., Harder, W. and Dijkhuizen, L. (1990). Metabolic regulation in the yeast *Hansenula polymorpha*. Growth of dihydroxyacetone kinase/glycerol kinase-negative mutants on mixtures of methanol and xylose in continuous cultures. *Yeast* **6,** 107–115.

Didion, T. and Roggenkamp, R. (1992). Targeting signal of the peroxisomal catalase in the methylotrophic yeast *Hansenula polymorpha*. *FEBS Letters* **303,** 113–116.

Douma, A. C., Veenhuis, M., de Koning, W., Evers, M. and Harder, W. (1985). Dihydroxyacetone synthase is localized in the peroxisomal matrix of methanol-grown *Hansenula polymorpha*. *Arch. Microbiol.* **143,** 237–243.

Elgersma, Y., Vos, A., van den Berg, M., *et al*. (1996). Analysis of the carboxy-terminal peroxisomal targeting signal 1 in a homologous context in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271,** 26375–26382.

Ellis, S. B., Burst, P. F., Koutz, P. J., Waters, A. F., Harpold, M. M. and Gingeras, T. R. (1985). Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. *Mol. Cell. Biol.* **5,** 1111–1121.

Faber, K. N., Harder, W., Ab, G. and Veenhuis, M. (1995). Review: Methylotrophic yeasts as factories for the production of foreign proteins. *Yeast* **11,** 1331–1344.

Fukui, S., Kawamoto, S., Yasuhara, S. and Tanaka, A. (1975). Microbody of methanol-grown yeasts. *Eur. J. Biochem*. **59,** 561–566.

Gellissen, G., Hollenberg, C. P. and Janowicz, Z. A. (1995). Gene expression in methylotrophic yeasts. *Bioprocess. Technol.* **22,** 195–239.

Goodman, J. M. (1985). Dihydroxyacetone synthase is an abundant constituent of the methanol-induced peroxisome of *Candida bodinii*. *J. Biol. Chem*. **260,** 7108–7113.

Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J. and Subramani, S. (1989). A conserved tripeptide sorts proteins to peroxisomes. *J. Cell. Biol.* **108,** 1657–1664.

Hofmann, K. H. and Babel, W. (1980). Dihydroxyacetone kinase of methanol-assimilating yeasts I. Regulation of dihydroxyacetone kinase from *Candida methylica* in situ. *Zeitschrift für allgemeine Mikrobiologie* **20,** 389–398.

Hofmann, K. H. and Babel, W. (1981). Dihydroxyacetone kinase of methanol-assimilating yeasts II. Partial purification and some properties of dihydroxyacetone kinase from *Candida methylica*. *Zeitschrift für allgemeine Mikrobiologie* **21,** 219–224.

Janowicz, Z. A., Eckart, M., Drewke, C., *et al*. (1985). Cloning and characterization of the *DAS* gene encoding the major methanol assimilatory enzyme from the methylotrophic yeast *Hansenula polymorpha*. *Nucl. Acids Res.* **13,** 3063–3082.

Kato, N., Yoshikawa, H., Tanaka, K., Shimao, M. and Sakazawa, C. (1988). Dihydroxyacetone kinase from a methylotrophic yeast, *Hansenula polymorpha* CBS 4732. *Arch. Microbiol.* **150,** 155–159.

Ledeboer, A. M., Edens, L., Maat, J., *et al*. (1985). Molecular cloning and characterization of a gene coding for methanol oxidase in *Hansenula polymorpha*. *Nucl. Acids Res.* **13,** 3063–3082.

Liu, H., Tan, X., Russell, K. A., Veenhuis, M. and Cregg, J. M. (1995). *PER3*, a gene required for peroxisome biogenesis in *Pichia pastoris*, encodes a peroxisomal membrane protein involved in protein import. *J. Biol. Chem*. **270,** 10940–10951.

McCollum, D., Monosov, E. and Subramani, S. (1993). The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger Syndrome cells. The PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family. *J. Cell. Biol.* **121,** 761–774.

Monosov, E. Z., Wenzel, T. J., Lüers, G. H., Heyman, J. A. and Subramani, S. (1996). Labeling of peroxisomes with green fluorescent protein in living *P. pastoris* cells. *J. Histochem. Cytochem*. **44,** 581–589.

Norbeck, J. and Blomberg, A. (1997). Metabolic and regulatory changes associated with growth of *Saccharomyces cerevisiae* in 1·4 M-NaCl. *J. Biol. Chem*. **272,** 5544–5554.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Terlecky, S. T., Nuttley, W. M., McCollum, D., Sock, E. and Subramani, S. (1995). The *Pichia pastoris* peroxisomal protein PAS8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal. *EMBO J.* **14,** 3627–3634.

Tikhomirova, L. P., Ikonomova, E. N., Fodor, I. I., Bystrykh, L. V., Aminova, L. R. and Trotsenko, Y. A. (1988). Transformation of methylotrophic yeast *Hansenula polymorpha*: Cloning and expression of genes. *J. Basic Microbiol.* **28,** 343–351.

van Dijken, J. P., Beardsmore, A. J. and Quayle, J. R. (1978). Dihydroxyacetone: an intermediate in the assimilation of methanol by yeasts? *FEMS Letters* **4,** 97–102.

van Dijken, J. P., Veenhuis, M. and Harder, W. (1982). Peroxisomes of methanol-grown yeasts. In Kindl, H. and Lazarow, P. (Eds), *Peroxisomes and Glyoxysomes*. *Annals N. Y. Acad. Sci.*, New York.

 

Veenhuis, M. and Harder, W. (1987). Metabolic significance and the biogenesis of microbodies in yeasts. In Fahimi, H. D. and Sies, H. (Eds), *Peroxisomes in Biology and Medicine*. Springer-Verlag, New York, pp. 436–458.

Waterham, H. R., Digan, M. E., Koutz, P J., Lair, S. V. and Cregg, J. M. (1997). Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* **186,** 37–44.

Wiemer, E. A. C., Lüers, G. H., Faber, K. N., Wenzel, T., Veenhuis, M. and Subramani, S. (1996). Isolation and characterization of Pas2p, a peroxisomal membrane protein essential for peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris*. *J. Biol. Chem.* **271,** 18973–18980.