Novel Genetic Selections for Peroxisome Biogenesis Mutants (*pex*) and the Isolation and Characterization of *PEX14* and Pex14p in *Pichia pastoris*

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Abstract

Novel Genetic Selections for Peroxisome Biogenesis Mutants (*pex*) and the Isolation and Characterization of *PEX14* and Pex14p in *Pichia pastoris*

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This thesis describes two positive selection schemes for the direct selection of peroxisome-biogenesis-defective (*pex*) mutants of the methylotrophic yeast *Pichia pastoris*. Both schemes take advantage of the observation that in *P. pastoris*, methanol-induced *pex* mutants have little or no activity for alcohol oxidase (AOX), the first enzyme in the methanol metabolic 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 to allyl alcohol selectively kills wild-type cells, resulting in an enrichment for *pex* mutants. The second scheme utilizes a *P. pastoris* strain that is defective in formaldehyde dehydrogenase (FLD1), an enzyme required to metabolize the formaldehyde generated by AOX's oxidation of methanol. AOX-induced *fld1* cells are sensitive to high levels of methanol due to the resulting toxic accumulation of formaldehyde while *fld1 pex* mutants, with little or no AOX activity, are not. These selections resulted in the isolation of *pex* alleles in previously identified *PEX* genes as well as the isolation of alleles in three novel *PEX* groups and two groups encoding putative transcription factors.

From one of those novel *PEX* groups, *PEX14* was cloned via functional complementation. We showed that Pex14p is peripherally associated with the cytosolic side of the peroxisomal membrane and is essential for the import of peroxisomal targeting signal (PTS) 1 and PTS2 proteins but not for the targeting of peroxisomal membrane proteins. We also found that Pex14p interacts directly with Pex5p, Pex7p, Pex13p and Pex17p, all components of the peroxisomal import machinery. Finally, we discovered that Pex14p is present as a mixture of phosphorylated and unphosphorylated forms and that the phosphorylated form of Pex14p is the predominant form found in complexes with Pex13p and Pex17p. We propose a trigger model for the function of phosphorylated Pex14p in which this event is key in regulating peroxisomal matrix protein import.

CHAPTER 1
INTRODUCTION

1.1 Peroxisomes: General Characteristics

Peroxisomes, present in virtually all eukaryotic cells, were the last of the major subcellular organelles to be discovered. Originally called microbodies by Rhodin [Rhodin, 1954], the name peroxisome came later from the finding that these organelles contained enzymes involved in the production and degradation of hydrogen peroxide (oxidases and catalase, respectively) [de Duve and Baudhuin, 1966]. The presence of at least one hydrogen peroxide-generating oxidase is the unifying feature that defines peroxisomes in all species.

Peroxisomes are unique among eukaryotic organelles in that peroxisomes are highly diverse, varying dramatically in size, abundance and enzyme content depending upon the metabolic needs of the organism or tissue [Veenhuis and Goodman, 1990]. In methylotrophic yeast grown on glucose, for example, peroxisomes are small and few in number but when shifted to methanol, undergo massive proliferation [Veenhuis and Harder, 1991]. In mammals, peroxisomes are involved in a variety of essential catabolic and anabolic pathways such as the oxidative degradation of very long-chain fatty acids, purines, amino acids, and pipecolic acid as well as the biosynthesis of plasmalogens, cholesterol, and bile acid [Wanders, 1999]. Peroxisomes are particularly abundant in human liver and kidney cells and are often located close to the sites of lipid synthesis such as the endoplasmic reticulum [Bock et al., 1980]. As demonstrated by Zellweger syndrome and other lethal peroxisomal biogenesis disorders, peroxisomes are indispensable for human survival [Subramani, 1997].

Glyoxysomes, specialized peroxisomes found only in plants, have the enzymatic capacity to convert fatty acids to carbohydrates, via the glyoxylate

pathway, in germinating fatty seeds [Olsen and Harada, 1995]. Another subset of peroxisomes, the glycosomes, is found only in kinetoplastid protozoa, which includes the trypanosomatid pathogens causing sleeping sickness and leishmaniasis [Flaspohler et al., 1997]. The glycosomal compartmentalization of the first six enzymes of glycolysis is hypothesized to be important for the high glycolytic rate of African trypanosomes [Opperdoes, 1987].

Morphologically, peroxisomes range in diameter from 0.1 to 1.0 μM and are bounded by a single peroxisomal membrane which is 4.5 to 8 nanometers thick [Beard and Allen, 1968; Cavalier-Smith, 1987]. Peroxisomes are often characterized in electron micrographs by the presence of a crystalline core. In methylotrophic yeasts, this core is composed mostly of alcohol oxidase, while in mammalian tissue it is primarily urate oxidase.

Because peroxisomes harbor no genome or protein synthetic machinery, all peroxisomal proteins are believed to be synthesized on free polysomes and then imported into the organelle post-translationally [Subramani, 1993]. The peroxisomal matrix is acidic (pH 5.8–6.0) relative to the cytosol (pH 7.1) [Nicolay et al., 1987]. While ATP hydrolysis appears to be required for import, the proton gradient that exists across the peroxisomal membrane is not required [Imanaka et al., 1987; Wendland and Subramani, 1993].

In the last decade, tremendous progress has been made in the elucidation of peroxisome biogenesis (protein import, organelle proliferation and segregation, and membrane lipid acquisition) and in understanding the contribution of peroxisomes to metabolic processes in yeast, plants and animals. With the recognition of its indispensable nature and its clinical significance with respect to human disorders, the peroxisome has moved to the biological center stage.

1.2 Selected Peroxisomal Metabolic Pathways

1.2.1 β-Oxidation of fatty acids

The presence of a fatty acid β-oxidation system outside of the mitochondria was first identified in glyoxysomes from germinating castor bean endosperm [Cooper

and Beevers, 1969] and later in peroxisomes from rat liver [Lazarow and de Duve, 1976], protozoans [Blum, 1973] and fungi [Veenhuis and Harder, 1987]. In fungi, peroxisomes are the sole site of β-oxidation [Mannaerts and van Veldhoven, 1996]. In plants, peroxisomes are capable of β-oxidation of fatty acids, but whether mitochondria participate remains controversial. In mammals, both peroxisomes and mitochondria are sites of β-oxidation of fatty acids. A primary role of the mammalian peroxisome is to shorten very long chain fatty acids (VLCFAs), C_{24} or greater, to long or medium chain fatty acids and then export these as acyl-CoAs to the mitochondrion for oxidation to acetyl-CoA or esterification [Farrell and Bieber, 1983; Lazarow, 1988]. VLCFAs are not efficiently oxidized in the mitochondria, most probably due to the mitochondria's lack of a very long chain acyl-CoA synthetase. Acetyl-CoA units, also products of chain shortening, may be sent to the mitochondrion or, when the cell has ample energy supplies, may be retained in the peroxisome and used in anabolic reactions, e.g., cholesterol and dolichol synthesis [Lazarow, 1982]. Lazarow and de Duve were the first to observe this partial β-oxidation, or chain-shortening, phenomenon performed in mammalian peroxisomes [Lazarow and de Duve, 1976]. Carbon-chain shortening in the mammalian peroxisome is thought to stop at an acyl chain length of approximately 8 carbon atoms since the peroxisomal acyl-CoA oxidase cannot oxidize acyl-CoA shorter than this [Hashimoto, 1996].

The essential function of peroxisomal β-oxidation is illustrated by the accumulation of VLCFAs, dicarboxylic acids and cholestanoic acid in patients with X-linked adrenoleukodystrophy, the most common peroxisomal disorder in humans [Osmundsen et al., 1991]. This disease is often lethal and results from a specific deficiency in peroxisomal VLCFA oxidation.

While long-chain fatty acids are oxidized preferentially in the mitochondria in mammalian cells, the following, in addition to VLCFAs, are predominantly or exclusively oxidized in mammalian peroxisomes: long-chain dicarboxylic fatty acids [Cerdan et al., 1988; Leighton et al., 1989], isoprenoid derived 2-methyl-branched fatty acids [Van Hove et al., 1991], eicosanoids such as prostaglandins [Schepers et al., 1988], branched chain fatty acids [Singh et al., 1990], and a number of

xenobiotic acyl compounds [Gatt et al., 1988]. The fate of most of these acyl-group shortened lipids, i.e., those derived from eiconsanoids, bile acids, xenobiotics, fat-soluble vitamins, is excretion; while the end product of β-oxidation of long chain dicarboxylic fatty acids is acetate [Leighton et al., 1989; Mannaerts and van Veldhoven, 1996].

The first step of peroxisomal β-oxidation of fatty acids is activation by a substrate- specific acyl-CoA synthetase, present on the peroxisomal membrane, to the corresponding fatty acyl-CoA ester, a reaction that requires both CoA and ATP [Shindo and Hashimoto, 1978; Mannaerts et al., 1982; Bhushan et al., 1986]. Acyl-CoAs are then transported across the peroxisomal membrane and oxidized by acyl-CoA oxidase, to 2-enoyl-CoA. The mechanism by which acyl-CoAs penetrate the peroxisomal membrane is unknown but, unlike the mitochondrial import, is not carnitine dependent. H_2O_2 is formed in this first oxidation reaction and then is converted to water and oxygen by catalase. 2-Enoyl-CoA is then converted to 3-ketoacyl-CoA in a two-step reaction catalyzed by bifunctional 2-enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase [Osumi and Hashimoto, 1978; Furuta et al., 1982]. Lastly, 3-ketoacyl-CoA is converted to acyl-CoA and acetyl-CoA by the activity of 3-oxoacyl-CoA thiolase [Miyazawa et al., 1981]. As previously mentioned, in mammals the oxidation is incomplete and results in chain shortening to approximately C8. However, in yeast the oxidation proceeds completely to acetyl-CoA as the final product [Kunau et al., 1988].

Another important feature of peroxisomal β-oxidation is the preference toward oxidation of unsaturated fatty acids using auxiliary enzymes. The rate of peroxisomal oxidation of unsaturated fatty acids is comparable to, or greater than that of the mitochondrion [Vamecq and Draye, 1989; Osmundsen et al., 1991]. A final noteworthy contribution of mammalian peroxisomal β-oxidation is the conversion of cholesterol to bile acids involved in the digestion of lipids [Pedersen, 1993].

In summary, in humans, peroxisomes are required not only for VLCFA chain shortening reactions, but also for the predominant or exclusive oxidation of many other acyl compounds. In yeast, peroxisomes are the exclusive site of β-oxidation.

1.2.2 Sterol biosynthesis

As mentioned in Section 1.2.1, it is thought that acetyl-CoA produced during peroxisomal β-oxidation is used in part for anabolic reactions such as cholesterol synthesis. Krisans and coworkers have shown that the conversion of mevalonate to farnesyl-diphosphate (FPP) occurs predominately in the peroxisome [Krisans, 1996] (Fig. 1.1B). Both mevalonate kinase and FPP synthase have been shown to be predominately, if not exclusively, peroxisomal [Biardi and Krisans, 1996].

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the key regulatory enzyme of cholesterol biosynthesis, was previously believed to reside exclusively in the endoplasmic reticulum (ER) but was later proven to be peroxisomal as well [Keller et al., 1985]. Finally, apolipoprotein E (apo E) is localized to the peroxisome. Apo E is thought to function in cholesterol transport and homeostasis [Hamilton et al., 1990].

In rat liver, peroxisomal cholesterol biosynthesis accounts for about 20% of total cholesterol synthesis [Ericsson et al., 1993]. Normal cholesterol synthesis in human cells does not take place without functional peroxisomes since patients suffering from peroxisomal deficiency diseases have low serum cholesterol levels [Hodge et al., 1991].

Dolichol, a polyisoprenoid involved in the process of membrane stability and permeability, is also central to the formation of *N*-glycosidically linked oligosaccharide chains prior to their transfer to polypeptide chains. Dolichol and cholesterol metabolism share several initial steps (acetate→mevalonate→FPP). Ericsson and coworkers showed that peroxisomes are able to synthesize dolichol from mevalonate and contribute approximately one-half of the total dolichol synthesized in rat liver [Ericsson et al., 1993].

1.2.3 Plasmalogen biosynthesis

As mentioned in Section 1.2.1, the most common fate of shortened acyl-CoAs is export to the mitochondrion for further oxidation. In contrast, in brain tissue the peroxisomal acyl-CoA products may instead be shuttled into the plasmalogen biosynthetic pathway [Masters and Crane, 1995]. Plasmalogens are ether-linked

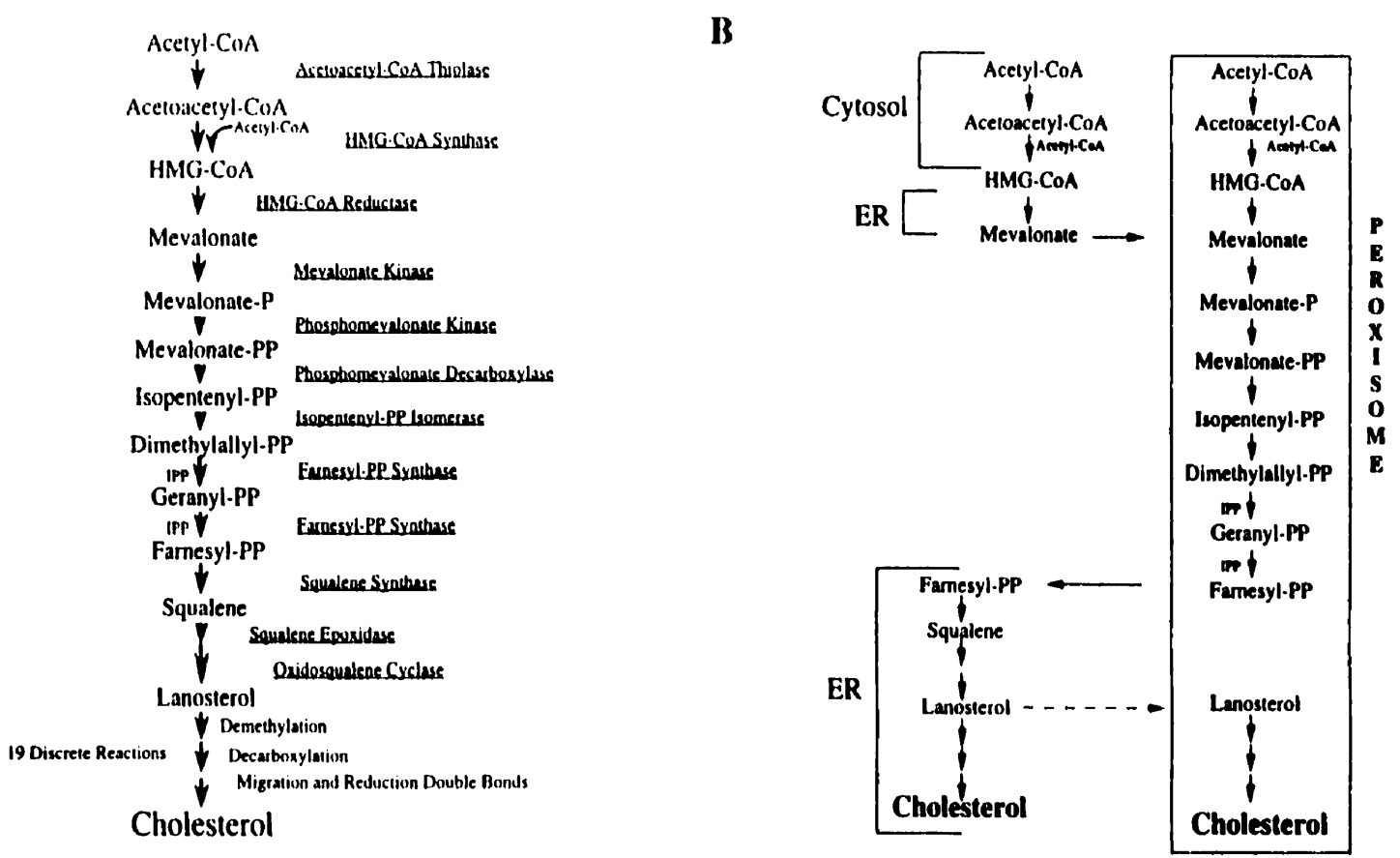

Figure 1.1 Pathway of peroxisomal cholesterol biosynthesis. (**A**) Enzymes of the cholesterol metabolism pathway. (**B**) Compartmentalization of cholesterol synthesis. The peroxisome contains enzymes for the conversion of acetyl-CoA to farnesyl diphosphate (FPP) and enzymes for the conversion of lanosterol to cholesterol. The conversion of acetyl-CoA to HMG-CoA also occurs in the cytosol. The further conversion of HMG-CoA to mevalonate occurs both in the ER and peroxisomes. However, the conversion of mevalonate to FPP is believed to occur predominately in the peroxisome. The further conversion of FPP to squalene occurs exclusively in the ER, and lastly the metabolism of lanosterol to cholesterol occurs in both the ER and peroxisomes. CoA, coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; P, phosphate; PP, diphosphate; IPP, isopentenyl-diphosphate.

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glycerolipids and comprise 5-20% of the phospholipid component of membranes in mammalian tissue. Plasmalogens are especially abundant in myelin of nervous tissue where they comprise more than 80% of the ethanolamine phospholipids. Several functions have been suggested for plasmalogens such as an antioxidant, arachidonic acid storage and a modulator of the physiochemical properties of biomembranes, i.e., modifier of the impulse propagation characteristics of nervous tissue [Garg and Haerdi, 1993].

The first steps of plasmalogen synthesis, resulting in the introduction of the characteristic ether linkage, are catalyzed by enzymes located exclusively within the peroxisome: dihydroxyacetone phosphate (DHAP) acyltransferase (DHAPAT), alkyl-DHAP synthase, and alkyl-DHAP reductase [Hajra and Bishop, 1982; Das et al., 1992; Hajra and Das, 1996]. The product of those reactions, alkyl-DHAP, is then subjected to a series of oxidation and reduction reactions in either the peroxisome or the ER to yield alkyl-glycerol-3-phosphate [Hajra and Das, 1996]. All subsequent reactions take place in the ER [Hajra and Das, 1996].

The importance of peroxisomes in plasmalogen synthesis became clear when tissues from Zellweger patients, known to lack peroxisomes, were shown to be severely deficient in ether-linked phosphoglycerides. Fibroblasts from these patients are specifically impaired in plasmalogen biosynthesis [Heymans et al., 1983].

1.2.4 Purine metabolism

The end product of purine metabolism varies from species to species. The series of enzymes necessary to convert purines to their final metabolic products has been progressively truncated as a result of evolutionary pressure [Yeldandi et al., 1996]. Urate oxidase, a peroxisomal enzyme responsible for the conversion of uric acid to allantoin, is present in most mammals except humans and hominoid mammals [Yeldandi et al., 1996]. Birds and some reptiles also lack urate oxidase. In rat hepatic parenchymal cells, urate oxidase is seen as a distinct crystalloid present within the peroxisomal matrix [Masters and Crane, 1995]. In the absence of urate oxidase, uric acid is excreted instead of allantoin. It is postulated that an evolutionary advantage of lacking urate oxidase lies in the antioxidant aspect of uric acid. A

relatively high level of serum uric acid in humans may protect against cancer, aging and other disorders caused by reactive oxygen intermediates [Ames et al., 1981].

1.2.5 Oxidation of amino acids

During starvation, amino acids serve as a major source of glucose via gluconeogenesis [Masters and Crane, 1995]. In the initial steps of gluconeogenic utilization of amino acids, peroxisomal amino acid oxidases deaminate amino acids to keto acids [Tolbert, 1981; Noguchi, 1987]. Human peroxisomes are also known to oxidize L-pipecolic acid, a lysine intermediate, to α-aminoadipic acid [Wanders et al., 1989].

1.2.6 Phytanic acid α-oxidation

The mechanism for phytanic acid α-oxidation has been resolved by Moser and coworkers who showed that phytanoyl-CoA, and not phytanic acid, is the true substrate for α-oxidation [Watkins et al., 1994]. A recently identified peroxisomal enzyme, phytanoyl-CoA hydroxylase, catalyzes the hydroxylation of phytanoyl-CoA to 2-hydroxyphytanoyl and is deficient in classical Refsum disease [Mihalik et al., 1995; Jansen et al., 1997].

1.2.7 Glyoxylate cycle

Discovered by Kornberg and Krebs [1957], the glyoxylate cycle provides a mechanism for the net conversion of two acetyl units to one molecule of succinate [Tolbert, 1981]. Succinate may then be converted to glucose or other cellular biosynthetic components. This conversion is brought about by peroxisomal isocitrate lyase, malate synthase and the mitochondrial enzymes of the tricarboxylic acid cycle. In germinating fat-bearing seed, the enzymes of the glyoxylate cycle are localized in a specialized peroxisome called the glyoxysome [Breidenbach and Beevers, 1967].

While the glyoxylate cycle was once considered to be absent in animals, Davis and coworkers showed that isocitrate lyase and malate synthase are present in several vertebrate tissues including rat liver and hibernating bear adipose tissue [Davis et al.,

1989]. The presence of the glyoxylate cycle enzymes in peroxisomes now appears to be nearly universal among eukaryotes.

1.2.8 Alcohol metabolism

In methylotrophic yeasts, such as *Pichia pastoris, Hansenula polymorpha* and *Candida boidinii*, the utilization of methanol as the sole carbon and energy source results in a massive proliferation of peroxisomes as well as the induction of peroxisomal matrix enzymes catalase (CAT), alcohol oxidase (AOX) and dihydroxyacetone synthase (DHAS) [Veenhuis et al., 1983; Douma et al., 1985].

In this pathway's initial step, methanol is converted to formaldehyde and hydrogen peroxide by the flavin-dependent AOX (Fig. 1.2). CAT subsequently converts hydrogen peroxide to water and oxygen. Formaldehyde may then enter either an energy-generating pathway (dissimilatory) or a cell biomass-generating (assimilatory) pathway. For energy, formaldehyde is first oxidized to formate and then to carbon dioxide by two cytosolic dehydrogenases. Both dehydrogenase reactions generate NADH which the cell's oxidative phosphorylation system uses to capture energy [Douma et al., 1985]. For the assimilatory pathway, formaldehyde is condensed with xylulose 5'-phosphate (Xu_5P) by peroxisomal DHAS to form glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone (DHA). GAP and DHA then exit the peroxisome and via a series of cytosolic enzymes, regenerate Xu_5P and one molecule of GAP for every three turns of the cycle. GAP then serves as the beginning carbon skeleton for synthesis of all the cell's organic compounds.

In both humans and yeasts, cytosolic alcohol dehydrogenase oxidizes ethanol to acetaldehyde which is then further metabolized in yeasts by the peroxisomal glyoxylate pathway enzymes along with the tricarboxylic acid cycle enzymes. In mammals, further metabolism of acetaldehyde (mainly its oxidation to acetate) takes place in the cytoplasm or mitochondria, with acetate then entering the bloodstream. Peroxisomes may contribute significantly to this oxidation, particularly when ethanol is at high concentrations and substrates such as fatty acids are available to generate H_2O_2 inside the peroxisome. In this case, ethanol elimination occurs via catalase [Oshino et al., 1973; van den Bosch, 1992; Masters and Crane, 1995].

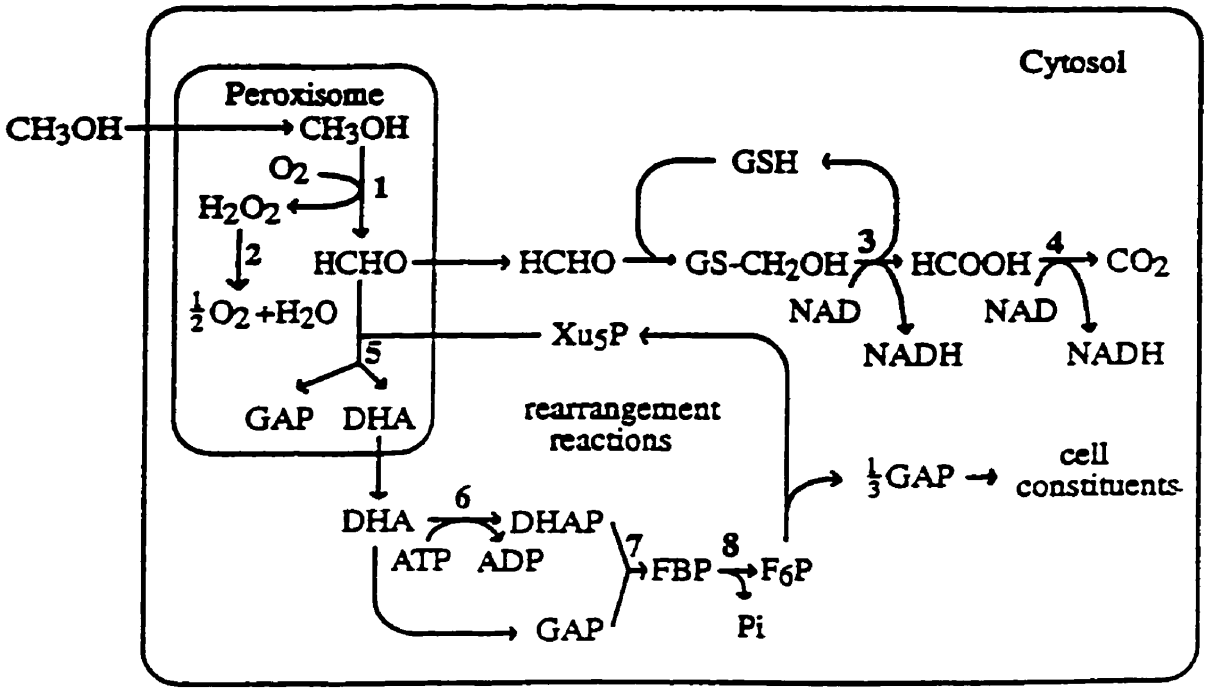

Figure 1.2 The methanol pathway in yeasts. Enzymes: 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-bisphosphate aldolase; 8, fructose 1,6-bisphosphatase. DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; FGP, fructose 6-phosphate; GAP, glyceraldehyde-3-phosphate; GSH, reduced glutathione; Xu_5P, xylulose 5-phosphate.

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1.2.9 Glycolytic pathway

Glycosomes are members of the microbody family of organelles which includes peroxisomes and glyoxysomes, but are found only in kinetoplastid protozoa such as African and American trypanosomes and Leishmania [Opperdoes, 1987; Opperdoes and Michels, 1993]. Like all members of the peroxisome family, they are characterized by a dense matrix, single membrane and lack of DNA [Purdue and Lazarow, 1994]. Some enzymes involved in β-oxidation of fatty acids [Hart and Opperdoes, 1984] and ether-lipid synthesis [Hajra, 1995] are also found in both glycosomes and peroxisomes.

Glycosomes harbor several unique enzyme pathways not found in other microbodies. These include the first six steps of glycolysis as well as enzymes involved in purine salvage and pyrimidine biosynthesis [Opperdoes, 1987]. It is hypothesized that compartmentalization of glycolytic enzymes serves to concentrate enzymes and their substrates, resulting in a high glycolytic rate in these protozoa [Opperdoes and Michels, 1993].

1.3 Peroxisome Biogenesis

Peroxisome biogenesis can be divided into four interrelated processes: (1) protein import; (2) membrane lipid acquisition; (3) proliferation; and (4) segregation.

1.3.1 Protein import

1.3.1.1 Matrix proteins.

1.3.1.1.1 Peroxisomal targeting signals. Because peroxisomes lack nucleic acids and ribosomes, it is thought that all peroxisomal proteins are synthesized on free cytoplasmic ribosomes and imported post-translationally into the organelle, although this has been directly demonstrated for only a few [Kamiryo et al., 1982; Lazarow and Fujiki, 1985; Borst, 1989]. As in the case of protein sorting to other organelles, the targeting of proteins to the peroxisomal matrix (and to the membrane which will be discussed in Section 1.3.1.2) is achieved through organelle-specific

signals called peroxisomal targeting signals (PTSs), short stretches of amino acids that are part of each polypeptide.

The first PTS, PTS1, was described in 1987 by Gould and co-workers on firefly luciferase [Gould et al., 1987]. PTS1 is the most widely used signal and is composed of the C-terminal tripeptide sequence SKL or conservative functional variants (S/C/A, K/R/H, L/M) [Gould et al., 1989]. PTS1 targets proteins to the peroxisomes of mammals [Motley et al., 1995], yeasts [Gould et al., 1989], plants [Trelease et al., 1996], and kinetoplastid protozoa [Blattner et al., 1992]. PTS1 is sufficient to target non-peroxisomal proteins to microbodies in virtually all eukaryotes [Gould et al., 1990]. In addition to the three C-terminal residues, protein context, i.e., additional sequences, may be necessary for efficient PTS1 targeting in some cases such as catalase A of *Saccharomyces cerevisiae* [Kragler et al., 1993].

PTS2, a nonapeptide (R/K, L/V/I, X_5, H/Q, L/A) located at or near the NH_2-terminus, is used by a smaller group of matrix proteins such as 3-ketoacyl CoA thiolase of mammals [Osumi et al., 1991] and yeasts [Glover et al., 1994b]. In some instances, the PTS2 is cleaved after import (rat thiolase and watermelon malate dehydrogenase) but not in others (yeast thiolase). The presequences in rat peroxisomal thiolase and watermelon malate dehydrogenase are sufficient to target non-peroxisomal proteins to the peroxisome [Swinkels et al., 1991; Gietl et al., 1994]. As with PTS1, PTS2 has been conserved across species as seen by the targeting of PTS2 proteins to peroxisomes in yeasts, humans [Faber et al., 1995; de Vet et al., 1997], plants [Gietl et al., 1994], and trypanosomes [Blattner et al., 1992]. Other PTSs for peroxisomal matrix proteins may exist but are not yet well defined since many matrix proteins have neither PTS1 nor PTS2.

1.3.1.1.2 PTS1 and PTS2 receptors. Through the use of yeast molecular genetics, i.e., the isolation of peroxisomal biogenesis (*pex*) mutants and *PEX* genes, receptor proteins that bind nascent peroxisomal matrix proteins in the cytoplasm for delivery to peroxisomes have been identified. The first of these receptor proteins to be found was encoded by the *P. pastoris PEX5* gene. *P. pastoris pex5* mutants have a PTS1-specific import defect and were used to isolate *PEX5* by functional complementation [McCollum et al., 1993]. *PEX5* was subsequently

identified in *S. cerevisiae* [van der Leij et al., 1993], *H. polymorpha* [van der Klei et al., 1995], *Yarrowia lipolytica* [Szilard et al., 1995], Chinese hamster ovary (CHO) cells [Otera et al., 1998] and human cells [Slawecki et al., 1995]. Interestingly, in both CHO and human cells, mutations in *pex5* result in defects in both PTS1 and PTS2 import.

In addition to the PTS1-pathway import-specific defect of yeast *pex5* mutants, a second key piece of evidence supporting a PTS1 receptor role for Pex5p was the discovery that the peroxin specifically binds polypeptides ending in a PTS1 but not other polypeptides [McCollum et al., 1993]. The predicted amino acid sequence of all Pex5ps contains repeating 34 amino acid motifs called the tetratricopeptide repeats (TPRs). The number of TPRs varies from six to eight, depending on the organism. Three TPRs of Pex5p have been shown to be necessary and sufficient for binding of PTS1 tripeptides in different species [McCollum et al., 1993; Dodt et al., 1995; Fransen et al., 1995; Terlecky et al., 1995].

*Pp*Pex5p has been reported to be localized to different regions of the cell. In *P. pastoris*, McCollum and coworkers reported Pex5p as tightly membrane bound while in *S. cerevisiae* and *H. polymorpha*, Pex5p was reported to be mainly cytosolic [McCollum et al., 1993; van der Klei et al., 1995; Elgersma et al., 1996a] while in *Y. lipolytica* Pex5p appeared to be mostly in the peroxisomal matrix [Szilard et al., 1995]. Human Pex5p was reported to be primarily cytosolic with a small amount associated with the peroxisomal membrane [Dodt and Gould, 1996]. Pex5ps various locations may be dependent on differences in cell growth conditions, methods of preparation of organellar materials, and/or the specific techniques used to localize the protein, i.e., fractionation versus microscopic methods. The favored model of Pex5p function is a cycling model in which Pex5p, bound to a PTS1 protein in the cytosol, then binds to a docking protein on the peroxisomal membrane followed by the import of the complex into the matrix. This cycling model explains the observation of Pex5p in multiple locations and is supported by experiments by Dodt and Gould in which they observed Pex5p accumulation on the peroxisome surface under conditions that inhibited peroxisomal protein translocation (low temperature or ATP depletion), and a return of Pex5p to the cytoplasm when translocation was restored [Dodt and Gould,

1996]. Additionally, these investigators observed that in CG7 human fibroblast cells (*pex10* mutants), Pex5p accumulates within the peroxisome, suggesting that Pex5p may enter the peroxisome at some point in the import cycle [Dodt and Gould, 1996].

The PTS2 receptor, Pex7p, was first identified in *S. cerevisiae* [Marzioch et al., 1994; Rehling et al., 1996; Zhang and Lazarow, 1996], and then in humans [Braverman et al., 1997; Purdue et al., 1997] and *P. pastoris* [Elgersma et al., 1998]. *pex7* mutants in all organisms import PTS1 proteins but are unable to import PTS2 proteins and have morphologically normal-looking peroxisomes. Like Pex5p's selective binding of its cargo protein, Pex7p binds the PTS2 sequence specifically [Rehling et al., 1996; Zhang and Lazarow, 1996]. Additionally, Pex7p has six copies of a motif called the WD (Trp-Asp) 40 repeat which, in other proteins, binds TPR proteins. As expected, Pex7p and Pex5p interact as shown by two-hybrid system studies [Gietel et al., 1994].

As with Pex5p, the subcellular location of Pex7p is uncertain. Results of Zhang and Lazarow [1996] suggested that most of Pex7p from *S. cerevisiae* is intraperoxisomal; they proposed that Pex7p pulls PTS2-proteins into the peroxisome. Kunau and co-workers reported a cytosolic localization for *S. cerevisiae* Pex7p [Erdmann et al., 1991; Marzioch et al., 1994] while Elgersma and coworkers found *P. pastoris* Pex7p to be both cytosolic and intraperoxisomal and propose a cycling model similar to that described for Pex5p [Elgersma et al., 1998].

1.3.1.1.3 Receptor docking proteins. Once the PTS receptors have bound to their respective matrix proteins, what mediates their association with the peroxisomal membrane prior to import? Pex13p has been identified as a potential docking protein for Pex5p in *S. cerevisiae* [Elgersma et al., 1996b; Erdmann and Blobel, 1996], and in *P. pastoris* and humans [Gould et al., 1996]. Pex13p, an integral membrane protein, interacts with Pex5p through its cytosolic C-terminus Src homology 3 (SH3) domain [Elgersma et al., 1996a; Erdmann and Blobel, 1996; Gould et al., 1996]. Additionally, Girzalsky and co-workers report a functional interaction, i.e., Pex13p and Pex7 co-immunoprecipitate but no direct interaction is

seen by two-hybrid studies, through the amino terminus of Pex13p with Pex7p [Girzalsky et al., 1999].

Pex14p, another putative docking protein for Pex5p and Pex7p, has been described as either a peripherally associated or integral peroxisomal membrane protein in *S. cerevisiae* [Albertini et al., 1997; Brocard et al., 1997], and an integral peroxisomal membrane protein in human, *H. polymorpha* and CHO cells [Komori et al., 1997; Shimizu et al., 1999; Will et al., 1999]. In *S. cerevisiae* and *P. pastoris*, Pex14p has been shown to interact not only with Pex5p and Pex7p but also with Pex13p, Pex17p and itself [Albertini et al., 1997; Brocard et al., 1997; Huhse et al., 1998; Girzalsky et al., 1999; Johnson, M.A., unpublished data].

1.3.1.1.4 Other peroxins required for import of matrix proteins. While a role in matrix protein import has been clearly shown for the PTS1 and PTS2 receptors Pex5p and Pex7p, respectively, and for the docking proteins Pex14p and Pex13p, a role for other peroxins in matrix import is suspected but not yet defined. For example, like *pex5* mutants, a *P. pastoris pex8* point mutant has only a PTS1 import deficiency [Liu et al., 1995]. *S. cerevisiae pex18* and *21* mutants are defective in PTS2 import only [Purdue et al., 1998] while in *pex20* mutants, only thiolase is mislocalized [Titorenko et al., 1998]. In *pex16* mutants, some PTS1 and PTS2 proteins are mislocalized but others are not [Eitzen et al., 1997]. These PTS-pathway mutant phenotypes suggest an import role yet to be elucidated. Finally, Pex17p has been shown to be part of an import complex containing Pex5p, Pex7p, Pex13p, and Pex14p in *P. pastoris* [Snyder et al., 1999b; this thesis].

1.3.1.1.5 Chaperones and cofactors. Experiments with permeabilized tissue culture cells have shown that import of peroxisomal matrix proteins requires ATP and is facilitated by HSP70 and HSP40 class cytoplasmic chaperones [Walton et al., 1994]. While the specific function for the HSPs is uncertain, the following hypotheses have been proposed [Subramani, 1996] as to their function: (1) stabilization of exposed PTS1 sequence prior to receptor binding; (2) participation in the assembly of PTS1-Pex5p-docking protein complexes; and (3) participation in the disassembly of PTS1-Pex5p-docking protein complexes.

1.3.1.1.6 General features of the matrix protein import mechanism. Unlike other organelles, general features of the peroxisomal matrix protein import mechanism have yet to be elucidated. Indeed, the current view of import is confusing. It was once thought that all peroxisomal matrix proteins entered the organelle as unfolded, unassembled monomers through narrow translocation pores similar to proteins entering the ER and mitochondrion. However, it was demonstrated that completely folded polypeptides, stabilized with disulfide bonds and chemically cross-linked, can be imported into the peroxisome matrix as well as gold particles up to 9 nm in diameter conjugated to human serum albumin-SKL [Walton et al., 1995]. Clearly, the translocation of very large proteins and particles into the peroxisome is possible. Other studies have shown that oligomeric peroxisomal proteins can be imported. Such "piggyback" import was shown for thiolase of *S. cerevisiae*, a homodimer [Glover et al., 1994a]. If the PTS2 of thiolase is removed, import of thiolase does not occur. However, if the PTS2-less thiolase is co-expressed with full-length thiolase (with its PTS2), then both are efficiently imported. This result indicates that PTS2-less thiolase dimerizes with normal thiolase in the cytoplasm and enters the peroxisome in at least a partially assembled state. Import of pre-assembled proteins has been confirmed with other matrix proteins including tetrameric human catalase [McNew and Goodman, 1996].

These and other reports of oligomeric import do not preclude the possibility that some peroxisomal proteins must be imported as monomers and assembled in the matrix. Waterham and co-workers have shown in *P. pastoris* that AOX, a homooctamer, is unable to assemble in the cytosol, but rather must be first targeted to the peroxisome before assembly begins [Waterham et al., 1997b].

Thus, the general question of how proteins are translocated across the peroxisomal membrane remains unanswered. There is no evidence for peroxisomal pores large enough to translocate folded and oligomeric proteins into the peroxisome, such as exist in nuclear membranes, and in fact work by the labs of Veenhuis and Tabak suggest that the permeability properties of peroxisomes are not consistent with the existence of large pores [Nicolay et al., 1987; van Roermund et al., 1995]. Models featuring transient pores and/or endocytosis have been proposed [McNew and

Goodman, 1994] but, while interesting, have not been supported by experimental evidence. It is known that import of matrix proteins occurs as a two-step process. Studies with permeabilized mammalian cells indicate that the matrix protein is first bound to the cytosolic face of the peroxisomal membrane and then translocated into the peroxisomal matrix in a step that requires ATP [Imanaka et al., 1987; Wendland and Subramani, 1993] but how translocation occurs remains a black box.

*1.3.1.2 **Membrane proteins.*** Targeting of peroxisomal membrane proteins is a distinct mechanism from that of the targeting of matrix proteins as shown by the fact that many *pex* mutant cells that are unable to import matrix proteins are still able to insert membrane proteins into peroxisomal remnants or ghosts [Erdmann and Blobel, 1996; Gould et al., 1996]. Targeting signals for membrane proteins (mPTSs) have been described for *C. boidinii* PMP47 [McCammon et al., 1994; Dyer et al., 1996], and Pex3p from *P. pastoris* [Wiemer et al., 1996], *H. polymorpha* [Baerends et al., 1996] and humans [Kammerer et al., 1998], as well as *S. cerevisiae* Pex15p [Elgersma et al., 1997] and *P. pastoris* Pex22p [Koller et al., 1999]. The only similarity among the reported mPTSs appears to be a short stretch of several basic amino acids. The mPTSs of *Pp*Pex3p and PMP47 are sufficient not only to target passenger proteins to the peroxisomal membrane but also to insert the proteins in the membrane, demonstrated by the fusion proteins' resistance to alkaline carbonate extraction.

In vitro studies indicate that the mPTS protein first binds to the peroxisomal membrane through some protenacious element and then inserts into the membrane bilayer [Diestelkotter and Just, 1993]. However, a new theory is emerging regarding targeting of a subset of peroxisomal membrane proteins. Evidence suggests that some peroxins are targeted to peroxisomes via the ER. Titorenko and Rachubinski showed in *Y. lipolytica* that Pex2p and Pex16p travel first to the ER and then to peroxisomes as evidenced by N-linked glycosylation on these peroxins [Titorenko and Rachubinski, 1998]. In addition, *S. cerevisiae* Pex15p is O-mannosylated, a post-translational event thought to be exclusive to the ER [Elgersma et al., 1997]. Also, the N-terminal 16

amino acids of *H. polymorpha* Pex3p are sufficient to target a chimeric protein to the ER [Baerends et al., 1996]. If the formation of ER coated vesicles is prevented by brefelden, Pex3p accumulates in the ER membrane [Salomons et al., 1997]. How do these proteins then reach peroxisomes? In 1978, Goldman and Blobel proposed that peroxisome bound vesicles may bud from the ER and either fuse with each other or with pre-existing peroxisomes [Goldman and Blobel, 1978].

Recently, *P. pastoris* Pex3p and Pex17p were shown to be required for insertion of membrane proteins [Wiemer et al., 1996; Snyder et al., 1999a,b]. *pex3* and *pex17Δ* mutants are unique among *pex* mutants in that no integral membrane protein-containing remnants are observed.

1.3.2 Membrane lipid acquisition and peroxisome proliferation

In the 1960s, it was thought that peroxisomes arose through budding from the ER [Higashi and Peters, 1963; Novikoff and Shin, 1964]. This theory fell by the wayside because detailed morphological studies failed to reveal direct contacts between the ER and peroxisomes [Lazarow and Fujiki, 1985]. Furthermore, evidence accumulated in the late 1970s and early 1980s that was incompatible with the ER origin theory and led to a growth and division model by Lazarow and Fujiki in 1985 that proposed new peroxisomes arise exclusively by budding or fission from pre-existing peroxisomes and not from the ER [Lazarow and Fujiki, 1985]. The budding model left open the question of the source of peroxisomal membrane lipids and predicted that cells that totally lost the organelles by mutation would not be able to regenerate them even if transformed back to wild-type since there would be no pre-existing peroxisomes from which to re-initiate peroxisome biogenesis.

Recent reports show that the ER does in fact play a direct role in supplying phospholipids to the peroxisomes and that peroxisomes can generate *de novo* [Kunau and Erdmann, 1998]. In 1998, Titorenko and Rachubinski suggested that ER-derived vesicles that contain subsets of peroxisomal membrane (Pex2p and Pex16p) and matrix (thiolase and catalase) proteins transfer phospholipids from the ER to the peroxisomal membrane. The principle phospholipids in eukaryotic peroxisomes are phosphatidyl choline and phosphatidyl ethanolamine, the same lipids found in ER

membranes [Subramani, 1993]. Evidence tying the ER to the peroxisome includes the findings that two peroxisomal membrane proteins, Pex2p and Pex16p, are N-glycosylated [Titorenko and Rachubinski, 1998] and Pex15p is O-mannosylated, suggesting trafficking via the ER. Secondly, the N-terminal 16 amino acid residues of Pex3p are able to target a chimeric reporter protein to the ER [Baerends et al., 1996]. Thirdly, incubation of exponentially growing *H. polymorpha* on methanol, in the presence of brefeldin A, prevents peroxisome formation and results in decoration of the ER with peroxisomal proteins [Salomons et al., 1997]. Fourthly, distinct Pex1p and Pex6p ER-derived vesicles have been identified as possible intermediates in peroxisome formation [Faber et al., 1998]. Finally, Kunau and coworkers have shown that coatomers, proteins involved in movement of vesicles from ER to their compartments, bind to peroxisomes and are essential for normal peroxisomal growth [Kunau and Erdmann, 1998; Passreiter et al., 1998]. In addition, Pex11p, a peroxin necessary for normal peroxisomal growth and proliferation, contains a potential coatomer binding site [Erdmann and Blobel, 1995; Marshall et al., 1996].

How do these ER-derived vesicles contribute phospholipids to peroxisomes? Pex1p and Pex6p may act to fuse these vesicles either with pre-existing peroxisomes, thereby allowing peroxisomal growth, or with other vesicles and thereby mature themselves into new peroxisomes [Faber et al., 1995].

The prediction of the peroxisome budding model, that peroxisomes only originate from pre-existing peroxisomes, is also in doubt. A study by Waterham and co-workers first suggested that peroxisomes may form *de novo* [Waterham et al., 1993]. This study made use of a novel temperature-sensitive *pex* mutant, *per13*, in which, at restrictive temperature, the cells were devoid of peroxisomes; but within one hour of shifting to permissive temperature, peroxisome formation was observed. Neither membrane proteins nor mature peroxisomal matrix proteins present in the cytosol prior to the temperature shift were incorporated into the newly formed peroxisomes, suggesting that the new organelles formed *de novo*. Recent work of South and Gould [1999] further supported the idea that peroxisomes can arise in the absence of pre-existing peroxisomes. In this study, a human *pex16* mutant cell, which lacks peroxisomes, was identified. Two to three hours after injection of the *PEX16*

gene into this mutant strain, peroxisomes were detected. Thus, the possibility of *de novo* synthesis appears to be a plausible alternative to the long-held belief that peroxisomes must arise only from pre-existing peroxisomes.

1.3.3 Segregation and movement via microtubules

During cell division, peroxisomes, like other organelles, must segregate to daughter cells. Recent data on peroxisome segregation, using GFP-PTS1 fusions, show that inheritance in mammalian cells is stochastic [Wiemer et al., 1997] rather than orderly as previously believed. Peroxisomes are not microtubule-associated during mitosis and appear to be randomly distributed to daughter cells. However, during interphase, most peroxisomes are closely associated with microtubules. It has been reported that 90-95% of peroxisomes exhibit a slow, microtubule-independent movement [Rapp et al., 1996; Wiemer et al., 1997]. The remaining 5-10% display rapid, saltatory movements that are microtubule-dependent. It has not been demonstrated why—when essentially all of the peroxisomes are microtubule-associated—only a small percentage move significantly, nor the physiological significance of the movement.

1.4 Human Peroxisomal Disorders

The essential nature of the peroxisome to humans is demonstrated by the existence of numerous peroxisomal disorders, many of which are lethal [Lazarow and Moser, 1995; Wanders, 1999]. Several classification systems of peroxisomal disorders are currently in use based on: (1) clinical parameters [Wanders et al., 1996]; (2) deficiency in one or more specific peroxisomal enzyme activities; (3) genetic criteria; and (4) a combination of one or more of these. For the following description of human peroxisomal disorders, the third classification system is used. Table 1.1 summarizes the enzymatic basis for and general biochemical abnormalities of peroxisomal disorders.

Table 1.1
Human Peroxisomal Disorders

Group	Peroxisomal Disorder	Enzymatic Basis (loss of function)	Additional biochemical abnormalities	
			VLCFA	*de novo* plasmalogen synthesis
A	Cerebro-hepato-renal syndrome (Zellweger)	Generalized	↑	↓
A	Neonatal adrenoleukodystrophy (NALD)	Generalized	↑	↓
A	Infantile Refsum disease (IRD)	Generalized	↑	↓
A	Hyperpipecolic acidaemia (HPA)	Generalized	↑	↓
B	RCDP Type 1 (PTS2-receptor defect)	Multiple	N	↓
B	Zellweger-like syndrome	Multiple	↑	↓
C	RCDP Type 2	DHAPAT	N	↓
C	RCDP Type 3	AlkylDHAP-synthase	N	↓
C	X-linked adrenoleukodystrophy (XALD)	ALDp	↑[a]	N
C	Acyl-CoA oxidase deficiency	Acyl-CoA oxidase	↑	N
C	Bifunctional protein deficiency	D-Bifunctional protein	↑	N
C	Peroxisomal thiolase (I) deficiency	Thiolase	↑	NF
C	Hyperoxaluria Type I	AGT	N	NF
C	Refsum disease (classic form)	Phytanoyl-CoA	N	N
C	Glutaric aciduria Type 3	Glutaryl-CoA oxidase	N	NF
C	Mevalonate kinase deficiency	Mevalonate kinase	N	NF
C	Acatalasaemia	Catalase	N	NF

DHAPAT, dihydroxyacetonephosphate acyltransferase; AGT, alanine glyoxylate aminotransferase; ALDp, adrenoleukodystrophy protein; RCDP, rhizomelic chondrodysplasia punctata; N, normal; NF, not found in the literature; ↑, elevated; ↓, deficient. [a] Except in some cases (Compiled from Wanders et al. [1996] and Wanders [1999].)

1.4.1 Group A

Group A is composed of the peroxisomal biogenesis disorders (PBDs) cerebro-hepato-renal (Zellweger) syndrome, neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and hyperpipecolic acidemia (HPA) [Wanders, 1999]. All are characterized by a generalized defect in peroxisome biogenesis, resulting in the apparent absence or near absence of peroxisomes [Lazarow and Moser, 1995]. Zellweger syndrome is generally considered to be the most severe and the prototypical group A disorder [Subramani, 1998; Wanders, 1999].

Zellweger syndrome is characterized by severe neurological and hepatic dysfunction, craniofacial abnormalities, hypotonia, glaucoma, impaired hearing, neonatal seizures, renal cysts and joint calcifications. Biochemically, the patients have high levels of plasma VLCFA, eicosanoids and phytanic acids. Low levels of cholesterol, plasmalogens and isoprenoids are noted as well as brain dysmyelination (hypomyelination) rather than progressive demyelination which will be discussed later relating to a group C disorder. The medical prognosis for affected individuals is bleak with death almost always occurring by age ten [Subramani, 1998]. Group A disorders fall into nine complementation groups (CG) based on complementation studies using fibroblast fusions: CG1-4, CG7, 9, 10 [Subramani, 1998] and CGJ [Matsuzono et al., 1999] and CGH [Shimozawa et al., 1999]. See Table 1.2 for *PEX* gene correlation to CGs.

A mouse model for Zellweger syndrome has recently been developed by Baes and co-workers [1997] through directed mutation of the mouse *PEX1* gene (CG1).

1.4.2 Group B

Rhizomelic chondrodysplasia punctata (RCDP) type I and Zellweger-like syndrome constitute group B. Cells from patients in this group have abnormal peroxisomes with several enzyme deficiencies [Wanders, 1999]. RCDP is characterized by severe growth defects, cataracts, rhizomelia, and mental retardation. While these individuals have normal levels of VLCFA, they are deficient in plasmalogens. In most RCDP patients, the primary defect is in PEX7 which encodes

Table 1.2

Genes Required for Peroxisome Biogenesis

PEX Gene	Former names	Characteristics of products	Interacting partners[a]
PpPEX1[1] *ScPEX1*[2] *HsPEX1*[3,4,5]	*PAS1* *PAS1* -------	AAA-type ATPase; 117 to 127 kDa; CG1	Pex6p
PpPEX2[6] *ScPEX2*[7] *YlPEX2*[8] *ChPEX2*[9] *LdPEX2*[10] *RnPEX2*[11] *HsPEX2*[12]	*PER6* *PAS5* *PAY5* *PAF1* ------- *PAF1* *PAF1*	IMP; *Yl* Pex2p is processed via ER[77]; C-terminal Zn-finger; N-glycosylated; 35 to 52 kDa; CG10	
PpPEX3[13] *ScPEX3*[14] *HpPEX3*[15] *HsPEX3*[16]	*PAS2* *PAS3* *PER9* -------	IMP; may regulate proliferation[78]; may recruit phospholipids via ER[79]; 42 to 52 kDa	Pex19p
PpPEX4[17] *ScPEX4*[18] *HpPEX4*[19]	*PAS4* *PAS2* -------	Ubiquitin-conjugating enzyme; located on the cytosolic side of the peroxisomal membrane; 21 to 24 kDa	Pex22p
PpPEX5[20] *ScPEX5*[21] *YlPEX5*[22] *HpPEX5*[23,24] *ChPEX5*[25] *HsPEX5*[26,27,28]	*PAS8* *PAS10* *PAY32* *PER3/PAH2* ------- *PXR1/PTS1R*	PTS1-receptor; contains 6-8 TPR motifs; cytosolic and peroxisome associated; 64 to 69 kDa; CG2	Pex13p, Pex14p, Pex8p[80] Pex7p

PpPEX6[29] *ScPEX6*[30] *YlPEX6*[31] *RnPEX6*[32] *HsPEX6*[33]	*PAS5* *PAS8* *PAY4* *PAF2* *PXAAA1*	AAA-type ATPase; cytosolic and vesicle associated; 112 to 127 kDa; CG4	Pex1p
PpPEX7[34,35] *ScPEX7*[36,37] *HsPEX7*[38]	------- *PAS7/PEB1* -------	PTS2-receptor; contains 7 WD-40 repeats; cytosolic and peroxisome associated; 37 to 42 kDa; CG11/RCDP	Pex13p, Pex14p, Pex18p, Pex21p, Pex5p
PpPEX8[39] *HpPEX8*[40] *YlPEX8*[41]	*PER3* *PER1* *PEX17*	Located on matrix side of peroxisome; contains both PTS1 and PTS2; 71 to 81 kDa	Pex5p
YlPEX9[42]	*PAY2*	IMP; cysteine rich region; 42 to 45 kDa	
PpPEX10[43] *HpPEX10*[44] *HsPEX10*[45]	*PAS7* *PER8* -------	IMP; C-terminal Zn-finger; 34 to 48 kDa; CG7	Pex19p
ScPEX11[46,47] *CbPEX11*[48]	*PMP27* *PMP30*	Peroxisome associated; involved in peroxisome proliferation; 24 to 32 kDa	Pex11p
PpPEX12[49] *ScPEX12*[50] *RnPEX12*[51] *HsPEX12*[52]	*PAS10* *PAS11* ------- -------	IMP; degenerative C-terminus Zn-finger motif; 40 to 48 kDa; CG3	
PpPEX13[53] *ScPEX13*[54] *HsPEX13*[55]	*PAS6* ------- -------	IMP; PTS receptor docking protein; C-terminal SH3 domain; 40 to 43 kDa; CGH[b]	Pex5p, Pex7p, Pex14p

PpPEX14[this study] *ScPEX14*[56,57,58] *HpPEX14*[59] *ChPEX14*[60] *HsPEX14*[61,62,63] *RnPEX14*[64]	------- ------- ------- ------- ------- -------	Yeast Pex14p interacts with Pex5p, Pex7p, Pex13p, Pex17p and itself; peripherally associated with the peroxisome in most yeasts; integral in human, CHO and rat; phosphorylated; 38 to 46 kDa; CG14[c]	Pex5p, Pex7p, Pex13p, Pex14p, Pex17p
ScPEX15[65]	*PAS21*	IMP; phosphorylated; O-glycosylated; targeted to ER; 44 kDa	
HsPEX16[66] *YlPEX16*[67]	------- -------	Associated on matrix face of peroxisome; processed via ER[79]; N-glycosylated; 39 to 45.5 kDa; CG9	
ScPEX17[68] *PpPEX17*[69]	*PAS9* -------	Associated with peroxisomes on the cytosolic side; selective impairment of matrix protein import in *Sc*; impairment of both matrix and integral membrane protein import in *Pp*; 23 kDa	Pex14p, Pex19p
ScPEX18[70]	-------	Required for PTS2 import; redundant with Pex21p; primarily cytosolic; 32 kDa	Pex7p, Pex21p
PpPEX19[71] *ScPEX19*[72] *HsPEX19*[73]	------- ------- -------	Associated with peroxisome on cytosolic side; *HsPEX19* and *ScPEX19* are farnesylated; 33 to 40 kDa; CGJ[b]	Pex3p, Pex10p, Pex17p
YlPEX20[74]	-------	Involved in oligomerization and targeting of thiolase; 47 kDa	
ScPEX21[75]	-------	Essential for PTS2 import; cytosolic; 33 kDa	Pex7p, Pex18p

PpPEX22[76]	-------	IMP; Essential for peroxisomal matrix protein import; anchors Pex4p; contains a 25-amino acid peroxisome membrane-targeting signal at its amino terminus; 21 kDa	Pex4p

Cb, Candida boidinii; Ch, Chinese hamster ovary cells (also CHO); *Hp, Hansenula polymorpha; Hs, Homo sapiens; Ld, Leishmania donovani; Pp, Pichia pastoris; Rn, Rattus norwegicus; Sc, Saccharomyces cerevisiae; Yl, Yarrowia lipolytica*; IMP, integral membrane protein; CG, human complementation group; AAA, ATPases associated with diverse cellular activities; TPR, tetratricopeptide repeat; WD40, tryptophan, aspartic acid with a 40 amino acid motif; RCDP, Rhizomelic chondrodysplasia punctata.

[a] Interaction as detected by the two-hybrid system and/or coimmunoprecipitation, seen in one or more species.

[b] Unique group but no complementation group (CG) number assigned.

[c] CG14 from CHO cell line.

(1) Erdmann et al., 1991
(2) Heyman et al., 1994
(3) Tamura et al., 1998
(4) Geisbrecht et al., 1998
(5) Collins and Gould, 1999
(6) Waterham et al., 1996
(7) Liu, Y. et al., 1996
(8) Eitzen et al., 1996
(9) Tsukamoto et al., 1994
(10) Flaspohler et al., 1997
(11) Tsukamoto et al., 1991
(12) Shimozawa et al., 1992
(13) Wiemer et al., 1996
(14) Hohfeld et al., 1991
(15) Baerends et al., 1996
(16) Soukupova et al., 1999
(17) Crane et al., 1994
(18) Wiebel and Kunau, 1992
(19) van der Klei et al., 1998
(20) McCollum et al., 1993
(21) van der Leij et al., 1993
(22) Szilard et al., 1995
(23) van der Klei et al., 1995
(24) Nuttley et al., 1995
(25) Otera et al., 1998
(26) Dodt et al., 1995
(27) Fransen et al., 1995
(28) Wiemer et al., 1995
(29) Spong and Subramani, 1993
(30) Voorn-Brouwer et al, 1993
(31) Nuttley et al., 1994
(32) Tsukamoto et al., 1995
(33) Yahraus et al., 1996
(34) Elgersma et al., 1998
(35) Purdue et al., 1999
(36) Marzioch et al., 1994
(37) Zhang and Lazarow, 1996
(38) Braverman et al., 1997
(39) Liu et al., 1995
(40) Waterham et al., 1994
(41) Smith et al., 1997
(42) Eitzen et al., 1995
(43) Kalish et al., 1995
(44) Tan et al., 1995b
(45) Warren et al., 1998
(46) Erdmann and Blobel, 1995
(47) Marshall et al., 1995
(48) Sakai et al., 1995

(49) Kalish et al., 1996
(50) Kunau and Hartig, 1992
(51) Ocumoto et al., 1998
(52) Chang et al., 1997
(53) Gould et al., 1996
(54) Elgersma et al., 1996a
(55) Gould et al., 1996
(56) Albertini et al., 1997
(57) Brocard et al., 1997
(58) Girzalsky et al., 1999
(59) Komori et al., 1997
(60) Shimizu et al., 1999
(61) Will et al., 1999
(62) Franzen et al., 1998
(63) Shimizu et al., 1999
(64) Shimizu et al., 1999
(65) Elgersma et al., 1997
(66) Honsho et al., 1998
(67) Eitzen et al., 1997
(68) Huhse et al., 1998
(69) Snyder et al., 1999 in press
(70) Purdue et al., 1998
(71) Snyder et al., 1999
(72) Gotte et al., 1998
(73) Matsuzono et al., 1999
(74) Titorenko et al., 1998
(75) Purdue et al., 1998
(76) Koller et al., 1999
(77) Titorenko et al., 1997
(78) Elgersma and Tabak, 1996
(79) Salomons et al., 1997
(80) Cregg lab, unpublished

the PTS2 receptor (CG11) [Braverman et al., 1997; Purdue et al., 1997]. ThusRCDP appears to be a PTS2 protein import pathway defect.

However, dihydroxyacetonephosphate acyltransferase is also deficient in RCDP type I, and has recently been shown to be a PTS1 protein [Ofman et al., 1998].

1.4.3 Group C

Group C diseases result from mutations in single genes whose products are peroxisomal enzymes (Table 1.1). X-linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder and its diversity ranges from the lethal childhood form (CCALD) to a milder, Addison-only form with no neurological involvement [Moser et al., 1995]. In all forms of X-ALD, VLCFAs accumulate in the plasma. In the most severe form, a demyelination of neurons occurs [Dubois-Dalcq et al., 1999]. The gene responsible for X-ALD codes for a peroxisomal membrane protein which may be involved in the uptake of VLCFAs across the peroxisomal membrane [Mosser et al., 1993]. It is unclear how accumulation of VLCFAs results in the demyelination process.

1.4.4 Amyotrophic lateral sclerosis (ALS)

Approximately 20% of familial ALS cases are a result of a defect in Zn/Cu superoxide dismutase (SOD). Keller and coworkers, demonstrated that this SOD is peroxisomal, contrary to its often reported cytosolic location [Keller et al., 1991]. ALS is characterized by degeneration of motor neurons in the cortex, brainstem and spinal cord. It is unclear how the loss of peroxisomal SOD activity, which converts oxygen radicals to molecular oxygen, results in the pathology of the disease. One possibility is that the accumulation of free oxygen radicals results in neuronal injury [McNamara and Fridovich, 1993].

1.5 Approaches to Studying Peroxisomal Biogenesis

The understanding of mechanisms controlling peroxisome biogenesis at the molecular level, and subsequently the underlying mechanisms of human peroxisomal

biogenesis disorders has been greatly facilitated by the isolation and characterization of peroxisome biogenesis (*pex*) mutants, especially in yeasts. Once a yeast *pex* mutant has been identified and characterized, the wild-type copy of the affected gene can be easily cloned and the *PEX* gene product characterized. Because *pex* genes have been conserved from yeasts to humans and because the fundamental molecular, cellular and regulatory mechanisms in yeasts are similar to humans, many labs, including our own, have focused on yeast model systems to investigate peroxisome biogenesis mechanisms. A key to the exploitation of yeasts is the ready isolation of *pex* mutants in these organisms. Yeast *pex* mutants have been isolated in *P. pastoris, S. cerevisiae, H. polymorpha* and *Y. lipolytica* using the following selection schemes.

1.5.1 Strategies for the isolation of yeast *pex* mutants

1.5.1.1 Negative metabolic screening schemes. Certain yeast species require functional peroxisomes for growth on either methanol (*H. polymorpha*), oleic acid (*S. cerevisiae* and *Y. lipolitica*) or both (*P. pastoris*), but not for growth on other substrates such as glucose or glycerol. Thus, the first *pex* mutants in each of these yeast species were isolated by screening populations of mutagenized cells for ones that could not grow on these peroxisome-requiring substrates [Erdmann et al., 1989; Cregg et al., 1990; Liu et al., 1992; Nuttley et al., 1993; Tan et al., 1995a].

1.5.1.2 Positive metabolic selection schemes. As opposed to looking for an inability to grow on methanol or oleate as described in 1.5.1.1, positive selections were subsequently developed that resulted in the growth of *pex* mutants under conditions in which wild-type cells could not grow. An H_2O_2-suicide selection scheme was developed for *S. cerevisiae* based on the principle that if catalase, a peroxisomal enzyme which degrades toxic H_2O_2, is inhibited either by mutation or 3-aminotriazole, then wild-type cells would die due to the accumulation of H_2O_2 while *pex* mutants would live due to the lack of β-oxidation pathway function and therefore the absence of H_2O_2 production [van der Leij et al., 1992; Zhang et al., 1993]. In

practice, this selection did not significantly enrich *pex* mutant populations, partly because this screen also selected for β-oxidation pathway mutants.

A second *S. cerevisiae* positive selection scheme circumvented these problems [Elgersma et al., 1993]. Wild-type cells were transformed with a plasmid encoding the bleomycin (phleomycin) resistance protein fused to a PTS1. After mutagenesis, the cells were grown in the presence of the toxic drug phleomycin. Wild-type cells expressing the fusion protein died because the bleomycin resistance protein was imported into peroxisomes and thus sequestered it from most of the cell. *pex* mutants expressing the fusion protein survived because the bleomycin resistance protein remained in the cytosol and was available to bind and inactivate phleomycin. This selection proved to be highly selective and efficient.

Two additional positive selection schemes, and the topics of chapter 2 of this thesis, were based on the fact that in *P. pastoris*, peroxisomal AOX must be imported into peroxisomes to assemble and become active [Waterham et al., 1997b]. In one scheme, allyl alcohol was provided to wild-type cells which die because AOX oxidizes allyl alcohol to acrolein, a toxic compound. *pex* mutants are resistant to this selection because they lack functional peroxisomes and thus have little or no active AOX. In the second screen, a *P. pastoris* strain was used which is defective in formaldehyde dehydrogenase (FLD), a methanol pathway enzyme required to metabolize formaldehyde, the product of AOX's metabolism of methanol. AOX-induced wild-type cells are sensitive to methanol, presumably due to the accumulation of formaldehyde, while *pex* mutants survive the selection because they have little active AOX and therefore are unable to metabolize methanol to formaldehyde.

*1.5.1.3 **Green fluorescence screening schemes.*** In *P. pastoris*, selection schemes have been developed which take advantage of a green fluorescent protein (EGFP). If excited by ultraviolet light, EGFP strongly fluoresces in living cells. In one scheme, an EGFP-PTS1 fusion was used [Kalish et al., 1996]. EGFP-PTS1 fusion proteins are targeted to the peroxisome in wild-type cells and produce a punctate bright fluorescence. In *pex* mutants, the EGFP-PTS1 protein is mislocalized

to the cytosol and results in a diffuse fluorescence pattern, thereby making screening for *pex* mutants by fluorescence microscopy possible. A second EGFP screening scheme used a peroxisomal integral membrane protein-EGFP fusion strain and fluorescence-activated cell sorting (FACS) [Snyder et al., 1999b]. They noted that insertion of the mPTS-EGFP fusion into the peroxisomal membrane quenched the fluorescence signal from EGFP and used this phenomenon to screen by FACS for mutant strains that had high levels of fluorescence. Among these mutants were *pex3* and *pex17* mutant which are defective in targeting of peroxisomal matrix and integral membrane proteins.

1.5.2 Identification of peroxisome-deficient mutants

While Section 1.5.1 delineated the strategies used for selection of *pex* mutants, in practice, it is common for non-*pex* mutants to be members of that pool as well. *P. pastoris* has the advantage over other yeasts of growing on both methanol and oleate. Since these metabolic pathways do not overlap, single enzyme-deficient mutants can be eliminated with this double growth substrate screen. However, even in *P. pastoris*, identification of *pex* mutants requires further biochemical (subcellular fractionation) and morphological (electron and fluorescence microscopic) evidence to support the peroxisome-associated substrate-utilization deficiency phenotype. Subcellular fractionation of peroxisomes involves disruption of the cell's plasma membrane under conditions that leave organelle membranes largely intact, and centrifugation at a force sufficient to sediment peroxisomes (20–30,000 $\times$ *g*). The resulting organelle pellet and cytosolic supernatant fractions are then assayed for selected peroxisomal enzymes such as CAT. In wild-type cells, CAT activity and protein are found in the peroxisomal pellet and cytoplasmic supernatant in approximately equal proportions due to the disruption of a significant portion of the organelle during fractionation. However, in *pex* mutants, CAT is found almost exclusively in the supernatant fraction [Liu et al., 1992]. As PTSs were defined, an expanded study of import could then be done to differentiate *pex* mutants based on their inability to import PTS1, PTS2 and/or mPTS proteins.

Microscopy is also required to verify the identified *pex* mutant. Until recently, electron microscopy was the only available tool. A more recently developed technique using fluorescence microscopy involves the *in vivo* use of EGFP fused to a PTS. Using this technique, large numbers of live cells can be rapidly examined for peroxisomes [Kalish et al., 1996; Johnson et al., 1999].

1.5.3 Cloning of wild-type *PEX* gene alleles in yeasts

1.5.3.1 Via functional complementation. After a yeast *pex* strain has been identified, the wild-type allele of that mutant gene is easily cloned using a method known as functional complementation. A genomic DNA library, prepared from wild-type yeast DNA inserted into a yeast-bacterial shuttle vector, is transformed into the mutant cells. The cells are then asked to grow on a peroxisome requiring substrate, either methanol or oleate. The transforming plasmid(s) is then recovered by transforming total genomic DNA from the transformed yeast cells into *E. coli*, then retransforming each recovered plasmid into the original mutant cells for verification of rescue. The *PEX* gene-containing inserts are then sequenced directly and the open reading frames (ORFs) identified *in silico*. Finally, ORFs harboring *PEX* gene candidates are subcloned and individually tested for *pex* mutant-complementing activity.

1.5.3.2 Via **in silico** *techniques.* The availability from data banks of all or significant portions of the DNA (or cDNA) sequences of selected organisms has greatly facilitated the identification of genes previously identified in other organisms. This is most frequently accomplished by comparing the predicted amino acid sequences of the known gene product to that of all ORFs or cDNAs in the data bases. This approach has been especially useful for *S. cerevisiae*, the first free living organism for which the entire genomic sequence was determined and humans where large cDNA sequence data bases (expressed sequence tags or EST data bases) are publicly available.

Two examples of this *in silico* gene identification that relate to this thesis are the cloning of *S. cerevisiae* and human *PEX14* orthologs. *ScPEX14* was found by searching *S. cerevisiae* data bases using the *H. polymorpha* Pex14p deduced amino acid sequence. A candidate was identified and this *Sc*ORF was then amplified by PCR and cloned into a plasmid vector [Albertini et al., 1997]. A human *PEX14* cDNA was found by using the BLAST algorithm to probe the human EST data base with the deduced *Sc*Pex14p amino acid sequence. The human *Pex14* cDNA was then fully sequenced and a full cDNA clone identified [Will et al., 1999].

A second, less specific, method of *in silico* screening for peroxisomal genes/proteins uses a computer algorithm called CoSMos (context sensitive motif searches) to identify sequence motifs in the *S. cerevisiae* data bases that are commonly found on peroxisomal genes and proteins [Geraghty et al., 1999]. Specifically, CoSMos searches these data bases for type 1 or type 2 peroxisomal targeting signals and for the oleate-response element consensus sequence while adding context sensitivity to these sequence motif searches, i.e., defines locations within the sequence for which motifs may be searched. This program successfully identified almost all known *S. cerevisiae* matrix peroxisomal proteins as well as putative new ones. The advantage of this program is its ability to find new proteins. Its disadvantages include false positives, the fact that peroxisomal matrix genes/proteins are identified, not necessarily *pex* genes, and that the sequence of the species of interest must be known in order to search.

1.5.3.3 Via DNA chip array analysis. DNA chip arrays, also known as DNA microarrays, are a recent addition to the tool box of gene cloning [Lockhart et al., 1996]. Like the *in silico* techniques described above, these methods are largely restricted to analysis of sequenced genomes. Oligonucleotides, approximately twenty bases long, are chemically synthesized and affixed in a light-directed spatially addressable fashion. Fluorescently labeled cDNA is then applied to the chip under conditions that allow stringent hybridization. The chip is washed to remove unhybridized and not-fully hybridized cDNA and read by an automated system that

quantifies how much cDNA has bound to each oligonucleotide sequence position. Our lab is testing cDNA from ethanol- and oleate-induced *S. cerevisiae* cells on an *S. cerevisiae* oligonucleotide chip to identify oleate-induced genes, some of which may be previously unidentified *PEX* genes. This technique has also been used to analyze expression in breast cancer cells [DeRisi et al., 1996]. Chip DNA arrays hold the promise of being powerful tools in the analysis of gene expression, identification of genes within a family, clinical diagnosis and perhaps the study of protein-DNA interaction.

1.5.4 Overview of peroxisomal biogenesis genes

Table 1.2 presents an overview of the twenty-two *PEX* genes that have been cloned to date and the characterization of their corresponding proteins. Eleven *PEX* genes (*PEX1, 2, 5, 6, 7, 10, 12, 13, 14, 16* and *19*) have human homologues identified which are members of peroxisomal biogenesis disorder complementation groups. Four (*PEX5, 7, 13,* and *14*) are known to be directly involved in PTS1 and/or PTS2 import while recently *PEX17* and *PEX3* in *P. pastoris* have been shown to be impaired in both matrix protein import and in insertion of integral membrane proteins. Nine (*PEX2, 3, 9, 10, 12, 13, 15, 17* and *22*) are integral membrane proteins (IMPS). Those thought to be involved in various aspects of peroxisomal proliferation include *PEX1, 3, 6, 11,* and *16*.

Recently, several post-translational modifications have been reported, including N-glycosylation of the *PEX2* and *PEX16* peroxins, O-glycosylation of the *PEX15* peroxin, and farnesylation of the *PEX19* peroxin. All of these modifications are interesting with respect to possible ties between peroxisomes and the ER. Lastly, *PEX14* and *PEX15* peroxins are both phosphorylated, with the function of phosphorylation yet to be elucidated.

1.5.5 *P. pastoris*, a model system for studying peroxisome biogenesis and human peroxisomal disorders

Yeast species, particularly *P. pastoris*, have been very useful as model systems in the study of peroxisomal biogenesis and human peroxisomal biogenesis disorders [Elgersma and Tabak, 1996]. Firstly, and most importantly, the primary sequences of yeast peroxins have been remarkably conserved through evolution, allowing researchers to use their sequences to identify human orthologs, making yeasts particularly productive model systems with which to further elucidate peroxisome biogenesis in eukaryotes. An example of the close ties between yeast and human genes was shown by the high similarity between human and *P. pastoris* Pex5p, the PTS1 receptor [Wiemer et al., 1995; Dodt and Gould, 1996]. To date, there are yeast homologues for all human genes implicated in peroxisomal disorders (Table 1.2).

Secondly, yeast *pex* mutants are ideal conditional mutants with respect to their inability to grow on peroxisome-requiring substrates such as oleate or methanol, but ability to grow at wild-type rates on other substrates such as glucose. Thus, yeast *pex* mutants are easily maintained and manipulated on non-restrictive substrates. *P. pastoris* has a distinct advantage over other yeast peroxisomal specific model systems since two unique substrates, methanol and oleate can be employed to identify *pex* mutants. In *P. pastoris*, almost all mutants that are specifically defective in growth on both methanol and oleate are *pex* mutants, whereas in other yeasts with only one peroxisome-requiring phenotype, less than 20% of mutants defective in that phenotype are *pex* mutants.

Thirdly, peroxisome proliferation in methylotrophic yeasts, such as *P. pastoris*, is massive on methanol, making it a convenient source of the organelles and their enzymes for biochemical studies. Conveniently, this proliferation can be tightly controlled by growth substrate manipulation [Veenhuis and Harder, 1991] and under induced conditions, peroxisomes are easily identified by microscopic techniques.

Fourthly, both classical and molecular genetic techniques for *P. pastoris* are well developed. Strains can easily be cultured as either haploids or diploids.

Working with a haploid strain greatly simplifies the genetic characterization of a mutant, e.g., facilitating the isolation of mutants, determining complementation groups, and working with recessive mutant alleles. Additionally, the building of deletion strains or adding or removing auxotrophic markers is simplified. Homologous recombination occurs at a high rate in *P. pastoris*, making the targeting of genes to specific locations in the genome efficient. Furthermore, introns are rare in *P. pastoris* genes; therefore, the cloning of genes by functional complementation (discussed in Section 1.5.4) is usually straightforward and efficient. Finally, *P. pastoris* has a relatively small genome size which is advantageous since comprehensive DNA libraries are easily constructed and ORFs represent a high proportion of the genome content, reducing the amount of DNA sequencing needed to identify genes of interest. Many molecular biological, biochemical and genetic techniques have been developed for work with *P. pastoris,* mostly in our laboratory [Higgins and Cregg, 1998]. These include transformation, deletion strain construction, complementation analysis, random spore analysis, subcellular fractionation, and expression plasmids designed to produce specific proteins in response to peroxisome inducing growth substrates.

Finally, *P. pastoris'* small size, rapid growth rate, inexpensive growth media and biological safety, contribute to *P. pastoris'* role as a convenient and productive model system to study peroxisome biogenesis and human peroxisomal disorders.

CHAPTER 2
POSITIVE SELECTION OF NOVEL PEROXISOME BIOGENESIS-DEFECTIVE MUTANTS OF THE YEAST *PICHIA PASTORIS*[*]

2.1 Introduction

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

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Johnson, M. A., Waterham, H. R., Ksheminska, G. P., Fayura, L. R., Lin Cereghino, J., Stasyk, O. V., Veenhuis, M., Kulachkovsky, A. R., Sibirny, A. A., and Cregg, J. M. (1999) Positive selection of novel peroxisome biogenesis-defective mutants of the yeast *Pichia pastoris*. *Genetics* **151,** 1379–1391.

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 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.

2.2 Materials and Methods

2.2.1 Strains, media and microbial techniques

P. pastoris strains used in this study are listed in Table 2.1. 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)

Table 2.1

***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 aox1Δ::SARG4 aox2Δ::Phis4*	Cregg et al., 1989
JC121	*pex1-1 his4*	Liu et al., 1992
JC116	*pex2-1 his4*	"
JC129	*pex3-1 his4*	"
JC127	*pex4-1 his4*	"
JC122	*pex5-1 his4*	"
JC128	*pex6-1 his4*	"
JC123	*pex8-1 his4*	"
JC125	*pex10-1 his4*	"
JC124	*pex12-1 his4*	"
JC130	*pex13-1 his4*	"
JC132	*mxr1 his4*	This study
JC140	*pexA arg4*	"
JC141	*pexB arg4*	"
JC142	*pexC arg4*	"
JC143	*mxr2 arg4*	"
JC145	*pex3 fld1*	"
JC146	*pex6 fld1*	"
JC147	*pex8 fld1*	"
JC148	*pex1-1 AOX1::P_{GAP}-AOX1:HIS4 his4*	"
JC149	*pex8-1 AOX1::P_{GAP}-AOX1:HIS4 his4*	"
JC150	*pex3-1 AOX1::P_{GAP}-AOX1:HIS4 his4*	"
JC151	*pex6-1 AOX1::P_{GAP}-AOX1:HIS4 his4*	"

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].

2.2.2 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., 1997a] 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′-CAGGAATTCTAACAG-GCACCTGAAGATAGGT-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′-TCGACGCCCAATCAACCGCAAAGTTATAA-ACCGGTA-3′ and 5′-AGCTTACCGGTTTATAACTTTGCGGTTGATTGGGCG-3′). The adapter was inserted into *Hin*dIII- and *Xho*I-digested pEGFP-C3 (Clontech Laboratories, Inc., Palo Alto, CA) which resulted 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′-CGGAATTCATGGTGAGCAAGGGCG-AGGAG-3′ and 5′-CAGAATTCGAAGCTTACCGGTTTATAACTTTGCGG-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′-TCAGGATCCATG-CCCAATAGGCTCATACC-3′ and 5′-GACCATGGCACCAACGAAAAAACCTGG-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].

2.2.3 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) 20 ml 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.

2.2.4 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 h 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 mini-centrifuge at 14,000 rpm and 4°. The supernatant was removed and stored on ice. For whole cell extracts, the post-disruption centrifugation step was omitted, and the supernatant was removed after the beads had settled.

2.2.5 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 h 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]propannesulfonic 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.

2.2.6 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., 1997b] 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).

2.2.7 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 h. 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 h at 30° with shaking. Slides were prepared by adding 10 μl of culture to a slide and affixing the coverslip with rubber cement.

2.2.8 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 occured, i.e., growth on methanol, the backcrossed strain did acquire the *FLD1* gene during the first backcross.

2.2.9 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].

2.3 Results

2.3.1 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 (Fig. 2.1) [Ciriacy, 1975; Sibirny et al., 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 which 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

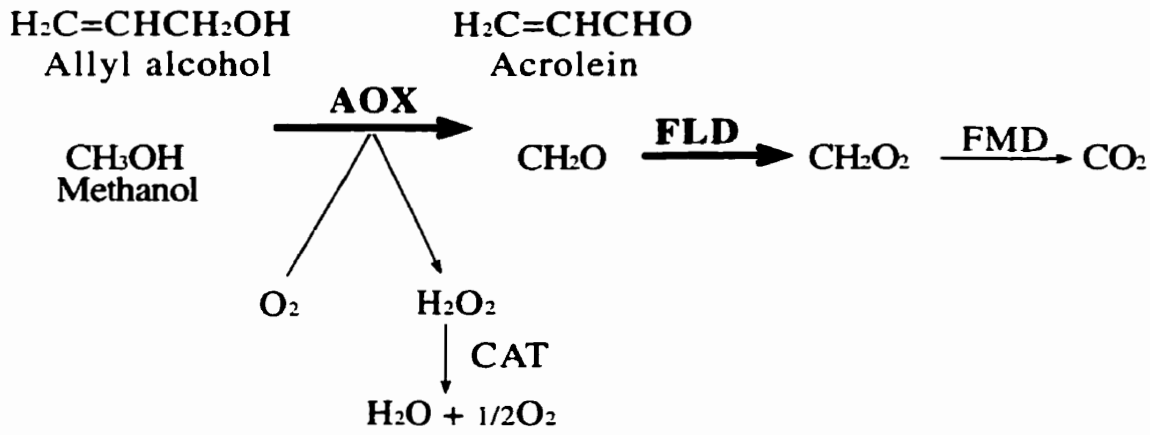


Figure 2.1 Metabolism of methanol and allyl alcohol in yeast. AOX, alcohol oxidase; CAT, catalase; FLD, formaldehye dehydrogenase; FMD, formate dehydrogenase.

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 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 (Fig. 2.2A 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 2.2). As described below, the remaining 5 Mut$^-$ Out$^-$ mutants represented two non-*PEX* complementation groups that

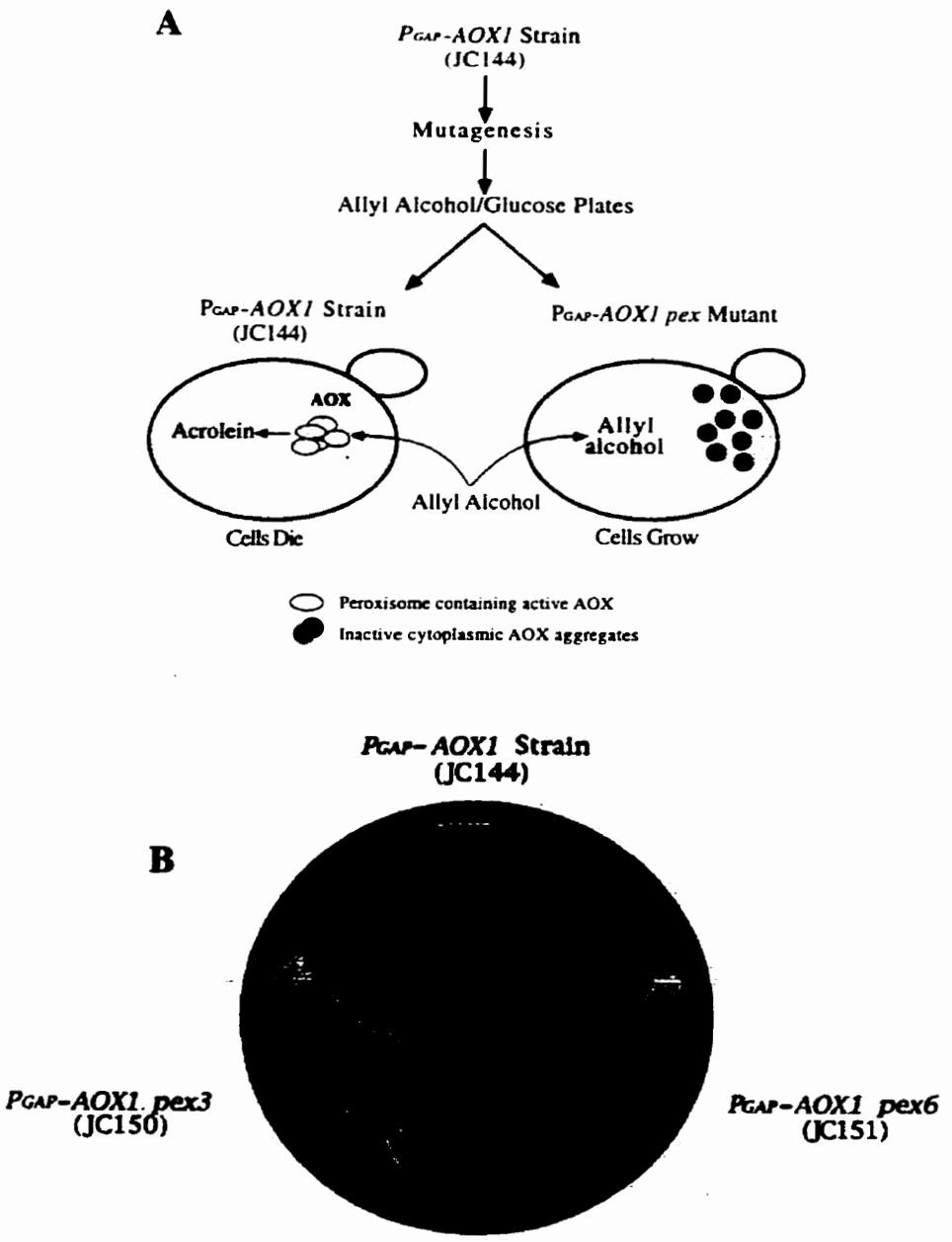


Figure 2.2 Allyl alcohol selection scheme for the isolation of *P. pastoris pex* mutants. (**A**) The parent strain constitutively expresses *AOX1* from P_{GAP}. Wild-type cells die as a result of the active AOX converting allyl alcohol to acrolein. *Pex* mutants grow due to the absence of active AOX. (**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.

Table 2.2

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].

we have tentatively named *MXR1* and *MXR2* and that appear to encode transcription factors required for expression of certain peroxisomal enzymes.

2.3.2 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.

2.3.3 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] (Fig. 2.1). However, in strains defective in FLD, toxic formaldehyde is not further metabolized and accumulates, killing these cells. The buildup 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 (Fig. 2.3A). To demonstrate this *pex* mutant selection scheme, Fig. 2.3B 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.

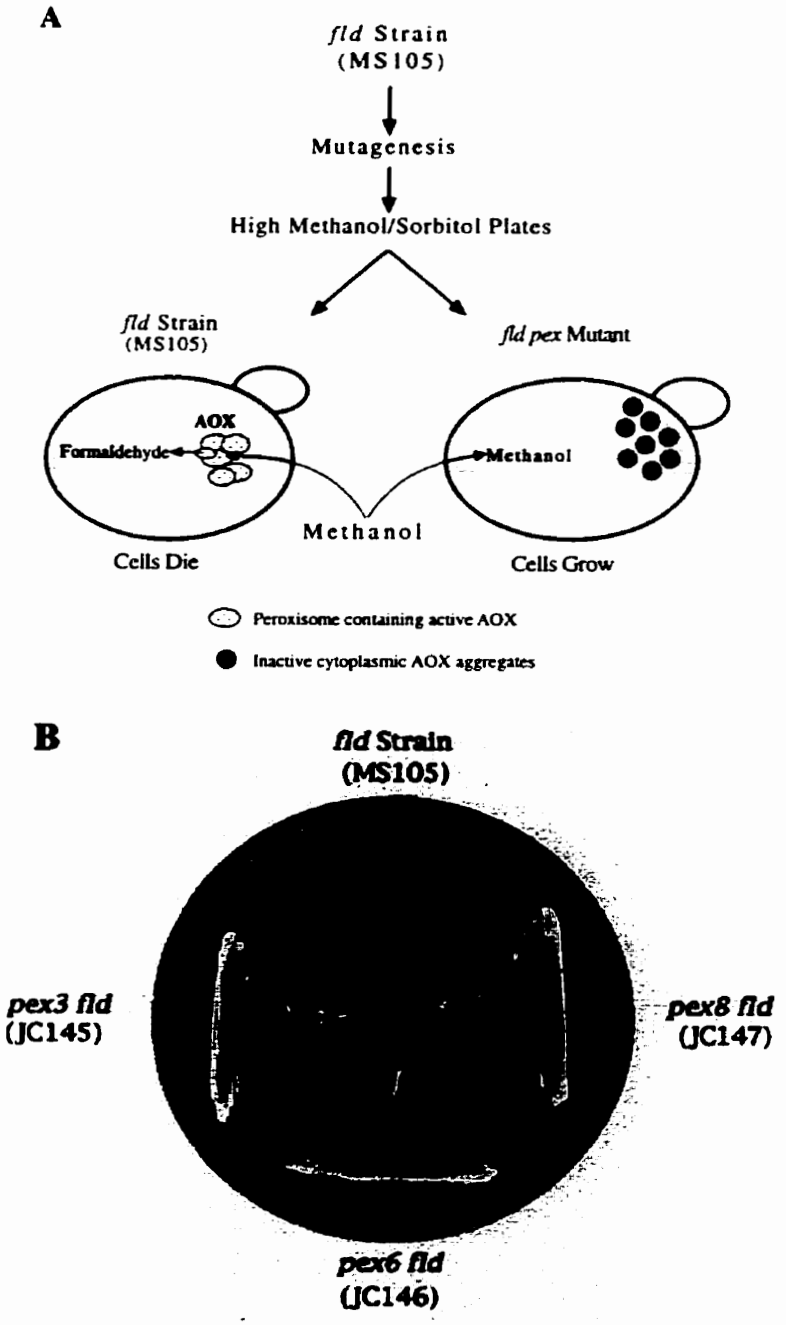


Figure 2.3 Methanol selection scheme for the isolation of *P. pastoris pex* mutants using an *fld1* mutant parent strain. **(A)** *fld1* non*pex* cells die due to accumulation of formaldehyde while *fld1 pex* mutants, which lack AOX, accumulate little or no formaldehyde and grow. **(B)** Growth of *fld1*, and *pex fld1* strains on high methanol-sorbitol plates.

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

2.3.4 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 2.2). 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 2.2). 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.

2.3.5 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.

2.3.6 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 2.3). Conversely, AOX activity, 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 2.4).

Methanol- and oleate-induced cells of *pexA*, *pexB*, and *pexC* were also examined by electron microscopy for peroxisomes and no normal peroxisomes were observed (Fig. 2.4; *pexB* not shown), confirming that each of these strains represents mutants in new *PEX* genes.

Table 2.3

Enzyme Activities in Induced Cells of New Mutant Strains[a]

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

[a] 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}/min/mg of protein induced on methanol; CAT = 127 ΔE_{240}/min/mg of protein induced on oleate; AOX = 1.75 μmol of product/min/mg of protein; acyl-CoA oxidase = 0.125 μmol of product/min/mg of protein). Wild-type β-lactamase activity was 0.81247 μmol of product/min/mg of protein. ND, not done; WT, wild type.

Table 2.4

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.

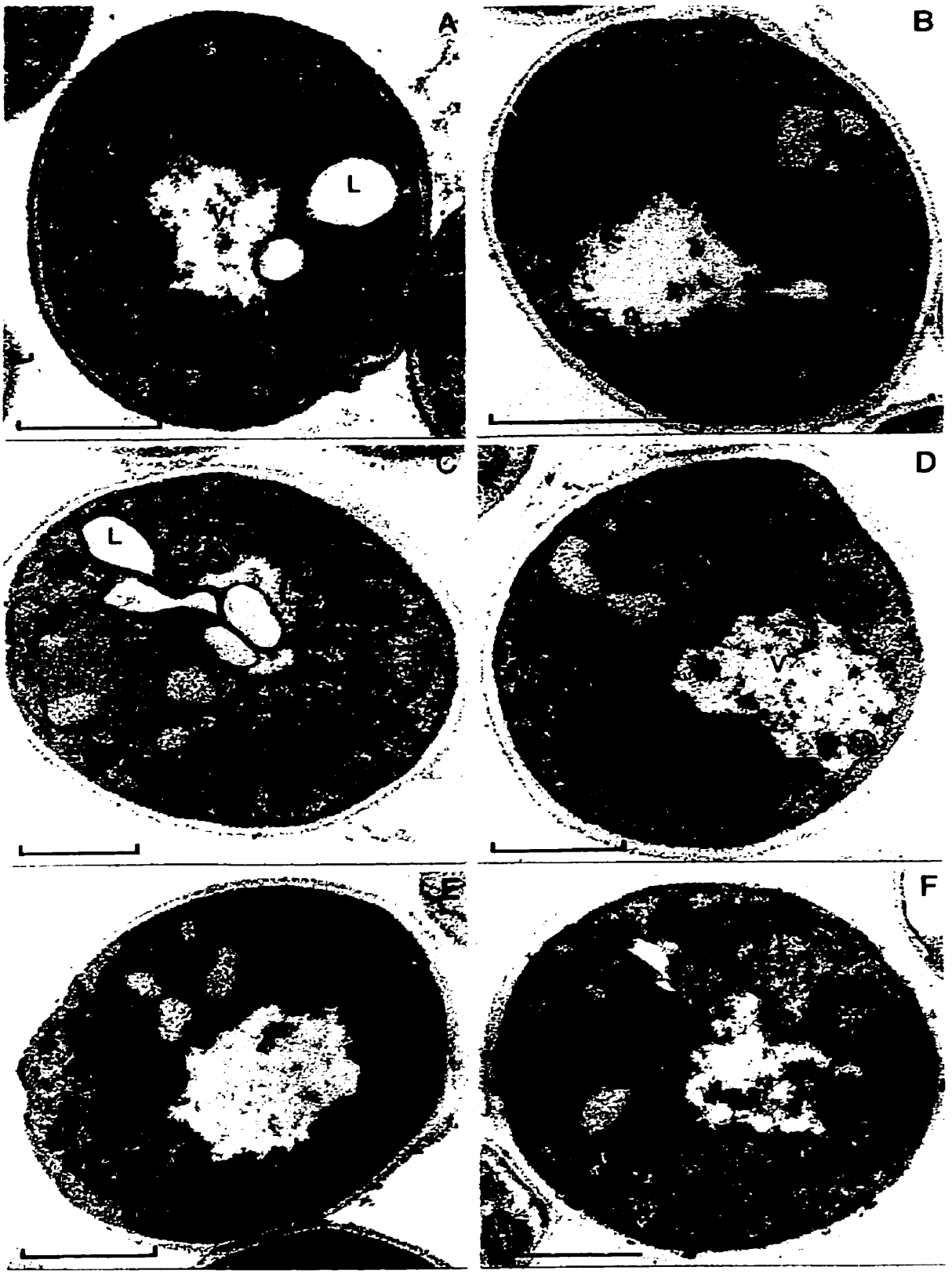
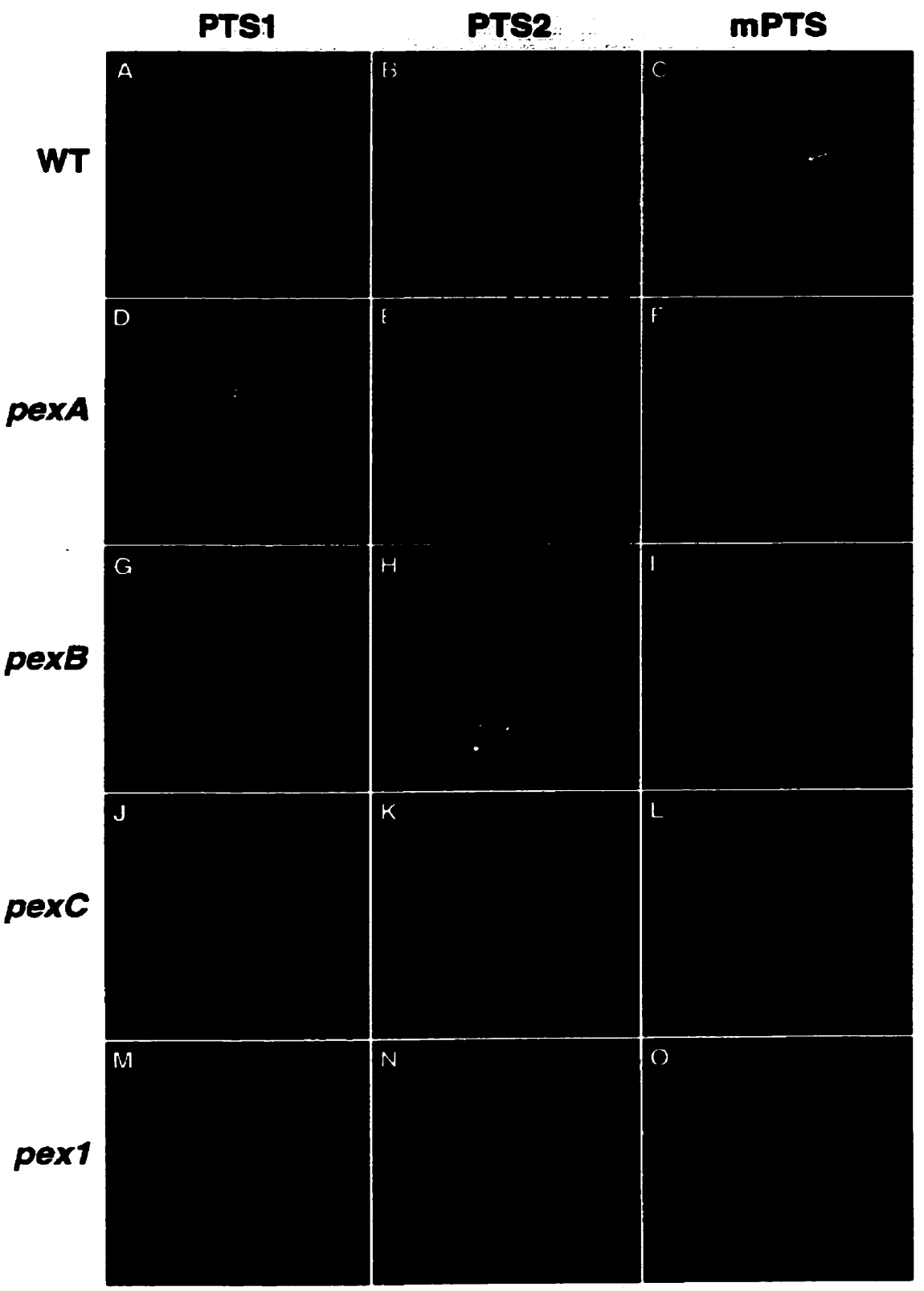

A
L
V
B
C
L
D
V
E
F

Figure 2.4 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.

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 proteins, 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 2.4), whereas in wild-type cells approximately 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 (Fig. 2.5A). However, in each of the *pex* mutants the entire cytoplasm fluoresced (Fig. 2.5D, 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 (Fig. 2.6). 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 (Fig. 2.5E, 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


PTS1
PTS2
mPTS
WT
pexA
pexB
pexC
pex1
A
B
C
D
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F
G
H
I
J
K
L
M
N
O

Figure 2.5 Subcellular location of EGFP-PTS1, PTS2-EGFP, 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) versus the cytosolic fluorescence in the mutants *pexA* (D), *pexB* (G), *pexC* (J), and *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)—versus 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. Wild-type (C) exhibits localization of fluorescence to the peroxisomal membranes seen as rings. Arrow denotes a cell in which four such rings are visible. Mutants *pexA* (F), *pexC* (L), and *pex1* (O) show fluorescence localized to what appears to be peroxisomal remnants while *pexB* (I) shows both cytosolic and peroxisomal localization.

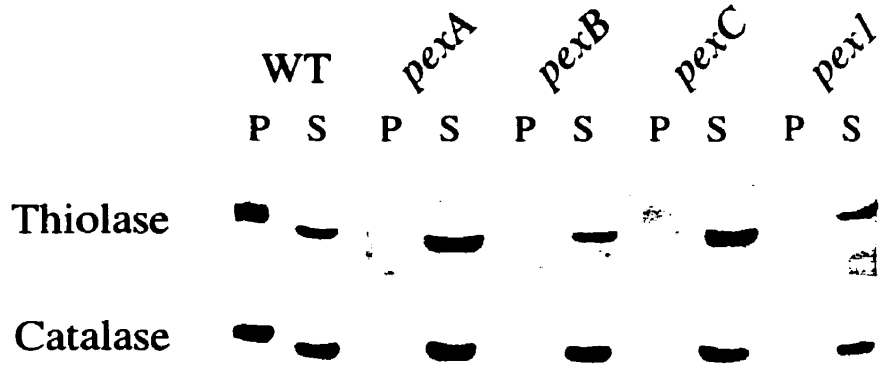


Figure 2.6 Subcellular localization of thiolase and catalase protein. Organelle pellet and cytosolic supernatant fractions obtained after subcellular fractionation of wild type, *pexA*, *pexB*, *pexC*, and *pex1*, induced on oleate, were analyzed by immunoblots with antibodies against thiolase and catalase. 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.

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 of wild-type methanol-induced cells, the Pex2p-EGFP protein localized to the peroxisomal membranes, which appeared as bright rings surrounding the organelles (Fig. 2.5C). 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 (Fig. 2.5F, 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 *pexA* and *pexC* mutants. In *pexB* cells, fluorescence also localized to punctate structures, but the intensity of the signal was significantly lower than in the other *pex* mutants and a low intensity cytoplasmic fluorescence was apparent (Fig. 2.5I). 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. Both had peroxisomal enzyme levels that were uncharacteristic of *pex* mutants (Table 2.3). 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 2.3) and protein [not shown] were low (Table 2.3). In *mxr2* cells, oleate- and

methanol-induced peroxisomal enzyme levels were low. These included activity levels for acyl-CoA oxidase and CAT (Table 2.3) and protein levels for thiolase and CAT [not shown] in oleate-induced cells, and activity (Table 2.3) 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 2.4). 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 (Fig. 2.4).

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 2.3), suggesting that *MXR1* and *MXR2* may be involved in transcription of *AOX1* and perhaps other methanol pathway genes.

2.4 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 2.2) [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 2.2). 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.

CHAPTER 3

THE *PICHIA PASTORIS* PEROXIN PEX14P INTERACTS WITH MULTIPLE PEROXINS, IS PARTIALLY PHOSPHORYLATED, AND THE PHOSPHORYLATED FORM INTERACTS PREFERENTIALLY WITH PEX13p

3.1 Introduction

Peroxisomes are organelles found in virtually all eukaryotic cells and are involved in a variety of essential catabolic and anabolic pathways [Subramani, 1998]. In mammals, including humans, these pathways include the oxidative degradation of very long-chain fatty acids and biosynthesis of plasmalogens, cholesterol and bile acids. As demonstrated by Zellweger syndrome and other lethal peroxisomal biogenesis disorders, peroxisomes are indispensable for human survival. These disorders are the consequence of defects in any one of at least thirteen different genes, termed *PEX* genes, whose products (*Pex* proteins or peroxins) are required for the biogenesis of the organelle [Wanders, 1999]. Certain yeast species including *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris* and *Yarrowia lipolitica* have been instrumental in identifying human *PEX* genes and understanding the role of their products in peroxisome biogenesis [Waterham and Cregg, 1997; Subramani, 1998]. In these yeasts, mutations in *pex* genes result in a specific inability to grow on oleic acid, methanol, or both. This has led to the isolation of over 20 different *pex* mutants and the subsequent isolation and characterization of their genes and protein products. All of the human *PEX* genes have yeast homologues, and the cloning of the human genes has been aided enormously by utilizing the amino acid sequences of the yeast Pex products to search the expressed sequence tag (EST) data bases *in silico* to discover their human counterparts [Dodt et al., 1996].

Protein import is a major component of the peroxisome biogenesis machinery [Erdmann et al., 1997; Waterham and Cregg, 1997; Subramani, 1998]. Because peroxisomes lack nucleic acids and ribosomes, all peroxisomal proteins are thought to be nuclear encoded, synthesized on free polysomes and post-translationally imported into the organelle via one of several peroxisomal targeting signals (PTSs) [Waterham and Cregg, 1997]. The first and most widely used signal, PTS1, is composed of a tripeptide sequence (SKL and conservative functional variants) present at the extreme carboxy terminus of many animal, plant, and yeast peroxisomal matrix proteins [Subramani, 1998; Geraghty et al., 1999]. PTS2, a second matrix targeting signal, is less common and is located on a nonapeptide (R/K,L/V/I,X_5,H/Q, L/A) near the NH_2-terminus [Subramani, 1998]. PTS2 is found on all 3-ketoacyl CoA thiolases from mammals and yeasts [Osumi et al., 1991; Glover et al., 1994; Rachubinski and Subramani, 1995]. Both PTS1 and PTS2 have been evolutionarily conserved from yeast to humans. Peroxisomal membrane targeting signals (mPTSs) are less well understood. mPTSs have been described for *Candida boidinii* PMP47, *P. pastoris*, *H. polymorpha* and human Pex3p, *S. cerevisiae* Pex15p, and *P. pastoris* Pex22p [McCammon et al., 1994; Baerends et al., 1996; Dyer et al., 1996; Wiemer et al., 1996; Elgersma et al., 1997; Kammerer et al., 1998; Koller et al., 1999]. The only similarity among these appears to be a small conserved region containing several positively charged residues. Little is known about mPTS receptors or other targeting/insertion components.

Recently, some of the mysteries regarding the import of peroxisomal matrix proteins have begun to unravel. A PTS1 receptor, Pex5p, was first described in *P. pastoris* and subsequently in *S. cerevisiae, H. polymorpha, Homo sapiens, Y. lipolytica* and Chinese hamster ovary (CHO) cells [McCollum et al., 1993; van der Leij et al., 1993; Dodt et al., 1995; Fransen et al., 1995; Szilard et al., 1995; van der Klei et al., 1995; Otera et al., 1998]. The *P. pastoris* Pex5p specifically recognizes and binds PTS1 sequences through its tetratricopeptide repeat (TPR) [Terlecky et al., 1995]. A PTS2 receptor, Pex7p, was first identified in *S. cerevisiae* and subsequently in *P. pastoris* and *H. sapiens* [Marzioch et al., 1994; Rehling et al., 1996; Zhang and Lazarow, 1996; Braverman et al., 1997; Elgersma et al., 1998].

Recently two putative PTS1- and PTS2-receptor docking proteins have been identified. These peroxins are located on the peroxisomal membrane surface and are believed to bind Pex5p and Pex7p carrying nascent proteins as a second step in matrix protein import. Pex13p, an integral membrane protein, binds Pex5p through its cytosolic Src homology 3 (SH3) domain in *S. cerevisiae, P. pastoris,* and in *H. sapiens* and Pex7p through a portion of its amino terminus that does not contain the SH3 domain [Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996; Girzalsky et al., 1999]. The second potential docking protein is Pex14p. In *S. cerevisiae*, Pex14p interacts with both PTS receptors, Pex5p, Pex7p, Pex13p, Pex17p and itself (homo-oligomerization) [Albertini et al., 1997; Brocard et al., 1997; Huhse et al., 1998; Girzalsky et al., 1999]. Human Pex14p interaction is limited to Pex5p, and possibly also Pex13p [Fransen et al., 1998; Will et al., 1999]. Pex14p in *S. cerevisiae* has been reported to behave as a peripherally associated peroxisomal membrane protein [Albertini et al., 1997; Girzalsky et al., 1999] and as either an integral membrane protein or tightly associated peroxisomal membrane protein [Brocard et al., 1997]. In *H. polymorpha*, humans and CHO cells, Pex14p behaved as an integral membrane protein [Komori et al., 1997; Fransen et al., 1998; Shimizu et al., 1999; Will et al., 1999]. Events occurring after Pex5p and Pex7p—carrying their respective PTS1 and PTS2-proteins—have docked with Pex13p and/or Pex14p have yet to be elucidated.

Here we report the cloning of the *P. pastoris PEX14* gene and the characterization of its product, Pex14p. We find that *P. pastoris* Pex14p is peripherally associated with the cytosolic side of the peroxisomal membrane. Pex14p is necessary for the import of both PTS1 and PTS2 proteins but not for the targeting of peroxisomal integral membrane proteins. Pex14p is found complexed with Pex5p, Pex7p, Pex13p, Pex17p, itself, and interestingly Pex8p—a previously unreported interaction. We also report that a portion of Pex14p is phosphorylated and that its interaction with Pex13p requires that phosphorylation. This interaction has important implications for its possible regulatory role in the import process.

3.2 Materials and Methods

3.2.1 Strains, media and microbial techniques

P. pastoris strains used in this study are listed in Table 3.1. Cultures (liquid and solid) were grown or induced at 30°C 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 0.5% (wt/vol) ammonium sulfate (Difco Laboratories Inc., Detroit, MI), supplemented with one of the following: 0.5% (vol/vol) methanol (YNM medium); 0.1-0.4% (wt/vol) glucose (YND medium); 0.2% (vol/vol) oleate plus 0.02% (vol/vol) Tween 40 and 0.05% (wt/vol) yeast extract (YNO medium). Sporulation/mating medium was 0.5% (wt/vol) sodium acetate, 1% (wt/vol) potassium chloride, and 1% (wt/vol) glucose. 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. The sporulation and mating procedures for classical genetic manipulation of *P. pastoris* have been described previously [Cregg et al., 1998]. Transformation of *P. pastoris* was done by electroporation according to Cregg and Russell [1998]. Cultivation of *Escherichia coli* strain DH5α and standard recombinant DNA techniques were performed essentially as described previously [Sambrook et al., 1989].

3.2.2 Cloning and sequence analysis of *PEX14*

To isolate the *PEX14* gene, the *P. pastoris pex14-1 his4* mutant JC400 was transformed with a *P. pastoris* genomic DNA library [Liu et al., 1995]. Following selection for histidine prototrophy on YND medium plates, transformants were collected from plates, pooled and inoculated into liquid YNM medium at a starting optical density of 0.1 at 600 nm (OD_{600}). The cultures were incubated with shaking for several days. Optical densities were measured daily and when cultures reached approximately 1.0 OD_{600}, the cells were harvested. Total DNA was extracted from the yeast cells and transformed into *E. coli* DH5α. Two of the recovered plasmids, pMJPEX14-6 and pMJPEX14-11, restored histidine prototrophy and methanol-growth

Table 3.1

Strains Used in This Study

Name	Genotype	Comments	Source
JC140	*pex14-1 arg4*		Johnson et al., 1999
JC227	*ade1 arg4*		Cereghino (Cregg)
JC400	*pex14-1 his4*		This study
JC401	*pex14-1 his4* (pMJKORF1)P_{PEX8}*PEX14*	rescued	This study
JC402	*pex14-1 his4* (pMJPHORF1)P_{AOX}*PEX14*	rescued	This study
JC403	*pex14Δ::SARG4 arg4*	deletion	This study
JC404	*pex14Δ::SARG4 arg4 his4*	deletion	This study
JC405	*pex14Δ::SARG4 arg4 ade4*	deletion	This study
JC406	*pex14Δ::SARG4 arg4 his4* (pMJKORF1)	*PEX14* rescued	This study
JC407	*pex14Δ::SARG4 arg4 his4* (pMJPHORF1)	*PEX14* rescued	This study
JC408	*pex14-1 his4* (pHW017)	P_{AOX}Luc	This study
JC409	*pex14Δ::SARG4 arg4 his4* (pHW017)	P_{AOX}Luc	This study
JC410	*pex14-1his4* (pMJJSORF1)(pHW017)	P_{AOX}Luc *PEX14* rescued	This study
JC411	*pex1 his4* (pHW017)	P_{AOX}Luc	This study
JC412	*his4* (pHW017)	P_{AOX}Luc	This study

JC413	*pex14-1 his4* (pOPGP1)	P_{PEX8}EGFP-PTS1	This study
JC414	*pex14Δ::SARG4 arg4 his4* (pOPGP)	P_{PEX8}EGFP-PTS1	This study
JC415	*pex14-1his4* (pMJJSORF1)(pOPGP)	P_{PEX8}EGFP-PTS1 *PEX14* rescued	This study
JC416	*pex1 his4* (pOPGP)	P_{PEX8}EGFP-PTS1	This study
JC417	*his4* (pOPGP)	P_{PEX8}EGFP-PTS1	This study
JC418	*pex14-1 his4* (pTW65)	P_{ACO}PTS2-EGFP	This study
JC419	*pex14Δ::SARG4 arg4 his4* (pTW65)	P_{ACO}PTS2-EGFP	This study
JC420	*pex14-1 his4* (pMJJSORF1) (pTW65)	P_{ACO}PTS2-EGFP rescued	This study
JC421	*Pex14-1 his4* (pLC303)	P_{AOX}mPTS-EGFP	This study
JC422	*pex14Δ::SARG4 arg4 his4* (pLC303)	P_{AOX}mPTS-EGFP	This study
JC423	*pex14-1 his4* (pMJKORF1) (pLC303)	P_{AOX}mPTS-EGFP *PEX14* rescued	This study
JC424	*pex1 his4* (pLC303)	P_{AOX}mPTS-EGFP	This study
JC425	*his4* (pLC303)	P_{AOX}mPTS-EGFP	This study
JC426	*ade1/ADE1 arg4/arg4 his4/HIS4*	Diploid strain	This study

upon transformation back into JC400. Restriction analysis showed the library inserts in pMJPEX14-6 and in pMJPEX14-11 to be approximately 6.3 kb and 9.2 kb, respectively. DNA sequencing of both strands of the insert in pMJPEX14-6 was performed using ADI 373 Automated Sequencer (PE Biosystem, Foster City, CA) at the Oregon Regional Primate Research Center, Beaverton, OR. Open reading frames (ORFs) were identified using MacVector software. The BLAST-BEAUTY Network Service of the National Center for Biotechnology Information was used to search for sequence similarities in protein data bases. Sequence alignments were done using MacVector ClustalW. ORF1 was subcloned under the control of the *P. pastoris PEX8* promoter into pK312 [Johnson et al., 1999] and transformed into JC400 to test for the ability of this ORF to complement methanol and oleate growth defects. COILS [Lupas et al., 1991] was used to search for coiled-coil motifs. The algorithms of Klein and coworkers [Klein et al., 1985] and Kyte and Doolittle [1982] were used to search for transmembrane domains. 1275 bp of *P. pastoris PEX14* sequence have been deposited in GenBank and were assigned the accession number AF200421.

3.2.3 Construction of a *PEX14* deletion strain

To delete the wild-type *PEX14* gene, the *S. cerevisiae ARG4* gene (*SARG4*) [Beacham et al., 1984] was amplified by polymerase chain reaction (PCR) with primers that contained 75 bp of *PEX14* 5′ flanking region along with 20 bp of *SARG4* 5′ sequence and 77 bp of *PEX14* 3′ flanking region along with 20 bp of *SARG4* 3′ sequence. The 5′ forward primer sequence was 5′-ATCATATTTAAGGCCCAT-CTTCCCCAACCTCGAGTAGTGTTTGTTGTTGTGCCATCTGTTATCAAGCCGTC CGAAAATAAATGGTTGGCGCAGGC-3′ and consisted of 75 bases immediately upstream of the *PEX14* methionine initiator codon ATG (–75 to –1) followed by 20 bases (–375 to –352) upstream of the *SARG4* ATG. The 3′ reverse primer sequence was 5′-ATTTCAATGCATAATGCGCCCAGAAGCTGAGCTTCTCAAGT-AAGTAACTTTCTATTACCGTTGATCAACAGCGATACATACGACTTTGGGAGG TTACAA-3′ and consisted of 77 bases downstream of *PEX14* (+26 to +102 from the translational stop codon) followed by 20 bases of *SARG4* 3′ flanking region (+327 to

+346 from the translational stop codon). The template used was pYM25 which is composed of a 3.1 kb *Hin*dIII fragment encoding the *SARG4* gene inserted into the HindIII site of pBR322 [Cregg et al., 1989]. PCR yielded a 2.2-kb product, consistent with the expected 2256-bp product (2104 bp of *SARG4* plus 75 bp of *PEX14* 5′ flanking region plus 77 bp of *PEX14* 3′ flanking region). This PCR product was transformed into the *P. pastoris arg4/arg4* diploid strain JC426 (see Table 3.1). Diploid arginine prototrophs were sporulated and spores germinated on YND (plus adenine and histidine) plates; these haploid cells were screened for ability to grow on YNM plates. Mut$^-$ strains were examined for correctly targeted genomic integration of *SARG4*, and deletion of *PEX14* by PCR. For PCR analysis, total genomic DNA was recovered from three putative *pex14Δ::SARG4* strains (JC403, JC404, JC405). PCR was performed using primer A, a forward primer (5′-CCCCAACCTCGAGTAGTG-3′) comprised of nucleotides -54 to -37 of the *PEX14* 5′ flanking region, in combination with either reverse primer B (5′-ACGTATTCTTTATGCTCTCA-3′) which is the complement of nucleotides 614 to 633 within the *PEX14* ORF or in combination with reverse primer C (5′-TGTATGAAACCAAATTCT-3′) which is the complement of nucleotides 1180 to 1196 of *SARG4*. PCR with primers A and B on wild-type genomic DNA as a template was predicted to yield a product composed of *PEX14* -54 to 633 (687 bp). In contrast, using primers A and B with *pex14Δ::SARG4* strain genomic DNA as a template should yield no PCR product. PCR with primers A and C and wild-type genomic DNA as a template should yield no PCR product, while primers A and C with *pex14Δ::SARG4* strain genomic DNA as a template should yield a PCR product of 1250 bp (54 bp of *PEX14* 5′ flanking plus 1196 bp of *SARG4*). All three putative *pex14Δ::SARG4* strains generated PCR products consistent with predicted PCR fragment sizes.

3.2.4 Preparation of anti-Pex14p antibodies

The carboxy-terminal 146 amino acids of Pex14p were expressed in *E. coli* as a fusion protein with maltose-binding protein (MBP) using the Protein Fusion and

Purification System supplied by New England Biolabs (Beverly, MA). To subclone this 446 bp fragment which included the *PEX14* translational stop codon and 5 additional 3′ bp, PCR was performed using primers that added a 5′ *Bam*HI site (5′-CGCGGATCCTCTGTACCAATAAGGACACAACTC) and a 3′ *Pst*I site (5′-TTAACTGCAGGACAACTCAGCTTTGAGCTGCCAACTG) to the *PEX14* fragment. The PCR product was inserted into *Bam*HI and *Pst*I cut pMAL-c2 resulting in pMJMEND-6. The MBP-PEX14 junction was sequenced to confirm that the *PEX14* sequence was in the correct reading frame with respect to MBP. The fusion protein was expressed and purified according to New England Biolabs protocols. Purified MBP-Pex14p fusion protein was then used to immunize rabbits (Josman Laboratories, Napa, CA). Crude serum had only one significant cross-reacting band of approximately 70 kDa and was used for immunoblotting directly.

3.2.5 Plasmid constructions

All plasmids used for this work are listed in Table 3.2.

pMJPHORF1, an *E. coli-P. pastoris* shuttle vector that expressed the *PEX14* ORF under the control of the *P. pastoris* alcohol oxidase1 gene promoter (P_{AOX1}), was constructed as follows: *Mfe*I sites were added to both ends of the *PEX14* ORF by PCR using as forward primer 5′-GGCGGCCAATTGATGTCCAGTATACGT-GAAGAAATG-3′, and as reverse primer 5′-CGATACCAATTGTCAGCTTTG-AGCTGCCAACTGCCAAG-3′. The PCR product was inserted at the *Eco*RI site of pHIL-A1 (Phillips Petroleum, Bartelsville, OK) The vector was linearized within *HIS4* by digestion with *Sal*I prior to transformation into *P. pastoris*.

pMJKORF1, a vector capable of expressing the *PEX14* ORF in *P. pastoris* under the control of the *P. pastoris PEX8* promoter (P_{PEX8}) [Liu et al., 1995], was constructed by inserting the *PEX14* PCR product described above for pMJPHORF-3 at the *Eco*RI site of pK312 [Johnson et al., 1999]. The vector was linearized within *HIS4* by digestion with *Sal*I prior to transformation into *P. pastoris*.

pMJJSORF1, a second vector capable of expressing the *PEX14* ORF under the control of P_{PEX8} was constructed by inserting the *PEX14* PCR product described above

Table 3.2

Plasmids Used in This Study

Name	Comment	Source
P. pastoris - *E. coli* shuttle vectors		
pMJPEX14-6	pYM8+Pp library fragment (6335) bp	This study
pMJPEX14-11	pYM8+Pp library fragment (9.2 kb)	This study
pMJPHORF1	pPHIL-A1+*PEX14* (P_{AOX})	This study
pMJKORF1	pK312+*PEX14* (P_{PEX8})	This study
pMJJSORF1	pJS1 (pPICZ-B but P_{PEX8})+*PEX14*	This study
pMJMEND-6	pMALc-2+#914-1360 of *PEX14*	This study
pHW017	pHIL-A1 Luc	Waterham et al., 1996
pOPGP	pK312 with EGFP-AKL (P_{PEX8})	Johnson et al., 1999
pLC303	pPICZ-B with mPTS-EGFP (P_{AOX})	Johnson et al., 1999
pTW65	pHIL-D2 with PTS2-EGFP (P_{ACO})	Johnson et al., 1999
pK312	pHIL-A1 but with P_{PEX8} in place of P_{AOX}	Johnson et al., 1999
p17HA	*PEX17* HA tagged	This study
p13HA	*PEX13* HA tagged	This study
pHA7	*PEX7* HA tagged	This study

S. cerevisiae two-hybrid system vectors		
p2HBD14	*Sc* two-hybrid binding domain (BD) containing full-length (FL) *PEX14*	This study
p2HAD14	*Sc* two-hybrid activating domain (AD) containing FL *PEX14*	This study
p2HB13	*Sc* two-hybrid BD containing FL *PEX13*	This study
p2HAD13	*Sc* two-hybrid AD containg FL *PEX13*	This study
pBD13sh3	*Sc* two-hybrid BD containing the SH3 domain of *PEX13*	This study
pAD13sh3	*Sc* two-hybrid AD containing the SH3 domain of *PEX13*	This study
p2H17	*Sc* two-hybrid BD containing FL *PEX17*	Snyder et al., 1999b
p2H17NB	*Sc* two-hybrid BD containing *PEX17* [1-124]	Snyder et al., 1999b
p2H17lum	*Sc* two-hybrid BD containing *PEX17* [1-59]	Snyder et al., 1999b
p2H17cyt	*Sc* two-hybrid BD containing *PEX17* [52-267]	Snyder et al., 1999b
E. coli expression vector		
pMJSORF7C	pSE380+*PEX14* (*E.coli* expression)	This study

for pMJPHORF1 at the *Eco*RI site of pJS1. pJS1 is the zeocine resistance selection vector pPICZ B (Invitrogen, San Diego, CA) with a *Bgl*II-*Eco*RI fragment containing P_{AOX} excised and replaced with a *Bam*HI-*Eco*RI fragment from pK312 carrying P_{PEX8}. The vector was linearized within *PEX14* by digestion with *Bgl*II prior to transformation into *P. pastoris*.

pMJSORF7C, an *E. coli* vector capable of expressing the *PEX14* ORF in *E. coli*, was constructed as follows: a PCR product composed of the *PEX14* ORF with an added 5′ *Nco*I site and a 3′ *Eco*RI site was made by PCR using the 5′ forward primer 5′-TAGCGTCCATGGCCAGTATACGTGAAGAAATG-3′ and the 3′ reverse primer 5′-CGATACCAATTGTCAGCTTTGAGCTGCCAACTGCCAAG-3′. The resulting PCR product was inserted into the IPTG inducible vector, pSE380 (Invitrogen) at its *Nco*I and *Eco*RI sites. The promoter-*PEX14* junction was sequenced to confirm that *PEX14* was properly inserted in the correct reading frame.

pTW65 is a vector that expresses the chimeric protein PTS2-EGFP under control of the *P. pastoris* acyl-CoA oxidase promoter (P_{ACO}) [Johnson et al., 1999].

Two-hybrid clones containing *PEX17* and sub-domains are described elsewhere [Snyder et al., 1999b]. A full-length clone of *PEX14* was amplified by PCR (Primers 2h14u [GCGGATCCATGTCCAGTATACGTGAAGAAATG] and 2h14d [GATCCTGCAGGCTTTGAGCTGCCAACTGCC]) and inserted as a *Bam*H1-*Pst*I fragment into pKNSD55 (two-hybrid binding domain vector) and pKNSD52 (two-hybrid activating domain vector) cut with *Bam*HI and *Pst*I, creating p2HBD14 and p2HAD14. Full length *PEX13* and the SH3 (247-380) domain were amplified by PCR and introduced into pKNSD55 and pKNSD52 as follows: *PEX13* (primers 2h13u [GTCCAGATCTATGAGACTCATCAGCTCC] and 2h13d [CGCGACTACTTT-ATGTCTTCATCTTCT]) were cut with *Spe*I and *Bgl*II and inserted into vectors cut with *Spe*I and *Bam*HI, creating p2HBD13 and p2HAD13; *PEX13*[SH3] (primers P13sh3u [GTCCAGATCTAAGAAATTAATTGCTCATCTTGC] and 2h13d) were cut with *Spe*I and *Bgl*II and cloned into vectors cut with *Spe*I and *Bam*HI, creating pBD13sh3 and pAD13sh3.

The strain expressing Pex17HAp is described elsewhere [Snyder et al., 1999b]. The HA epitope was cut from the *PEX17HA* plasmid [Snyder et al., 1999b] by cutting with *Xma*I and *Pst*I and was cloned into pIB1 (a gift of Ben Glick, University of Chicago) cut with the same, creating pIBHA. *PEX13* was amplified by PCR with primers TAG13u (GCGCCAATTGACACTTTCACCCCGCGTTTG) and TAG13dn (GCGCCCCGGGTGTCTTCATCTTCTGAAATTCTG), cut with *Mfe*I and *Xma*I and cloned into pIBHA cut with *Eco*RI and *Xma*I, creating p13HA. This plasmid was linearized by cutting with *Sal*I and integrated into the *HIS4* locus of the *pex13*Δ strain [Elgersma et al., 1996], creating strain SWS13HA. Pex13HAp complements the *pex13*Δ strain for growth on methanol and oleate. *HAPEX7* was created by two-step PCR. Primers TAG7u (GCGCAGATCTTACATGCCCGG-GCGCATCTTTTAC) and HA7d (CGTTTGTTTGGAACTTAAACATGCGG-CCGCACTGAGCAG) were used to amplify the *HA* tag and primers TAG7d (GCGCCAATTGTTACTGTTGTCTCTGTGTATTC) and HA7u (CTGCTC-AGTGCGGCCGCATGTTTAAGTTCCAAACAAACG) were used to amplify *PEX7*. The two products were gel purified and combined as a template for PCR with primers TAG7u and TAG7d, creating the full-length *HA-PEX7*. This was cut with *Bam*HI and *Eco*RI and cloned into p21.43 [Snyder et al., 1999a] cut with *Bgl*II and *Eco*RI, creating pHA7. pHA7 was linearized with *Sal*I and integrated into the *HIS4* locus of the *pex7*Δ strain [Elgersma et al., 1998], creating SWS7HA which was complemented for growth on oleate.

3.2.6 Preparation of cell lysates

To prepare *P. pastoris* cell lysates, cells were precultured in YPD and harvested at approximately 1.0 OD_{600}units. For mutant strains, 50 OD_{600} units were resuspended in 50 ml of either methanol or oleate medium and then induced for 6 h at 30°C. For wild-type strains, 25 OD_{600} units were suspended in 50 ml of medium and processed as described for the mutant strains. Cells were harvested and washed twice by centrifugation with ice-cold 50 mM potassium phosphate buffer (pH 7.0) and stored as frozen pellets at -20°C. Cell pellets were thawed and suspended 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 added to 1/3 volume of buffer. The mixture was then vortexed at 4°C at high speed for 10 minutes followed by a 1-minute centrifugation in a minicentrifuge at 14,000 rpm and 4°C. The cell-free lysate supernatant was collected and stored on ice.

3.2.7 Subcellular fractionation and sucrose density gradient centrifugation

Cells were pregrown in YPD medium, transferred during logarithmic growth phase (1–1.5 OD_{600}) by centrifugation into YNO or YNM medium and induced for 6 h at 30°C. Subcellular fractionation of these cells was performed as previously described [Liu et al., 1995], with the following modifications: (1) 10 mM Na_2SO_3 was used in place of DTT; (2) incubation with zymolyase ranged from 15 to 45 minutes; (3) following treatments with zymolyase, 10 μl of 1 M PMSF was added; (4) following the collection of protoplasts by centrifugation, a wash using MOPS buffer (pH 7.2) was added to remove any remaining zymolyase; (5) the MES buffer contained 1.2 M sorbitol (instead of 0.6 M); (6) 0.5 mM EDTA, 1 mM PMSF, 1 mM leupeptin and 1 mM apoprotinin were also added to the MES buffer; and (7) the final centrifugation was performed at 30,000 $\times$ *g* (instead of 20,000) for 30 minutes. The sucrose density gradient was prepared as previously described [Waterham et al., 1996].

3.2.8 Biochemical methods

Peroxisomal alcohol oxidase (AOX) [Verduyn et al., 1984; van der Klei et al., 1990], catalase (CAT) [Ueda et al., 1990], and mitochondrial cytochrome *c* oxidase [Douma et al., 1985] activities were assayed at 30°C according to published procedures. Activities were expressed as μmol of product/minute/mg of protein for AOX, and cytochrome *c* oxidase. Activity for CAT were expressed as ΔE_{240}/minute/mg of protein. Protein concentrations were determined with the Pierce bicinchoninic acid protein assay kit (Rockford, IL) with bovine serum albumin as a standard. Transfer of protein onto nitrocellulose after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) [Laemmli, 1970] was performed using the

Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell as indicated by the manufacturer (Bio-Rad, Hercules, CA).

Immunoblotting experiments were performed with specific polyclonal (unless otherwise stated) antibodies against *P. pastoris* Pex14p, Pex5p, Pex8p, AOX, CAT, or thiolase (a gift from W. H. Kunau, Ruhr University, Bochum, Germany), or monoclonal antibodies against HA. Primary antibodies and dilutions used were as follows: α-Pex5 (1:5000), α-Pex8p (1:10,000), α-Pex14p (1:10,000), rat-α-HA (1:1000). The secondary antibody used was either protein A conjugated horseradish peroxidase antibody (Bio-Rad), goat-anti-rat conjugated horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) or goat-anti-rabbit horseradish peroxidase conjugated antibody preabsorbed against rat sera (Jackson ImmunoResearch Laboratories). Detection was done using either an ECL (Amersham Corporation, Arlington Heights, IL) or Western Light kit (Tropix, Bedford, MA) according to the manufacturers' protocols.

3.2.9 Fluorescence microscopy

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

3.2.10 Extractions of protein from membranes

For carbonate extractions, 1 mg of oleate-induced cell lysate was adjusted to a final concentration of 0.1 M Na_2CO_3, pH 11.5, plus 1mM PMSF, 1 mM leupeptin and 1 mM aprotinin and incubated for 30 min. on ice followed by centrifugation at 100,000 $\times$ g for 60 min. For high salt extractions, 1 mg of oleate-induced cell lysate was adjusted to a final concentration of 0.5 M KCl, 0.01 M Tris-HCl, pH 8 and 1 mM PMSF, incubated on ice for 30 min. and centrifuged at 100,000 $\times$ g for 60 min.

For all extraction experiments, both resulting pellet and supernatant fractions were trichloroacetic acid (TCA) precipitated and acetone washed. Equal protein was loaded in all lanes for SDS-PAGE.

3.2.11 Proteinase K treatment

Subcellular fractionation of oleate induced cells was performed as described above except that no protease inhibitors were added following the centrifugation of the spheroplasts. Following the 30,000 × *g* spin, the organellar pellet was resuspended in disruption buffer (5 mM MES, 0.5 mM EDTA, 1.2 M sorbitol pH 6.0). A volume containing 100 μg total protein per sample was then treated as follows: (1) proteinase K was added to result in one of the following concentrations: 0.0, 0.01, 0.05, 0.1, or 0.3 mg/ml; (2) additional disruption buffer was added to bring the volume of each sample to 350 μl; (3) samples were incubated on ice for 20 min; (4) 4 mM PMSF was added to stop proteinase K reactions; (5) samples were TCA precipitated, acetone washed, and resuspended in 40 μl of 1X SDS-PAGE sample loading buffer. 15 μl of each sample was loaded on each of two SDS-PAGE gels to immunoblot with Pex14p antibodies or CAT antibodies.

3.2.12 Luciferase measurement

Luciferase activity was determined using 10 μl of sample cell lysate plus 50 μl of luciferase buffer (TB101 Luciferase Assay System, PROMEGA, Madison, WI) at room temperature according to the PROMEGA protocol. Chemiluminescence was measured for 10 seconds following a 2-second delay on a Turner Designs TD20/20 Luminometer (PROMEGA). Relative light units (RLU) were determined per microgram of total protein and used to calculate percent activity (relative to total activity) in pellet and supernatant fractions.

3.2.13 Phosphatase treatment

Phosphatase treatments were done in a manner similar to one previously described [Elgersma et al., 1997]. Cells from 50 ml 6 h oleate-induced cultures ($OD_{600} \approx 1$) were centrifuged, washed and frozen as cell pellets at -20°C. The

pellets were resuspended in 400 μl of 50mM potassium phosphate buffer, pH 7.0, lysed with glass beads, and centrifuged as described above for preparation of cell lysates. SDS and β-mercaptoethanol were added to final concentrations of 0.5% and 1%, respectively, and the resulting solution was then boiled for 5 min. 10 μl of this solution was added to 40 μl of potato acid phosphatase (PAP) buffer (50 mM MES, pH 6.0; 1 mM DTT, 1 mM PMSF, 1 mM leupeptin, 1 mM aprotinin) containing 1 U of PAP (Boehringer Mannheim, Indianapolis, IN). Samples were incubated for 18 h at 28°C, TCA precipitated and acetone washed prior to SDS-PAGE and immunoblotting.

3.2.14 Two-hybrid analysis

Cloning vectors, tester strains and screening by two-hybrid analysis were performed as described previously [Faber et al., 1998].

3.2.15 Immunoprecipitation

Immunoprecipitation and crosslinking with DSP (dithiobis[succinimidyl propionate]) (Pierce) were performed from 5 OD_{600} units of oleate-grown cells as described previously [Rieder and Emr, 1997]. For immunoprecipitations, 10 μl of affinity-purified anti-Pex14p antisera (purified according to Harlow and Lane, 1988] was used and 2 μl of anti-HA was used per immunoprecipitation.

To visualize Pex14p on an immunoblot following immunoprecipitation with either Pex13HAp or Pex17HAp or HA-Pex7p, the secondary antibody used was a goat-anti-rabbit horseradish peroxidase-conjugated antibody that was pre-absorbed against rat sera (Jackson ImmunoResearch Laboratories).

3.2.16 Miscellaneous methods

Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) was used for scanning X-ray films developed from immunoblots and negatives of photographs from EGFP fluorescence microscopy. The scans were then imported into Freehand (Macromedia Inc., San Francisco, CA) or Adobe Page Maker to arrange and add

text. Electron microscopy was performed as previously described [Waterham et al., 1996].

3.3 Results

3.3.1 Cloning and identification of the *P. pastoris PEX14* gene

The *P. pastoris PEX14* gene was cloned by functional complementation of the *P. pastoris per14-1 his4* mutant (JC400) using a *P. pastoris* genomic DNA library [Liu et al., 1995]. This mutant was one of a collection of peroxisome-deficient (*pex*) mutants recently isolated in our lab using a novel positive screen [Johnson et al., 1999]. Library transformants were first selected for histidine prototrophy (His^+) and subsequently for restored ability to grow on methanol (Mut^+). Two plasmids, pMJPEX14-6 and pMJPEX14-11, were recovered that transformed the *pex14-1 his4* strain simultaneously to His^+ and Mut^+ at high frequency, indicating that both plasmids most likely contained the *PEX14* gene. Restriction analysis revealed that pMJPEX14-6 contained a smaller *P. pastoris* genomic DNA fragment (6.3 kb) and this fragment was sequenced in its entirety. Three long open reading frames (ORFs) were revealed as *PEX14* gene candidates. ORF2 potentially encoded a polypeptide with 65% identity and 78% similarity to the *S. cerevisiae* homoaconitase. The putative ORF3 product showed no sequence similarity to any other protein in the data bases. The putative ORF1 product was identified as a possible ortholog of the *H. polymorpha* and *S. cerevisiae PEX14* products, and thus, was a good candidate for the complementing gene [Albertini et al., 1997; Brocard et al., 1997; Komori et al., 1997]. To test this further, ORF1 was subcloned into a vector under the control of *P. pastoris PEX8* promoter and the resulting vector, pMJKORF1, was transformed into the *pex14-1 his4* strain. All His^+ transformants were also Mut^+, indicating that ORF1 contained the complementing gene.

ORF1 was predicted to encode a polypeptide of 425 amino acids with a calculated molecular mass of 47 kDa. Alignment of the deduced amino acid sequence of ORF1 to known Pex14 proteins showed 52% identity and 66% similarity to *H.*

polymorpha Pex14p and 29% identity and 41% similarity to *S. cerevisiae* Pex14p (Fig. 3.1). Recently, the putative human ortholog of *PEX14* was described [Fransen et al., 1998, Will et al., 1999]. The *P. pastoris* ORF1 product showed 15% identity and 22% similarity to *H. sapiens* Pex14p. In addition to amino acid sequence similarity, the yeast Pex14ps all share a conserved class II SH3-ligand binding motif (xPPLPxR) which has been shown in other proteins to facilitate interaction with SH3 domains (Fig. 3.1, indicated by overline) [Feng et al., 1994]. Interestingly, Pex14p from human, CHO, and rat do not have this motif [Shimizu et al., 1999; Will et al., 1999]. All putative Pex14ps also contain two predicted coiled-coil motifs located in approximately the same position in their sequences (loosely aligned with *Pp*Pex14 amino acids 130–250) (Fig. 3.1, between arrowheads). The coiled-coil motif is thought to be a structural element for oligomerization [Lupas et al., 1991]. The putative *P. pastoris* Pex14p showed no potential transmembrane regions [Klein et al., 1985; Kyte and Doolittle, 1982], peroxisomal targeting signals (PTS1 or PTS2), or other identifiable motifs. These results strongly indicated that we had cloned the *P. pastoris* ortholog of *PEX14*.

To confirm that ORF1 is the authentic *PEX14* gene, rather than a suppressor, and to examine the phenotype of a true *P. pastoris PEX14* null mutant, an ORF1 deleted *P. pastoris* strain was constructed. To construct this strain, a DNA fragment in which all of ORF1 was replaced by a fragment containing the *S. cerevisiae ARG4* gene (*SARG4*) was first constructed using the superprimer PCR method [Shoemaker et al., 1996]. The 2256 bp *pex14Δ::SARG4* PCR product was transformed into a JC426 *arg4/arg4* diploid strain of *P. pastoris*. The resulting Arg^+ transformants were sporulated and tested for ability to grow on methanol. Approximately 6% of the spore products were Mut^-. Several of the Mut^- spore products were examined by PCR to confirm proper insertion of the *pex14Δ::ARG4* fragment and deletion of *PEX14* (Fig. 3.2). One *pex14Δ::SARG4 ade1* strain, JC405, was then crossed with the original *pex14-1 his4* strain. Prototrophic diploids were selected on minimal glucose plates and tested for ability to grow on methanol. All were found to be

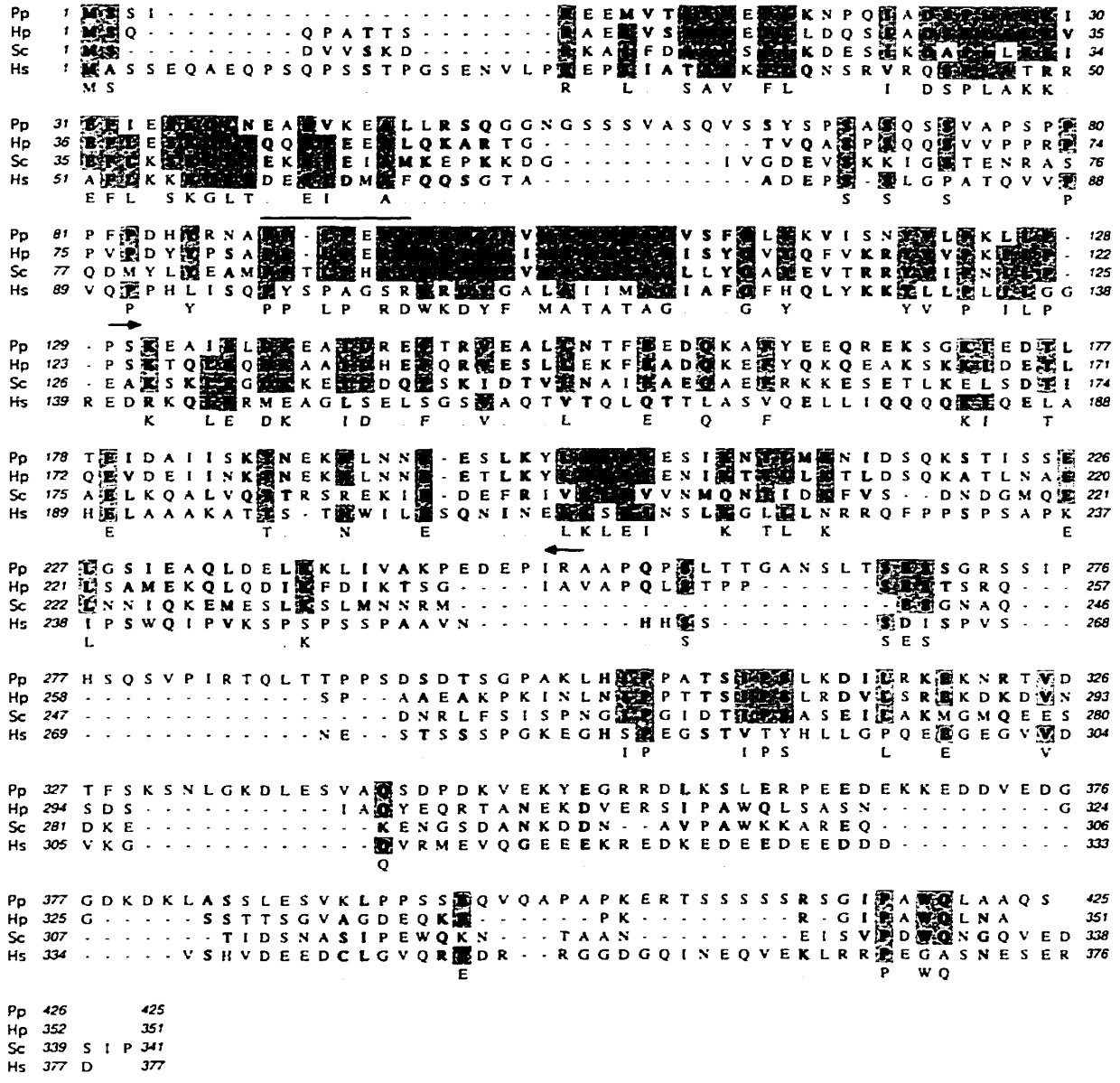


Figure 3.1 Alignment of the predicted amino acid sequences of Pex14p of *P. pastoris* (Pp), *H. polymorpha* (Hp), *S. cerevisiae* (Sc), and *H. sapiens* (Hs) using MacVector ClustalW. Amino acid residues identical in at least three sequences are shaded in dark gray; similar amino acids are shown in light gray. Similar residues are defined as follows: M=V=I=L; A=S=C; T=S=A; K=R=Q; N=T; E=D; E=Q; H=N; Q=H; N=G; F=Y. Hyphens represent spaces. Class II SH3 ligand binding domain, xPPLPxR [Feng et al., 1994], is overlined. A predicted coiled-coil region is indicated between arrows.

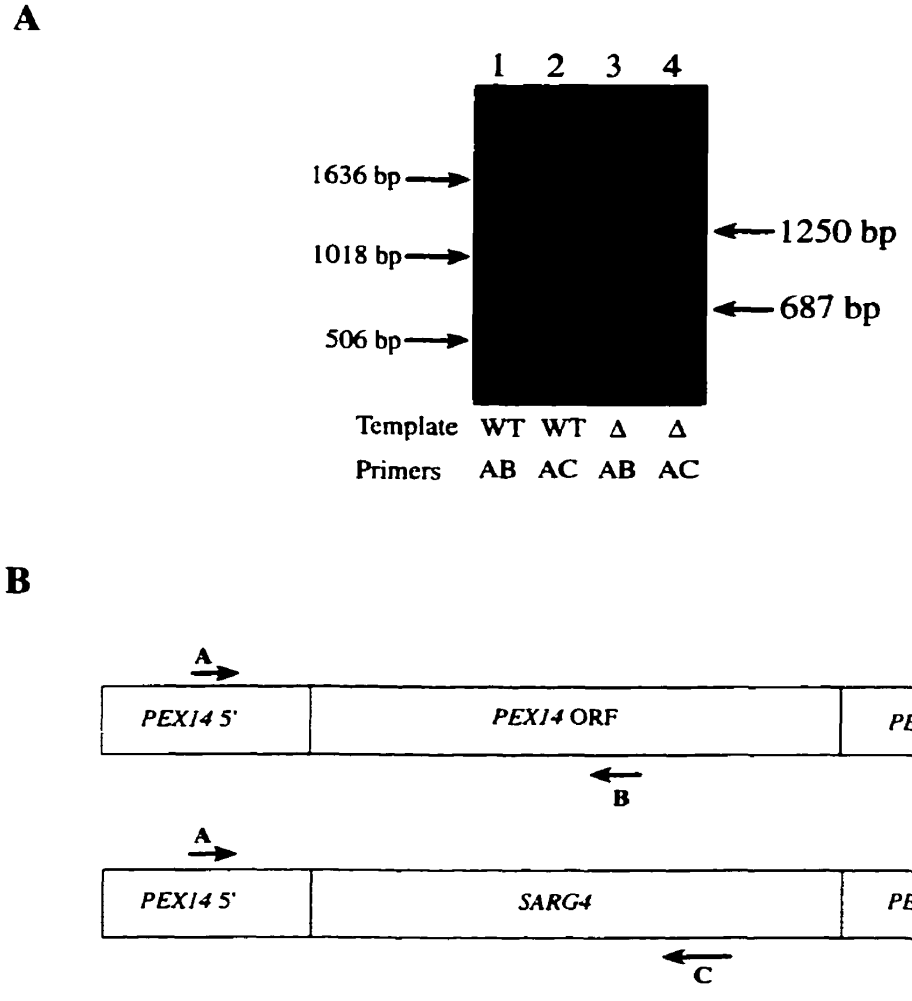


Figure 3.2 Verification of *PEX14* deletion in strain JC405 by polymerase chain reaction (PCR). (**A**) PCR products are as follows: lane 1, wild-type *P. pastoris* genomic DNA used as the template (WT) with primers A (5′ PEX14 flanking region -54 to -37) and B (complement of bases 614 to 633 of *PEX14* ORF) resulting in a predicted PCR product size of 687 bp; lane 2, WT used as the template with primers A and C (complement of bases 1180–1196 of *SARG4* ORF); lane 3, deletion strain genomic DNA used as the template (Δ) with primers A and B; lane 4, Δ used as the template with primers A and C resulting in a predicted PCR product size of 1280 bp. (**B**) Diagram of WT and Δ*PEX14* allelels showing approximate hybridization sites of primers A, B, and C.

Mut$^-$. The diploids were then sporulated and all of the approximately 600 spore products examined were found to be Mut$^-$ as well. These results indicated that the *pex14-1* and *pex14Δ* alleles are tightly linked and most probably alleles of the same gene. Based on these results, we concluded that we had cloned the authentic *P. pastoris PEX14* gene.

To confirm that we had identified the correct *in vivo* methionine initiator ATG for Pex14p, we transformed the *pex14Δ* strain with pMJKORF1, a vector containing the *PEX14* ORF under the control of the *PEX8* promoter. A transformant was induced on methanol and cell extracts prepared and subjected to SDS-PAGE and immunoblotting with Pex14p antibodies (see section on Pex14p localization for characterization of Pex14p antibodies). The Pex14ps synthesized by the P_{PEX8}*PEX14*-transformed *pex14Δ* strain and wild-type were identical in mobility (Fig. 3.3). Since use of the next in-frame ATG in either the N- or C-terminal directions by wild-type *P. pastoris* would have resulted in significantly longer or shorter Pex14p polypeptides, we concluded that we had identified the correct translational start site for Pex14p.

3.3.2 *P. pastoris pex14* mutants lack normal peroxisomes but contain peroxisomal remnants

To investigate the function of Pex14p, the phenotypic and morphological characteristics of the *pex14-1* and *pex14Δ* strains were examined and compared to wild-type *P. pastoris*. Both of the *pex14* mutants grew at a similar rate to the wild-type on glucose, glycerol or ethanol, but were specifically unable to grow on methanol or oleate [data not shown]. These mutant phenotypes were completely reversed in the *pex14-1* strain transformed with a vector containing *PEX14* under control of the *PEX8* promoter (*PEX14* rescued strain) [Liu et al., 1995]. Because peroxisomes are required for metabolism of both oleate and methanol in *P. pastoris*, these results suggested that the *pex14-1* and *pex14Δ* strains lacked functional peroxisomes [Liu et al., 1992]. Electron micrographs of methanol- and oleate-induced cells of the *pex14-1* and *pex14Δ* strains revealed the absence of normal-

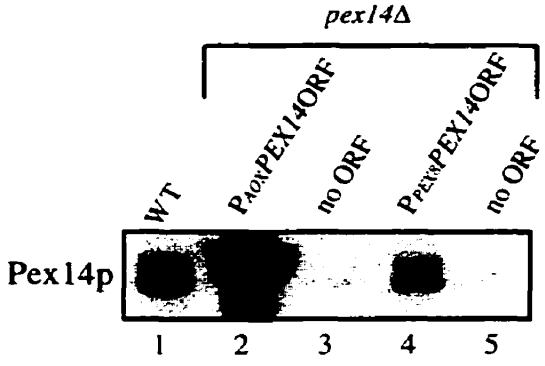


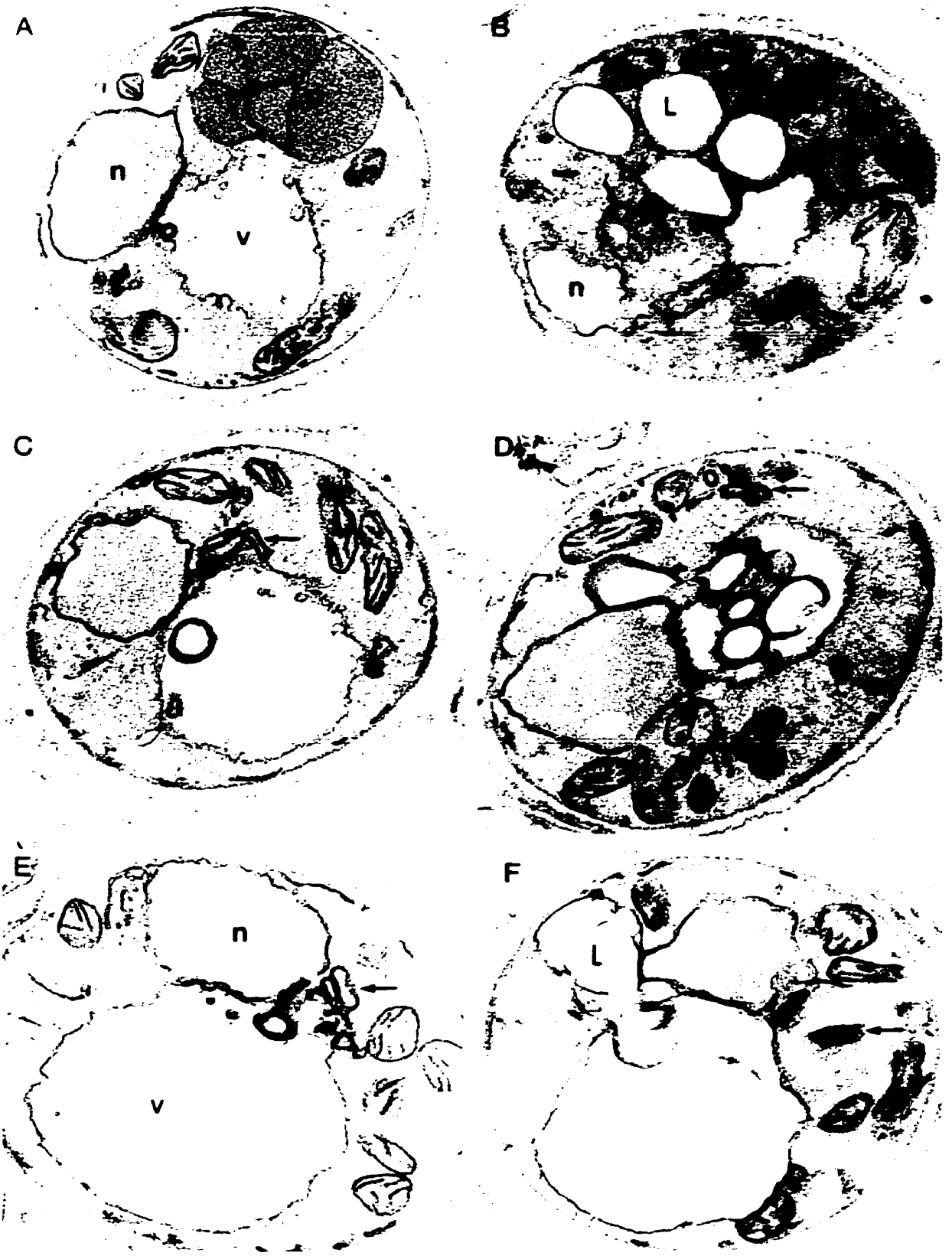
Figure 3.3 Confirmation of correct *in vivo* methionine initiator ATG for Pex14p. Cell lysates of methanol-grown cells were analyzed by immunoblot with Pex14p antibodies. Each lane was loaded with 20 μg of total protein. Lane 1 contains WT *P. pastoris*. Lanes 2–5 contain the *pex14*Δ strain transformed with: (lane 2) pMJPHORF1, which results in overexpression of *PEX14*; (lane 3) pMJPHORF1 without *PEX14*; (lane 4) pMJKORF1, which results in expression of *PEX14* controlled by the *PEX8* promoter; (lane 5) pMJKORF1 without *PEX14*.

appearing peroxisomes (Fig. 3.4). Instead, small vesicular structures were induced that appeared similar to peroxisomal remnants or ghosts observed in other *P. pastoris pex* mutants [Liu et al., 1992] (Fig. 3.4C, D, E, and F, respectively). That these vesicular structures are most likely peroxisomal remnants was supported by fluorescence microscopy studies of *PEX14* mutants expressing a known peroxisomal integral membrane protein (Pex2p) [Waterham et al., 1996] fused to green fluorescent protein, EGFP, which showed a punctate pattern typical of remnant structures in *P. pastoris pex* mutants [Johnson et al., 1999] (Fig. 3.5F and I). These peroxisome morphology defects were restored to normal in either methanol- or oleate-grown cells of the *PEX14* rescued strain (Fig. 3.4G and H; Fig. 3.5J, K, and L).

3.3.3 Pex14p is required for import of PTS1 and PTS2 proteins but not for the targeting of mPTS proteins

The function of specific PTS pathways in the *pex14-1* and *pex14*Δ mutants was investigated through a combination of subcellular fractionation experiments and EGFP-based fluorescence microscopy studies. For subcellular fractionation, cells were induced, spheroplasted, homogenized and centrifuged at 30,000 × *g*. The resulting organellar (primarily peroxisomal and mitochondrial) pellet and cytosolic supernatant fractions were then assayed for selected peroxisomal proteins either by enzyme assay or immunoblotting. Cytochrome *c* oxidase, a mitochondrial marker protein, was used as a control to confirm the general integrity of the organelles in the pellet. In all of the subcellular fractionation experiments, the *pex14* mutants behaved the same, while the *PEX14* rescued strain behaved the same as the wild-type strain.

To investigate the PTS1 import pathway in *pex14-1*- and *pex14*Δ-derived subcellular fractionations, methanol-induced cells were first assayed for alcohol oxidase (AOX), a known PTS1 protein. As seen for other *P. pastoris pex* mutants, AOX activity in the mutants was negligible [Liu et al., 1992; Johnson et al., 1999] [data not shown]. Next, fractions from both methanol- and oleate-induced mutants cells were assayed for activity for catalase (CAT), a putative PTS1 protein. CAT activity was found to be distributed approximately equally between the pellet and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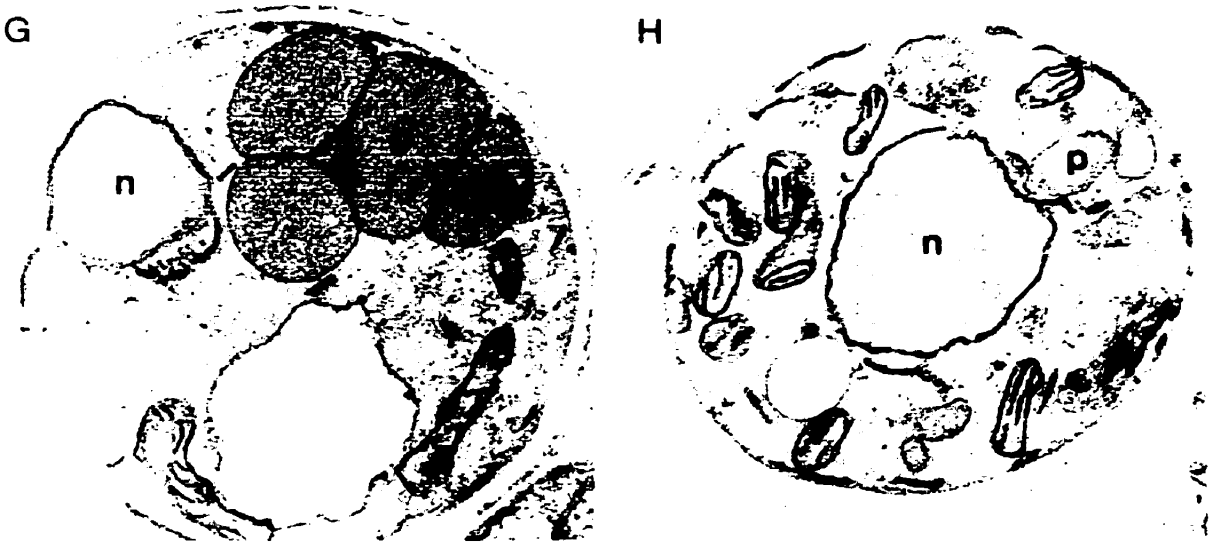
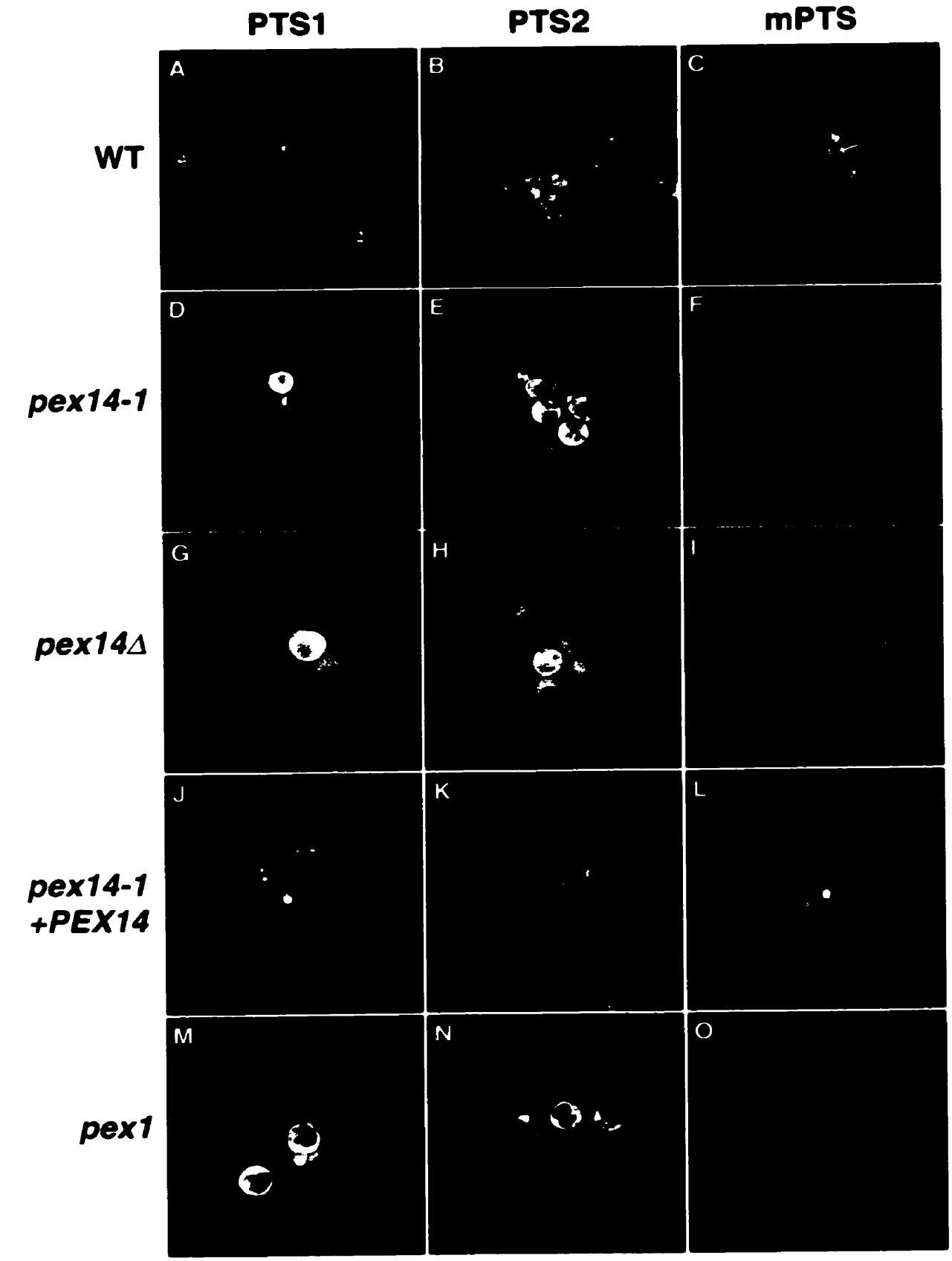


Figure 3.4 Electron micrographs showing subcellular morphology of WT, *pex14-1*, *pex14Δ*, and *pex14-1+PEX14* (rescued) strains. **(A)** Proliferation of large clusters of peroxisomes in methanol-induced WT cells. **(B)** Proliferation of small or disperse peroxisomes in oleate-induced wild-type cells. **(C,D)** Lack of recognizable peroxisomes in *pex14-1* methanol- and oleate-induced cells, respectively (peroxisomal remnants indicated by arrow). **(E,F)** Lack of recognizable peroxisomes in *pex14Δ* strain induced in either methanol or oleate, respectively (peroxisomal remnants indicated by arrow). **(G, H)** Restored peroxisomes in methanol- and oleate-induced *PEX14* rescued cells, respectively. P, peroxisome; N, nucleus; V, vacuole; L, lipid body.

A


PTS1
PTS2
mPTS
A
B
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WT
D
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pex1

B

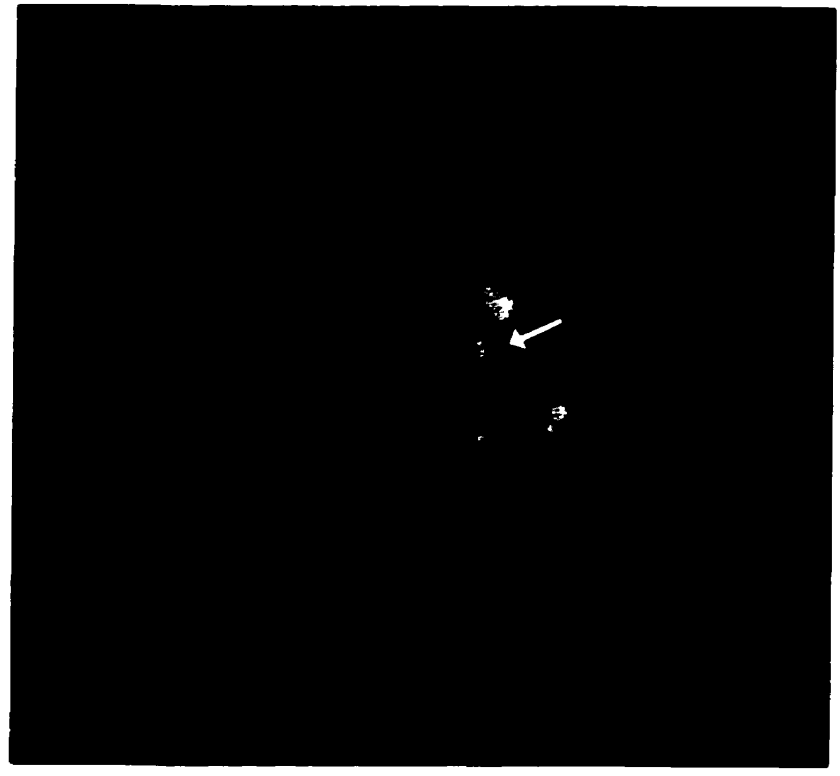

Figure 3.5 Subcellular location of EGFP-PTS1, PTS2-EGFP, and mPTS-EGFP in WT, *pex14-1*, *pex14Δ*, and *PEX14* rescued strains. (**A**) (A, D, G, J, M) Cells expressing PTS1-EGFP on methanol. Note the clusters of strongly fluorescing peroxisomes in WT (A) and rescued (J) strains versus the cytosolic fluorescence in the *pex14-1* (D), *pex14Δ* (G), and *pex1* control (M) strains. (B,E, H, K, N) Cells expressing PTS2-EGFP on oleate. As with EGFP-PTS1 strains, the WT cells (B) and rescued cells (K) exhibited a punctate pattern, although this pattern was less pronounced than in A due to induction on oleate, versus the diffuse pattern seen in *pex14-1* (E), *pex14Δ* (H) and *pex1* (M) strains. (C, F, I, L, O) Cells expressing mPTS-EGFP on methanol. WT (C) and the rescued (L) strains exhibited localization of fluorescence to the peroxisomal membranes, seen as rings. Arrow denotes a cell in which rings are visible. *pex14-1* (F), *pex14Δ* (I), and *pex1* (O) show fluorescence localized to peroxisomal remnants. (**B**) WT cells expressing mPTS-EGFP on methanol (C) enlarged to show peroxisomal membrane rings (indicated by arrow).

supernatant fractions of the wild-type strain (due to typical leakiness of peroxisomes) but was found almost entirely in the supernatant fractions of the mutants (Table 3.3) (methanol data not shown]. To further investigate PTS1 import, the fate of two PTS1 reporter proteins was followed in the *pex14* mutants. One reporter was luciferase (Luc), a known PTS1 protein [Gould et al., 1987, Keller et al., 1987]. The second was green fluorescent protein (EGFP) fused to a peptide ending in the PTS1 AKL (EGFP-PTS1) [Johnson et al., 1999]. As can be seen in Table 3.3, Luc was mislocalized in oleate-induced *pex14* mutants. Similarly, EGFP-PTS1 was mislocalized in methanol-induced mutants (Fig. 3.5D and G) but properly targeted to peroxisomes in the wild-type and rescued strains (Fig. 3.5A and J). These experiments demonstrate that *P. pastoris pex14* mutants cannot import PTS1 proteins.

The PTS2 pathway was investigated in the same manner using subcellular fractions from oleate-induced *pex14* mutant cells by examining the fate of the PTS2 enzyme, thiolase, and a PTS2-EGFP fusion protein [Gietl et al., 1994; Glover et al., 1994). Fractions immunoblotted for thiolase showed that thiolase was mislocalized to the cytosolic supernatant of the *pex14* mutants (Fig. 3.6). Interestingly, the *pex14Δ* mutant had very low levels of thiolase. In the wild-type and rescued strains, thiolase was found primarily in the pellet fractions. Similarly, fluorescence microscopy of oleate-induced cells showed PTS2-EGFP mislocalized to the cytoplasm in the *pex14-1* and *pex14Δ* strains (Fig. 3.5E and H, respectively) while the PTS2-EGFP was localized to the peroxisomes in wild-type cells (Fig. 3.5B). These data demonstrate that, as with PTS1 import, import of PTS2 proteins are defective in both *P. pastoris pex14* strains.

Finally, the function of the integral membrane protein targeting signal (mPTS) pathway was examined by fluorescence microscopy using strains expressing EGFP fused to the C-terminus of Pex2p, a known peroxisomal integral membrane protein [Waterham et al., 1996; Johnson et al., 1999]. As can be seen in Fig. 3.5C, the mPTS-EGFP is targeted to the peroxisomal membrane in methanol-induced wild-type cells (note ring structures). In methanol-induced cells of *pex14-1* and *pex14Δ*, mPTS-EGFP was targeted to the peroxisomal remnants (Fig. 3.5F, I, and O, respectively).

Table 3.3

Distribution of Peroxisomal Enzyme Activities in Organelle Pellet and Cytosolic Supernatant Fractions After Subcellular Fractionation of Oleate-Induced *P. pastoris* Cells

Fraction		Cyto *c* ox	%	CAT	%	Luc	%
WT	P	0.260	99	39.00	57	0.4300	49
	S	0.002	1	30.00	43	0.4500	51
pex14-1	P	0.300	95	0.07	1	0.0010	1
	S	0.015	5	18.00	99	0.1300	99
*pex14*Δ	P	0.370	96	0.83	2	0.0054	9
	S	0.015	4	45.00	98	0.0560	91
pex14-1 +*PEX14*	P	0.790	97	40.00	63	0.3200	55
	S	0.012	3	24.00	37	0.2600	45
pex1	P	0.400	95	0.44	5	0.0010	1
	S	0.020	5	8.20	95	0.1200	99

P, pellet; S, supernatant; WT, wild-type; Cyto *c* ox, cytochrome *c* oxidase; CAT, catalase; Luc, luciferase.

Activity units: CAT, ΔE_{240} per minute per milligram of protein; Cyto. *c* ox, μmol of product per minute per milligram of protein; Luc, relative light units per microgram of total protein.

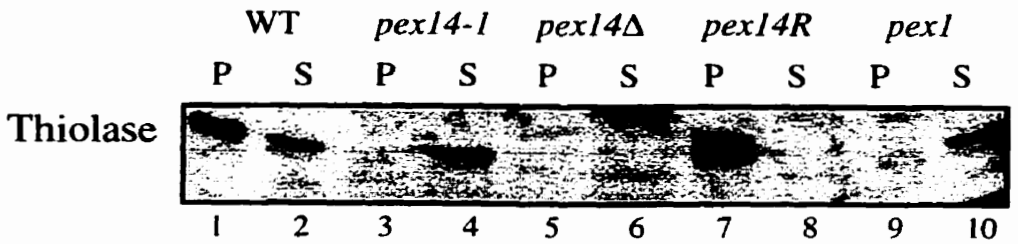

Figure 3.6 Subcellular localization of thiolase. Organelle pellet and cytosolic supernatant fractions obtained after subcellular fractionation of oleate-induced WT, *pex14-1*, *pex14Δ*, *pex14* rescued (R), and *pex1* strains analyzed by immunoblotting with antibodies against thiolase. 20 μg of total protein was loaded in each lane.

These data suggest that Pex14p is not necessary for the proper targeting of mPTS proteins.

3.3.4 Pex14p is a membrane-associated protein located on the cytoplasmic side of the peroxisome

To visualize Pex14p, polyclonal antibodies were raised in rabbits against the 153 C-terminal amino acids of Pex14p. Crude extracts prepared from wild-type *P. pastoris* cells and subjected to SDS-PAGE and immunoblotting showed a major band, at approximately 58 kDa, that was not present in extracts from the *pex14Δ* strain (Fig. 3.7). At least two anti-Pex14p antibody reacting bands were typically observed: a major 58 kDa band which represented at least 90% of Pex14p, and a slower-migrating band of approximately 60 kDa that represented less that 10% of Pex14p. The origin of the larger Pex14p species is discussed in the next section. Although both bands are visible regardless of carbon source, the overall level of Pex14p was greatest from lysates of wild-type cells induced on oleate (Fig. 3.7). From these immunoblots, we estimated that Pex14p was induced approximately 10-fold and 20-fold in methanol- and oleate-induced cells, respectively, relative to glucose-grown cells. We performed a visual search for the oleate response element consensus sequences, $CGG(N_3)TNA(N_{7-13})(G/C)CG$ or $CGG(N_{6-12})TNA(N_3)(C/G)CG$, and found neither to be present in the available 75 bp of *PEX14* upstream sequence [Geraghty et al., 1999].

Pex14p localization in *P. pastoris* was investigated by first subjecting oleic acid-induced wild-type cells to subcellular fractionation. Pex14p was found primarily in the resulting pellet fractions with a significant portion also seen in the supernatant fraction (Fig. 3.8A). Some degradation was typically apparent, most likely a result of proteolysis of Pex14p during the spheroplasting incubations.

To determine whether the Pex14p material in the pellet fraction was peroxisomal, pellet material was further fractionated by centrifugation through a sucrose-density gradient. The resulting fractions were then assayed for CAT and cytochrome *c* oxidase activities (Fig. 3.8B) [Waterham et al., 1996]. Immunoblotting

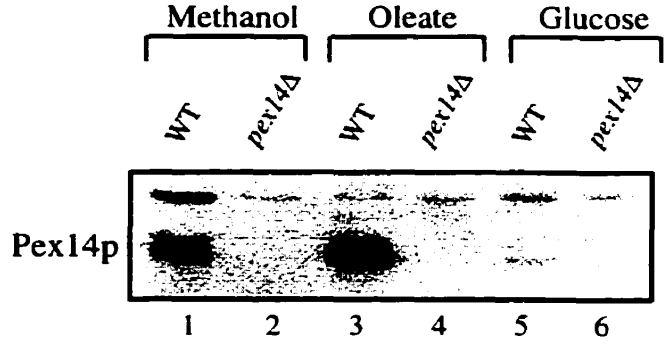

Figure 3.7 Expression of Pex14p induced on methanol, oleate, and glucose. Cell lysates of WT and *pex14*Δ strains were induced on each of the following carbon sources: methanol, oleate, and glucose. 20 μg of total protein was loaded in each lane and, after SDS-PAGE, analyzed by immunoblotting with Pex14p antibodies.

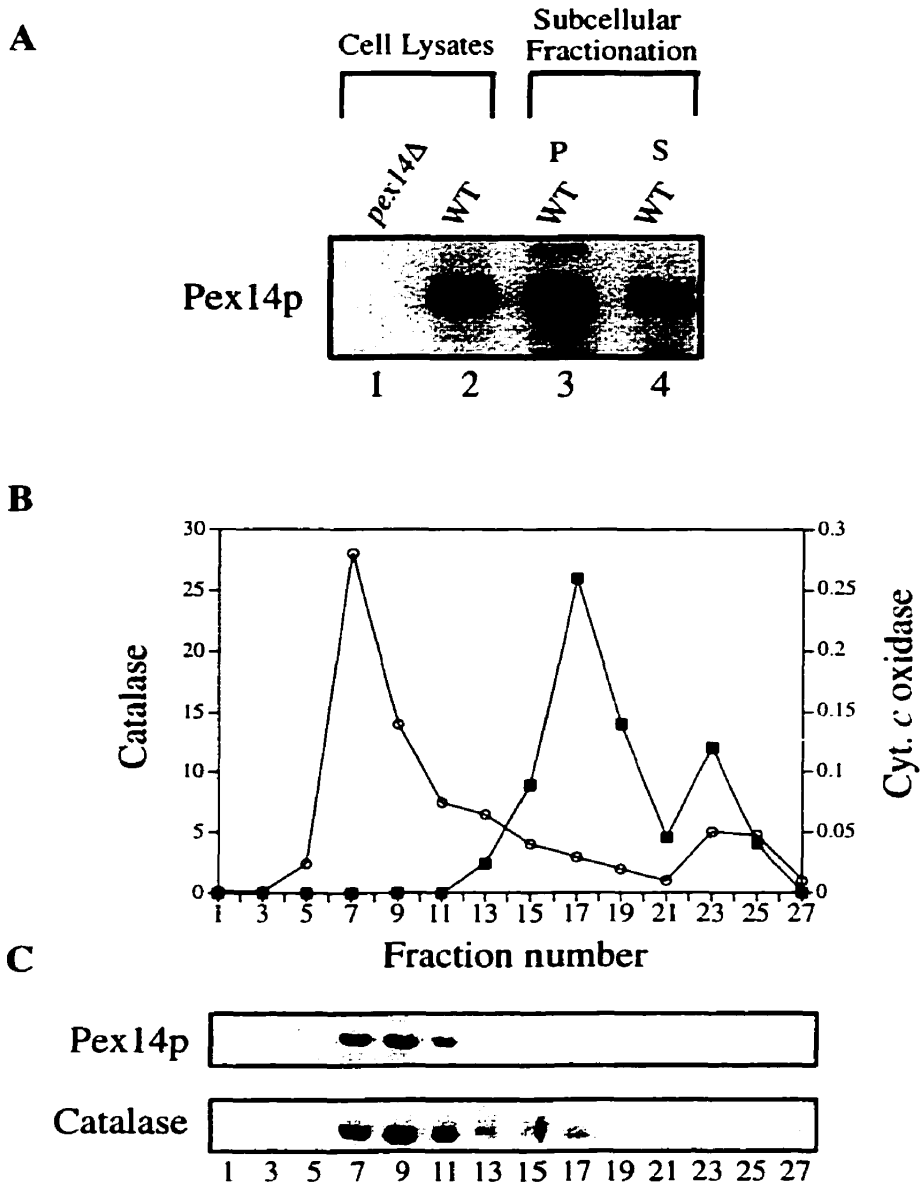

Figure 3.8 Pex14p is a peroxisomal protein. (**A**) Organelle pellet (P, lane 3) and cytoplasmic supernatant (S, lane 4) obtained after subcellular fractionation of oleate-grown WT cells were analyzed by immunoblot with Pex14p antibodies. Each lane contained 20 μg of total protein. (**B**) Sucrose density gradient profile of the organellar pellet obtained from oleate-grown WT cells. Fractions collected from the gradient were assayed for peroxisomal CAT (○) and mitochondrial cytochrome *c* oxidase (■) activities. (**C**) Equal volumes of odd-numbered fractions of the sucrose density gradient in panel B were analyzed by immunoblotting with the Pex14p and CAT antibodies.

of fractions from this gradient with Pex14p and CAT antibodies showed that Pex14p co-sedimented with CAT indicating that Pex14p is a peroxisomal protein (Fig. 3.8C).

The nature of the association of Pex14p with the organelle was examined by subjecting cell lysates of oleate induced wild-type cells to extraction with carbonate (pH 11.5). Pex14p was fully extractable under these conditions as observed by the presence of Pex14p in the post-extraction 100,000 × *g* supernatant (Fig. 3.9A), suggesting that Pex14p was not an integral membrane protein. As a control, an aliquot of the same lysate was subjected to the same procedure but without extraction with carbonate at pH 11.5. This mock extraction failed to remove Pex14p from the membrane [data not shown]. We next subjected extracts to extraction with high salt (0.5 M KCl) and observed proportions of Pex14p in post-100,000 × *g* pellet and supernatant fractions that were similar to those of the unextracted controls (Fig. 3.9B). These results suggested that Pex14p is tightly associated with the peroxisomal membrane or perhaps one or more other peroxisomal membrane proteins. The portion of Pex14p found in cytosolic supernatant fractions may represent a cytoplasmic Pex14p species, or more likely, the inadvertent removal of a portion of Pex14p from peroxisomal membranes during the extraction procedures.

To determine whether Pex14p is located on the cytoplasmic or matrix side of the peroxisome, the organellar pellets resulting from subcellular fractionation were incubated with increasing concentrations of proteinase K, and the sensitivity of Pex14p to proteolytic cleavage was examined via immunoblotting. As can be seen in Fig. 3.9C, Pex14p was sensitive to proteinase K at the lowest concentration tested but remained stable in the absence of proteinase K. In contrast, CAT, a matrix protein, was insensitive to proteinase K even at the highest concentration. These results indicate that Pex14p is exposed on the cytoplasmic face of peroxisomes.

3.3.5 A portion of Pex14p is phosphorylated

Throughout our studies of Pex14p, we noted that Pex14p appeared on immunoblots as at least two distinct species, a major species of 58 kDa and a minor species at approximately 60 kDa (e.g., Fig. 3.3). Neither of these bands was present in the *pex14Δ* strain. One explanation for the multiple bands was that the faster-

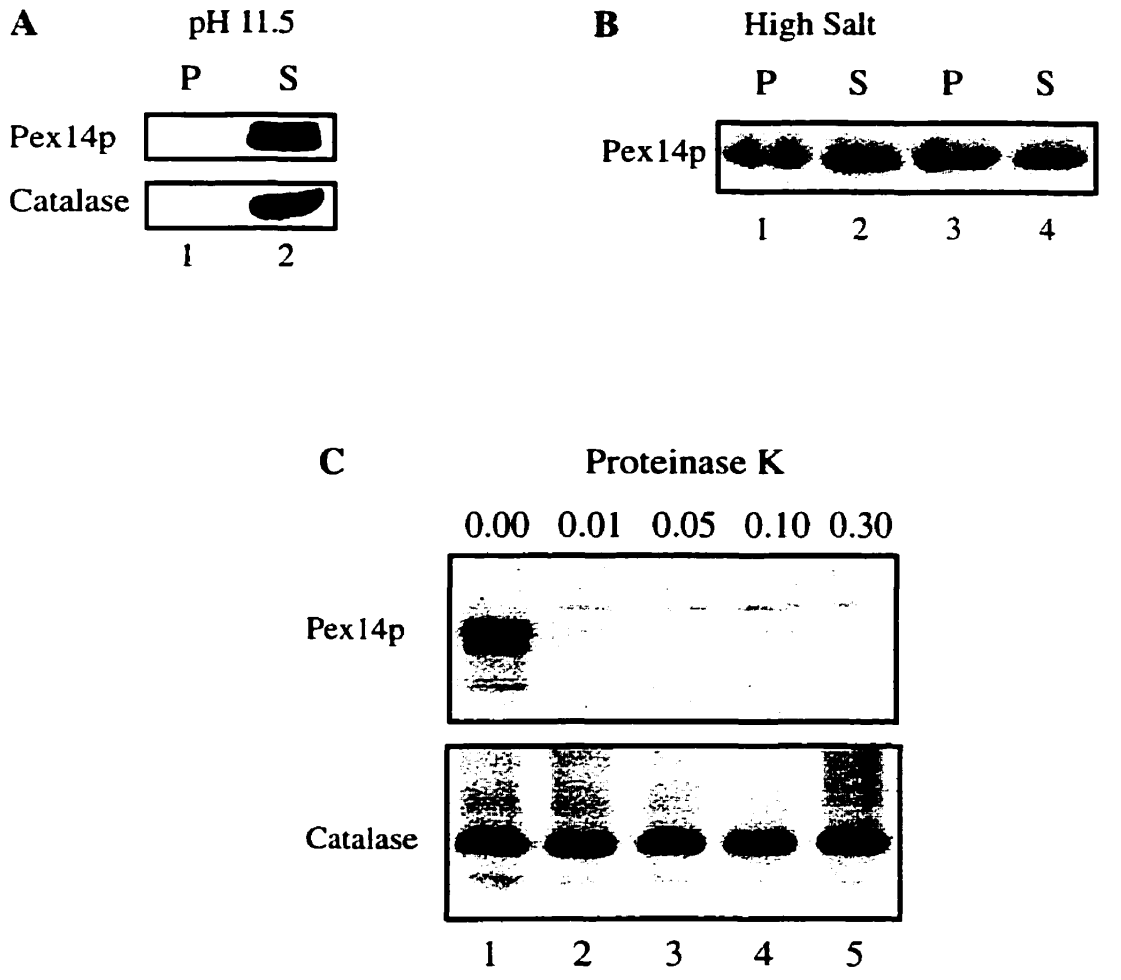

Figure 3.9 Pex14p is peripherally associated with the cytoplasmic surface of the peroxisome. (**A**) 1 mg total protein from cell lysates of oleate-grown WT cells was extracted with 0.1 M sodium carbonate (pH 11.5), centrifuged at 100,000 × *g*, and equal amounts of protein were loaded in each lane. The fractions were analyzed by immunoblotting with Pex14p antibodies. (**B**) 1 mg of oleate-grown WT cell lysates was extracted with 0.5 M potassium chloride, centrifuged, and equal amounts of protein were loaded in each lane (lanes 1 and 2). Lanes 3 and 4 were subjected to the same procedure except no extraction step was performed. (**C**) 100-μg aliquots of organellar pellet from a subcellular fractionation of oleate-induced WT cells were subjected to digestion with selected concentrations of proteinase K. The concentrations shown are mg of proteinase K per ml of reaction. Equal volumes were then loaded on two SDS-PAGE gels and immunoblotted with either CAT or Pex14p antibodies.

migrating species was a degradation product of the slower migrating species. To examine this possibility, we expressed the full *PEX14* ORF in *E. coli* and compared the size of the bacterial Pex14p to that of the *P. pastoris* wild-type cells. As seen in Fig. 3.10A, only the faster-migrating band was present in *E. coli* extracts. Because *E. coli* was unlikely to modify *P. pastoris* Pex14p, the bacterially synthesized 58 kDa protein most likely represented full-length unmodified Pex14p. This result suggested that the minor 60 kDa species may be a modified form of Pex14p. Expression of *PEX14* under either the *PEX8* or *AOX1* promoters in the *pex14*Δ strain resulted in Pex14p migrating in the same manner as wild-type Pex14p (Fig. 3.3). Therefore, the larger species did not appear to be the result of the use of an alternative upstream start site in *PEX14*.

We next examined the possibility that the slower-migrating band was a phosphorylated form of Pex14p. For this we treated wild-type oleate-induced cell lysates with potato acid phosphatase (PAP). With phosphatase incubations, the higher molecular mass species disappeared (Fig. 3.10B). No change in mobility was observed for the main lower molecular mass species. Thus, we conclude that the larger species is a phosphorylated form of Pex14p.

The relative proportions of phosphorylated and non-phosphorylated Pex14p forms were compared in glucose-, methanol-, and oleate-grown cells and were approximately the same under all three growth conditions (Fig. 3.7). Furthermore, there did not appear to be a significant difference in the proportion of Pex14p forms seen in any of our post-extraction pellet and supernatant fractions, suggesting the tightness of Pex14p interaction with the peroxisomal membrane (or another peroxin on the surface of the membrane) is not controlled by the phosphorylation state of Pex14p.

3.3.6 Pex14p interacts with Pex5p, Pex7p, Pex8p, Pex13p, Pex17p and itself

To identify protein–protein interactions between Pex14p and other peroxins, the yeast two-hybrid system was used. When expressed as a DNA-binding domain fusion, Pex14p interacted with Pex5p-, HAPex7p-, and Pex14p-activation domain fusions as judged by transcriptional activation of both the *HIS3* and *LacZ* reporter

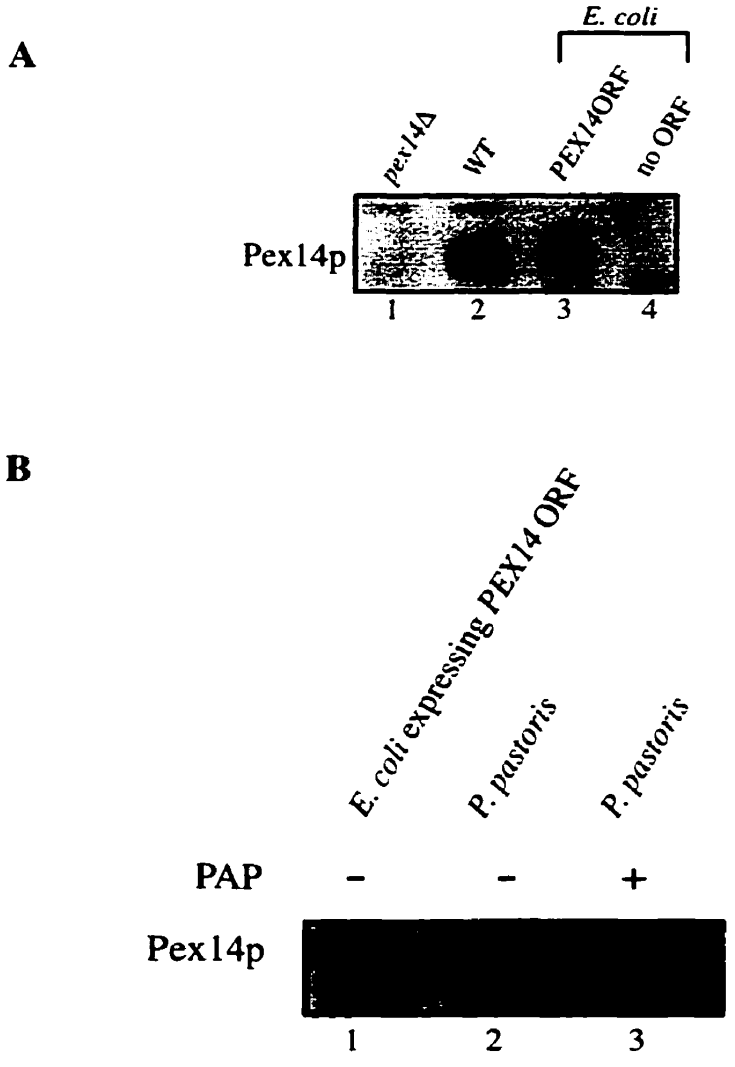

Figure 3.10 Pex14p expressed in *E. coli* and phosphatased yeast Pex14p are each a single 58-kDa species. (**A**) Cell lysates (20 μg) from oleate-induced WT and *pex14Δ* were analyzed by immunoblotting with Pex14p antibodies (lanes 1 and 2, respectively). An approximately equal amount of anti-Pex14p reacting material from extracts from an *E. coli PEX14* ORF expression strain was loaded in lane 3. Extracts from an *E. coli* strain containing the same vector but without *PEX14* ORF was loaded in lane 4. (**B**) Phosphate treatment of *P. pastoris* extracts. Equal volumes of oleate-grown WT cell lysates were loaded in lanes 2 (without phosphatase treatment) and 3 (phosphatase treated). An approximately equal amount of anti-Pex14p reacting material from the *E. coli* PEX14 ORF expression strain was loaded in lane 1. All lanes were analyzed by immunoblotting using Pex14p antibodies. Note the unmodified Pex14p in lane 1 has migrated to approximately the same position as the phosphatased Pex14p in lane 3.

genes (Fig. 3.11A). The Pex14p DNA-binding domain did not, in concert with the empty activation domain, activate the reporter genes. Interaction was also observed between Pex14p in an activation domain fusion with a cytosolic carboxy-terminal fragment (55–267) of Pex17p [Snyder et al., 1999b], while no activation was seen if the membrane-spanning region (35–54) of Pex17p was the DNA-binding domain fusion partner (Fig. 3.11B). Pex17p fragments composed of amino acids 1–59 and 1–142 were also tested with Pex14p and resulted in no activation [data not shown]. Thus, *Pp*Pex14p interacts specifically with the carboxy-terminal fragment of Pex17p (amino acids 57–267).

A Pex14p activation domain fusion also activated the two-hybrid system when paired with a Pex13p SH3 domain DNA-binding domain fusion (Fig. 3.11C), in agreement with the research of others [Albertini et al., 1997; Brocard et al., 1997; Fransen et al., 1998].

No two-hybrid interaction was seen between Pex14p and all other known *P. pastoris* peroxins (Pex1p, Pex2p, Pex3p, Pex4p, Pex6p, Pex10p, Pex12p, Pex19p and Pex22p).

The interactions identified using the two-hybrid system were confirmed by co-immunoprecipitation. HA-tagged versions of Pex17p, Pex13p and Pex7p were created and shown to fully complement pex17Δ, pex13Δ, and pex7Δ mutant strains, respectively [data not shown]. Oleate-induced *PEX13HA*, *PEX17HA*, *HAPEX7*, *PEX8* and *PEX5* cell extracts were prepared, crosslinked with the cleavable crosslinker, DSP, and immunoprecipitated using the anti-Pex14p antisera. Following immunoprecipitation, a reducing agent was used to dissociate the crosslinked proteins. The crosslinking reaction appeared to be specific. The anti-Pex14p antisera did not immunoprecipate either Pex19p or Pex2p, peroxins that are not part of Pex14p-containing complexes [data not shown]. The immunoprecipitated materials were separated by SDS-PAGE and immunoblotted using monoclonal anti-HA antibodies or polyclonal antibodies against selected peroxins. As can be seen in Fig. 3.12, Pex13HAp, Pex17HAp, HAPex7p, and Pex5p each co-immunoprecipitated with Pex14p. Together with the two-hybrid results, these data suggest that Pex14p is in a

A

pAD/pBD	HIS+/-	β-gal+/-
C/14	-	-
5/14	+	+
7/14	+	+
14/14	+	+
13/14	-	-
17/14	-	-

B

pAD/pBD	HIS+/-	β-gal+/-
C/17FL(1-267)	-	-
14/17FL(1-267)	-	-
C/17(55-267)	-	-
14/17(55-267)	+	+
17(55-267)/14	-	-

C

pAD/pBD	HIS+/-	β-gal+/-
C/13FL(1-380)	-	-
14/13FL(1-380)	-	-
C/13(SH3)	-	-
14/13(SH3)	+	+
13(SH3)/14	-	-

Figure 3.11 Two hybrid analysis of interaction between Pex14p and Pex5p, Pex7p, Pex13p, Pex17p, and itself. The indicated hybrid protein constructs were tested for trans-activation of the *HIS3* gene, resulting in growth on medium lacking histidine, and *LacZ*, resulting in the production of β-galactosidase as described in Section 3.2. Numbers refer to amino acids from Pex17p (**B**) or Pex13p (**C**). SH3 refers to the Src homology 3 domain of Pex13p. FL denotes full length. pAD refers to the transcriptional activation-domain fusion constructs. pBD refers to the DNA-binding domain fusion constructs. C refers to the presence of empty DNA-binding or activation domain plasmids in the two hybrid strains.

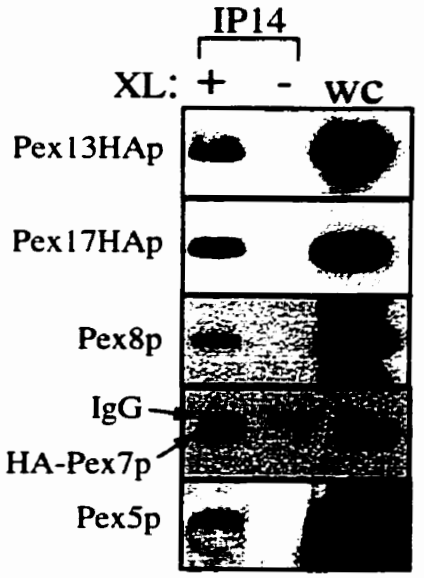

Figure 3.12 Crosslinking and coimmunoprecipitation of Pex5p, Pex7p, Pex8p, Pex13p, and Pex17p with anti-Pex14p antibodies. Immunoprecipitations with affinity-purified Pex14p antibodies from crosslinked (+) or non-crosslinked (–) extracts of oleate-grown cells expressing Pex8p, Pex5p, Pex13HAp, Pex17HAp, or HAPex7p were analyzed by immunoblotting. Pex13HAp, Pex17HAp, and HAPex7p were immunoblotted with anti-HA. Pex5p and Pex8p were immunobloted with anti-Pex5p and Pex8p antibodies, respectively. XL refers to crosslinker. IP14 refers to immunoprecipitation with Pex14p antibodies. Whole-cell lysates (ws) were loaded ($0.033A_{600}$) as a control for immunoblotting.

complex with—and most likely directly physically interacts with—Pex5p, Pex7p, Pex13p, Pex17p and itself.

Interestingly, Pex8p was also detected as a member of the immunoprecipitation complex (Fig. 3.12). The presence of Pex8p in a complex with Pex14p had not previously been reported. Attempts to detect interaction between Pex14p and Pex8 via the two-hybrid system gave negative results. A Pex8p-binding domain fusion and a Pex14p-activating domain fusion, both of which were able to strongly activate the system with other two-hybrid partners, gave no response above a low background when paired with each other [data not shown and Fig. 3.12A, B, and C, respectively). It is unclear from these results whether Pex8p and Pex14p are components of the same complex but do not directly interact, or whether they directly interact *in vivo* but that this interaction was not revealed by the two-hybrid system for unknown reasons.

3.3.7 Only the phosphorylated form of Pex14p precipitates with Pex13p

Insight into the functional significance of the phosphorylation of Pex14p was gained through further co-immunoprecipitation studies. As before, oleate-induced cells of the Pex13HAp-, Pex17HAp-, and HA-Pex7p-expressing strains were lysed and immunoprecipitated with HA antibodies. The precipitate was then subjected to SDS-PAGE and immunoblotting for Pex14p. The molecular weight of Pex14p is coincident with IgGs unavoidably brought into the immunoprecipitation with the HA antibodies. The secondary antibody used in immunoblotting nonspecifically binds to these IgGs, resulting in the masking of Pex14p on an immunoblot. In an effort to circumvent this problem, we used goat anti-rabbit peroxidase-conjugated secondary antibodies pre-absorbed against rat sera. By doing so, we reduced the amount of binding to the IgGs and, although a light band of IgGs was still present, we were able to clearly observe Pex14p. We found that only the phosphorylated form of Pex14p immunoprecipitated with Pex13HAp (Fig. 3.13B). The same experiment with Pex17HAp also showed a strong enrichment for the phosphorylated form of Pex14p, although some non-phosphorylated Pex14p was clearly present as well (Fig. 3.13A). We could not rule out the possibility that the DSP preferentially crosslinked the

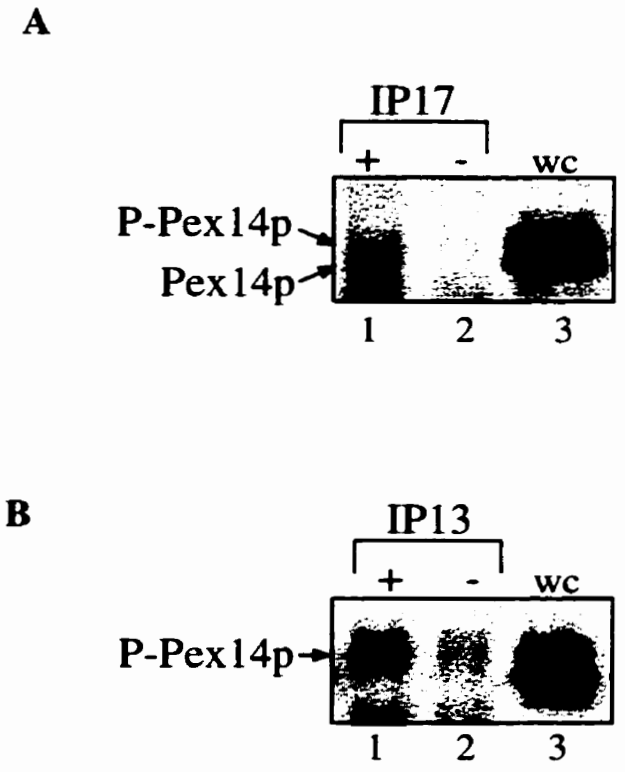

Figure 3.13 Immunoprecipitation of Pex14p with HA monoclonal antibodies in Pex17HAp and Pex13HAp expressing strains. (**A**) Pex14p immunoprecipitated using HA antibodies and extracts prepared from the Pex17HAp expression strains. In the whole-cell extract (wc), the major form of Pex14p present is the unmodified form (lane 3), while approximately equal amounts of both forms of Pex14p (unmodified and phosphorylated) interacted with Pex17HAp. (**B**) Pex14p immunoprecipitated with HA antibodies from extracts of the Pex13HAp expression strain. Whole-cell extract (wc) (lane 3); immunoprecipitated material (lane 1). XL, crosslinking; +, with crosslinking; -, without crosslinking; IP17, immunoprecipitated with HA antibodies from Pex17HAp strains; IP13, immunoprecipitated with HA antibodies from Pex13HAp strain; P-Pex14p, phosphorylated Pex14p.

phosphorylated form of Pex14p. However, based on the chemistry of DSP, it is not an expected result of this crosslinking agent. In addition, the fact that the Pex17HAp also precipitated significant amounts of non-phosphoryplated Pex14p suggests that DSP must not be absolutely selective for phosphorylated Pex14p.

Although the Pex14p antibodies co-immunoprecipitated HAPex7p, no Pex14p was visible on the immunoblot resulting from co-immunoprecipitation with HA-Pex7. We also attemped, using biotinylated Pex14p in concert with avidin-alkaline phosphatase (used in place of secondary antibodies) to visualize Pex14p co-immunoprecipitated by either Pex5 or Pex8 antibodies, but were unsuccessful. Fig. 3.14 schematically summarizes the Pex14p interactions we observed.

3.4 Discussion

This report describes the identification and characterization of the *P. pastoris* peroxin Pex14p, a protein essential for peroxisome biogenesis in this yeast. Orthologs of this peroxin have been described in yeasts (*S. cerevisiae* and *H. polymorpha*) and mammals (humans and CHO cells) [Albertini et al., 1997; Brocard et al., 1997; Komori et al., 1997; Fransen et al., 1998; Shimizu et al., 1999; Will et al., 1999]. In each of these previous reports, and this work, evidence is consistent with the hypothesis that Pex14p serves an essential role in the import of peroxisomal matrix proteins as a receptor docking protein. This evidence consists primarily of the observations that Pex14p is located on the surface of the peroxisomal membrane and that Pex14p directly interacts with the PTS1 protein receptor, Pex5p [Albertini et al., 1997; Brocard et al., 1997; Huhse et al., 1998; Girzalsky et al., 1999]. Also consistent with a role for Pex14p in matrix protein import is the observation that the *pex14* mutants in each of these organisms are defective in the import of both PTS1 and PTS2 proteins, while the targeting of mPTS proteins appears normal.

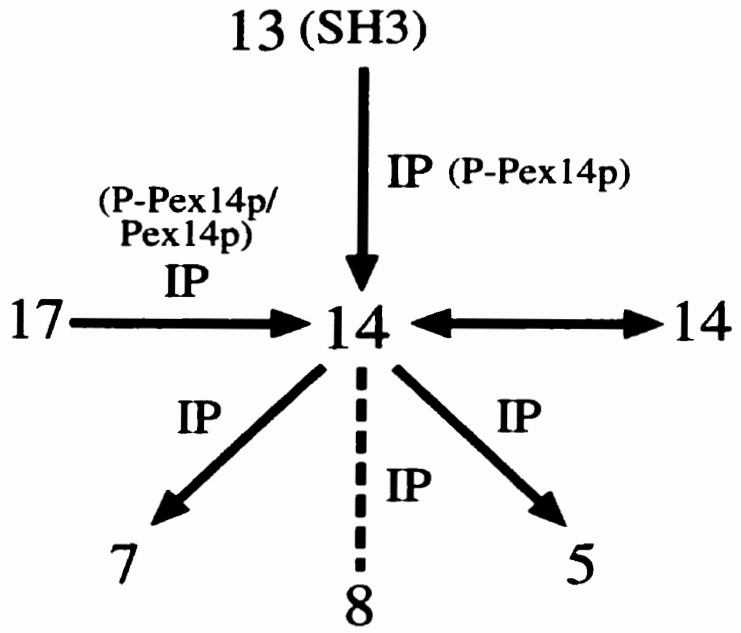

Figure 3.14 Schematic representation of interactions between Pex14p and other peroxins. Arrow heads represent peroxins in activation domain and arrow tail represents peroxins in DNA binding domains in the two-hybrid system. Dashed line indicates that no two-hybrid interaction was observed. IP, immunoprecipitation; P-Pex14p, phosphorylated Pex14p; SH3, Src homology 3 domain.

3.4.1 Comparison of Pex14p orthologs

P. pastoris Pex14p shares these and other similarities with Pex14ps of the other species. As expected, the predicted amino acid sequence of *Pp*Pex14p is closest yin similarity to those of the other yeast Pex14ps (29% identical and 41% similar to *Sc*Pex14p and 52% identical and 66% similar to *Hp*Pex14p) relative to those from mammals (15% identical and 22% similar to *Hs*Pex14p and 16% identical and 22% similar to *Cl*Pex14p) [Albertini et al., 1997; Brocard et al., 1997; Komori et al., 1997; Fransen et al., 1998; Shimizu et al., 1999; Will et al., 1999]. Interestingly, PpPex14p at 425 amino acids is 55 to 70 amino acids longer than the others. Alignment of the Pex14 polypeptides indicates that the "extra" residues are primarily confined to the C-terminal region of *Pp*Pex14p, a region that is not strongly conserved among Pex14ps. The "extra" amino acids include numerous charged residues. A similar situation exists with the *P. pastoris* peroxin Pex2p which contains an acidic residue-rich C-terminal extension of approximately 100 residues that is not seen in other fungal or animal Pex2p sequences [Waterham et al., 1996]. In the case of Pex2p, deletion of its acid tail has no discernible effect on Pex2p function [our unpublished results]. The significance of the "extra" amino acids on Pex14p, if any, is unknown.

The *P. pastoris* Pex14p sequence contains potential secondary structures that are also present in other Pex14ps. All known Pex14p sequences predict the presence of a coiled-coil motif, a motif shown in other proteins to be involved in homo-oligomerization [Lupas et al., 1991]. Since most (all except human) Pex14ps, including *Pp*Pex14p, homo-oligomerize (as judged by the yeast two-hybrid system results), the coiled-coil motif may play a role in their oligomerization, although this has not been directly demonstrated. A second predicted secondary structure that appears universal to yeast Pex14ps is a class II SH3 ligand motif, a ligand known to be bound by Src homology 3 (SH3) domains [Feng et al., 1994]. Such an SH3 domain exists in the yeast Pex13ps and has been shown both *in vivo* and *in vitro* to be important for binding of Pex14p to Pex13p in *S. cerevisiae* [Girzalsky et al., 1999]. One function of the SH3 ligand binding motif appears to be the tethering of Pex14p to

the outer surface of the peroxisome through Pex13p. Here, we demonstrate that *P. pastoris* Pex13p also binds Pex14p through its SH3 domain. Interestingly, and as discussed further below, the mammalian Pex14ps lack an SH3 ligand binding domain and interact with Pex13p only weakly [Fransen et al., 1998], if at all [Will et al., 1999].

Although all known Pex14ps, including PpPex14p, are located on the outer surface of the peroxisome, the nature of their association with the peroxisomal membrane appears to differ. The mammalian Pex14ps, including those of CHO cell, rat and human origin, behave as integral membrane proteins as judged by their resistance to extraction from the peroxisomal membrane by carbonate at high pH [Fransen et al., 1998; Shimizu et al., 1999; Will et al., 1999]. In yeasts, the carbonate extraction results are more complicated. Komori and coworkers reported that *Hp*Pex14p is resistant to extraction, while Brocard and coworkers reported that *Sc*Pex14p is also resistant; but Albertini and coworkers reported that *Sc*Pex14p is fully extractable [Albertini et al., 1997; Brocard et al., 1997; Komori et al., 1997]. Further studies recently reported by Girzalsky and coworkers strongly support a model in which *S. cerevisiae* Pex14p is tethered to the surface of the peroxisome, at least in part by its association with Pex13p, a peroxisomal integral membrane protein, and not by direct insertion of the peroxin into the membrane [Girzalsky et al., 1999]. The most compelling evidence is their finding that, in a *S. cerevisiae pex13* deletion strain, *Sc*Pex14p is no longer associated with the peroxisomal membrane but is cytoplasmic. Through mutations in regions encoding the Pex13p SH3 and the Pex14p SH3 ligand binding domains, they further show that these regions are important, although not essential, for the association of Pex14p with peroxisomal membrane. These results leave little doubt that Pex14p in *S. cerevisiae* is not an integral membrane protein. Our results with *P. pastoris* Pex14p are most consistent with those of Albertini and coworkers and Girzalsky and coworkers [Albertini et al., 1997; Girzalsky et al., 1999]. We find that *Pp*Pex14p is completely extracted by carbonate at high pH. In fact, approximately half of *Pp*Pex14p is typically removed from peroxisomes during subcellular fractionation. As with *Sc*Pex14p, *Pp*Pex14p binds to

*Pp*Pex13p through its SH3 ligand binding domain as demonstrated by our two-hybrid interaction study. The integral nature of Pex14p in mammals in conjunction with the absence of an SH3 ligand binding domain leads to an interesting model of eukaryotic evolution in which higher eukaryotes appear to have "traded in" their ability to bind to the surface of peroxisomes through Pex13p, an ability still dominant in fungi, for the more direct peroxisome membrane association of an integral membrane protein.

3.4.2 The *P. pastoris* import complex contains Pex14p, Pex17p, Pex13p, Pex7p, Pex5p, and Pex8p

In *S. cerevisiae*, three other peroxins, in addition to Pex13p, have been identified as members of a Pex14p-containing matrix protein import complex. These proteins are the PTS1 and PTS2 receptors, Pex5p and Pex7p, and the peripheral peroxisomal membrane protein Pex17p [Albertini et al., 1997; Brocard et al., 1997; Huhse et al., 1998; Girzalsky et al., 1999]. By both two-hybrid and co-immunoprecipitation methods, we also observe these three peroxins in *P. pastoris* Pex14p-containing complexes. Additionally, we show for the first time that *Pp*Pex14p interacts specifically with the carboxy-terminal fragment of Pex17p (amino acids 57–267).

We also identified a potential new member of this complex, the *P. pastoris* peroxin Pex8p. In co-immunoprecipitation experiments, the presence of Pex8p as part of a Pex14p-containing complex is consistently observed. However, we do not see direct interaction between Pex8p and Pex14p via two-hybrid analysis. The possible presence of Pex8p in the putative import complex is surprising since this peroxin is located primarily in the peroxisomal matrix (*Hp*Pex8p/*Hp*Per1p), [Waterham et al. 1994; our unpublished results], while the import complex itself is located on the cytoplasmic surface of the organelle. A reasonable explanation for our co-immunoprecipitation results is that they are an artifact of the procedure. For example, in the cell-free extracts used for immunoprecipitations, peroxisomes are disrupted and the normally matrix-localized Pex8p could bind to the import complex through Pex5p [Pex8p is known to have a PTS1 [Liu et al., 1995]]. On the other hand, Pex8p may actually be a transient component of the complex. A role for Pex8p

in matrix protein import was suggested by our lab based on the phenotype of one of our *P. pastoris pex8* mutant alleles, *pex8-1*, which is specifically defective in the import of PTS1 proteins [Liu et al., 1995]. *PEX8* is the only gene, other than the PTS receptor genes, in which a mutation specifically affects one PTS-import pathway, clearly marking Pex8p as a member of the matrix protein import machinery. It may be that Pex8p, or the import complex itself, cycles from one side of the peroxisome to the other. Further studies are in progress to elucidate the Pex8p's role, if any, in import.

3.4.3 Cascade versus complex activation models of docking protein function

In yeasts, a consensus picture is emerging in which a matrix protein import complex exists on the surface of the peroxisome; the complex is composed of directly interacting Pex13p and Pex14p (the latter as at least a homo-dimer), each with its own direct binding sites for both Pex5p and Pex7p [although the possibility of indirect binding of Pex13p to Pex7p has not been entirely eliminated [Girzalsky et al., 1999]]. Pex17p appears to join the complex by direct interaction with Pex14p only [Huhse et al., 1998; Snyder et al.,1999b]. In addition to being a member of the matrix protein import complex, *Pp*Pex17p appears to play a role in the insertion of intergral membrane proteins [Snyder et al., 1999b].

In mammals (human, rat and CHO cells), the organization of this complex is not as well defined, with conflicting results obtained in different systems and/or with different techniques. All report that Pex14p is an integral peroxisomal membrane protein primarily facing the cytoplasm and all report that Pex14p binds directly with Pex5p. Using a ligand blot assay, Fransen and coworkers reported binding (albeit weak) between human Pex14p and Pex13p; in contrast Will and coworkers, also working with the human peroxins and with the same technique, did not see this interaction [Fransen et al., 1998; Will et al., 1999]. With regard to potential Pex14p/Pex7p and Pex14p/Pex14p interactions, Will and coworkers, using a number of *in vivo* and *in vitro* methods, detected neither while Shimizu and coworkers, using an *in vitro* binding method along with recombinant rat Pex14p and human Pex7p, and rat and CHO Pex14ps, saw both [Shimizu et al., 1999; Will et al., 1999]. A

mammalian ortholog of Pex17p has yet to be described. Further studies are needed to determine whether the organization of this import complex actually varies between mammals or whether the observed differences are the consequence of the different techniques employed.

Setting aside the unresolved issues involving the putative mammalian import complex, how might this PTS-docking system work in the yeast import complex? Since Pex13p and Pex14p can bind both PTS receptors, the simplest model would be that Pex13p and Pex14p each perform the docking function independently of the other. However, this model also predicts that mutants defective in either *pex13* or *pex14* should be capable of at least some matrix protein import when, in fact, such mutants are completely defective in PTS1- and PTS2-protein import [Elgersma et al., 1996; Gould et al., 1996; Komori et al., 1997; Girzalsky et al., 1999]. Two general models can accommodate this complication. One is a docking cascade model in which a PTS receptor with its nascent protein cargo docks first with one specific docking protein, for example, Pex14p; and then, in a second step, passes the PTS receptor-protein cargo on to Pex13p. When Pex17p binds to this complex is another interesting question. Pex17p and Pex14p co-immunoprecipitate with both PTS receptors in the absence of Pex13p, suggesting that perhaps the complex of Pex14p, Pex5p or Pex7p, and Pex17p forms prior to binding with Pex13p [Huhse et al., 1998]. The other model is a single-step complex activation model in which the PTS receptor plus cargo must dock with both Pex13p and Pex14p, and perhaps Pex17p, simultaneously to activate the complex for the next step in translocation.

3.4.4 A trigger model for the function of Pex14p phosphorylation

We have discovered that *P. pastoris* Pex14p exists in two forms. The majority of Pex14p (>90%) is unmodified while a small portion of the protein (<10%) is phosphorylated. During the preparation of this report, Komori and coworkers published work showing that a portion (~50%) of *H. polymorpha* Pex14p is also phosphorylated [Komori et al., 1999]. Pex14p is the second phospho-peroxin described in yeasts after *S. cerevisiae* Pex15p, a peroxisomal integral membrane protein of unknown function that is also *O*-glycosylated [Elgersma et al., 1997]. To

elucidate a potential function for Pex14p phosphorylation, we performed further studies. Komori and coworkers observed that the relative amounts of modified and unmodified Pex14p forms seemed to vary with growth conditions in *H. polymorpha* [Komori et al., 1999]. However, we did not observe this in *P. pastoris*. The relative proportion of the two forms is not significantly different in glucose-, oleate-, and methanol-grown cells, although the amount of both Pex14p forms increases significantly on the latter two peroxisome-inducing substrates. Because a good portion ($\sim$50%) of Pex14p appears in supernatant fractions during standard subcellular fractionation experiments, we entertained the idea that the tightness of Pex14p binding to the peroxisome might be related to its phosphorylation state. However, the proportion of the *Pp*Pex14p forms in pellet and supernatant fractions is not significantly different from each other or from that seen in cell-free extracts.

Lastly, we examined whether the phosphorylation state of Pex14p affects Pex14p's ability to associate in a complex (or complexes) with other peroxins. This was achieved by expressing HA-tagged versions of selected Pexps in *P. pastoris*, immunoprecipitating complexes that contained them, and observing the relative amounts of the two Pex14p forms via SDS-PAGE and immunoblotting. For Pex13p, the results are striking. Virtually all of the co-immunoprecipitated Pex14p is the phosphorylated form. A more variable but strong enrichment for phosphorylated Pex14p is also seen upon co-immunoprecipitation with Pex17p. Unfortunately, we were not able to extend these results to Pex5p, Pex7p, and Pex8p due to the relatively small amount of Pex14p immunoprecipitated (as predicted by the small amount of Pex5p, Pex7p and Pex8p immunoprecipitated by Pex14p antibodies) and difficulty of observing Pex14p on immunoblots through the IgGs present in samples.

What does the selective immunoprecipitation of phosphorylated Pex14p mean for the function of Pex14p and the import complex? We propose a simple trigger model in which Pex14p is phosphorylated by an unknown kinase in response to binding of a PTS receptor (loaded with its PTS-protein cargo). The phosphorylation event serves as the signal that triggers Pex14p, and perhaps other components of the import complex, to lock on to Pex17p and Pex13p and proceed with the next step in the protein translocation process. In a cascade model of Pex14p function, this step

could be the transfer of loaded PTS receptors to Pex13p. In a complex activation model, phosphorylation of Pex14p could be the trigger for translocation itself. This model of Pex14p function in response to phosphorylation provides a testable framework to further investigate the intriguing role of Pex14p phosphorylation in matrix protein import.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary of Research

This thesis describes the identification and characterization of *P. pastoris* peroxisome-biogenesis-deficient mutants (*pex* mutants) as well as the cloning of *PEX14* and the characterization of its corresponding peroxin, Pex14p.

Chapter 2 describes the identification and characterization of *P. pastoris pex* mutants. We used two novel positive selection schemes for the direct selection of *pex* mutants. Both schemes are based on the observation that AOX, the first enzyme in the methanol metabolism pathway, is active only when sequestered within functional peroxisomes, which *pex* mutants lack. In the first selection scheme, mutagenized cells are exposed to allyl alcohol, which is not toxic to cells itself but when metabolized by AOX, the resulting compound, acrolein, is toxic. Wild-type cells are selectively killed, while *pex* mutants, with little or no active AOX, survive. The second screen utilized an *fld1 P. pastoris* strain that is sensitive to exposure to high concentrations of methanol due to its inability to metabolize formaldehyde, the product of the first step in the metabolism of methanol, performed by AOX. *pex* cells survive this selection due to their lack of AOX activity while wild-type cells die.

Through complementation testing and random spore analysis, we determined that we had isolated mutants with alleles in most of the previously identified *P. pastoris PEX* genes as well as three novel *PEX* groups. To verify the mutants as *pex* mutants, biochemical (subcellular fractionation) and morphological (electron and fluorescence microscopy) studies were performed. Strains in all three new *PEX* groups were verified to be *pex* mutants. Additionally, two genes encoding potential transcription factors were identified.

Chapter 3 describes the cloning of the wild-type allele, via functional complementation, of one of the mutants, *pex14*, identified in Chapter 2. The isolated genomic DNA fragment was sequenced and, of the three ORFs identified *in silico*, the ORF1 product was identified as an ortholog of the *S. cerevisiae* and *H. polymorpha* Pex14ps. *PEX14* was subcloned and genetic evidence obtained that showed it is the authentic rescuing gene of mutant *pex14-1*. Electron microscopy revealed that *pex14* mutants (both *pex14-1* and *pex14Δ* mutants) lacked recognizable peroxisomes. Morphologically and functionally normal peroxisomes were restored upon transformation of the *pex14* mutants with *PEX14*.

Pex14p was shown, using sucrose gradients and the peroxisomal marker enzyme CAT, to be peroxisomal. High pH and high salt extraction, as well as protease protection experiments showed Pex14p to be peripherally associated with the cytoplasmic side of the peroxisome. Subcellular fractionation and EGFP targeting studies demonstrated that Pex14p is necessary for PTS1 and PTS2 import but not for integral membrane targeting. In the yeast two-hybrid system, Pex14p interacts directly with itself as well as with the following components of the peroxisome import machinery: Pex5p, the PTS1 receptor; Pex7p, the PTS2 receptor; Pex13p, the docking receptor for both Pex5p and Pex7p; and Pex17p, recently shown by Snyder and coworkers in *P. pastoris* to be involved in both matrix and integral membrane protein targeting [Snyder et al., 1999b]. Except for homodimerization, these interactions were confirmed by co-immunoprecipitation experiments. Additionally, Pex14p was shown via immunoprecipitation to form a complex with Pex8p, a matrix protein that also interacts with Pex5p. Interestingly, Pex14p is phosphorylated and primarily the phosphorylated form of the Pex14p is found in complexes containing Pex13p and Pex17p.

These data demonstrate that Pex14p is an essential component of the peroxisome import machinery. We propose a model of Pex14p function in which phosphorylation of Pex14p acts as a triggering event signaling readiness of the import complex for import.

4.2 Future Directions

Although we have shown Pex14p to be an essential component of the peroxisomal import machinery, there are still many unanswered questions regarding the function of Pex14p. Firstly, as discussed in Chapter 3, we were unable to determine if Pex5p, Pex7p, and Pex8p interact predominantly with the phosphorylated form of Pex14p as we observed with Pex13p and Pex17p. We propose restructuring this co-immunoprecipitation in order to address that issue. Secondly, we wish to determine the functional significance and the nature of the phosphorylation of Pex14p. Thirdly, by investigating the topology of the import complex, with specific consideration of how Pex14p binds to its partners, we hope to gain insight into how the import mechanism works. Fourthly, we wish to further explore the possible association of Pex8p with the import complex. Finally, we wish to explore the significance of Pex14p's "extra" amino acids with the expectation that these results may provide clues to the differences observed across species.

4.2.1 Do Pex5p, Pex7p and Pex8p preferentially interact with the phosphorylated form of Pex14p?

As was discussed in Chapter 3, Pex13p and Pex17p preferentially interact with the phosphorylated form of Pex14p but we were unable, due to technical problems, to determine if the same was true for Pex5p, Pex7p and Pex8p. The most significant technical problem was that the size of Pex14p is coincident with IgGs which are present in large amounts after immunoprecipitation. These IgGs then nonspecifically interact with the secondary antibodies used for immunoblotting. The result is that Pex14p is obscured by the IgGs on the immunoblot. This problem may be most pronounced for Pex5p, Pex7p and Pex8p because each of these proteins is thought to interact with Pex14p only transiently, whereas Pex13p and Pex17p are believed to be permanent components of the import complex with Pex14p. To circumvent this problem, we propose to change the size of Pex14p by tagging it with EGFP, thereby increasing its mass above that of the IgGs. Following the construction of an *EGFP-PEX14* gene, we would transform the construct into a *pex14Δ* strain and verify that

the EGFP tag does not interfere with Pex14p function, as determined by restoration of a wild-type phenotype to the transformed strain. We would also need to confirm that we could distinguish between phosphorylated and unphosphorylated forms of EGFP-Pex14p on our immunoblot by running a phosphatase experiment as described in Chapter 3 of this thesis. Co-immunoprecipitations would then be performed using anti-Pex5p, -Pex7p, and -Pex8p antibodies and the precipitated material immunoblotted for EGFP-Pex14p.

4.2.2 What is the functional significance of the phosphorylation of Pex14p?

To determine the functional significance of Pex14p's phosphorylation, we propose generating a mutant Pex14p that is not phosphorylated and investigating the effect of lack of phosphorylation on peroxisomal protein import. Although the sites of phosporylation are usually hydroxyl groups of context-specific serine (S), threonine (T), or tyrosine (Y) residues, others—aspartate (D), histidine (H), and lysine (K)—are also known to be phosphorylated [Creighton, 1984]. The Prosite ProfileScan for phosphorylation sites was unable to narrow down the large number of potential phosphorylation residues on Pex14p (88 S/T/Y and 63 D/H/K sites). Thus, few clues are available regarding the identity of the phosphorylation site(s) within Pex14p. Elgersma and coworkers considered S and T to be likely phosphorylation sites in their *S. cerevisiae* Pex15p and found that when extra S and T residues were added to the protein, they observed a "drastic increase in phosphorylation" [Elgersma et al., 1997]. As a first step to identifying which residues are phosphorylated in Pex14p, we propose engineering these same additions in *Pp*Pex14p.

Secondly, the specific types of residues that are phosphorylated in Pex14p (serine vs threonine vs tyrosine) may be determined using antibodies specific to each type of phosphorylation. Rabbit anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine polyclonal antibodies are each described to be specific to their respective targets and non-reactive to other phosphoamino acid residues (CHEMICON International, Inc., Temecula, CA). We would test the anti-phosphoamino acid antibodies on immunoblots generated from peroxisomal fractions from a sucrose gradient. Other peroxisomal phosphoproteins in addition to Pex14p, such as Pex15p,

should not be a problem as long as their masses differ from Pex14p and there are not too many of them associated with peroxisomes. If phosphoproteins, in addition to Pex14p, are a problem with respect to visualization of Pex14p, we would immunoprecipitate Pex14p with anti-Pex14p antibodies and then blot with each of the three anti-phosphoamino acid antibodies. Alternately, we could immunoprecipitate *P. pastoris* extracts or sucrose gradient peroxisomal fractions with anti-phosphoamino acid antibodies and then immunoblot with anti-Pex14p antibodies. As discussed earlier, EGFP-*PEX14* would be used to circumvent the IgG masking problem. As a control to verify that we are visualizing Pex14p on the immunoblot, we would run a duplicate experiment using a *pex14Δ* strain. As a positive control for the ability of the anti-phosphoamino acid antibodies to precipitate serine, threonine or tyrosine phosphoproteins, we would blot for Pex15p on the immunoblots resulting from each of the three co-immunoprecipitations. The results of Elgersma and coworkers suggest that immunoprecipitation with anti-phosphoserine and anti-phosphothreonine antibodies should bring down Pex15p [Elgersma et al., 1997].

We could also use a protease degradation approach to narrow down which portion(s) of Pex14p is phosphorylated. C- and N-terminally tagged Pex14p would be subjected to partial digestions with proteases. By looking at the degradation products on an immunoblot and noting what fragments are phosphorylated, we can narrow the location of the site(s) of phosphorylation to a region of Pex14p. We would also express selected portions of *PEX14* in wild-type or in *pex14Δ* strains of *P. pastoris* and observe whether they are phosphorylated or not. As a word of caution, the conformation of each Pex14p fragment is important since a misfolded fragment can be a poor substrate for the kinase [Creighton, 1984].

Finally, through *in vitro* site directed mutagenesis, we could identify the phosphorylation site(s) by changing selected amino acids in the phosphorylated region to alanine and noting which change eliminated phosphorylation of Pex14p. A *PEX14* gene specifically mutated to change the phosphorylated amino acid to alanine would then be expressed in our *pex14Δ* strain and the strain investigated for the following: (1) general ability to utilize peroxisome requiring carbon sources (methanol and oleate); (2) peroxisomal morphology via electron microscopy; (3) PTS1 and PTS2

import pathway function; (4) localization of Pex14p; (5) interaction of nonphosphorylated Pex14p with Pex13p, Pex17p, Pex8p, Pex7p, and Pex5p by co-immunoprecipitation. Two-hybrid experiments may not be valuable using *PEX14A* since we do not know whether *P. pastoris* Pex14p expressed as a fusion protein and targeted to the nucleus in *S. cerevisiae* is phosphorylated.

How much of Pex14p is phosphorylated? Judging from immunoblots, it appears that approximately 10% of Pex14p is phosphorylated but we do not know if that is an artifact of our procedures (e.g., a phosphatase removes most phosphorylation during extract preparation).

4.2.3 What is the Pex14p kinase?

To identify the Pex14p kinase, it may be possible to assay extracts from oleate-induced wild-type *P. pastoris* for kinase activity against Pex14p. The assay for kinase activity may be performed by incubating cell-free extracts with ^{32}P-ATP, immunoprecipitating the extracts with anti-Pex14p antibody and measuring kinase activity by observing ^{32}P-Pex14p by autoradiography. With such an assay, standard protein chromatography methods could be used to purify a Pex14p kinase. A portion of the kinase would then be sequenced, and the gene identified *in silico* from the *S. cerevisiae* data base, or oligonucleotides would be synthesized to clone the gene from a *P. pastoris* library. We would then be able to construct a *P. pastoris* Pex14p *kinase*Δ strain in which we would study the resulting effects on peroxisome biogenesis (as discussed in Section 4.2.2 above).

4.2.4 What regions of Pex14p bind to each of the known interacting proteins?

Girzalsky and coworkers have shown in *S. cerevisiae* that a mutation in either the SH3 ligand binding motif of Pex14p or the SH3 domain of Pex13p abolished two-hybrid interaction of Pex14p with Pex13p but not with other binding partners [Girzalsky et al., 1999]. Is the same true for *P. pastoris*? Similar site directed mutagenesis studies can be performed to answer that question. Additionally, if binding of Pex13p does occur through the SH3 ligand binding domain of Pex14p,

what effect does abolishing this binding have on PTS1 and PTS2 import? This question would be addressed by examining PTS1 and PTS2 protein import as described in Chapter 3.

To extend the examination of binding domains of Pex14p to Pex5p, Pex7p, Pex17p, and itself, two-hybrid experiments could be done using truncated versions of each protein. For example, the coiled coil domain of Pex14p, which was identified using the COILS algorithm, is thought to facilitate the binding of Pex14p to itself but this has not been demonstrated experimentally. This segment of Pex14p would be the initial target used to investigate the homodimerization. Once the binding regions of each protein have been narrowed down using truncated versions of each in two-hybrid studies, *in vitro* mutagenesis may be performed to further define essential amino acids in these domains. Observing the effects of mutations in the binding domains of these Pexps on the import of peroxisomal matrix proteins may give some clues to the topology of the import complex and the mechanism by which it translocates proteins into the peroxisome.

While we know the resulting phenotypes of yeast strains deleted for each of the above peroxins (i.e., *pex14*Δ abolishes PTS1 and PTS2 import, *pex17*Δ additionally abolishes integral membrane import; *pex5*Δ abolishes PTS1 import; *pex7*Δ abolishes PTS2 import), the phenotypes of the binding mutants may also be interesting due to their specific loss of function. For example, the abolition of binding of Pex14p to one or more of its partners may result in loss of Pex14p phosphorylation.

4.2.5 Do Pex14p and Pex8p interact directly?

Because Pex8p is a matrix protein, we found it surprising that Pex8p is immunoprecipitated by Pex14p antibodies. It is possible that Pex14p was binding indirectly to Pex8p through Pex5p, a binding partner of both Pex8p and Pex14p. We could investigate this possibility by generating cell lysates from a *P. pastoris pex5*Δ strain and repeating the immunoprecipitation experiment. The precipitation of Pex8p by Pex14p, in the absence of Pex5p, would suggest that Pex8p interacts with Pex14p via another member of the import complex or by direct binding to Pex14p itself.

Although no interaction was seen between Pex8p and Pex14p by two-hybrid analysis, only full length Pex proteins were used and perhaps a truncated version of one or both may result in interaction as we observed for Pex13p and Pex17p interactions with Pex14p. If direct interaction is seen between Pex14p and Pex8p, it would suggest that Pex8p is at least transiently part of the import complex and that either Pex8p cycles out to the import complex or the complex, specifically Pex14p, cycles into the matrix.

4.2.6 What is the physiological relevance of *Pp*Pex14p's "extra" amino acids?

We noted from the Pex14p predicted amino acid sequence that it is fifty-five to seventy amino acids longer than Pex14ps from *S. cerevisiae*, *H. polymorpha*, CHO or human. Are these "extra" amino acids, found mostly in the carboxy-half of Pex14p, physiologically relevant? As a first experiment to address this question, we propose transforming the *P. pastoris pex14*Δ strain with the *H. polymorpha* and *S. cerevisiae* *PEX14* genes and testing for complementation in the following ways: (1) ability to grow on methanol or oleate; (2) ability of Pex14p to become phosphorylated; (3) ability of Pex14p to achieve wild-type localization; (4) ability of Pex14p to interact with its binding partners; (5) ability of heterologous Pex14ps to import both PTS1 and PTS2 proteins. If all of those criteria are met, perhaps the additional amino acids have no essential function in peroxisomal biogenesis.

However, if neither of the foreign yeast *PEX14* genes complements a *P. pastoris pex14*Δ strain, it may be that the "extra" amino acids of *P. pastoris* Pex14p are essential in *P. pastoris*. To examine this further, the specific "extra" amino acids needed from Pex14p for function in *P. pastoris* can be identified by constructing a mutant *P. pastoris* Pex14p in which sequences encoding the "extra" amino acid regions are deleted, and examining this effect of the deleted amino acids on peroxisome function in our *P. pastoris pex14*Δ strain.

4.2.7 Does Pex14p play a role in thiolase assembly?

We suspect Pex14p may play an added role in the assembly/import of thiolase. Two pieces of evidence support a Pex14p/thiolase connection: (1) in *pex14*Δ strains, thiolase is not just mislocalized in the cytoplasm but is absent; (2) Pex14p is highly induced on oleate relative to glucose and methanol, and thiolase is a β-oxidation pathway enzyme. Perhaps Pex14p, which is probably at least a dimer in the import complex, facilitates the dimerization of thiolase in the cytoplasm by holding two thiolase monomers in close proximity. Without this event (as in a *pex14*Δ strain), unstable thiolase monomers are degraded in the cytosol. The two-hybrid system may be used to test for direct interaction between Pex14p and thiolase and, if results are positive, to map the site of interaction. To specifically test the Pex14p aided oligomerization of thiolase hypothesis, a *pex14* strain in which Pex14p is mutated for its ability to homodimerize would be constructed and immunoblotted for thiolase. If Pex14p cannot dimerize itself, it may not be able to aid in the assembly of thiolase and the unstable thiolase would be degraded. We propose looking at thiolase levels in a *P. pastoris pex13* strain which, in *S. cerevisiae*, is mislocalized Pex14p to the cytoplasm [Girzalsky et al., 1999]. If Pex14p is similarly mislocalized in *P. pastoris*, we could determine, by thiolase levels, if Pex14p must be peroxisome membrane associated to aid in thiolase assembly/stability.

Another interesting gene is *Y. lipolytica PEX20* which, like *PEX14*, is highly induced on oleate and is required for folding or oligomerization of thiolase and for its targeting to the peroxisome [Titorenko et al., 1998]. In the *pex20-1* mutant strain, thiolase is mislocalized to the cytosol, whereas the import of other peroxisomal proteins is unaffected. Anti-thiolase and anti-Pex20p antibodies both immunoprecipitated two radiolabeled proteins with kDas corresponding to Pex20p and thiolase. Those proteins could then be decorated with anti-Pex20p and anti-thiolase antibodies. Titorenko and coworkers also showed that the binding between Pex20p and thiolase does not require the PTS2 of thiolase. We could perform co-immunoprecipitation experiments as above, and if interaction is observed, then repeat the experiments in a *pex7*Δ mutant, i.e.,we know that *Pp*Pex14p interacts directly

with *Pp*Pex7p, and thiolase binds Pex7p. Pex20p is thought to bring thiolase to the peroxisomal membrane as a heterotetrameric complex, composed of two polypeptides of each protein. The possibility that *Pp*Pex14p plays a docking role for the Pex20p-thiolase complex can be examined after the *P. pastoris PEX20* gene is cloned.

Shimizu and coworkers have recently assigned *PEX14* to a CHO cell line complementation group, CG14 [Shimizu et al., 1999]. It will be interesting to compare the metabolic and physiological anomalies, particularly relating to thiolase levels, in cells from CHO-line CG14 with our *P. pastoris pex14* mutants.

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BIOGRAPHICAL SKETCH

Monique Johnson was born on February 10, 1955, in Moline, IL. She received her Bachelor of Science degree in biology from Illinois State University in 1977. In 1983, she received a Master's degree in School Administration and Supervision from Roosevelt University, Chicago, IL. From 1977 to 1993, she taught middle school and high school biology and chemistry. In September of 1993, she began work on her Ph.D. at Oregon Graduate Institute of Science and Technology under the direction of Dr. James M. Cregg. In November of 1999, she will begin a postdoctoral position at Oregon Health Sciences University, with Dr. Susan Hayflick, investigating the genetic basis of neurodegenerative diseases.