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## An Efficient Screen for Peroxisome-Deficient Mutants of *Pichia pastoris*

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**We describe a rapid and efficient screen for peroxisome-deficient (*per*) mutants in the yeast *Pichia pastoris*. The screen relies on the unusual ability of *P. pastoris* to grow on two carbon sources, methanol and oleic acid, both of which absolutely require peroxisomes to be metabolized. A collection of 280 methanol utilization-defective (*Mut*<sup>-</sup>) *P. pastoris* mutants was isolated, organized into 46 complementation groups, and tested for those that were also oleate-utilization defective (*Out*<sup>-</sup>) but still capable of growth on ethanol and glucose. Mutants in 10 groups met this phenotypic description, and 8 of these were observed by electron microscopy to be peroxisome deficient (*Per*<sup>-</sup>). In each *per* mutant, *Mut*<sup>-</sup>, *Out*<sup>-</sup>, and *Per*<sup>-</sup> phenotypes were tightly linked and therefore were most likely due to a mutation at a single locus. Subcellular fractionation experiments indicated that the peroxisomal marker enzyme catalase was mislocalized to the cytosol in both methanol- and oleate-induced cultures of the mutants. In contrast, alcohol oxidase, a peroxisomal methanol utilization pathway enzyme, was virtually absent from *per* mutant cells. The relative ease of *per* mutant isolation in *P. pastoris*, in conjunction with well-developed procedures for its molecular and genetic manipulation, makes this organism an attractive system for studies on peroxisome biogenesis.**

Virtually all eukaryotic cells harbor single-membrane-bound organelles called peroxisomes. They are the site of hydrogen peroxide-generating oxidative reactions in cells and, almost without exception, contain the heme enzyme catalase to break down this reactive compound (1, 24). Enzymes found within the peroxisome matrix are involved in a variety of important metabolic pathways. However, the specific pathways vary significantly depending upon the organism. In humans, peroxisomal enzymes are known to play an essential role in a number of anabolic and catabolic pathways, particularly in lipid metabolism (26). The importance of peroxisomes to humans is dramatically demonstrated by a family of lethal genetic disorders called Zellweger syndrome in which peroxisomes appear to be absent from cells (26, 50).

In recent years, basic features of peroxisome biogenesis have emerged. Peroxisomes do not appear to be synthesized *de novo* or to arise by budding from the endoplasmic reticulum as do secretory pathway organelles. Instead, peroxisomes form by budding from preexisting peroxisomes (48). Proteins destined for peroxisomal localization are synthesized on cytosolic or free ribosomes and are posttranslationally imported (14). To date, two peroxisomal targeting signals (PTSs) have been defined. A few peroxisomal enzymes, including rat 3-ketoacyl-coenzyme A (CoA) thiolase, are directed to the organelles by an amino-terminal signal sequence that is proteolytically cleaved upon import, a feature reminiscent of signal sequences on proteins destined for import into the endoplasmic reticulum, mitochondrion, or chloroplast (29, 39). However, the more commonly utilized PTS is a tripeptide sequence, serine-lysine-leucine (and a few conservative variants), located at the carboxy termi-

nus of many peroxisomal proteins (18, 19, 21). Additional PTS systems may exist as well. As observed for other organelles, peroxisomal protein translocation requires ATP hydrolysis (20). In addition, a proton gradient exists across the peroxisomal membrane (28), although its role in protein import, if any, is not clear.

To date, genetic methods have not been extensively utilized to investigate peroxisomes. In fact, the first description of a peroxisomal genetic defect was Zellweger syndrome (16). Cell fusion results indicate that the syndrome is a consequence of mutations in any one of at least six different genes (3, 26, 30). Although Zellweger cells were once thought to be completely devoid of peroxisomes, recent studies have revealed the presence of single-membrane-bound vesicles containing peroxisomal membrane proteins (25, 32, 33). Since these structures, termed peroxisomal ghosts, are without most matrix enzymes, it appears that the primary defect in Zellweger cells is a general inability to import these enzymes (33, 49). Peroxisome-deficient mutants in Chinese hamster ovary cell lines (41, 51, 52) and in the yeasts *Saccharomyces cerevisiae* (13) and *Hansenula polymorpha* (8, 9) have also been reported. In *S. cerevisiae*, two peroxisome-deficient mutants were identified among a collection of mutants defective in ability to grow on oleate, a carbon source whose metabolism requires a peroxisomal  $\beta$ -oxidation system in yeasts. Similarly, two *H. polymorpha* peroxisome-deficient mutants were isolated from a collection of mutants defective in ability to grow on methanol (8).

We are pursuing a genetic approach toward understanding peroxisome biogenesis and have selected the methylotrophic yeast *Pichia pastoris* as a model system. For these studies, *P. pastoris* presents certain advantages. First, peroxisomes are absolutely required for the metabolism of methanol as a consequence of the presence in the peroxisome of three

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TABLE 1. *P. pastoris* strains used

Strain	Genotype	No. of alleles	Source or reference
JC100	Wild type		NRRL Y-11430, <sup>a</sup> 6
GS115	<i>his4</i>		NRRL Y-15851, 6
GS190	<i>arg4</i>		NRRL Y-18014, 4
MS105	<i>fld<sup>b</sup></i>		5
JC101	<i>per1</i>	5	This study
JC105	<i>per2</i>	6	This study
JC111	<i>per3</i>	1	This study
JC112	<i>per4</i>	1	This study
JC113	<i>per5</i>	1	This study
JC114	<i>per6</i>	1	This study
JC115	<i>per7</i>	3	This study
JC118	<i>per8</i>	5	This study
JC126	<i>out1</i>		This study

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<sup>b</sup> Formaldehyde dehydrogenase defective.

pathway specific enzymes, alcohol oxidase (AOX), catalase, and dihydroxyacetone synthase (46). Second, methanol growth is robust, and mutants that are defective in its utilization are easily identified through standard replica plate screening methods. Third, peroxisomes in methanol-induced cells become numerous and massive (46, 48), a useful feature since the primary means of identifying peroxisome-deficient mutants is by direct electron microscopic examination. Fourth, *P. pastoris* also grows on oleic acid (36). Thus, *P. pastoris* is unique in that two easily observable phenotypes are available to screen for peroxisome-deficient mutants. Fifth, techniques for both classical and molecular genetic manipulation of this organism are highly developed (4, 6, 7, 10).

Our general strategy is to first isolate a comprehensive collection of peroxisome-deficient yeast mutants and then utilize the mutants to clone the affected genes by complementation. In this report, we describe an efficient screen for the isolation of peroxisome-deficient mutants (*per* mutants) of *P. pastoris*. We report the identification of *per* mutants defective in eight different genes and the effects of the mutations on the presence and subcellular localization of selected peroxisomal matrix enzymes.

## MATERIALS AND METHODS

**Strains, media, and growth conditions.** *P. pastoris* strains used in this study are listed in Table 1. Yeast strains were grown in either YPD (1% yeast extract, 2% peptone, 2% glucose) or YNB (0.67% yeast nitrogen base without amino acids) medium supplemented with one of the following carbon sources: 0.4% glucose, 0.5% glycerol, 0.5% ethanol, 0.5% methanol, or 0.1% oleic acid. Oleic acid medium was additionally supplemented with 0.5% Tween 40 to solubilize oleate. Amino acids were added to 50 µg/ml as required. Sporulation medium contained 0.5% sodium acetate, 1% potassium chloride, and 1% glucose. To measure growth of *P. pastoris* strains on oleic acid, cells were precultured in YPD to an optical density at 600 nm (OD<sub>600</sub>) of approximately 1.0 and transferred by centrifugation to YNB medium with Tween 40 and oleate at an initial OD<sub>600</sub> of about 0.1. Since *P. pastoris* grows to a significant extent on Tween 40 alone, parallel cultures of each strain were incubated in YNB medium with Tween 40 but without oleate. Oleate growth was judged as the difference in growth between cultures with and without oleate. To prepare methanol-induced cells, each

strain was precultured in YPD medium and shifted by centrifugation into YNB plus methanol medium. Methanol utilization-defective (Mut<sup>-</sup>) strains were inoculated into methanol medium at a starting OD<sub>600</sub> of 0.5 and harvested at the times indicated in the text. Mut<sup>+</sup> strains were inoculated at an OD<sub>600</sub> of 0.1 and harvested at an OD<sub>600</sub> of about 1.0. Oleate-induced cells were prepared in the same manner except for the substitution of YNB medium plus Tween 40 and oleic acid.

**Mutant isolation.** The procedure for mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was described in Cregg et al. (8), except that cells were grown at 30°C. Mutagenized cultures were brought to 30% in glycerol and stored frozen at -70°C until used. NTG treatment resulted in the death of approximately 90% of the cells, and freezing killed an additional 90%. To isolate Mut<sup>-</sup> mutants, mutagenized frozen cultures were thawed, washed twice in 25 ml of sterile water, and spread on YNB agar plates with 0.1% glucose at a dilution that produced approximately 500 colonies per plate. After 3 days at 30°C, colonies which formed were replica plated onto two sets of YNB plates; the first was supplemented with methanol, and the second was supplemented with glucose. After 3 days of incubation at 30°C, colonies which failed to grow on methanol were isolated and retested for tightness of Mut<sup>-</sup> phenotype, reversion rate, and glucose growth rate.

**Genetic analysis.** Mating and sporulation procedures were modified versions of those previously described (7). For complementation testing of the Mut<sup>-</sup> strain collection, each mutant to be tested was patched onto a YPD agar plate (up to 16 strains per plate) and spread onto a second YPD plate at a density sufficient to form a lawn (~10<sup>7</sup> cells per plate). After incubation at 30°C overnight, each patch plate was paired with a lawn plate, and the plates were serially replica plated onto sporulation agar medium to initiate mating. Cells were incubated at 30°C for 2 days on sporulation plates and then replica plated onto YNB agar supplemented with methanol to select for Mut<sup>+</sup> complementing diploid cells. After incubation for at least 3 days at 30°C, plates were scored. As a control for reversion, the procedure was also performed in parallel with plates that contained individual unmixed strains.

Procedures for backcrossing and random spore analysis in *P. pastoris* began with mating of desired sets of mutants and the selection of diploids as described for complementation analysis, except that mating was allowed to proceed for only one day at 30°C prior to replica plating onto an appropriate diploid selection medium. The purpose of the limited mating period was to ensure that diploids did not have time to sporulate prior to transfer to selection medium. Diploid colonies resulting from each mating were transferred onto a fresh plate of selection medium agar, incubated at 30°C overnight, transferred onto YPD plates, and then incubated overnight again. Diploid colonies were then streaked onto sporulation plates and incubated for 3 to 4 days at 30°C.

Spore analysis procedures were essentially as described in Gleason and Sudbery (15). Material from the spore plates was transferred to 1 ml of sterile water and mixed. To preferentially kill remaining vegetative cells, an equal volume of ethyl ether was added to the spore suspensions, which were then mixed vigorously and allowed to stand at room temperature for about 20 min. The spore preparations were then serially diluted with water, and aliquots of selected dilutions were spread on YPD plates. After incubation for 2 to 3 days at 30°C, the resulting colonies were picked

and streaked onto sets of plates containing agar media appropriate for phenotypic determination.

**Preparation of cell-free extracts.** To prepare cell-free extracts, 50 OD<sub>600</sub> units of each culture was washed twice by centrifugation with ice-cold 50 mM potassium phosphate buffer (pH 7.0), resuspended in 400  $\mu$ l of the same buffer, and transferred to a glass tube (13 by 100 mm) containing 0.5 g of 0.5-mm-diameter acid-washed glass beads. The mixture was vigorously mixed for 1 min by using a bench-top vortexer and held on ice for at least 1 min. This cycle of mixing and cooling was repeated a total of four times for each sample. Extracts were then transferred to a 1.5-ml microcentrifuge tube. Extract which remained trapped in the glass beads was recovered by washing the beads with 300  $\mu$ l of cold buffer and adding this wash to the extract. To remove cell debris, extracts were centrifuged for 5 min at approximately 10,000  $\times$  g. The top 400  $\mu$ l of supernatant was then transferred to a fresh microcentrifuge tube and held on ice for assay.

**Cell fractionation.** Protoplasts were prepared, lysed, and subjected to differential centrifugation by a modification of the procedure described by Kimiryo et al. (22). Approximately 500 OD<sub>600</sub> units of induced culture was washed twice with 10 ml of distilled water, once in 10 ml of MOPS buffer plus dithiothreitol (DTT) [5 mM K 3-(*N*-morpholino)propanesulfonate (pH 7.2), 0.5 M KCl, 10 mM DTT], and once in 10 ml of MOPS buffer without DTT. Cells were then converted to protoplasts by the addition of 0.8 mg of Zymolyase 100T (ICN, Costa Mesa, Calif.) and incubation at 30°C for 45 min. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation at 3,000  $\times$  g for 8 min and osmotically lysed by gentle resuspension in 5 ml of sorbitol-MES buffer [5 mM K 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0), 0.5 mM EDTA, 0.6 M sorbitol, 0.1% ethanol]. Resulting mixtures were then homogenized in a Potter-Elvehjem tissue grinder. Samples were centrifuged at 3,000  $\times$  g for 10 min to remove unbroken cells and other debris. Supernatants were collected and subjected to a second centrifugation at 20,000  $\times$  g for 20 min. The resulting peroxisome-containing pellets were resuspended in 500  $\mu$ l of sorbitol-MES buffer and, along with supernatants, were held on ice until assay.

**Miscellaneous methods.** Total protein was measured by the method of Bradford with bovine serum albumin as a standard (2). AOX (43), catalase (42), acyl-CoA oxidase (11), and fumarase (40) activities were measured according to published procedures. Specific activities for AOX, acyl-CoA oxidase, and fumarase were expressed as follows: 1 U was defined as 1  $\mu$ mol of product per min per mg of protein. Catalase specific activity was expressed as  $\Delta E_{240}$  per minute per milligram of protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting procedures were performed as previously described (23, 31). Nitrocellulose filters were incubated with affinity-purified rabbit antibody against AOX of *P. pastoris* (a gift from M. Gleeson, SIBIA, La Jolla, Calif.). Protein-antibody complexes were visualized by using alkaline phosphatase-conjugated goat anti-rabbit antibody and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a substrate (31). Purified *P. pastoris* AOX was purchased from Sigma Chemical Co., St. Louis, Mo. Yeast cell sections were prepared for electron microscopy as described by Cregg et al. (8) or by McConnell et al. (27).

## RESULTS

**Mutant isolation and screening.** Cultures of wild-type *P. pastoris* were subjected to NTG mutagenesis and stored frozen until use. To isolate methanol utilization-defective (Mut<sup>-</sup>) mutants, aliquots of mutagenized cultures were thawed, spread on minimal glucose agar, and after incubation to allow colony formation, were screened by replica plating for growth on methanol. Approximately 0.5% of cells surviving mutagenesis and freezing were Mut<sup>-</sup>, and 280 such mutants were collected.

The Mut<sup>-</sup> collection was organized into complementation groups. *P. pastoris* is a homothallic haploid yeast and initiates its sexual cycle in response to nitrogen starvation (7, 10). For complementation testing, each Mut<sup>-</sup> mutant was mated pairwise with each of the other mutants as described under Materials and Methods, and complementing diploids were selected by growth on methanol medium agar. A few mutants failed to complement any of the other mutants and were not examined further. These mutants may have harbored secondary mutations affecting mating or dominant Mut<sup>-</sup> alleles. The complementation results indicated that the collection represented mutations in approximately 46 different genes whose products are required for growth on methanol. From previous studies, the specific genetic defects were known for two groups. One group was defective in *AOX1*, the primary source of AOX in *P. pastoris* (7). The other group was defective in *FLD*, which encodes formaldehyde dehydrogenase, a cytosolic methanol pathway enzyme required for energy generation from methanol (5).

Representative Mut<sup>-</sup> strains from each complementation group were further examined for utilization of oleic acid and ethanol, carbon sources that along with methanol are indicators of the state of peroxisomes in yeasts. Oleate metabolism has been shown to require a peroxisomal  $\beta$ -oxidation system (13, 47). In contrast, peroxisomes are not required for growth on ethanol (8, 13, 37). Thus, we anticipated that peroxisome-deficient mutants of *P. pastoris* would be phenotypically Mut<sup>-</sup> and oleic acid-utilization defective (Out<sup>-</sup>) but still ethanol-utilization proficient (Eut<sup>+</sup>). Because *P. pastoris* grows much more readily on ethanol than oleate, the Mut<sup>-</sup> collection was first screened for growth on ethanol. Of the 46 Mut<sup>-</sup> groups, 34 were observed to be Eut<sup>+</sup>.

Oleate is a relatively poor growth substrate for *P. pastoris*. Therefore, to best quantify growth, mutant strains were cultured in liquid oleate medium and culture densities were determined by spectrophotometry. Particular attention was paid to cell growth attributable to Tween 40, which was present in oleate medium to help solubilize the fatty acid (13). Typical results for wild-type *P. pastoris* and an oleic acid utilization-defective (Out<sup>-</sup>) strain are shown in Fig. 1. Within 24 h after shift to medium without oleate, both wild-type and mutant cultures had increased in density to approximately the same extent, the result of growth on Tween 40 and residual YPD. In the presence of oleate, the wild-type culture reached a cell density of approximately four times that of the same culture without oleate. In contrast, Out<sup>-</sup> mutants showed no further increase in culture density above that observed in medium without oleate. Representatives of each Mut<sup>-</sup> but Eut<sup>+</sup> group were examined for oleic acid utilization, and 10 groups were found that were both Mut<sup>-</sup> and Out<sup>-</sup> but Eut<sup>+</sup>, the phenotype expected of peroxisome-deficient mutants. One representative of each of the 10 groups was crossed three to five times against *his4* and *arg4*, but otherwise wild-type *P. pastoris* strains and the resulting backcrossed strains were analyzed further.

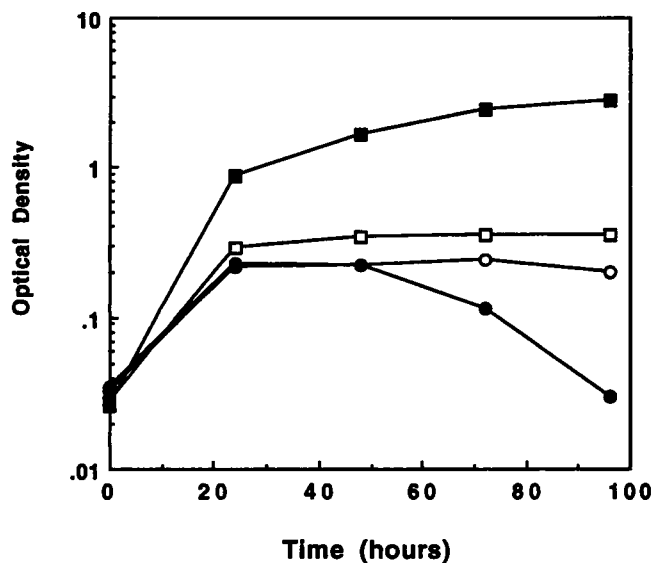


FIG. 1. Growth of *P. pastoris* on oleic acid. Time 0 is point at which YPD precultures were shifted to oleic acid growth test medium. Strains shown are JC100 (wild type) in medium with (■) or without (□) oleate and JC101 (*per1*) in medium with (●) or without (○) oleate.

**Identification of peroxisome-deficient mutants.** The 10 peroxisome-deficient candidates were subjected to extensive electron microscopic examination for the presence of peroxisomes. In preparation for this, each mutant was precultured in YPD medium and then incubated for approximately 20 h in medium containing methanol, conditions that lead to an increase in both the size and the number of peroxisomes in wild-type *P. pastoris*. Under electron microscopic examination, most sections of methanol-induced wild-type cells contained readily observable clusters of large peroxisomes (Fig. 2A). Sections derived from 2 of the 10 mutants contained peroxisomes that were comparable in size, number, and morphology to those observed in wild-type *P. pastoris* cells (not shown). The remaining eight mutants appeared to be peroxisome deficient in that cell sections derived from each were devoid of peroxisomes of the sort normally observed (Fig. 2B). In seven of the eight mutants, a few cell sections that contained small, irregularly shaped, single-membrane-bound vesicles were observed (Fig. 2C and D). The proportion of sections that displayed these vesicles varied between strains. One of the mutants, JC111, also contained cytosolic proteinaceous aggregates in addition to small membranous vesicles (Fig. 2E). Finally, the eighth mutant, JC101, appeared to be completely peroxisome deficient in that not even abnormal vesicles were observed. Peroxisome deficiency in these mutants was not an indirect consequence of their inability to metabolize methanol, since other *Mut*<sup>-</sup> mutants were capable of inducing peroxisomes that were morphologically indistinguishable from those observed in wild-type cells (not shown). We concluded that these eight mutant groups were peroxisome deficient (*Per*<sup>-</sup>).

The eight *Per*<sup>-</sup> strains were examined genetically to determine whether *Mut*, *Out*, and *Per* phenotypes were linked. Each mutant was crossed with a *his4 P. pastoris* strain, and 10 to 20 of the resulting spore progeny were selected at random and analyzed for phenotype. All progeny from these crosses were either *Mut*<sup>-</sup> *Out*<sup>-</sup> *Per*<sup>-</sup> or *Mut*<sup>+</sup>

*Out*<sup>+</sup> *Per*<sup>+</sup>, a result which demonstrated that the three phenotypes were tightly linked and therefore, likely to be a consequence of a defect in a single gene in each mutant. These genes were named *PER1* through *PER8* and are listed along with their mutant alleles in Table 1.

**Peroxisomal enzymes in *per* mutants.** The presence of selected peroxisomal matrix enzymes was investigated. For these experiments, the eight *per* mutants along with control strains were incubated in either methanol- or oleate-containing medium, prepared as cell-free extracts, and assayed for catalase, AOX, and acyl-CoA oxidase activities. Upon shifting to either medium, each *per* mutant induced approximately the same level of catalase as wild-type *P. pastoris* (Fig. 3A and B). Acyl-CoA oxidase, a  $\beta$ -oxidation pathway enzyme, reached specific activity levels in the *per* mutants that were only approximately 10% of those seen in wild-type *P. pastoris* (Fig. 4A). However, their reduced activity could be attributed to a general inability to utilize oleate, since *Out*<sup>-</sup> mutants with normal peroxisomes also displayed similarly reduced levels of acyl-CoA oxidase (Fig. 4A).

AOX presented a more complicated picture. In wild-type *P. pastoris*, shift to methanol resulted in a rapid 1,000-fold increase in AOX-specific activity (Fig. 4B). In contrast, AOX activity in each of the *per* mutants was much lower. At 4 h after shift to methanol, the peak of activity in the *per* mutants, AOX activity ranged from 0.4% of that of the wild type to undetectable (<0.0005 U). As a control for a general effect of carbon starvation on *Mut*<sup>-</sup> strains, activity was also measured in several *Mut*<sup>-</sup> but *Per*<sup>+</sup> *P. pastoris* mutants. AOX activity in these *Per*<sup>+</sup> strains was less than that in wild-type *P. pastoris* but not nearly as low as that observed in each of the *per* mutants (Fig. 4B).

Active AOX is a homo-octamer which, in wild-type methylotrophic yeast cells, is transported into peroxisomes as an inactive monomer where it is then assembled into the active form (17). It was possible that in our *per* mutants, AOX was synthesized but remained in an inactive form in the cytosol. To investigate whether inactive AOX might be accumulating in the mutants, cell-free extracts were prepared from methanol-induced cells and probed for AOX protein by using anti-AOX polyclonal antibodies. Figure 5 shows an example of the results observed in these experiments. Each lane contained a 10- $\mu$ g aliquot of extract (Fig. 5, lower panel). Extracts prepared from *P. pastoris* wild-type and *fld* mutant strains (lanes 1 and 3; AOX specific activities of 1.1 and 0.15 U/mg of protein, respectively) showed strong anti-AOX reacting bands at 72 kDa, the known molecular mass of AOX monomer (12). (The relative band intensities in the two samples do not appear to be proportional to activity because of saturation of the filter by the wild-type sample.) As a negative control, an extract prepared from glucose-grown wild-type cells which contained no AOX activity showed no detectable AOX protein (lane 2). Finally, lanes 4 and 5 contained samples of extracts prepared from methanol-induced cultures of *per1* and *per3* strains (AOX specific activities of 0.003 and 0.03 U/mg of protein, respectively) and showed only very small amounts of AOX protein. Together, the immunoblot experiments revealed no significant differences between AOX activity and protein levels, suggesting that large amounts of inactive AOX protein were not present in the *per* mutants.

**Subcellular localization of peroxisomal enzymes.** Each *per* mutant was examined for the subcellular location of selected peroxisomal matrix enzymes by differential centrifugation. Methanol- or oleate-induced cells of each mutant and control strains were protoplasted, homogenized, and after a low-

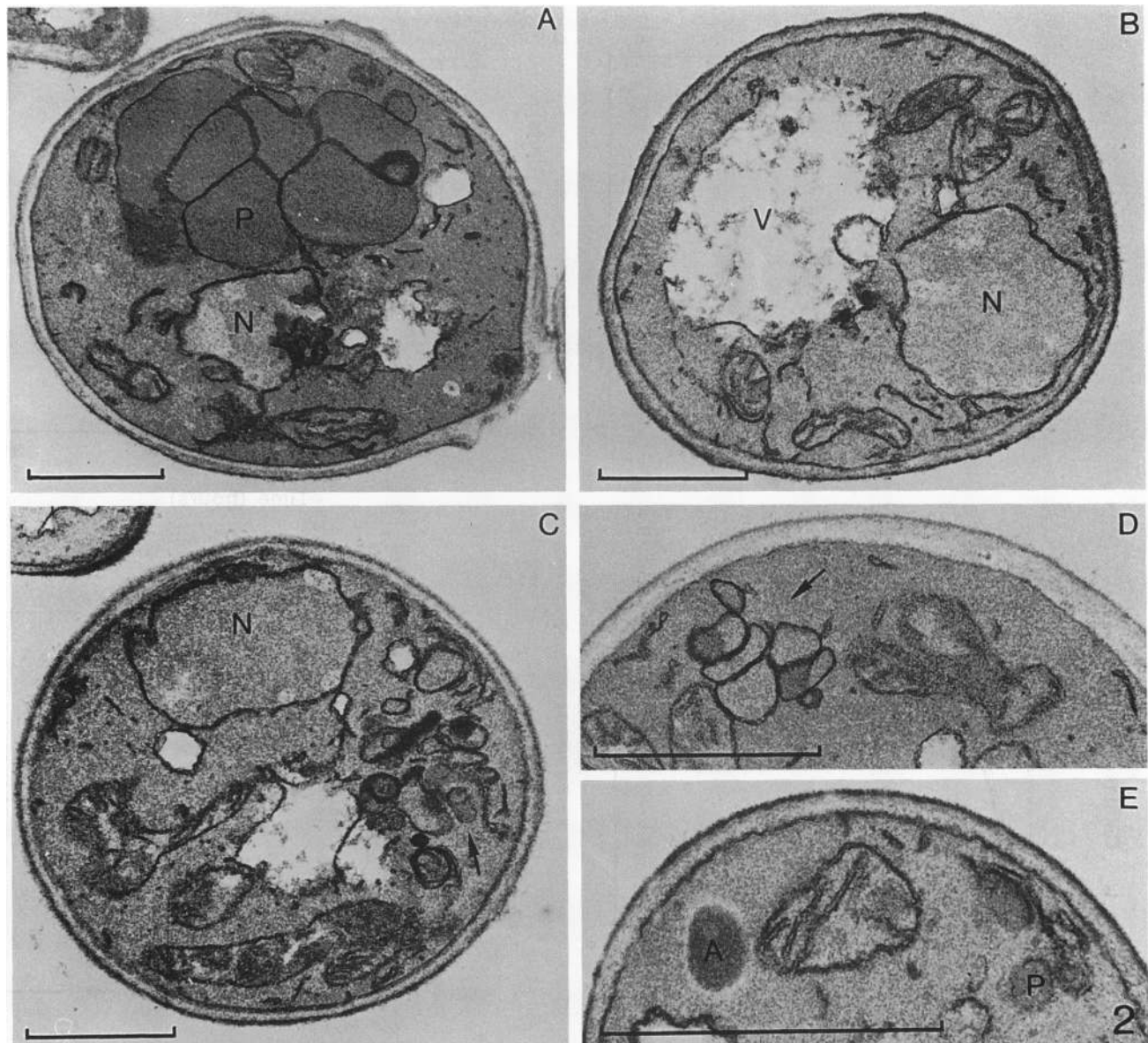


FIG. 2. Morphology of selected methanol-induced *P. pastoris* strains. Each strain was precultured in YPD, transferred to methanol-containing medium, and harvested after approximately 20 h. Strains shown are as follows: A, JC100 (wild type); B, JC101 (*per1*); C and D, JC111 (*per3*); E, JC112 (*per4*). Abbreviations: A, aggregate; N, nucleus; P, peroxisome; V, vacuole. Arrows point to abnormal membrane-bounded vesicles. Bar, 1  $\mu\text{m}$ .

speed centrifugation to remove unbroken cells and other debris, centrifuged at  $20,000 \times g$ , a force sufficient to sediment small organelles such as mitochondria and peroxisomes (22). Enzyme-specific activities were then determined in supernatant and pellet fractions and expressed as a ratio. As shown in Table 2, catalase ratios from methanol- or oleate-induced cultures of  $\text{Per}^+$  control strains were low, demonstrating that the majority of the enzyme was sedimentable. Addition of Triton X-100 to 0.05% to homogenates prior to centrifugation caused a dramatic shift of catalase to supernatant fractions, a result that suggested that sedimentable catalase was present within a membrane-enclosed vesicle. In contrast to control strains, *per* mutant ratios were high, suggesting that the enzyme was localized primarily in

the cytosol. The high ratios did not appear to be a result of an increased sensitivity of *per* cells to the fractionation procedure or to unequal handling during preparation, since activity ratios for fumarase, a mitochondrial marker enzyme, remained low.

Attempts to determine AOX activity ratios from mutant subcellular fractions were largely unsuccessful because of their low levels of activity (Table 2). However, it was clear that any AOX activity in *per* mutants was highly sedimentable. As observed for catalase, addition of Triton X-100 prior to  $20,000 \times g$  centrifugation resulted in release of AOX to supernatant fractions (Table 2). Thus, the small amount of AOX activity in *per* mutants appeared to be within a membrane-bounded structure.

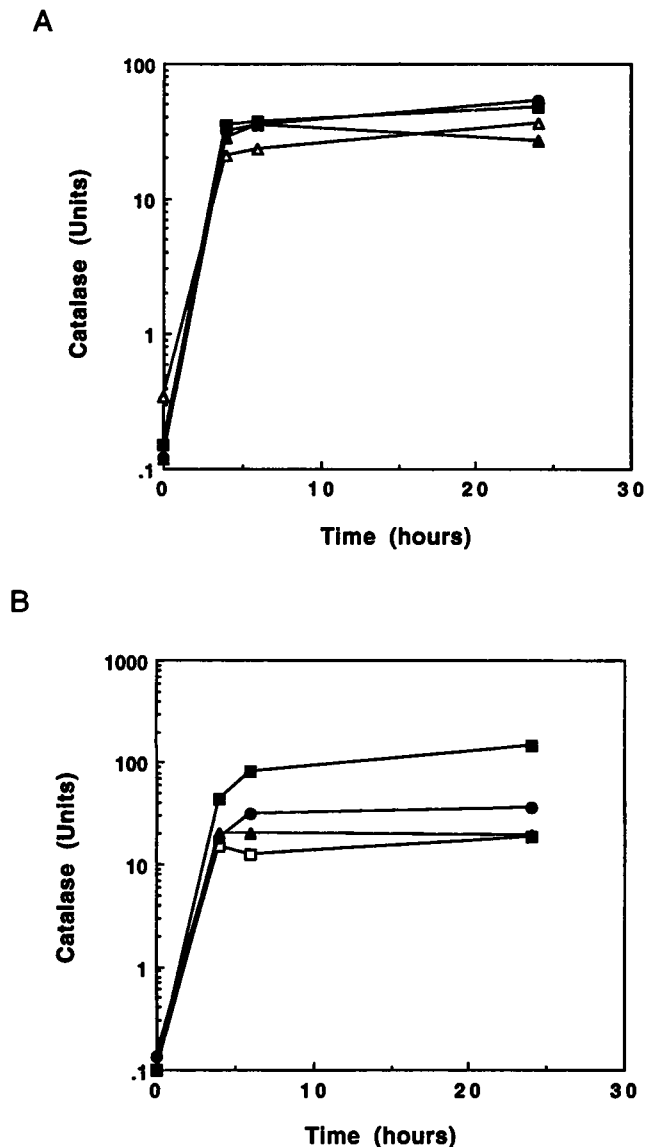


FIG. 3. Induction of catalase activity in peroxisome-deficient *P. pastoris* strains. Catalase activities in cell-free extracts of cultures incubated in methanol (A) and oleate (B) media. Time 0, cultures were shifted from YPD to induction media. Catalase activity is expressed as  $\Delta E_{240}$  per minute per milligram of protein. Strains shown are JC100 (wild type) (■), MS105 (*fld*) (△), JC126 (*out1*) (□), JC101 (*per1*) (●), and JC105 (*per2*) (▲).

## DISCUSSION

Our laboratory is interested in understanding peroxisome biogenesis and function at the molecular level. Toward that end, we have initiated a genetic approach with the methylotrophic yeast *P. pastoris* as a model system. In this report, we describe a critical tool required for these investigations, a rapid and efficient procedure for isolating peroxisome-deficient mutants (*per* mutants) of this yeast. In addition, we report the identification and initial characterization of a significant collection of *per* mutants. The key to the success of the mutant isolation scheme is the ability of *P. pastoris* to utilize two unusual carbon sources, methanol and oleic acid, both of which are dependent on peroxisomes for their

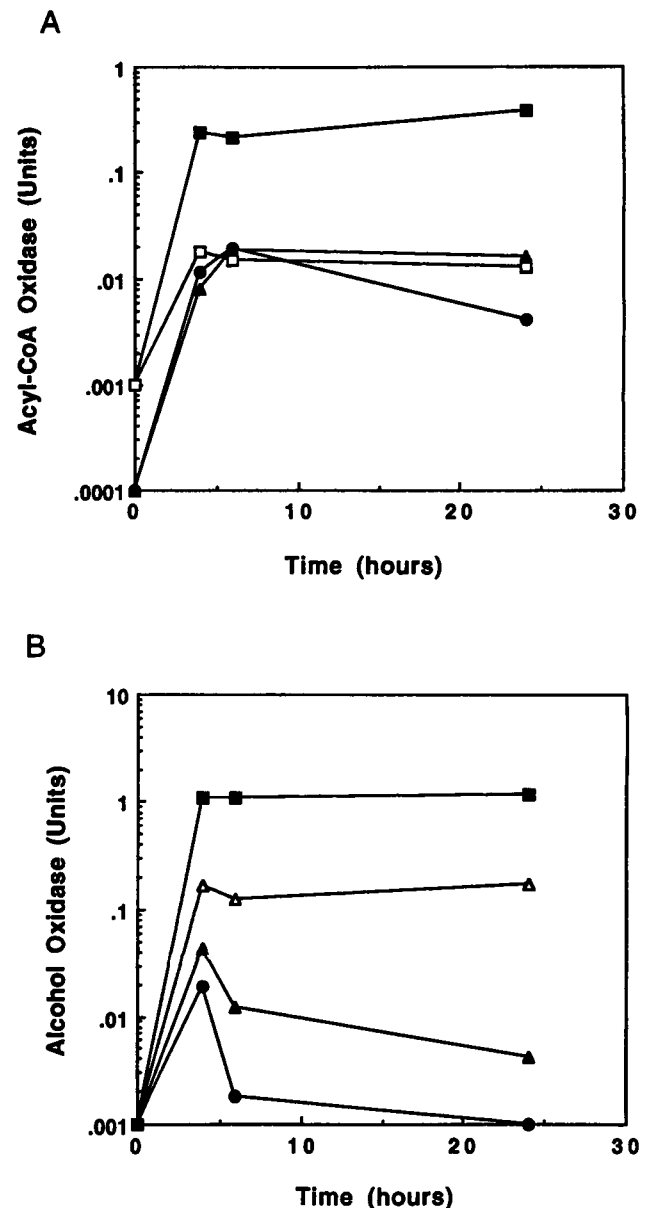


FIG. 4. Induction of acyl-CoA oxidase and AOX activities in *P. pastoris per* mutants. (A) Acyl-CoA oxidase activity at selected times after shift to oleate medium; (B) AOX activity after shift to methanol medium. Culture conditions and strain symbols are the same as described in the legend to Fig. 3.

metabolism (8, 13). By selecting for mutants that are simultaneously methanol- and oleate-utilization defective ( $Mut^-$  and  $Out^-$ , respectively), most mutants affected in nonperoxisomal genes are eliminated from consideration. The effectiveness of this screen is a major advantage, since the primary method of identifying peroxisome-deficient mutants is direct examination of the organelles by electron microscopy, a process that is tedious and time-consuming when applied to large mutant collections. To our knowledge, *P. pastoris* is unique in having two easily observable growth phenotypes linked to the state of peroxisomes.

Another aspect of the *per* mutant isolation scheme is testing mutants for growth on other carbon sources such as



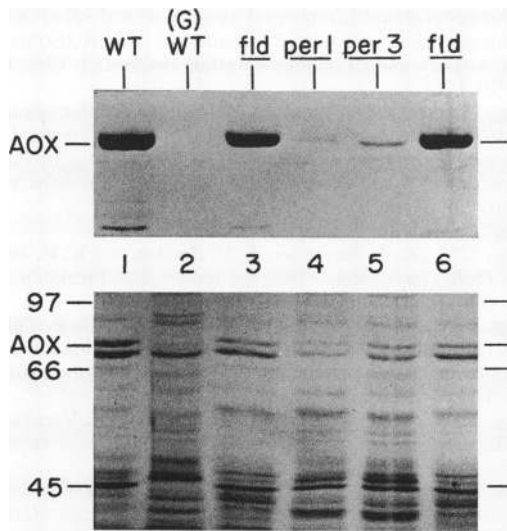


FIG. 5. Detection of AOX protein in cell-free extracts of methanol-induced *P. pastoris per* strains. Extracts were prepared, reduced, denatured, and separated by electrophoresis through a 10% polyacrylamide gel. Each lane contains 10  $\mu$ g of the following samples: lanes 1 and 2, methanol- and glucose-grown wild-type *P. pastoris*, respectively; lanes 3 and 6, methanol-induced MS105 (*fld*); lanes 4 and 5, methanol-induced JC101 (*per1*) and JC111 (*per3*), respectively. The lower panel is a gel in which proteins were stained with Coomassie blue dye. The upper panel is an immunoblot of the same samples reacted with polyclonal anti-AOX antibodies.

ethanol or acetate. Peroxisome-deficient mutants in three yeast species—*S. cerevisiae*, *H. polymorpha*, and *P. pastoris*—have now been described, and all are competent in metabolism of  $C_2$  substrates (8, 13, 37). This is somewhat surprising since  $C_2$  growth requires glyoxylate pathway enzymes which are located in peroxisomes (glyoxysomes). In *H. polymorpha*, glyoxysomes are clearly affected in *per* mutants, since pathway enzymes isocitrate lyase and malate synthase are mislocalized to the cytosol (8, 37). The continued ability of *per* mutants to utilize ethanol and other carbon sources is particularly useful in eliminating regulatory mutants that are defective in ability to derepress alternate carbon source utilization pathways. Such mutants fail to proliferate peroxisomes when induced and therefore appear to be peroxisome deficient (35).

We applied this phenotypic screen to a collection of 280 *Mut*<sup>-</sup> *P. pastoris* mutants representing approximately 46 complementation groups and found 10 groups that are *Mut*<sup>-</sup> and *Out*<sup>-</sup> but capable of growth on ethanol and glucose. Electron microscopic examination of methanol-induced cells of representative strains from these groups revealed that eight are grossly deficient in peroxisomes (*Per*<sup>-</sup>) while two contain normal-appearing peroxisomes (*Per*<sup>+</sup>). The existence of *Mut*<sup>-</sup> and *Out*<sup>-</sup> but *Per*<sup>+</sup> strains suggests that genes whose products are specifically required for growth on methanol and oleate exist but are not directly involved in peroxisome function. On the other hand, it may be that these mutants are affected in peroxisome function but in a way that does not alter the morphology of the organelle. More importantly, considering that 8 of 10 groups are *per* mutants, the screen is remarkably efficient. Additional *P. pastoris* peroxisome-deficient mutants have been isolated by using this screen (36), and complementation results indicate that the 2

TABLE 2. Enzyme distribution after differential centrifugation

Strain and treatment <sup>a</sup>	Sp act <sup>b</sup> under following growth condition <sup>c</sup> :				
	Methanol			Oleate	
	Catalase	AOX	Fumarase	Catalase	Fumarase
JC100 (wild type)					
None	0.4	0.26	0.13	0.80	0.16
+TX	5.2	4.8	5.2	30.5	8.2
MS105( <i>fld</i> )					
None	0.27	0.16	1.0	—	—
+TX	17.1	26.7	9.6	—	—
JC126( <i>out1</i> )					
None	—	—	—	1.6	0.2
+TX	—	—	—	25.3	17.3
JC101( <i>per1</i> )					
None	9.5*	0.07*	0.10*	31.7	0.17
+TX	12.0*	6.4*	2.8*	20.2	6.2
JC105( <i>per2</i> )					
None	7.0*	0.04*	0.37*	16.3	0.28
+TX	7.1*	15.1*	7.1*	17.9	10.6
No treatment					
JC111 ( <i>per3</i> )	11.3	—	0.41	23.3	0.15
JC112 ( <i>per4</i> )	6.2	—	0.42	22.0	0.03
JC113 ( <i>per5</i> )	12.8	—	0.77	27.6	0.08
JC114 ( <i>per6</i> )	8.1	—	0.54	24.2	0.34
JC115 ( <i>per7</i> )	6.7	—	0.40	16.5	0.04
JC118 ( <i>per8</i> )	10.1	—	0.33	27.8	0.17

<sup>a</sup> +TX, Triton X-100 added to 0.05% prior to final centrifugation.

<sup>b</sup> Data are expressed as the ratio of the specific activities present in the 20,000  $\times$  g supernatant and pellet fractions. —, not determined.

<sup>c</sup> Cultures were induced in oleate medium for 5 h or in methanol medium for 20 h with the exception of those designated with an asterisk, which were incubated for 5 h in methanol medium.

collections represent mutations in 12 different peroxisomal genes (5).

Subcellular fractionation experiments provided independent evidence that the 8 *per* mutants are peroxisome deficient. In both methanol- and oleate-induced cells of each mutant, catalase activity was located almost entirely in the 20,000  $\times$  g supernatant fraction, a result that suggests the enzyme is mislocalized to the cytosol. In contrast, catalase activity is present primarily in 20,000  $\times$  g pellet fraction in samples prepared from *Per*<sup>+</sup> control strains. It should be noted that, even in these control strains, a significant portion of catalase was consistently present in supernatant fractions. This may be an artifact caused by peroxisome leakage or breakage during the fractionation procedure. In *H. polymorpha*, similar catalase ratios are obtained despite the fact that catalase is exclusively peroxisomal in this yeast (45). However, it is also possible that, like *S. cerevisiae*, *P. pastoris* harbors both cytosolic and peroxisomal catalases (44).

Interestingly, AOX, a peroxisomal methanol pathway enzyme, is virtually absent in methanol-induced cells of each *per* mutant. Precedents for this result have been reported for certain matrix enzymes in peroxisome-deficient Chinese hamster ovary (CHO) cells and Zellweger fibroblasts (38, 51). For two of these enzymes, acyl-CoA oxidase and thiolase, pulse-labeling experiments indicated that the enzymes are synthesized but, as a consequence of their failure to be imported, are rapidly degraded in the cytosol (34, 38). If this is also the case with AOX in our *per* mutants, it would



be the first description of this phenomenon in a yeast. Experiments aimed at examining the fate of AOX in the *per* mutants are in progress.

The small amount of AOX that is present in *per* mutants is sedimentable. Furthermore, treatment of *per* cell homogenates with Triton X-100 prior to centrifugation releases AOX into the supernatant, a result that suggests the enzyme is present within a membrane-bound structure. The simplest explanation for these observations is that the *per* mutations are slightly "leaky," and as a result, a small amount of AOX is imported into a few remaining peroxisomes where it assembles into active enzyme.

The *P. pastoris per* mutants share a number of similarities with peroxisome-deficient mutants described for *S. cerevisiae*, *H. polymorpha*, CHO, and human Zellweger cell lines (8, 13, 26, 51). First, in all five species, peroxisome deficiency results from recessive mutations in any one of a number of different genes. Second, catalase as well as certain other matrix enzymes are active but appear to be mislocalized to the cytosol. Third, normal peroxisomes are virtually absent from the cells, and in their place, abnormal single-membrane-enclosed vesicles are observed. In peroxisome-deficient CHO mutants and in Zellweger cell lines, it has been shown that these vesicles are without most matrix enzymes but do contain peroxisomal membrane proteins (25, 32, 33, 41, 51). The presence of these peroxisomal remnants, termed ghosts, has led to the idea that the mutants are defective in a major matrix protein import system (33). We have yet to establish whether the vesicles we observe in the *P. pastoris per* mutants are the equivalent of peroxisomal ghosts.

In summary, the mechanisms responsible for peroxisome biogenesis remain a major unsolved mystery in cell biology. We believe that a combined genetic and biochemical attack is best able to solve that mystery. We have demonstrated that mutants with defects in peroxisome function are easily isolated in the yeast *P. pastoris* because of the fortuitous presence of two peroxisome-dependent carbon source utilization pathways in this organism. The existence of *P. pastoris per* mutants along with efficient transformation vectors and host strains for this yeast have enabled us to take the next major step in our studies, the cloning of *P. pastoris PER* genes by complementation. To date, we have isolated genes *PER3*, *PER4*, and *PER6* (5). The DNA sequences of these and other *PER* genes are expected to provide useful insights into the function of their products and their roles in peroxisome biogenesis.

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