Positive Selection of Novel Peroxisome Biogenesis-Defective Mutants of the Yeast *Pichia pastoris*

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ABSTRACT

We have developed two novel schemes for the direct selection of peroxisome-biogenesis-defective (*pex*) mutants of the methylotrophic yeast *Pichia pastoris*. Both schemes take advantage of our observation that methanol-induced *pex* mutants contain little or no alcohol oxidase (AOX) activity. AOX is a peroxisomal matrix enzyme that catalyzes the first step in the methanol-utilization pathway. One scheme utilizes allyl alcohol, a compound that is not toxic to cells but is oxidized by AOX to acrolein, a compound that is toxic. Exposure of mutagenized populations of AOX-induced cells to allyl alcohol selectively kills AOX-containing cells. However, *pex* mutants without AOX are able to grow. The second scheme utilizes a *P. pastoris* strain that is defective in formaldehyde dehydrogenase (FLD), a methanol pathway enzyme required to metabolize formaldehyde, the product of AOX. AOX-induced cells of *fld1* strains are sensitive to methanol because of the accumulation of formaldehyde. However, *fld1 pex* mutants, with little active AOX, do not efficiently oxidize methanol to formaldehyde and therefore are not sensitive to methanol. Using these selections, new *pex* mutant alleles in previously identified *PEX* genes have been isolated along with mutants in three previously unidentified *PEX* groups.

PEROXISOMES are organelles found in virtually all eukaryotic cells and are characterized by the presence of catalase and at least one hydrogen peroxide-generating oxidase (Waterham and Cregg 1997). The organelles are enclosed by a single membrane and vary dramatically in size, abundance, and enzyme content depending upon the organism, cell type, and environmental conditions. In mammals, peroxisomes are involved in a variety of essential catabolic and anabolic pathways such as the oxidative degradation of long-chain fatty acids, purines, amino acids, and pipecolic acid as well as the biosynthesis of plasmalogens, cholesterol, and bile acid. As demonstrated by Zellweger syndrome and other lethal peroxisomal biogenesis disorders, peroxisomes are indispensable for human survival (Subramani 1997).

Because peroxisomes lack nucleic acids and ribosomes, all peroxisomal proteins must be nuclear encoded. Peroxisomal proteins are synthesized on free polysomes and post-translationally imported into the organelle via peroxisomal targeting signals (PTS) (Waterham and Cregg 1997). Two classes of matrix protein PTSs have been characterized. The first and most common, PTS1, is composed of a tripeptide sequence (SKL and conservative variants) present at the extreme carboxy terminus of many animal, plant, and yeast peroxisomal matrix proteins (de Hoop and AB 1992; Subramani 1993). The second, less common PTS, PTS2, has a consensus sequence of RLX_5H/QL and is located near the amino terminus of matrix proteins such as 3-ketoacyl-coenzyme A thiolase of mammals (Osumi *et al.* 1991; Rachubinski and Subramani 1995) and yeast (Glover *et al.* 1994). Some matrix proteins appear to have neither targeting signal, suggesting that other matrix protein PTSs have yet to be discovered. In addition to PTSs, specific components of the matrix protein import pathway have been elucidated, including a PTS1 receptor (Pex5p; McCollum *et al.* 1993), a PTS2 receptor (Pex7p; Rehling *et al.* 1996; Zhang and Lazarow 1996), and putative PTS receptor docking proteins (Pex13p and Pex14p; Elgersma *et al.* 1996; Erdmann and Blobel 1996; Gould *et al.* 1996; Albertini *et al.* 1997; Komori *et al.* 1997). Much less is known about the targeting signals of peroxisomal membrane proteins (PMPs). Evidence suggests that PMPs are targeted and inserted by a mechanism that is independent of that for matrix proteins. Recently, an internal hydrophilic 20-amino acid loop was demonstrated to be necessary and sufficient for targeting of a PMP from *Candida boidinii* (Dyer *et al.* 1996).

Certain yeast species have served as productive model

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systems for investigations of peroxisome biogenesis. In yeasts, peroxisome proliferation is markedly induced by growth on carbon sources such as oleic acid and methanol, making them a convenient source of the organelles and their enzymes for biochemical studies. Furthermore, our lab and others have shown that yeasts require peroxisomes only for metabolism of these carbon sources and not others (*e.g.*, glucose) and that yeast mutants with defects in peroxisome biogenesis (*pex* mutants) can be found among collections of strains that are specifically defective in growth on methanol and/or oleate (Erdmann *et al.* 1997; Waterham and Cregg 1997). These observations have resulted in the isolation of numerous *pex* mutants in more than a dozen *PEX* genes in each of four yeast species: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, and *Yarrowia lipolytica* (Erdmann *et al.* 1997; Waterham and Cregg 1997). The yeast *PEX* genes have been isolated and their products characterized to provide important insights into peroxisome biogenesis mechanisms (Waterham and Cregg 1997). Importantly, because the primary sequences of their products have been conserved through evolution, they have been used to identify their human *PEX* homologues in the databases, many of which have subsequently been shown to be genes affected in patients with peroxisome biogenesis disorders (Subramani 1997).

Among yeasts, *P. pastoris* is unique in that it is able to grow on either methanol or oleic acid. With *P. pastoris*, virtually all mutants specifically defective in growth on these two peroxisome-requiring substrates are *pex* mutants. Nevertheless, the need to screen ever larger collections of strains for mutants in new *PEX* genes is laborious and time consuming. More direct means to select for *P. pastoris pex* mutants have been needed. Here, we describe two highly efficient positive selection schemes for the isolation of *P. pastoris pex* mutants. Their utilization has resulted in the isolation of a large number of new alleles of previously identified *PEX* genes, novel mutants in two genes encoding potential transcription factors, and *pex* mutants in three novel complementation groups.

MATERIALS AND METHODS

Strains, media, and microbial techniques: *P. pastoris* strains used in this study are listed in Table 1. JC144 was constructed by integration of vector pHW011 into the alcohol oxidase 1 (*AOX1*) locus of GS200. Cultures (liquid and agar) were grown or induced at 30° in YPD medium [1% (wt/vol) yeast extract, 2% (wt/vol) peptone, 0.4% (wt/vol) glucose] or minimal media containing 0.17% (wt/vol) yeast nitrogen base without amino acids and with ammonium sulfate (Difco Laboratories Inc., Detroit), 0.5% (wt/vol) supplemented with one of the following: 0.5% (vol/vol) methanol (YNM medium); 0.1–0.4% (wt/vol) glucose (YND medium); 0.5% (vol/vol) ethanol (YNE medium); 0.2% (vol/vol) oleate plus 0.02% (vol/vol) Tween 40 and 0.05% (wt/vol) yeast extract (YNO medium for induction experiments); 0.1% (vol/vol) oleate and 0.05% Tween 40 (YNO medium for growth experiments); and 0.4% (wt/vol) glucose plus 0.5 mm allyl alcohol (AAD medium). Methanol-sorbitol medium was 12.0% (vol/vol) methanol, 0.2% (wt/vol) sorbitol, 0.2% (wt/vol) yeast extract, and 0.4% (wt/vol) peptone (MSY medium). Sporulation/mating medium was 0.5% (wt/vol) sodium acetate, 1% (wt/vol) potassium chloride, and 1% (wt/vol) glucose. Alcohol oxidase (AOX) activity assay medium was 50 mm Tris-HCl, pH 8.0, 0.1% (wt/vol) digitonin, 0.04% (wt/vol), 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS; Sigma, St. Louis), 0.02% (wt/vol) peroxidase, and 0.5% (vol/vol) methanol. For solid medium, agar was added to 2% (wt/vol). For growth of auxotrophic strains, requisite amino acids were added to a final concentration of 50 μg/ml. Cultivation of *Escherichia coli* strain DH5α and standard recombinant DNA techniques were performed essentially as described previously (Sambrook *et al.* 1989).

Plasmid constructions: pHW011, an *E. coli-P. pastoris* shuttle vector capable of expressing the *P. pastoris AOX1* gene under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter (P_{GAP}), was constructed as follows: (1) The *AOX1* gene was amplified from plasmid pPG5.4 (Cregg *et al.* 1989; Koutz *et al.* 1989) by the polymerase chain reaction (PCR) using a forward primer composed of the first 18 bases of the *AOX1* open reading frame preceded by an *Eco*RI site (5′-GAATTCATGGCTATCCCCGAAGAG-3′) and a 19-base reverse primer complementary to a region located 247–266 bases downstream of the *AOX1* stop codon, which includes a genomic *Hin*dIII site and *AOX1* terminator sequence (5′-AAGCTTGCACAAACGAACG-3′). (2) After digestion with *Eco*RI and *Hin*dIII, the *AOX1* gene fragment was subcloned in *Eco*RI-*Hin*dIII-digested pBS-SK$^-$ (Stratagene, La Jolla, CA) and subsequently as an *Eco*RI-*Cla*I fragment into *Eco*RI-*Cla*I-digested vector pHW010 (Waterham *et al.* 1997) to produce vector pHW011. Prior to electrotransformation into *P. pastoris* strain GS200, pHW011 was linearized at a *Bgl*II site in the *AOX1* gene.

pOPGP-1, an *E. coli-P. pastoris* shuttle vector capable of expressing a peroxisomal targeted red-shifted form of the green fluorescent protein (EGFP) under control of the *P. pastoris PEX8* gene promoter (P_{PEX8}), was constructed. As a first step, a P_{PEX8} expression vector was made by replacing the *AOX1* gene promoter (P_{AOX1}) fragment in the *P. pastoris* expression vector pHIL-A1 (Phillips Petroleum, Bartelsville, OK) with a DNA fragment containing P_{PEX8}. The P_{PEX8} fragment was obtained from vector pYT4 (Liu *et al.* 1995) by digestion with *Bgl*II. The termini of the *Bgl*II fragment were made blunt ended by treatment with the Klenow fragment of DNA polymerase I and the fragment was inserted into *Sma*I- and *Eco*RV-digested pBS-SKII$^-$ (Stratagene). The resulting plasmid was then digested with *Eco*RI, Klenow treated, and ligated to remove an *Eco*RI site within the P_{PEX8} fragment. This plasmid was then used in PCR to produce a P_{PEX8} fragment that contained the *Bam*HI site of pBS-SKII$^-$ at its 5′ terminus and introduced an *Eco*RI site on its 3′ terminus using the oligonucleotides 5′-AACAGCTATGACCATG-3′ and 5′-CAGGAATTCTAACAGGC ACCTGAAGATAGGT-3′. The PCR product was digested with *Bam*HI and *Eco*RI and inserted into *Bgl*II- and *Eco*RI-digested pHIL-A1 to produce the *PEX8* promoter-driven expression vector pK312. To generate an *EGFP* gene whose product is targeted to peroxisomes, two complementary oligonucleotides were first synthesized, which formed an adapter fragment encoding the last nine amino acids and stop codon of Pex8p (including the PTS1 sequence AKL) with a *Hin*dIII site at the 3′ terminus and a *Sal*I site at the 5′ terminus (5′-TCGACGC CCAATCAACCGCAAAGTTATAAACCGGTA-3′ and 5′-AGC TTACCGGTTTATAACTTTGCGGTTGATTGGGCG-3′). The adapter was inserted into *Hin*dIII- and *Xho*I-digested pEGFP-C3 (Clontech Laboratories, Inc., Palo Alto, CA), which re-

TABLE 1

***P. pastoris* strains used**

Strain	Genotype	Source
GS115	*his4*	Cregg *et al.* (1998)
GS190	*arg4*	Cregg *et al.* (1998)
GS200	*his4 arg4*	Waterham *et al.* (1996)
JC144	*AOX1::*P_{GAP}*−AOX1:HIS4 his4 arg4*	This study
MS105	*fld1*	Shen *et al.* (1998)
MC100-3	*arg4 his4 aox1*Δ*::SARG4 aox2*Δ*::Phis4*	Cregg *et al.* (1989)
JC121	*pex1-1 his4*	Liu *et al.* (1992)
JC116	*pex2-1 his4*	Liu *et al.* (1992)
JC129	*pex3-1 his4*	Liu *et al.* (1992)
JC127	*pex4-1 his4*	Liu *et al.* (1992)
JC122	*pex5-1 his4*	Liu *et al.* (1992)
JC128	*pex6-1 his4*	Liu *et al.* (1992)
JC123	*pex8-1 his4*	Liu *et al.* (1992)
JC125	*pex10-1 his4*	Liu *et al.* (1992)
JC124	*pex12-1 his4*	Liu *et al.* (1992)
JC130	*pex13-1 his4*	Liu *et al.* (1992)
JC132	*mxr1 his4*	This study
JC140	*pexA arg4*	This study
JC141	*pexB arg4*	This study
JC142	*pexC arg4*	This study
JC143	*mxr2 arg4*	This study
JC145	*pex3 fld1*	This study
JC146	*pex6 fld1*	This study
JC147	*pex8 fld1*	This study
JC148	*pex1-1 AOX1::*P_{GAP}*-AOX1:HIS4 his4*	This study
JC149	*pex8-1 AOX1::*P_{GAP}*-AOX1:HIS4 his4*	This study
JC150	*pex3-1 AOX1::*P_{GAP}*-AOX1:HIS4 his4*	This study
JC151	*pex6-1 AOX1::*P_{GAP}*-AOX1:HIS4 his4*	This study

sulted in a chimeric gene encoding EGFP with the last nine amino acids of Pex8p fused in frame to its carboxyl terminus (EGFP-PTS1). A PCR was performed that amplified the *EGFP-PTS1* fusion with an *Eco*RI site immediately 5′ of *EGFP* and included an *Eco*RI site located 3′ of the fusion gene using oligonucleotides 5′-CGGAATTCATGGTGAGCAAGGGCGAG GAG-3′ and 5′-CAGAATTCGAAGCTTACCGGTTTATAACT TTGCGG-3′. The *EGFP-PTS1* fragment was then digested with *Eco*RI and inserted into the unique *Eco*RI site of pK312 to produce pOPGP-1. The *PTS2-EGFP* vector, pTW65, expresses the chimeric protein under control of the *P. pastoris* acyl-CoA oxidase promoter; it was a gift from Dr. Suresh Subramani (University of California, San Diego, La Jolla, CA) and was described in Elgersma *et al.* (1998). A vector that encoded a chimeric protein composed of the 460 (out of 461) N-terminal-most amino acids of *P. pastoris* Pex2p fused to the N terminus of EGFP and expressed under control of P_{AOX1} was constructed as follows. First, a 750-bp *Nhe*I-*Hin*dIII fragment containing the *EGFP* gene from plasmid pEGFP-C3 (Clontech) was ligated into *Xba*I- and *Hin*dIII-digested pUC18 to create pUC18-EGFP. Second, a DNA fragment encoding amino acids 1–460 of Pex2p was generated by PCR using oligonucleotides 5′-TCA GGATCCATGCCCAATAGGCTCATACC-3′ and 5′-GACCATGG CACCAACGAAAAAACCTGG-3′ as primers. This resulted in a fragment with a *Bam*HI site at the 5′ terminus and an *Nco*I site at the 3′ terminus. The *PEX2* fragment was digested with these enzymes and inserted into the same sites in pUC18-EGFP to create pUC18-*PEX2-EGFP*. Third, this vector was digested with *Sma*I and *Xho*I, and an ~2-kb fragment containing the *PEX2-EGFP* fusion was ligated into *Pml*I- and *Xho*I-digested pPICZB (Invitrogen, Carlsbad, CA) to create pLC303. The vector was linearized within the *AOX1* promoter region by digestion with *Pme*I prior to transformation into *P. pastoris*. The luciferase gene vector pHWO17 expresses the gene under control of P_{AOX1} and was described in Waterham *et al.* (1996).

Mutagenesis: The procedure for mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) was as described by Cregg *et al.* (1990) with the following modifications: (1) Cells were cultured in YND plus arginine; (2) NTG was dissolved in acetone (10 mg NTG/ml acetone) and used fresh; and (3) prior to mutagenesis, cultures were divided into three equal portions and aliquots treated with 0.02, 0.04, or 0.08 mg NTG/ml of cells. These treatments resulted in the killing of 67, 90, and 96% of cells, respectively. Glycerol (to 30%) was added to mutagenized cultures and cultures were frozen at −70° until use. Freezing killed an additional 90% of cells surviving mutagenesis.

For ultraviolet-light (UV) mutagenesis, the following procedure was followed: (1) Cells were pregrown on YPD medium to an OD_{600} of ~1. (2) Cells were harvested and resuspended in sterile water at an OD_{600} of 0.3. (3) Twenty milliliters of the culture in water were transferred to a petri dish and irradiated for 30–40 sec with gentle shaking (this UV treatment resulted in the death of 90–99% of the cells). (4) Under dim light to minimize photoreactivation repair, 100-μl aliquots of culture were spread on agar plates.

Preparation of cell-free and whole-cell extracts: To prepare cell-free extracts, cells were precultured in YPD and harvested at ~1 OD_{600}. For mutant strains, 50 OD_{600} units were resuspended in 50 ml of either YNM or YNO medium and induced

for 6 hr at 30°. For wild-type strains, 25 OD_{600} units were resuspended in 50 ml and processed as described for mutant strains. Cells were harvested and washed twice with ice-cold 50 mm potassium phosphate buffer, pH 7.0, and then frozen at −20°. Cells were thawed and resuspended in 400 μl of the same buffer along with 0.5 μl of 1 m phenylmethanesulfonyl fluoride (PMSF). Cold 0.5-mm-diameter acid-washed glass beads were then added to ~1/3 volume of buffer, and the mixture vortexed at 4° at high speed for 10 min, followed by a 20-min centrifugation in a minicentrifuge at 14,000 rpm and 4°. The supernatant was removed and stored on ice. For whole-cell extracts, the postdisruption centrifugation step was omitted, and the supernatant was removed after the beads had settled.

Subcellular fractionation: Cells were pregrown in YPD medium and transferred during logarithmic growth phase (1–1.5 OD_{600}) by centrifugation into YNO or YNM medium and induced for 6 hr at 30°. Subcellular fractionations were performed as described by Liu *et al.* (1992) with the following modifications: (1) 10 mm Na_2SO_3 was used in place of dithiothreitol (DTT). (2) Incubation with Zymolyase was for 15–45 min. (3) Immediately following treatment with Zymolyase, 10 μl of 1 m PMSF was added. (4) Following the collection of protoplasts by centrifugation, a wash using 5 mm 3-[*N*-morpholino]propanesulfonic acid, 0.5 m KCl, 10 mm Na_2SO_3, pH 7.2 buffer was done to remove Zymolyase. (5) MES buffer was composed of 5 mm MES, pH 6.0, 1.2 m sorbitol, 0.5 mm EDTA, 0.1% ethanol, 0.21 mg/ml NaF, 1 mm PMSF, 1 mm leupeptin, and 1 mm aprotinin. The final centrifugation was performed at 30,000 × *g* for 30 min.

Biochemical methods: Peroxisomal AOX (Verduyn *et al.* 1984; van der Klei *et al.* 1990), catalase (CAT; Ueda *et al.* 1990), acyl-CoA oxidase (Dommes *et al.* 1981), mitochondrial cytochrome *c* oxidase (Douma *et al.* 1985), and β-lactamase (Waterham *et al.* 1997) activities were assayed at 30° according to published procedures. Activities were expressed as micromoles of product/min/mg of protein for AOX, cytochrome *c* oxidase, acyl-CoA oxidase, and β-lactamase. Activities for catalase were expressed as ΔE_{240}/min/mg of protein. Protein concentrations were determined using the Pierce (Rockford, IL) Bicinchoninic acid protein assay kit with bovine serum albumin as a standard. Transfer of protein to nitrocellulose filters after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Mini trans-blot electophoretic transfer cell (Bio-Rad Laboratories, Richmond, CA) as indicated by the manufacturer. Immunoblotting experiments were performed with specific polyclonal antibodies to AOX, catalase, or thiolase (a gift from W. H. Kunau, Ruhr University, Bochum, Germany) using either the Western Light Kit (Tropix, Bedford, MA) or the color development assay using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and *p*-nitroblue tetrazolium chloride (NBT; Bio-Rad Laboratories).

Fluorescence microscopy: Strains transformed with either pOPGP-1 or pLC303 were grown overnight in YND (0.1% glucose) plus 0.5% glycerol to 1 OD_{600}, inoculated into YNM to a starting OD_{600} of 1.0, and grown at 30° with shaking for 4–6 hr. Strains transformed with pTW65 were precultured in YPD and grown to 1 OD_{600}, inoculated into YNO for induction at a starting OD_{600} of 1, and grown for 12 hr at 30° with shaking. Slides were prepared by adding 10 μl of culture to a slide and affixing the coverslip with rubber cement.

Genetic methods: Complementation testing was done as described by Cregg *et al.* (1998). Procedures for backcrossing and random spore analysis were as described in Liu *et al.* (1992). To verify that spore products of the first backcrossed formaldehyde dehydrogenase (*fld1*)-derived strains were wild type with respect to the *FLD1* gene, the progeny were crossed with an *fld1* strain and replica plated to YNM plates. If complementation occurred, *i.e.*, growth on methanol, the backcrossed strain did acquire the *FLD1* gene during the first backcross.

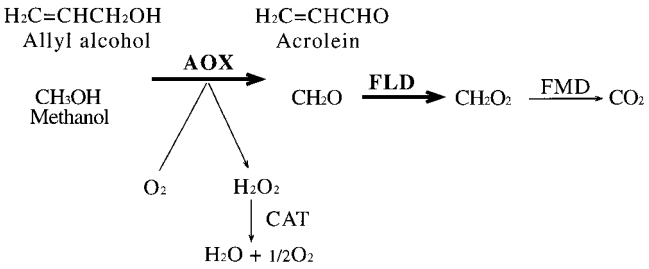

Figure 1.—Metabolism of methanol and allyl alcohol in yeast. AOX, alcohol oxidase; CAT, catalase; FLD, formaldehyde dehydrogenase; FMD, formate dehydrogenase.

Miscellaneous methods: Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) was used for scanning photographs of plates and X-ray films developed from immunoblots. The scans were then imported into Freehand (Macromedia Inc., San Francisco) to add text. Adobe Photoshop was also used for scanning EGFP expression negatives. These scans were then imported into Adobe PageMaker to arrange and add text. Electron microscopy was performed as described in Waterham *et al.* (1996).

RESULTS

Selection for *pex* mutants using allyl alcohol: Allyl alcohol is a nontoxic substrate that is oxidized by AOX, a peroxisomal matrix enzyme and the first enzyme in the yeast methanol metabolic pathway, to acrolein, a substance that is toxic (Figure 1; Ciriacy 1975; Sibirny *et al.* 1988, 1989). Thus, methanol-grown wild-type *P. pastoris* cells with high levels of AOX are sensitive to allyl alcohol because of its conversion to acrolein. In previous studies, we showed that, in methanol-induced *P. pastoris pex* mutants that lack functional peroxisomes, AOX is synthesized but does not assemble and remains in the cytoplasm as misfolded inactive aggregates (Liu *et al.* 1992; Waterham *et al.* 1997). Thus, methanol-induced *pex* mutants should be relatively insensitive to allyl alcohol, providing a potential means of selectively growing *pex* mutants in mutagenized cultures. However, AOX is typically present only in *P. pastoris* cells grown or induced on methanol, and *pex* mutants cannot grow on methanol. To circumvent this problem, a *P. pastoris* strain was constructed that expresses AOX on glucose medium. This strain, JC144, was made by transforming *P. pastoris* GS200 with pHW011, a vector that expresses the *P. pastoris AOX1* gene under the control of the constitutive P_{GAP}. In glucose-grown cells of both wild-type and *pex* strains transformed with this plasmid, AOX protein was present. However, in JC144, AOX was active, while in *pex* mutants it was not. The levels of AOX activity in JC144 were ~100-fold lower than in methanol-grown wild-type *P. pastoris* but more than 100-fold higher than those typically seen in glucose-grown cells of this yeast; subcellular fractionation studies showed that the AOX

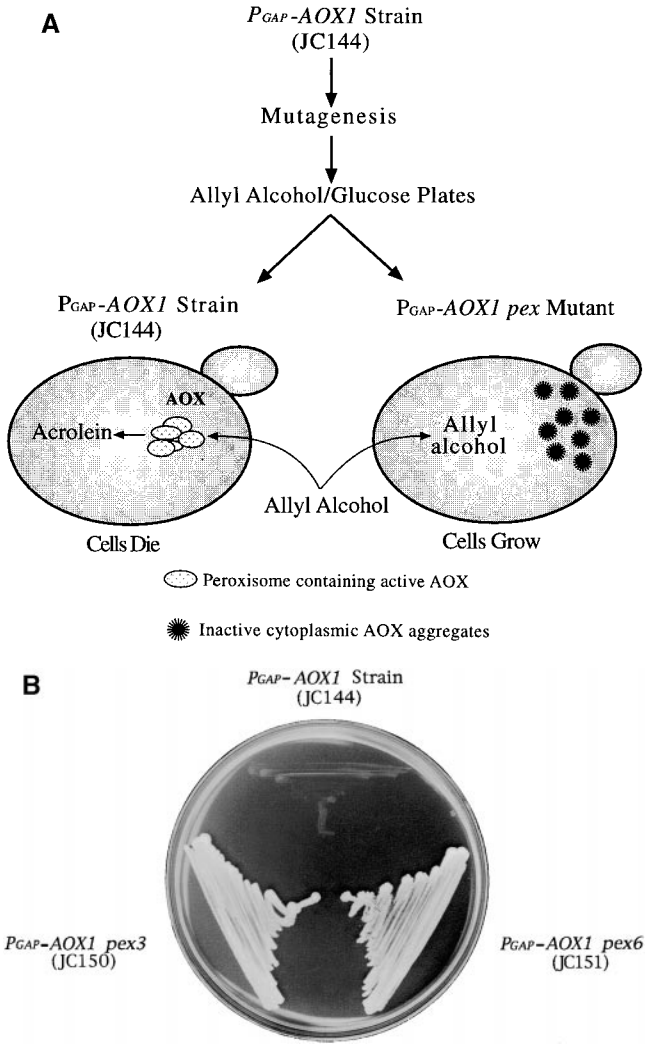

Figure 2.—Allyl alcohol selection scheme for the isolation of *P. pastoris pex* mutants. (A) The parent strain constitutively expresses *AOX1* from P_{GAP}. Wild-type cells die as a result of the active AOX converting allyl alcohol to acrolein. *Pex* mutants grow due to the absence of active AOX. (B) Growth of strains expressing AOX on allyl alcohol-glucose plates. All strains were transformed with pHWO11 and then spread on an allyl alcohol-glucose plate.

activity was localized to peroxisomes (data not shown). Importantly, JC144 was sensitive to 0.5 mm allyl alcohol on allyl alcohol dextrose (AAD) plates, while *P. pastoris pex* mutants transformed with pHW011 were not (Figure 2, A and B). Thus, using JC144 as the parent strain for mutagenesis and AAD plates, it was possible to select for *pex* mutants and against the parent strain and other types of mutants that continued to express and assemble active AOX.

To isolate *P. pastoris pex* mutants, cultures of strain JC144 were subjected to mutagenesis with NTG and spread on AAD plates. The optimal concentration of mutagenized cells was empirically determined to be $\sim 2 \times 10^6$ cells/ml. Higher concentrations resulted in fewer colonies, possibly due to a "neighbor effect" (*i.e.*, killing of cells by diffusion of acrolein from active AOX-containing cells to those without active AOX). The resulting colonies were further screened as described in Liu *et al.* (1992). Briefly, 2951 allyl alcohol-resistant colonies were tested first for methanol utilization (Mut) by replica plating. The resulting Mut$^-$ were then tested for oleic acid utilization (Out) in liquid oleic acid medium. Thirty-eight colonies were identified that were defective in growth on both of these peroxisome-requiring substrates. Complementation tests demonstrated that 33 of the Mut$^-$ Out$^-$ belonged to previously identified *PEX* gene groups (Table 2). As described below, the remaining 5 Mut$^-$ Out$^-$ mutants represented two non-*PEX* complementation groups that we have tentatively named *MXR1* and *MXR2* and that appear to encode transcription factors required for expression of certain peroxisomal enzymes.

Efficiency of the allyl alcohol selection scheme: To determine the efficiency of the allyl alcohol selection scheme, samples of the same NTG-mutagenized cultures of JC144 were subjected to selection on AAD plates and to the random negative screening method (Gould *et al.* 1992; Liu *et al.* 1992). Under optimal conditions, 1.2% of the colony population on the AAD plates were *pex* mutants, whereas only ~0.09% of the cells surviving mutagenesis were *pex* mutants in the population prior to selection. Thus, the allyl alcohol selection provided an ~13-fold enrichment in *pex* mutants.

Selection of *pex* mutants using an *fld1* strain and high methanol: In wild-type *P. pastoris* cells growing on methanol, formaldehyde, the product of AOX oxidation of methanol, is further oxidized to formate and then carbon dioxide by two nicotinamide adenine dinucleotide-dependent dehydrogenases (Veenhuis and Harder 1987; Shen *et al.* 1998; Figure 1). However, in strains defective in FLD, toxic formaldehyde is not further metabolized and accumulates, killing these cells. The build-up of formaldehyde does not occur in methanol-induced *fld1 pex* mutants, because these strains have little active AOX and, therefore, cannot convert methanol to formaldehyde. Thus, *fld1 pex* mutants survive exposure to methanol. However, neither *fld1* nor *pex* mutants grow on methanol. As described above, most carbon sources other than methanol repress methanol metabolic pathway enzyme synthesis. An exception is sorbitol. In medium containing a mixture of 12% methanol and 0.2% sorbitol (MSY), wild-type and *fld1* strains contained methanol pathway enzymes at levels similar to those observed when methanol is the sole carbon source. Thus sorbitol could be used as a carbon source that would allow for the selective growth of *fld1 pex* strains (Figure 3A). To demonstrate this *pex* mutant selection scheme, Figure 3B shows an MSY plate on which selected strains had been streaked. As expected, wild-type (not shown) and *fld1 pex* strains grew on this medium while the *fld1* strain did not.

To select for *pex* mutants using this scheme, UV- or

TABLE 2

***P. pastoris* mutants isolated by positive screens**

Complementation group	Negative screen[a]	Positive schemes[b]		
		Allyl alcohol	High methanol/*fld1*	High methanol/WT
pex1	5	4	25	1
pex2	1	0	2	1
pex3	0	6	16	2
pex4	3	7	3	0
pex5	6	5	14	1
pex6	5	7	17	1
pex8	1	1	12	0
pex10	1	1	13	0
pex12	1	2	3	0
pex13	0	0	16	1
Novel *pex* mutants[c]				
pexA	0	0	17	4
pexB	0	0	4	0
pexC	0	0	7	0
Potential transcription mutants				
mxr1	0	3	0	0
mxr2	0	2	0	0

[a] Liu *et al.* (1992).
[b] This study.
[c] The names *pexA*, *pexB*, and *pexC* are temporary. Once the gene products have been identified, an appropriate number will be assigned to each according to accepted guidelines (Distel *et al.* 1996).

NTG-mutagenized cultures of the *fld1* strain were spread on MSY plates. After incubation, the resulting large colonies were streaked onto YPD plates, incubated at 30° for 24 hr, and then replica plated onto a second MSY plate to confirm their ability to grow on the methanol-sorbitol medium. Strains able to grow on MSY plates were further tested for their ability to grow on oleate, and strains that were Out$^-$ were collected for further study.

Efficiency of the *fld1*/high-methanol selection scheme: A total of 582 methanol-resistant strains were examined for Out phenotype and 293 (50%) were Out$^-$. A sample of 149 of the methanol-resistant and Out$^-$ mutants was subjected to complementation analysis and found to represent new alleles of each of the 10 previously identified *PEX* gene groups plus 3 new complementation groups. As described below, these new groups were determined to be defective in previously unidentified *PEX* genes (Table 2). Thus, at least 25% of strains arising from the selection procedure were *pex* mutants. This represented an ~278-fold enrichment over the percentage of *pex* mutants in the nonenriched control cell population.

To determine whether the *fld1* mutant strain was required for the high-methanol scheme to work, we also performed the selection using the same conditions except with *P. pastoris* strains that were wild type with respect to their ability to use methanol [GS115 (*his4*) and GS190 (*arg4*)]. Out of 340 mutants derived from these two strains that were resistant to the high methanol concentration, we identified 11 *pex* mutants representing six different *PEX* gene complementation groups (Table 2). Thus, at 3.2%, the high-methanol selection scheme generated a strong (~35-fold) enrichment for *pex* mutants even without the use of the *fld1* mutant strain. However, the *fld1* strain resulted in a further 8-fold enrichment for *pex* mutants.

Genetic analysis of mutants in new complementation groups: Isolated mutants (described above) that did not fall into prevously identified *PEX* gene groups—including eight reported by our group in Liu *et al.* (1992) and two additional groups unique to a collection described by Gould *et al.* (1992)—were first backcrossed at least three times. Complementation studies with the backcrossed derivatives revealed that the strains represented recessive alleles in five new complementation groups.

Biochemical and morphological characterization of the new mutant groups: Representatives of each new group, along with wild-type and *pex1* control strains, were examined for the presence, activity, and subcellular location of selected peroxisomal enzymes. In total-cell extracts prepared from methanol-induced cells of three of the mutants (temporarily named *pexA*, *pexB*, and *pexC*), near wild-type levels of catalase (CAT) activity were observed (Table 3). Conversely, AOX activity,

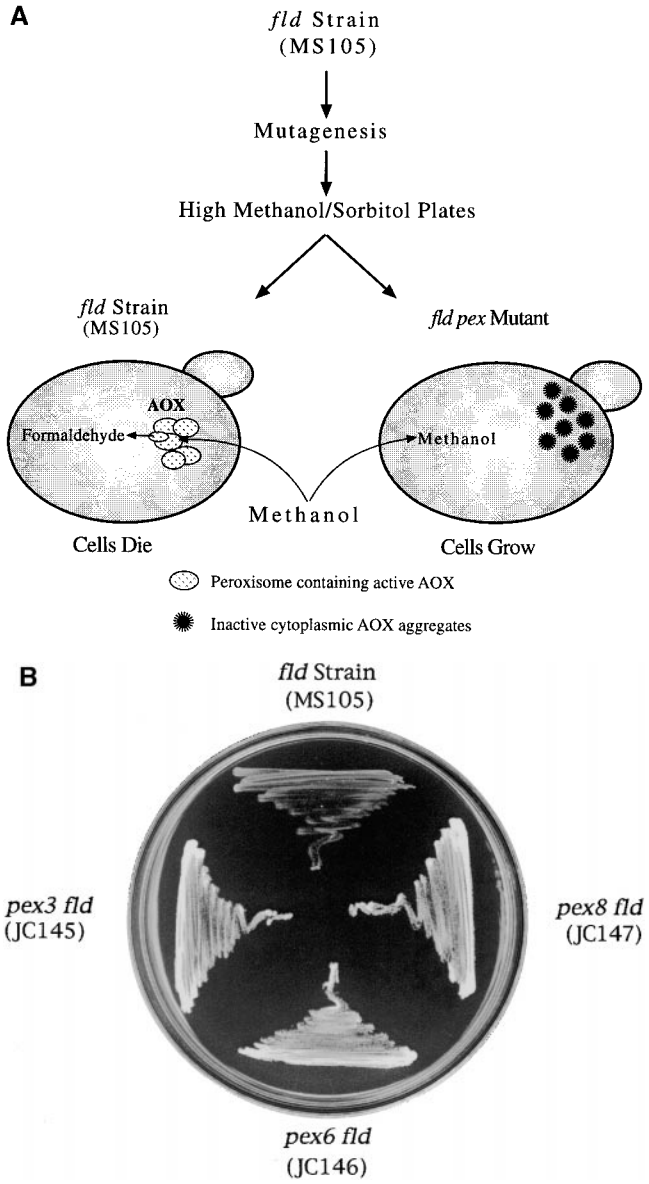

Figure 3.—Methanol selection scheme for the isolation of *P. pastoris pex* mutants using an *fld1* mutant parent strain. (A) *fld1* non*pex* cells die due to accumulation of formaldehyde while *fld1 pex* mutants, which lack AOX, accumulate little or no formaldehyde and grow. (B) Growth of *fld1* and *pex fld1* strains on high methanol-sorbitol plates.

TABLE 3

Enzyme activities in induced cells of new mutant strains

Strain	Enzyme	Methanol	Oleate
WT	CAT	100	100
	AOX	100	ND
	acyl-CoA oxidase	ND	100
	β-lactamase	100	ND
pex1	CAT	122	166
	AOX	0	ND
	acyl-CoA oxidase	ND	5
	β-lactamase	58	ND
pexA	CAT	91	137
	AOX	0	ND
	acyl-CoA oxidase	ND	3.8
pexB	CAT	71	142
	AOX	0	ND
	acyl-CoA oxidase	ND	8
pexC	CAT	75	135
	AOX	0.06	ND
	acyl-CoA oxidase	ND	5
mxr1	CAT	17	47
	AOX	0	ND
	acyl-CoA oxidase	ND	143
	β-lactamase	0.05	ND
mxr2	CAT	12.7	22
	AOX	0	ND
	acyl-CoA oxidase	ND	0
	β-lactamase	0.09	ND

Enzyme activities were measured in total-cell extracts. Percentage of CAT, AOX, acyl-CoA oxidase, and β-lactamase activities is reported relative to that of wild type. Actual wild-type enzyme activities for CAT, AOX, and acyl-CoA oxidase are an average of three experiments (CAT = 99 ΔE_{240}/minute/mg of protein induced on methanol; CAT = 127 ΔE_{240}/min/mg of protein induced on oleate; AOX = 1.75 μmol of product/minute/mg of protein; acyl-CoA oxidase = 0.125 μmol of product/minute/mg of protein). Wild-type β-lactamase activity was 0.81247 μmol of product/minute/mg of protein. ND, not done; WT, wild type.

which is typically low or absent in *pex* mutants (Liu *et al.* 1992; Waterham and Cregg 1997), was also low in these strains. In immunoblots prepared from these strains, AOX protein was also low in two of the strains (*pexA* and *pexB*) but nearly normal in *pexC* (data not shown). When induced on oleate medium, CAT activity and thiolase protein were near wild-type levels, while acyl-CoA oxidase activity was present at low but significant levels. All of these results were characteristic of previously isolated *pex* strains (Gould *et al.* 1992; Liu *et al.* 1992).

Results of subcellular fractionations with each of the three strains were also typical of those observed for other *pex* mutants. Methanol- and oleate-induced cells of each of the three strains were subjected to subcellular fractionation into a pellet fraction containing mostly mitochondria and peroxisomes, and a cytosolic supernatant fraction. Whereas ~50% of CAT activity was present in the organellar pellet from wild-type cells, most CAT activity from the *pexA*, *pexB*, *pexC*, and *pex1* strains was present in the supernatant fraction (Table 4).

Methanol- and oleate-induced cells of *pexA*, *pexB*, and *pexC* were also examined by electron microscopy for peroxisomes and no normal peroxisomes were observed (Figure 4; *pexB* not shown), confirming that each of these strains represents mutants in new *PEX* genes.

The three new *pex* mutant groups were further characterized with regard to the function of their peroxisomal targeting signal (PTS) pathways. Three PTS pathways have been defined in yeasts and other eukaryotes: two, PTS1 and PTS2, are specific to peroxisomal matrix pro-

TABLE 4

Distribution of peroxisomal enzyme activities after subcellular fractionation

C source	Strain	Fraction	Cytochrome *c* oxidase (%)	Catalase (%)	Luciferase (%)
Oleic acid	WT	P	99	57	48
		S	1	43	52
	pexA	P	95	1	1
		S	5	99	99
	pexB	P	97	3	3
		S	3	97	97
	pexC	P	97	9	6
		S	3	91	94
	pex1	P	95	5	1
		S	5	95	99
	mxr1	P	94	56	
		S	6	44	
	mxr2	P	98	27	
		S	2	73	
Methanol	WT	P	99	50	
		S	1	50	
	pexA	P	95	8	
		S	5	92	
	pexB	P	97	3	
		S	3	97	
	pexC	P	97	3	
		S	3	97	
	pex1	P	98	4	
		S	2	96	
	mxr1	P	99	33	
		S	1	67	
	mxr2	P	98	30	
		S	2	70	

P, pellet; S, supernatant.

teins, and the third, mPTS, is specific to peroxisomal integral membrane proteins (Waterham and Cregg 1997). To examine each pathway, the fate of selected proteins with well-characterized targeting signals was determined. For the PTS1 pathway, we expressed two heterologous PTS1 proteins, luciferase and an EGFP-PTS1 chimeric protein (Gould *et al.* 1987; Kalish *et al.* 1996; Monosov *et al.* 1996). After subcellular fractionation of oleate-induced cells of each of the new *pex* mutants, luciferase activity was found predominantly in the supernatant fractions (Table 4), whereas in wild-type cells ~50% of luciferase activity was present in the pellet fraction. These results were similar to those seen for CAT activity and indicated that the PTS1 pathway was defective in all three *pex* mutants. Further support for this conclusion was obtained by fluorescence microscopy of EGFP-PTS1-expressing methanol-induced cells of the mutants. In wild-type cells, a tight cluster of strongly fluorescing peroxisomes was observed (Figure 5A). However, in each of the *pex* mutants the entire cytoplasm fluoresced (Figure 5D, G, J, and M). To investigate PTS2 pathway function, the fates of two PTS2 proteins, *P. pastoris* thiolase and a PTS2-EGFP chimeric protein, were examined in each mutant (Glover *et al.* 1994; Elgersma *et al.* 1998). Subcellular fractions from oleate-induced cells of each mutant showed thiolase protein predominantly in the supernatant fractions, whereas the majority of thiolase protein was present in the pellet fraction from wild-type cells (Figure 6). Furthermore, fluorescence microscopy of PTS2-EGFP-expressing oleate-induced cells of each *pex* mutant showed a diffuse cytoplasmic fluorescence pattern typical of PTS2 pathway-defective *pex* mutants (Figure 5, E, H, K, and N; Elgersma *et al.* 1998). Thus, both PTS1- and PTS2-matrix protein import pathways are defective in all three new *pex* mutant groups.

To examine mPTS pathway function, we expressed a Pex2p-EGFP chimeric protein in each of the new *pex* mutants. Pex2p is an integral membrane protein in *P. pastoris* and other organisms (Waterham *et al.* 1996). Evidence for proper targeting of the Pex2p-EGFP chimera was provided by the observation that the *PEX2-EGFP* gene fully complemented a *P. pastoris pex2*-deletion strain with regard to methanol- and oleate-growth phenotypes and restoration of peroxisomal matrix protein targeting (not shown). In fluorescence microscopy

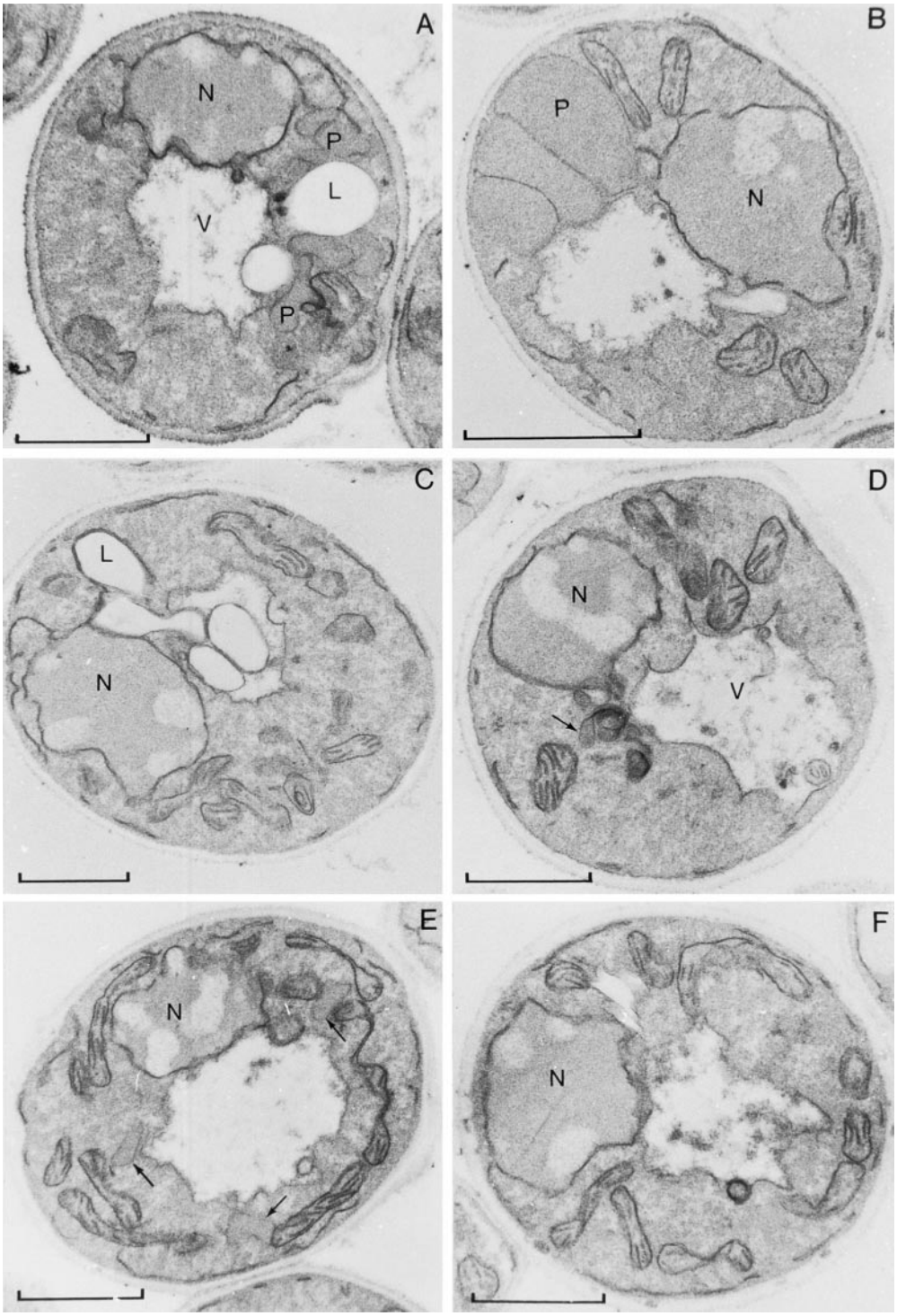

Figure 4.—Electron micrographs showing subcellular morphology of selected *P. pastoris* strains. (A) Proliferation of small peroxisomes in oleate-induced wild-type cells. (B) Proliferation of large peroxisomes in methanol-induced wild-type cells. (C) Lack of recognizable peroxisomes in *pexA* oleate-induced cells. (D) Lack of recognizable peroxisomes in *pexC* methanol-induced cells (peroxisomal remnants indicated by arrow). (E) Peroxisomes (indicated by arrows) in oleate-induced *mxr1*. (F) Lack of recognizable peroxisomes in methanol-induced *mxr1*. P, peroxisome; N, nucleus; V, vacuole; L, lipid body. Bar, 1.0 μm.

of wild-type methanol-induced cells, the Pex2p-EGFP protein localized to the peroxisomal membranes, which appeared as bright rings surrounding the organelles (Figure 5C). In cells of the *pexA*, *pexC*, and control *pex1* strains, this normal peroxisomal membrane fluorescence pattern was not observed. Instead, most cells contained one or two brightly fluorescing punctate structures (Figure 5, F, L, and O). This pattern is typical of peroxisomal remnants or ghosts, residual peroxisome-related matrix protein import-incompetent vesicular structures that are common to most *pex* mutants (Gould *et al.* 1996) and indicated that these mutants have peroxisomal remnants and that the Pex2p-EGFP protein was properly targeted to the remnants. Thus, unlike the matrix protein import pathways, the mPTS pathway is functional in *pex A* and *pexC* mutants. In *pexB* cells, fluorescence also localized to punctate structures, but the intensity of the signal was significantly lower than

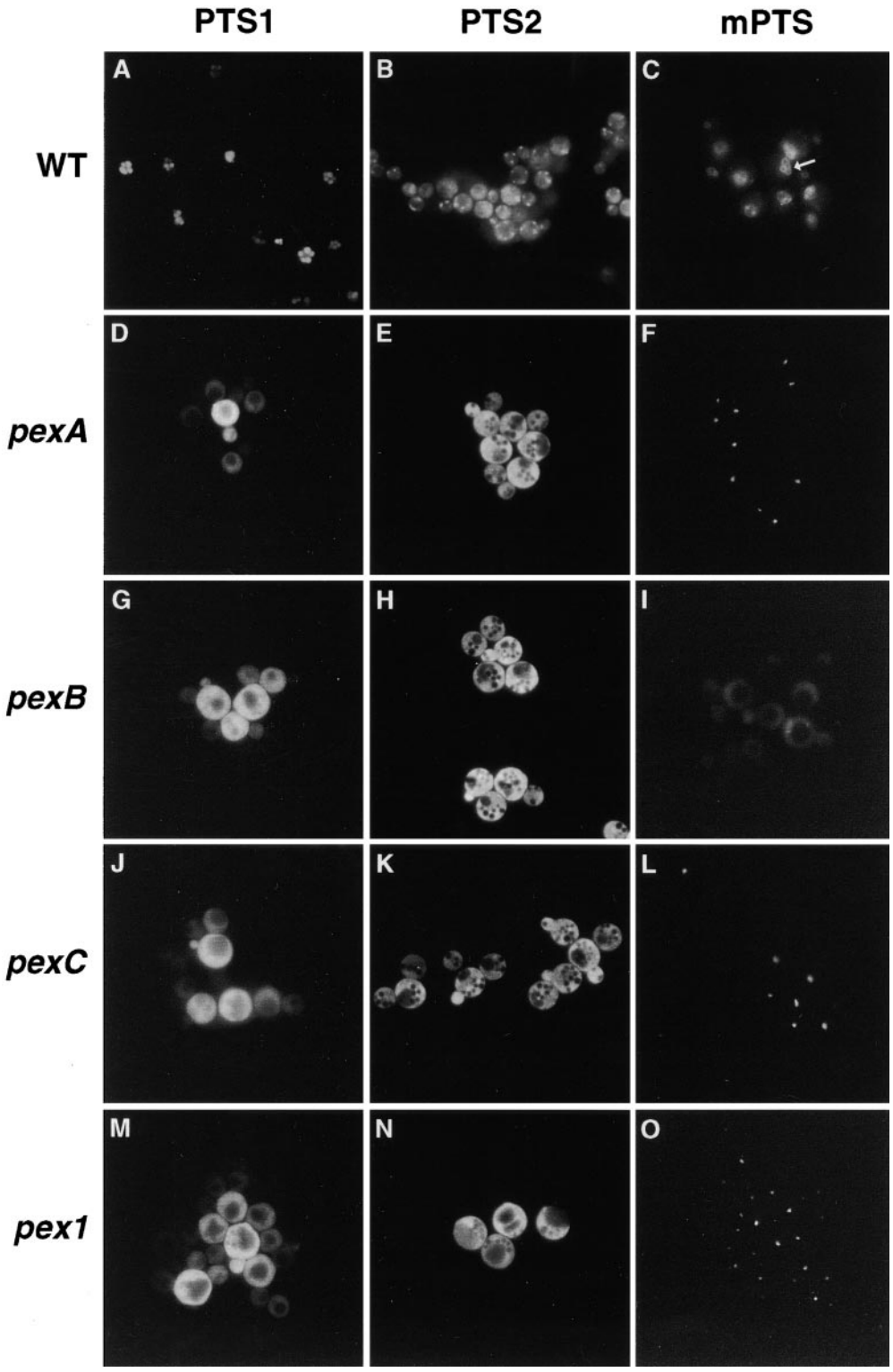

Figure 5.—Subcellular location of EGFP-PTS1, PTS2-EGFP, and mPTS-EGFP in new *pex* mutants. (A, D, G, J, M) Cells expressing PTS1-EGFP on methanol. Note the clusters of strongly fluorescing peroxisomes in wild type (A) *vs.* the cytosolic fluorescence in the mutants *pexA* (D), *pexB* (G), *pexC* (J), *pex1* (M). (B, E, H, K, N) Cells expressing PTS2-EGFP on oleate. As in EGFP-PTS1, the wild-type cells (B) exhibit a punctate pattern—although less pronounced than A—*vs.* the diffuse pattern seen in the mutants *pexA* (E), *pexB* (H), *pexC* (K), and *pex1* (N). (C, F, I, L, O) Cells expressing mPTS-EGFP on methanol. (C) Wild type exhibits localization of fluorescence to the peroxisomal membranes seen as rings. Arrow denotes a cell in which four such rings are visible. Mutants (F) *pexA*, (L) *pexC*, and (O) *pex1* show fluorescence localized to what appears to be peroxisomal remnants while (I) *pexB* shows both cytosolic and peroxisomal localization.

in the other *pex* mutants and a low intensity cytoplasmic fluorescence was apparent (Figure 5I). This result was not specific to one *pexB* transformant because each of 10 *pexB* strains transformed with the *PEX2-EGFP* expression vector showed the same pattern. One possible explanation for this result is that the *pexB* mutant contains peroxisomal remnants but is partially defective in membrane protein targeting. A second possibility is that the membrane protein-targeting system may be fully functional but the remnants are too small to accommodate all the Pex2p-EGFP protein present in *pexB* mutant cells.

The remaining two complementation group representatives (tentatively named *mxr1* and *mxr2*) were examined as described above but with different results.

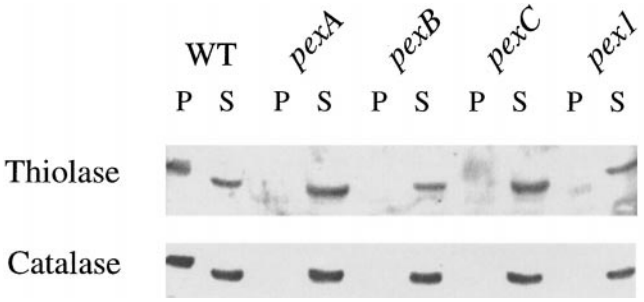

Figure 6.—Subcellular localization of thiolase and catalase protein. Organelle pellet and cytosolic supernatant fractions obtained after subcellular fractionation of wild type, *pexA*, *pexB*, *pexC*, and *pex1*, induced on oleate, were analyzed by immunoblots with antibodies against thiolase and catalase. For thiolase, 20 μg of total protein was loaded in the wild-type lane while 40 μg of total protein was loaded in each of the other lanes. For catalase, 20 μg of total protein was loaded in each lane.

Both had peroxisomal enzyme levels that were uncharacteristic of *pex* mutants (Table 3). In extracts prepared from oleate-induced cells of *mxr1*, levels of acyl-CoA oxidase activity, CAT activity, and thiolase protein were similar to those in wild-type cells. However, in methanol-induced cells of the strain, levels of AOX and CAT activity (Table 3) and protein (not shown) were low (Table 3). In *mxr2* cells, oleate- and methanol-induced peroxisomal enzyme levels were low. These included activity levels for acyl-CoA oxidase and CAT (Table 3) and protein levels for thiolase and CAT (not shown) in oleate-induced cells, and activity (Table 3) and protein (not shown) for AOX and CAT in methanol-induced cells of the mutant. Subcellular fraction studies showed that CAT activity in homogenates prepared from methanol- or oleate-induced cells of *mxr1* and *mxr2* was primarily located in pellet fractions (Table 4). Taken together, these results suggested that *mxr1* and *mxr2* mutants contained functional peroxisomes. However, except for oleate-induced cells of *mxr1*, the organelles were likely to be much smaller than normal due to the low levels of matrix proteins. These conclusions were supported by electron microscopic examination of *mxr1* and *mxr2*. In methanol-induced cells of *mxr1* and *mxr2* and oleate-induced cells of *mxr2*, the large, numerous peroxisomes typical of wild-type *P. pastoris* were absent. However, in oleate-induced cells of *mxr1*, peroxisomes were readily apparent but somewhat smaller and fewer in number than in wild-type cells (Figure 4).

We considered the possibility that *MXR1* and *MXR2* were genes required for expression of methanol pathway (*MXR1* and *MXR2*) and oleate pathway enzymes (*MXR2* only). Preliminary evidence for this hypothesis was obtained by introducing a vector that expresses *E. coli* β-lactamase under control of the *AOX1* promoter of *P. pastoris* into each mutant (Waterham *et al.* 1997). The ability of the mutants to induce expression of β-lactamase from this promoter in response to methanol was then assessed. Both *mxr1* and *mxr2* induced only very low levels of β-lactamase relative to either wild-type or *pex1* strains (Table 3), suggesting that *MXR1* and *MXR2* may be involved in transcription of *AOX1* and perhaps other methanol pathway genes.

DISCUSSION

The *P. pastoris pex* mutant selection schemes described here compare favorably with three schemes previously reported for selection of *S. cerevisiae pex* mutants. van der Leij *et al.* (1992) reported the use of the catalase inhibitor 3-amino-1,2,4-triazol (3-AT) as a *pex* mutant selective agent. The basic concept for the selection is that, without catalase, oleate-induced *S. cerevisiae* cells (containing peroxisomal β-oxidation pathway enzymes) should accumulate lethal levels of H_2O_2. However, mutants that lacked a functional β-oxidation system, including *pex* mutants, should survive. A scheme reported by Zhang *et al.* (1993) is based on the same H_2O_2 toxicity principle except that a parent strain defective in catalase (both cytoplasmic catalase T and peroxisomal catalase A) is employed. Using this same general strategy, we attempted to select for *pex* mutants using a *P. pastoris* mutant defective in catalase activity as the parental strain and selection plates containing methanol and sorbitol. However, for reasons that are unknown, the procedure did not result in a significant enrichment for *pex* mutants.

A third selection scheme described by Elgersma *et al.* (1993) makes use of a parental strain that expresses a chimeric polypeptide composed of the bleomycin resistance protein fused to peroxisomal luciferase (Ble-Luc). In the parental Ble-Luc strain, the fusion protein is targeted to peroxisomes via the luciferase PTS1. As a result, the strain is sensitive to phleomycin, a toxic compound and Ble ligand. However, *pex* mutants derived from the Ble-Luc strain are resistant to the drug because, without peroxisomes, the Ble-Luc protein is no longer sequestered and is free to bind the drug. The most effective of the three methods appears to be the phleomycin resistance scheme where ~10% of colonies surviving the drug are *pex* mutants (Elgersma *et al.* 1993). This number is comparable to the frequency of *pex* mutants from our selection methods (~1.2% of colonies arising after the allyl alcohol selection and ~25% of colonies surviving the methanol selection).

Comparison of the types and frequencies of mutants generated by our *P. pastoris* enrichment schemes reveals interesting similarities and differences. As expected, most of the *pex* mutants isolated by the selection schemes represent new alleles in *PEX* genes previously identified by the negative screening method (Table 2; Gould *et al.* 1992; Liu *et al.* 1992). Even the relative frequencies with which specific *pex* alleles are found are generally similar. However, there are some striking differences as

well. The allyl alcohol selection resulted in the isolation of five mutants in two genes (tentatively named *MXR1* and *MXR2*), which were not seen with either the negative screen or the methanol selection methods. Although the *mxr* mutants grow on neither methanol nor oleic acid, they are clearly not *pex* mutants, because catalase fractionates to the crude organellar pellet in differential centrifugation experiments with these strains. Thus, they represent the only mutants that are specifically defective in growth on these peroxisome-requiring substrates that are not *pex* mutants. The low levels of peroxisomal matrix proteins in *mxr1* (methanol-utilization enzymes only) and *mxr2* (both methanol- and oleate-utilization enzymes) suggest these mutants may be defective in a factor required for the transcription of matrix enzymes. Further studies on these mutants and the affected genes are in progress. The *fld1*/high methanol selection method preferentially enriches for mutations in some *PEX* genes such as *PEX3*, *PEX13*, and *PEXA* (each at ~11% of all *pex* mutants isolated) relative to either the negative screening or allyl alcohol methods (see Table 2). Importantly, the high methanol selection procedure succeeded in yielding numerous alleles in each of three new *P. pastoris PEX* gene groups. [Because we do not know whether these new groups are homologues of *PEX* genes previously identified in other organisms, we have temporarily named them *PEXA*, *PEXB*, and *PEXC*. Once their genes have been cloned, the sequence of their products will be used to assign them to a new or existing *PEX* homologue group according to the guidelines described in Distel *et al.* (1996).] Although the reason for the differences in frequencies and types of mutants resulting from the different *pex* mutant selection schemes is unclear, it is apparent that the schemes have subtle but distinct biases that influence the frequency of *pex* mutants that are found. Thus, to maximize our chances of identifying mutants in novel *PEX* genes, we believe the best strategy is the continued application of both schemes.

The identification of genes required for peroxisome biogenesis (*PEX* genes) through the isolation of yeast *pex* mutants has revolutionized our understanding of this intriguing organelle (Waterham and Cregg 1997). Furthermore, the amino acid sequences predicted by yeast *PEX* genes have been utilized to identify their human counterparts, several of which have recently been shown to be defective in patients with the lethal peroxisomal biogenesis disorder Zellweger syndrome (Subramani 1997). The isolation of yeast mutants in novel *PEX* genes will undoubtedly provide further insights into peroxisomal biogenesis mechanisms as well as the identification of additional Zellweger genes.

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LITERATURE CITED

Albertini, M., P. Rehling, R. Erdmann, W. Girzalsky, J. A. K. W. Kiel *et al.*, 1997 Pex14p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. Cell **89:** 83–92.

Ciriacy, M., 1975 Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae*. Isolation and genetic analysis of *adh* mutants. Mutat. Res. **29:** 315–326.

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

Cregg, J. M., I. J. van der Klei, G. J. Sulter, M. Veenhuis and W. Harder, 1990 Peroxisome-deficient mutants of *Hansenula polymorpha*. Yeast **6:** 87–97.

Cregg, J. M., S. Shen, M. Johnson and H. Waterham, 1998 Classical genetic manipulation, Chapter 2, pp. 17–26 in *Methods in Molecular Biology, Pichia Protocols 1998*, edited by D. R. Higgins and J. M. Cregg. Humana Press Inc., Totowa, NJ.

de Hoop, M. J., and G. AB, 1992 Import of proteins into peroxisomes and other microbodies. Biochem. J. **286:** 657–669.

Distel, B., R. Erdmann, S. J. Gould, G. Blobel, D. I. Crane *et al.*, 1996 A unified nomenclature for peroxisome biogenesis factors. J. Cell Biol. **135:** 1–3.

Dommes, V., C. Baumgart and W.-H. Kunau, 1981 Degradation of unsaturated fatty acids in peroxisomes: existence of a 2,4-dieonyl-CoA reductase pathway. J. Biol. Chem. **256:** 8259–8262.

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

Dyer, L. M., J. A. McNew and J. M. Goodman, 1996 The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop. J. Cell Biol. **133:** 269–280.

Elgersma, Y., M. van den Berg, H. F. Tabak and B. Distel, 1993 An efficient positive selection procedure for the isolation of peroxisomal import and peroxisome assembly mutants of *Saccharomyces cerevisiae*. Genetics **135:** 731–740.

Elgersma, Y., L. Kwast, A. Klein, T. Voorn-Brouwer, M. van den Berg *et al.*, 1996 The SH3 domain of the *Saccharomyces cerevisiae* peroxisomal membrane protein Pex13p functions as a docking site for Pex5p, a mobile receptor for the import of PTS1-containing proteins. J. Cell Biol. **135:** 97–109.

Elgersma, Y., M. Elgersma-Hooisma, T. Wenzel, J. M. McCaffery, M. G. Farquhar *et al.*, 1998 A mobile PTS2 receptor for peroxisomal protein important in *Pichia pastoris*. J. Cell Biol. **140:** 807–820.

Erdmann, R., and G. Blobel, 1996 Identification of Pex13p, a peroxisomal membrane receptor for the PTS1 recognition factor. J. Cell Biol. **135:** 111–121.

Erdmann, R., M. Veenhuis and W.-H. Kunau, 1997 Peroxisomes: organelles at the crossroads. Trends Cell Biol. **7:** 400–406.

Glover, J. R., D. W. Andrews, S. Subramani and R. A. Rachubinski, 1994 Mutagenesis of the amino targeting signal of the *Saccharomyces cerevisiae* 3-ketoacyl-CoA thiolase reveals conserved amino acids required for the import into peroxisomes *in vivo*. J. Biol. Chem. **269:** 7558–7563.

Gould, S. J., G.-A. Keller and S. Subramani, 1987 Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. J. Cell Biol. **105:** 2923–2931.

Gould, S. J., D. McCollum, A. P. Spong, J. A. Heyman and S. Subramani, 1992 Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast **8:** 613–628.

Gould, S. J., J. E. Kalish, J. C. Morrell, J. Bjorkman, A. J. Urquhart *et al.*, 1996 Pex13p is an SH3 protein of the peroxisome membrane and a docking factor for the predominantly cytoplasmic PTS1 receptor. J. Cell Biol. **135:** 85–95.

Kalish, J. E., G.-A. Keller, J. C. Morrell, S. J. Mihalik, B. Smith *et al.*, 1996 Characterization of a novel component of the peroxisomal protein import apparatus using fluorescent peroxisomal proteins. EMBO J. **15:** 3275–3285.

Komori, M., S. W. Rasmussen, J. A. K. W. Kiel, R. J. S. Baerends, J. M. Cregg *et al.*, 1997 The *Hansenula polymorpha PEX14* gene encodes a novel peroxisomal membrane protein essential for peroxisome biogenesis. EMBO J. **16:** 44–53.

Koutz, P., G. R. Davis, C. Stillman, K. Barringer, J. Cregg *et al.*, 1989 Structural comparison of the *Pichia pastoris* alcohol oxidase genes. Yeast **5:** 167–177.

Liu, H., X. Tan, M. Veenhuis, B. McCollum and J. M. Cregg, 1992 An efficient screen for peroxisome-deficient mutants of *Pichia pastoris*. J. Bacteriol. **174:** 4943–4951.

Liu, H., X. Tan, K. Russell, M. Veenhuis and J. M. Cregg, 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., E. Monosov and S. Subramani, 1993 The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal 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. A., T. J. Wenzel, G. H. Luers, J. A. Heyman and S. Subramani, 1996 Labeling of peroxisomes with green fluorescent protein in living *P. pastoris* cells. J. Histochem. Cytochem. **44:** 581–589.

Osumi, T., T. Tsukamoto, S. Hata, S. Yokota, S. Miura *et al.*, 1991 Amino-terminal presequence of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisomal targeting. Biochem. Biophys. Res. Commun. **181:** 947–954.

Rachubinski, R. A., and S. Subramani, 1995 How proteins penetrate peroxisomes. Cell **83:** 525–528.

Rehling, P., M. Marzioch, F. Niesen, E. Wittke, M. Veenhuis *et al.*, 1996 The import receptor for the peroxisomal targeting signal 2 (PTS2) in *Saccharomyces cerevisiae* is encoded by the *PAS7* gene. EMBO J. **15:** 2901–2913.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shen, S., G. Sulter, T. W. Jeffries and J. M. Cregg, 1998 A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. Gene **216:** 93–102.

Sibirny, A. A., V. I. Titorenko, M. V. Gonchar, V. M. Ubiyvovk, G. P. Ksheminskaya *et al.*, 1988 Genetic control of methanol metabolism in yeast. J. Basic Microbiol. **28:** 293–319.

Sibirny, A. A., O. P. Vitvitskaya, A. R. Kulachkovsky and V. M. Ubiyvovk, 1989 Selection and properties of the mutants of methylotrophic yeast *Hansenula polymorpha* defective in alcohol oxidase. Mikrobiologiya (Moscow) **58:** 751–759.

Subramani, S., 1993 Protein import into peroxisomes and biogenesis of the organelle. Annu. Rev. Cell Biol. **9:** 445–478.

Subramani, S., 1997 *PEX* genes on the rise. Nat. Genet. **15:** 331–333.

Ueda, M., S. Mozaffar and A. Tanaka, 1990 Catalase from *Candida boidinii* 2201. Methods Enzymol. **188:** 463–465.

van der Klei, I. J., L. V. Bystrykh and W. Harder, 1990 Alcohol oxidase from *Hansenula polymorpha* CBS 4732. Methods Enzymol. **188:** 420–422.

van der Leij, I., M. van den Berg, R. Boot, M. Franse, B. Distel *et al.*, 1992 Isolation of peroxisome assembly mutants from *Saccharomyces cerevisiae* with different morphologies using a novel positive selection procedure. J. Cell Biol. **119:** 153–162.

Veenhuis, M., and W. Harder, 1987 Metabolic significance and biogenesis of microbodies in yeasts, pp. 436–458 in *Peroxisomes in Biology and Medicine*, edited by H. D. Fahimi and H. Sies. Springer-Verlag, Berlin.

Verduyn, C., J. P. van Dijken and W. A. Scheffers, 1984 Colorometric alcohol assays with alcohol oxidase. J. Microbiol. Methods **2:** 15–25.

Waterham, H. R., and J. M. Cregg, 1997 Peroxisome biogenesis. BioEssays **19:** 57–66.

Waterham, H. R., Y. de Vries, K. A. Russell, W. Xie, M. Veenhuis *et al.*, 1996 The *Pichia pastoris PER6* gene product is a peroxisomal integral membrane protein essential for peroxisome biogenesis and has sequence similarity to the Zellweger syndrome protein PAF-1. Mol. Cell. Biol. **16:** 2527–2536.

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

Waterham, H. R., K. A. Russell, Y. de Vries and J. M. Cregg, 1997 Peroxisomal targeting, import, and assembly of alcohol oxidase in *Pichia pastoris*. J. Cell Biol. **139:** 1419–1431.

Zhang, J. W., and P. B. Lazarow, 1996 Peb1p (Pas7p) is an intraperoxisomal receptor for the NH_2-terminal, Type 2, peroxisomal targeting sequence of thiolase: Peb1p itself is targeted to peroxisomes by an NH_2-terminal peptide. J. Cell Biol. **132:** 323–334.

Zhang, J. W., Y. Han and P. B. Lazarow, 1993 Novel peroxisome clustering mutants and peroxisome biogenesis mutants of *Saccharomyces cerevisiae*. J. Cell Biol. **123:** 1133–1147.

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