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The Peroxin Pex19p of *Yarrowia lipolytica*

by

Gareth Robert Lambkin

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Cell Biology

Edmonton, Alberta

Spring 2001

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***The Peroxin Pex19p of Yarrowia lipolytica*** submitted by Gareth Robert Lambkin in partial fulfillment of the requirements for the degree of Master of Science.

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Date: JANUARY 22, 2001

Abstract

This thesis details the isolation and preliminary characterization of the peroxin gene *PEX19* of the yeast *Yarrowia lipolytica*. The *PEX19* gene was isolated from a genomic library by its ability to rescue growth on oleic acid of a mutant *Y. lipolytica* strain. The peroxin Pex19p is associated with peroxisomes in wild-type cells and contains a putative farnesylation sequence. Expression of Pex19p is not significantly induced upon growth of cells on oleic acid. Cells lacking Pex19p contain vesicular structures similar to peroxisomes in morphology, density, and protein complement, but these structures do not exhibit normal peroxisomal function as mutant cells are unable to grow on oleic acid. Mutant cells also mislocalize many matrix proteins to the cytosol. These data are consistent with a role for Pex19p in peroxisomal membrane protein stability.

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During my two and a half years of study in the Rachubinski lab, I learned a lot about what it means to be a scientist; about how to put together a logical, coherent argument; about how important it is to say only what you mean, and mean what you say; and about integrity in research, as well as in other areas of life. I was also exposed to many different people from many different cultures, which increased my understanding of the world and the way it works. I admire the strong sense of community that scientists create around themselves. It provides an atmosphere of collective well-being, of collective and personal growth, and of mutual admiration and understanding. I am very pleased that I was afforded the opportunity to be a part of such a positive experience.

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cells.

List of Abbreviations

200KgP	200 000 X *g* pellet
200KgS	200 000 X *g* supernatant
20KgP	20 000 X *g* pellet
20KgS	20 000 X *g* supernatant
AAA ATPase	ATPase associated with diverse cellular activities
ADP	adenosine diphosphate
ALDP	adrenoleukodystrophy protein
ALDRP	adrenoleukodystrophy related protein
amp	ampicillin
AOX	acyl-CoA oxidase
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CHO	chinese hamster ovary
CIP	calf intestinal phosphatase
CoA	coenzyme A
CSM	complete supplements mixture
D	dextrorotary
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidyl triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence labelling
EDTA	ethylenediaminetetraacetic acid
EMS	ethylmethanesulfonate
ER	endoplasmic reticulum
EST	Expressed Sequence Tag
g	gravity
G6PDH	glucose-6-phosphatase dehydrogenase
HRP	horseradish peroxidase
HSLAS	Health Sciences Laboratory Animal Services
ICL	isocitrate lyase
IgG	immunoglobulin G
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
λ	wavelength
L	levorotary
MBP	maltose binding protein
MES	2-(*N*-morpholino)ethanesulfonic acid
MOPS	3-(*N*-morpholino)propanesulfonic acid
mPTS	membrane peroxisomal targeting signal
mRNA	messenger ribonucleic acid
MUT	mutant

NTG	1-methyl-3-nitro-1-nitrosoguanidine
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBD	peroxisome biogenesis disorder
PCR	polymerase chain reaction
Pex	peroxin
PEX	peroxin gene
PINS	protease inhibitors
PMP	peroxisomal membrane protein
PMSF	phenylmethylsulfonylfluoride
PNS	post-nuclear supernatant
Pr	primer
PTS	peroxisomal targeting signal
SDS	sodium dodecyl sulfate
SH3	Src-homology 3
TCA	trichloroacetic acid
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
THI	3-ketoacyl-CoA thiolase
TPR	tetratricopeptide repeat
TSR	template suppression reagent
v	volume
w	weight
YNB	yeast nitrogen base

1.0 Introduction

1.1 Organelle Biogenesis

The eukaryotic cell differs from the prokaryotic cell in many ways. Most notably, eukaryotic cells use lipid membranes to form separate compartments, called organelles, within the body of the cell (*e.g.* the endoplasmic reticulum [ER] and secretory pathway organelles, mitochondria, the nucleus, peroxisomes, vacuoles or lysosomes, and, in plants, chloroplasts). These compartments allow certain sets of reactions to be separated from other reactions, essentially creating areas of specialization within the cell, and providing eukaryotes with a level of metabolic control unavailable to prokaryotes. Every membrane-bound organelle in a particular cell type has a characteristic copy number, size, and position, which reflect its cellular function (Warren and Wickner, 1996). Concomittant with the development of organelles was the development of systems of traffic for proteins, nucleic acids, metabolites, and other small substances between organelles and between the cell and its environment.

As cells also grow and multiply, there developed a system of organelle growth, multiplication, and inheritance (Warren and Wickner, 1996). This system often involves fission and fusion of homotypic and heterotypic vesicles (South and Gould, 1999), allowing organelles to grow, proliferate, and partition to daughter cells. Organellar membranes themselves cannot be synthesized *de novo*, and all membranes that are linked by vesicle-mediated transport (including the Golgi apparatus, the plasma membrane, endosomes, lysosomes, and secretory

granules) originate in large part from proteins and lipids that are synthesized in the ER (Warren and Wickner, 1996). It is not clear how the membranes of some organelles (*i.e.* mitochondria and peroxisomes) recruit phospholipids, although the ER has been suggested to play a role (Vance and Shiao, 1996; Titorenko and Rachubinski, 1998a).

It has been proposed that there are two inheritance strategies used by organelles: stochastic and ordered (Warren and Wickner, 1996). The stochastic strategy relies on an organelle being present in multiple copies more or less randomly dispersed throughout the cytoplasm. The ordered strategy encompasses a number of disparate mechanisms, including alignment with the spindle pole.

1.2 The Peroxisome

Peroxisomes, together with the glyoxysomes of plants and the glycosomes of trypanosomes, constitute the microbody family of organelles (de Duve, 1996). Peroxisomes are single membrane-bound organelles having a diameter of 0.1 μm to1.0 μm that house reactions which vary in accordance with the organism, cell type, developmental stage, and physiological condition. The peroxisomal membrane forms a selective permeability barrier, consisting mainly of phosphatidylcholine and phosphatidylethanolamine, at levels largely resembling those of the ER (van Veldhoven and Mannaerts, 1994). By definition, peroxisomes contain at least one H_2O_2-producing oxidase and H_2O_2-decomposing catalase (de Duve and Baudhuin, 1966). They do not contain any DNA or protein synthesis machinery. Proliferation of the organelle is often induced when cells

are exposed to certain growth media that contain substrates that are metabolized in peroxisomes (Tanaka *et al.*, 1982; Dommes *et al.*, 1983; Veenhuis *et al.*, 1987; Goodman *et al.*, 1990; van den Bosch *et al.*, 1992). Functions that have been conserved from yeasts to humans include the β-oxidation of fatty acids, the decomposition of hydrogen peroxide by catalase, and the synthesis of ether lipids (for reviews, see van den Bosch *et al.*, 1992; Subramani, 1993, 1996, 1997; Lazarow and Moser, 1994; Erdmann *et al.*, 1997). Peroxisomes are also responsible for the oxidation of methanol in methylotrophic yeasts (Kunau *et al.*, 1987; Veenhuis, 1992).

Proteins that are required for the proper functioning and biogenesis of this organelle have collectively been termed peroxins (Distel *et al.*, 1996). The genes of 23 peroxins (*PEX* genes) have been cloned by various groups in various organisms, such as several species of yeast, Chinese hamster ovary cells, and cultured human fibroblasts (Subramani, 1997, 1998; Götte *et al.*, 1998; Purdue *et al.*, 1998; Titorenko *et al.*, 1998; Koller *et al.*, 1999; Brown *et al.*, 2000). Table 1.1 lists the peroxins whose genes have been cloned to date.

1.3 Peroxisome Biogenesis

The biogenesis of peroxisomes involves an intricate series of events. These events include the recruitment of lipids and phospholipids; the synthesis, sorting, and assembly/activation of matrix and membrane proteins; as well as organelle multiplication and inheritance.

Table 1.1 Peroxins and their characteristics

Peroxin	Features	References
Pex1p	member of the AAA[a] family of ATPases; cytosolic and/or vesicle-associated; interacts with Pex6p; required for ER-to-peroxisome transit of Pex2p and Pex16p in *Y. lipolytica*	Erdmann *et al.*, 1991; Heyman *et al.*, 1994; Portsteffen *et al.*, 1997; Reuber *et al.*, 1997; Faber *et al.*, 1998; Geisbrecht *et al.*, 1998; Tamura *et al.*, 1998a, 1998b; Titorenko and Rachubinski, 1998b
Pex2p	C_3HC_4 zinc-binding integral PMP; glycosylated in *Y. lipolytica*; interacts with Pex19p	Tsukamoto *et al.*, 1991; Shimozawa *et al.*, 1992b; Berteaux-Lecellier *et al.*, 1995; Eitzen *et al.*, 1996; Titorenko *et al.*, 1996; Waterham *et al.*, 1996; Faust and Hatten, 1997; Titorenko and Rachubinski, 1998b; Snyder *et al.*, 2000
Pex3p	integral PMP; interacts with Pex19p	Höhfeld *et al.*, 1991; Baerends *et al.*, 1996; Wiemer *et al.*, 1996; Götte *et al.*, 1998; Kammerer *et al.*, 1998; Snyder *et al.*, 1999a, 2000; Soukupova *et al.*, 1999; Hettema *et al.*, 2000
Pex4p	ubiquitin-conjugating enzyme; peripheral PMP associated with outer face of the peroxisomal membrane or cytosolic or vesicle-associated; interacts with Pex22p; may be important for Pex5p recycling in *H. polymorpha*	Wiebel and Kunau, 1992; Crane *et al.*, 1994; van der Klei *et al.*, 1998; Koller *et al.*, 1999

Pex5p	PTS1 import receptor; 7 carboxyl-terminal TPR motifs; cytosolic and/or peroxisome-associated; 2 genes identified in tobacco; short and long isoforms in mammals generated from alternative splicing of mRNA; cycling receptor in humans	McCollum *et al.*, 1993; van der Leij *et al.*, 1993; Dodt *et al.*, 1995; Fransen *et al.*, 1995; Nuttley *et al.*, 1995; van der Klei *et al.*, 1995; Wiemer *et al.*, 1995; Baes *et al.*, 1997; Brickner *et al.*, 1998; Braverman *et al.*, 1998; Kragler *et al.*, 1998; Otera *et al.*, 1998; Wimmer *et al.*, 1998; de Walque *et al.*, 1999; Schliebs *et al.*, 1999
Pex6p	member of the AAA family of ATPases; peripheral PMP; cytosolic or vesicle-associated; interacts with Pex1p; required for ER-to-peroxisome transit of Pex2p and Pex16p in *Y. lipolytica*	Spong and Subramani, 1993; Voorn-Brouwer *et al.*, 1993; Nuttley *et al.*, 1994; Tsukamoto *et al.*, 1995; Yahraus *et al.*, 1996; Faber *et al.*, 1998; Geisbrecht *et al.*, 1998; Tamura *et al.*, 1998b; Titorenko and Rachubinski, 1998b
Pex7p	cytosolic and/or peroxisomal PTS2 import receptor; 6 WD-40 repeats; interacts with Pex18p and Pex21p	Marzioch *et al.*, 1994; Zhang and Lazarow, 1995, 1996; Rehling *et al.*, 1996; Braverman *et al.*, 1997; Motley *et al.*, 1997; Purdue *et al.*, 1997; Elgersma *et al.*, 1998; Schumann *et al.*, 1999
Pex8p	peroxisomal; localized to matrix or associated peripherally with the inner side of the peroxisomal membrane	Waterham *et al.*, 1994; Liu *et al.*, 1995; Smith *et al.*, 1997

Pex9p	integral PMP	Eitzen *et al.*, 1995
Pex10p	C_3HC_4 zinc-binding integral PMP; interacts with Pex19p; required for human Pex5p recycling; human orthologue interacts with Pex12p	Kalish *et al.*, 1995; Tan *et al.*, 1995; Dodt and Gould, 1996; Okumoto *et al.*, 1998a; Warren *et al.*, 1998; Snyder *et al.*, 1999a, 2000; Chang *et al.*,1999
Pex11p	integral or peripheral PMP; promotes proliferation; no apparent involvement in protein import; α (inducible) and β (constitutive) isoforms identified in mammals; may recruit ARF and coatomer; Pex11β interacts with Pex19p	Erdmann and Blobel, 1995; Marshall *et al.*, 1995; Sakai *et al.*, 1995; Abe and Fujiki, 1998; Abe *et al.*, 1998; Lorenz *et al.*, 1998; Passreiter *et al.*, 1998; Schrader *et al.*, 1998; Sacksteder *et al.*, 2000
Pex12p	C_3HC_4 zinc-binding integral PMP; interacts with Pex5p and Pex10p; required for human Pex5p recycling	Dodt and Gould, 1996; Kalish *et al.*, 1996; Chang *et al.*, 1997; Okumoto and Fujiki, 1997; Okumoto *et al.*, 1998; Chang *et al.*, 1999
Pex13p	SH3 domain-containing integral PMP; part of import receptor docking complex; interacts with Pex5p, Pex7p, Pex14p, Pex19p	Elgersma *et al.*, 1996a; Erdmann and Blobel, 1996; Gould *et al.*, 1996; Björkman *et al.*, 1998; Fransen *et al.*, 1998; Girzalsky *et al.*, 1999; Liu *et al.*, 1999; Shimozawa *et al.*, 1999; Snyder *et al.*, 2000
Pex14p	WD repeat-containing integral or peripheral PMP; phosphorylated in *H. polymorpha*; part of receptor docking complex; interacts	Albertini *et al.*, 1997; Brocard *et al.*, 1997; Komori *et al.*, 1997,

	with Pex5p, Pex7p, Pex13p, Pex17p, Pex19p	1999; Fransen *et al.*, 1998; Scliebs *et al.*, 1999; Shimizu *et al.*, 1999; Will *et al.*, 1999; Sacksteder *et al.*, 2000
Pex15p	integral PMP; phosphorylated	Elgersma *et al.*, 1997
Pex16p	integral or peripheral PMP; involved in proliferation; glycosylated in *Y. lipolytica*	Eitzen *et al.*, 1997; Honsho *et al.*, 1998; Lin *et al.*, 1999; South and Gould, 1999
Pex17p	peripheral PMP associated with the outside of the peroxisomal membrane; part of a receptor docking complex; interacts with Pex14p, Pex19p	Albertini *et al.*, 1997; Smith *et al.*, 1997; Huhse *et al.*, 1998; Snyder *et al.*, 1999b, 2000
Pex18p	PTS2-specific import factor; primarily cytosolic; interacts with Pex7p; related to Pex21p	Purdue *et al.*, 1998
Pex19p	sometimes farnesylated protein; cytosolic and peroxisome-associated; interacts with Pex2p, Pex3p, Pex10p, Pex11β, Pex13p, Pex17p, Pex22p, ALDP, ALDRP, PMP70	Braun *et al.*, 1994; James *et al.*, 1994; Götte *et al.*, 1998; Matsuzono *et al.*, 1999; Snyder *et al.*, 1999a, 1999b, 2000; Gloeckner *et al.*, 2000; Hettema *et al.*, 2000; Sacksteder *et al.*, 2000
Pex20p	thiolase- (or PTS2-) specific import factor; cytosolic and peripherally associated with outside of membrane of high-speed pelletable peroxisomes	Titorenko *et al.*, 1998
Pex21p	PTS2-specific import factor; cytosolic; interacts with Pex7p; related to Pex18p	Purdue *et al.*, 1998
Pex22p	integral PMP; anchors Pex4p to the peroxisomal membrane; interacts with Pex19p	Koller *et al.*, 1999; Snyder *et al.*, 2000

Pex23p	integral PMP	Brown *et al.*, 2000

[a]The following abbreviations are used: AAA ATPase, ATPase associated with diverse cellular activities; ALDP, adrenoleukodystrophy protein; ALDRP, adrenoleukodystrophy related protein; ARF, ADP-ribosylation factor; ER, endoplasmic reticulum; PMP, peroxisomal membrane protein; PTS, peroxisome targeting signal; SH3, src-homology 3; TPR, tetratricopeptide repeat

1.3.1 Protein Targeting Signals (PTSs)

Peroxisomal matrix and membrane proteins are encoded in the nucleus and are synthesized on cytosolic polysomes (Lazarow and Fujiki, 1985; Subramani, 1993, 1998), the one exception being the rat liver membrane protein Pmp50p (Bodnar and Rachubinski, 1991). Most matrix proteins are targeted to peroxisomes by one of two types of peroxisome targeting signals (PTSs). PTS1 is a carboxyl-terminal tripeptide (SKL and conserved variants: S/A/C-K/R/H-L/M) (Gould *et al.*, 1987, 1989; Motley *et al.*, 1995; Elgersma *et al.*, 1996b), whereas PTS2 is an amino-terminal nonapeptide (R/K-L/V/I-X_5-Q/H-L/A) (Swinkels *et al.*, 1991; Glover *et al.*, 1994b; Waterham *et al.*, 1994; Faber *et al.*, 1995). The PTS1 receptor (Pex5p) and PTS2 receptor (Pex7p) molecules have been identified (McCollum *et al.*, 1993; van der Leij *et al.*, 1993; Marzioch *et al.*, 1994; Zhang and Lazarow, 1995, 1996; Terlecky *et al.*, 1995). The putative docking proteins for the PTS1 and PTS2 receptors have been identified, namely Pex13p and Pex14p, respectively (Erdmann *et al.*, 1997; Subramani, 1998). Pex13p is an SH3 domain-containing integral membrane protein, whereas Pex14p is a peripheral membrane protein containing WD repeats. Both SH3 domains and WD repeats are reported to be important for protein-protein interactions (Musacchio *et al.*, 1994; Smith *et al.*, 1999). Pex17p has also been found to associate with these complexes by residing at the outer periphery of the peroxisomal membrane and by directly interacting with Pex14p (Smith *et al.*, 1997; Huhse *et al.*, 1998).

An interesting feature of peroxisomes is their ability to import oligomeric proteins (Glover *et al.*, 1994a; McNew and Goodman, 1994, 1996; Subramani,

1998; Titorenko *et al.*, 1998), stably folded proteins (Walton *et al.*, 1995; Häusler *et al.*, 1996), branched polypeptides (Walton *et al.*, 1992), and even 9 nm gold particles (Walton *et al.*, 1995). These represent relatively new phenomena in the realm of organellar protein import. Only in the case of the nucleus has the import of oligomeric proteins and large particles been shown to occur (Allen *et al.*, 2000).

The targeting of peroxisomal membrane proteins (PMPs) has remained somewhat of a mystery. A few PMPs have been shown to be translated on free polysomes (Fujiki *et al.*, 1984; Suzuki *et al.*, 1987; Bodnar and Rachubinski, 1991), but no targeting mechanisms have been elucidated. A few peroxins have been found to undergo *N*- or *O*-linked glycosylation, thus suggesting that these proteins are sorted through the ER (Baerends *et al.*, 2000). A membrane targeting signal (mPTS) of Pex3p of the yeast *Pichia pastoris* has been shown to reside within the first 40 amino acids (Wiemer *et al.*, 1996). The mPTS of the *Saccharomyces cerevisiae* orthologue of Pex3p seems to reside within the amino-terminal half (Baerends *et al.*, 2000), and that of Pmp47p of *Candida boidinii* within a 20-amino acid stretch between transmembrane domains 4 and 5 (Dyer *et al.*, 1996; McNew and Goodman, 1996). In all cases, the mPTSs are in close proximity to a hydrophobic region or regions of the protein (Baerends *et al.*, 2000).

1.3.2 Current Models of Peroxisome Biogenesis

Peroxisomes were originally thought to be derived from the ER (Novikoff and Shin, 1964). These thoughts were abandoned, however, when it was found that peroxisomal enzymes were imported from the cytosol (Lazarow *et al.*, 1982). Since that time, peroxisomes have commonly been thought to arise only by growth and fission of pre-existing peroxisomes (Lazarow and Fujiki, 1985; Borst, 1986, 1989), although recent lines of evidence suggest that this is perhaps not the case (Waterham *et al.*, 1993; Honsho *et al.*, 1998; South and Gould, 1999). For instance, extra-chromosomal expression of *PEX16* in fibroblasts from patients with Zellweger syndrome, a peroxisomal biogenesis disorder (PBD), restored peroxisome biogenesis in cells where there were no detectable peroxisomal remnants (Honsho *et al.*, 1998; South and Gould, 1999). Also, a *Hansemula polymorpha* temperature-sensitive mutant, *pex13-6*^{ts}, lacking detectable peroxisomal remnants, is able to regenerate peroxisomes after a shift to the permissive temperature (Waterham *et al.*, 1993).

Several lines of evidence implicating the ER in peroxisome biogenesis have been elucidated by Rachubinski and co-workers (Titorenko *et al.*, 1997 and Titorenko and Rachubinski, 1998b). They found that strains of *Yarrowia lipolytica* carrying mutations in the *PEX1* and *PEX6* genes extensively proliferate ER membranes. Pex1p and Pex6p are members of the *N*-ethylmaleimide-sensitive fusion (NSF)-protein family of ATPases associated with diverse cellular activities (AAA ATPases), which has been shown to be essential for the fusion of vesicles in the assembly of various organelles (Patel and Latterich, 1998). Pex1p

and Pex6p have also been found to be associated with what have been called pre-peroxisomal structures in *P. pastoris* (Faber *et al.*, 1998).

Also, in *Y. lipolytica*, the peroxisomal membrane proteins Pex2p and Pex16p are subject to *N*-linked core glycosylation within the ER lumen, but apparently they do not pass through the Golgi apparatus (Titorenko and Rachubinski, 1998b). Pex15p of *S. cerevisiae*, another membrane protein, is *O*-mannosylated in the ER, but does not pass through the Golgi apparatus, either (Elgersma *et al.*, 1997). Mutations in the *Y. lipolytica SEC238*, *SRP54*, *PEX1*, and *PEX6* genes cause defects in the exit of some secretory proteins from the ER, as well as significantly delay or prevent the exit of Pex2p and Pex16p from the ER (Titorenko *et al.*, 1997; Titorenko and Rachubinski, 1998b).

Taken together, these lines of evidence implicate the ER as a central player in the peroxisome biogenetic pathway. Erdmann *et al.* (1997) and Titorenko and Rachubinski (1998a) have proposed that vesicular elements containing peroxisomal proteins bud from the ER and fuse to form peroxisomes, probably through the action of Pex1p and Pex6p. Matrix proteins are then imported from the cytosol, along with additional membrane proteins. Vesicles continue to bud from the ER and fuse with peroxisomes to allow the addition of phospholipids, as peroxisomes are largely devoid of lipid-synthesizing enzymes (Lazarow and Fujiki, 1985). Peroxisomes continue to grow and can also undergo fission to form new peroxisomes.

1.4 Human Peroxisomal Biogenesis Disorders

The importance of peroxisomes in human growth and development is underlined by a group of genetic disorders known as the peroxisome biogenesis disorders (PBDs), including Zellweger syndrome, rhizomelic chondrodysplasia punctata, infantile Refsum disease, hyperpipecolic acidemia, and adrenoleukodystrophy (for reviews, see Lazarow and Moser, 1994; Fujiki, 1997). Patients with PBDs usually share a range of clinical symptoms, including severe mental retardation, plasmalogen deficiency, hypotonia, neuronal migration defects, hepatic dysfunction, and retinopathy. There are 16 genetically different peroxisomal disorders that have been identified to date, and these are classified into three types (Fujiki, 1997). They include: Type 1, in which peroxisomes are virtually absent and a generalized impairment of peroxisomal functions is evident (cerebrohepatorenal Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease); Type 2, in which peroxisomes are present but several peroxisomal functions are impaired (rhizomelic chondrodysplasia punctata and combined peroxisomal β-oxidation enzyme deficiency); and Type 3, in which peroxisomes are present and only a single peroxisomal function is impaired (X-linked adrenoleukodystrophy, hyperoxaluria type 1, acyl-CoA oxidase deficiency, multifunctional enzyme deficiency, thiolase deficiency, dihydroxyacetonephosphate acyltransferase deficiency, alkyl dihydroxyacetonephosphate synthetase deficiency, Refsum disease, and acatalasemia).

Attention has been turned to understanding the molecular mechanisms behind these diseases. Fibroblasts cultured from patients with PBDs and Chinese hamster ovary cell mutants which phenotypically resemble these fibroblasts have been isolated and shown to be very useful for the investigation of these disorders, as well as for peroxisome biogenesis in general (Zoeller and Raetz, 1986; Tsukamoto *et al.*, 1990; Shimozawa *et al.*, 1992a; Lazarow and Moser, 1994). Much progress has also been made using yeast as a model system. Thirteen human orthologues of yeast peroxins have been identified by screening the Expressed Sequence Tag (EST) databases (Fujiki, 1997), and several of these have been shown to complement the peroxisome deficiencies of cells of patients with PBDs (Subramani, 1997, 1998).

1.5 Yeast As a Model System

1.5.1 Yeast As a Model System

Working under the proposition that the principles of peroxisome biogenesis have been conserved between lower eukaryotes and higher eukaryotes, many investigators have found that yeasts are excellent model systems with which to study peroxisome biogenesis. Not only can the induction and protein composition of peroxisomes be readily manipulated by varying growth conditions, but in the absence of peroxisomes, yeasts are still found to be viable (Veenhuis, 1992; Subramani, 1993). Yeast cells can also be cultured as both haploid or diploid cells, which facilitates the generation and characterization of

mutants (Elgersma and Tabak, 1996). Also, in contrast to mammalian cells in which β-oxidation takes place in both peroxisomes and mitochondria, β-oxidation in yeasts occurs only in peroxisomes (Kawamoto *et al.*, 1978; Tanaka *et al.*, 1982; Ueda *et al.*, 1985; Kunau *et al.*, 1987). This simplified pathway has made studying the process of peroxisome assembly and its components easier. Finally, many recombinant DNA techniques are available for yeast, which makes the introduction and deletion of genes possible (Elgersma and Tabak, 1996).

It is also hoped and has, in fact, been demonstrated, that research in yeast has led and will continue to lead to a discovery of the molecular mechanisms behind human PBDs. Several human ESTs have been identified that are orthologues of yeast proteins involved in peroxisome biogenesis (Subramani, 1997; Abe *et al.*, 1998; Honsho *et al.*, 1998; Okumoto *et al.*, 1998; South and Gould, 1999).

Several protocols have been developed for generating and isolating yeast mutants that are defective in peroxisome biogenesis (Erdmann *et al.*, 1989; Cregg *et al.*, 1990; Gould *et al.*, 1992; Nuttley *et al.*, 1993). Yeast cultures are exposed to powerful mutagens in order to generate mutations in the genome. They are then grown on media containing carbon sources that are metabolized only in peroxisomes, and those that are deficient in peroxisome biogenesis or in the relevant metabolic pathway are identified by lack of growth on a suitable substrate. A genomic library is then transformed into the yeast to find a piece of DNA that will rescue the mutant phenotype and complement the mutation. This

gene is then isolated, and further genetic and biochemical studies are conducted. This system has provided a quick and easy way to identify *PEX* genes.

1.5.2 Yarrowia lipolytica As a Model System

The yeast *Y. lipolytica* is currently being used as a model organism for studies on protein secretion and dimorphism, as well as for peroxisome biogenesis (Barth and Gaillardin, 1997). It is well adapted for growth on fats, fatty acids, and alkanes (Barth and Gaillardin, 1997), and exhibits a high degree of peroxisome proliferation when fed solely long-chain fatty acids such as oleic acid. Cells containing mutations affecting peroxisome biogenesis are unable to grow on media containing oleic acid as the sole carbon source and are designated as exhibiting the ole$^-$ phenotype. Identification of peroxisome biogenesis mutants of *Y. lipolytica* has also been made rapid by the incorporation of an immunofluorescence assay designed to show the localization of PTS1-containing proteins (Nuttley *et al.*, 1993). PTS1-containing proteins are often mislocalized to the cytosol in *pex* mutants. Genomic libraries have been constructed in the *Y. lipolytica* expression vector pINA445, and an efficient and rapid electroporation procedure has been developed for the complementation of these mutants (Nuttley *et al.*, 1993).

1.6 Overview of This Study

The gene encoding the peroxin Pex19p has been isolated in a variety of organisms (Braun *et al.*, 1994; James *et al.*, 1994; Götte *et al.*, 1998; Matsuzono

et al., 1999; Snyder *et al.*, 1999). It has been shown to be a mainly cytosolic protein that can interact with a number of PMPs (Götte *et al.*, 1998; Snyder *et al.*, 1999a, 1999b, 2000; Gloeckner *et al.*, 2000; Sacksteder *et al.*, 2000). Every orthologue of Pex19p contains a concensus sequence for the addition of a lipid farnesyl moiety at its extreme carboxyl-terminus (see Omer and Gibbs, 1994, for a review of protein prenylation), although the physiological relevance of this sequence seems to vary between organisms. In the absence of a functional *PEX19* gene, growth on carbon sources metabolized by peroxisomes is compromised. Peroxisomal matrix proteins are mislocalized to the cytosol, and peroxisomes and peroxisomal remnants appear to be absent in the majority of cell types. In *P. pastoris*, small vesicles are observed that are not present in wild-type cells (Snyder *et al.*, 1999a, 1999b), and these have been postulated to represent peroxisome precursors. The absence of peroxisome remnants has led to the hypothesis that Pex19p acts early on in peroxisome biogenesis.

This thesis details the isolation and cloning of the *PEX19* gene and the preliminary characterization of its protein product Pex19p in *Y. lipolytica*. *Y. lipolytica* cells without a functional *PEX19* gene are no longer able to grow using oleic acid as a carbon source, as it is normally metabolized by β-oxidation in peroxisomes. They also mislocalize the majority of peroxisomal matrix proteins to the cytosol. However, in contrast to *PEX19* mutants of other organisms, electron microscopy reveals the presence of morphologically detectable peroxisome-like structures in *Y. lipolytica pex19* cells. Some of these structures are of the same density as wild-type peroxisomes, although the protein

complements differ. Pex19p itself is associated with peroxisomes in wild-type cells.

2.0 Materials and Methods

All percentages are weight per volume (w/v) unless otherwise stated.

2.1 Materials

All chemicals, enzymes, and kits were used according to the manufacturers' specifications and in accordance with protocols set out by Environmental Health and Safety of the University of Alberta and WHMIS.

2.1.1 Chemicals and Reagents

acetic acid, glacial	Fisher
acetone	Anachemia
acrylamide	GibcoBRL
agar	DIFCO
agarose, electrophoresis grade	GibcoBRL
agarose, SeaKem GTG	FMC BioProducts
albumin, bovine serum (BSA)	NEB
L-amino acids	Sigma
ammonium bicarbonate	BDH
ammonium persulfate	BDH
ampicillin	Sigma
antipain dihydrochloride	Roche
aprotinin	Roche
benzamidine hydrochloride	Sigma
Bio-Rad protein assay dye reagent	Bio-Rad
boric acid	Caledon
Brij-35 (polyoxyethylene 23-lauryl ether)	Sigma
bromophenol blue	BDH
chloroform	BDH
complete supplements mixture (CSM) minus amino acids	BIO101
Coomassie Brilliant Blue R-250	ICN
dithiothreitol (DTT)	ICN
DNA, sodium salt, from salmon testes	Sigma
DNA ladder, 1kb	GibcoBRL
ethanol, 100%	Commercial Alcohols
ethanol, 95%	Commercial Alcohols
ethyl methanesulfonate (EMS)	Sigma

ethylenediaminetetraacetic acid (EDTA)	Sigma
Freund's adjuvant (complete and incomplete)	Sigma
formaldehyde	BDH
formamide	BDH
D-glucose	BDH
glycerol	BDH
hydrogen peroxide (30%)	Sigma
isopropanol	Anachemia
isopropyl β-D-thiogalactopyranoside (IPTG)	Vector Biosystems
leupeptin	Roche
L-malic acid	Sigma
malt extract	DIFCO
maltose	BDH
β-mercaptoethanol	BDH
methanol	Anachemia
N,N'-methylenebisacrylamide	GibcoBRL
2-(*N*-morpholino)ethanesulfonic acid (MES)	Sigma
3-(N-morpholino)propanesulfonic acid (MOPS)	BDH
oleic acid	Fisher
pepstatin A	Sigma
peptone	DIFCO
phenol, buffer-saturated	GibcoBRL
phenylmethylsulfonylfluoride (PMSF)	Roche
Ponceau S red	Sigma
potassium chloride	BDH
potassium phosphates	BDH
N-propyl gallate	Sigma
prestained molecular weight markers	NEB
Sephadex G-50	Amersham-Pharmacia
silver nitrate	BDH
skim milk	
sodium carbonate	BDH
sodium chloride	BDH
sodium dodecyl sulfate (SDS)	Sigma
sodium fluoride	Sigma
sodium sulfite	Sigma
sodium thiosulfate	Sigma
sorbitol	BDH
sucrose	BDH
N,N,N',N'-tetramethylethylenediamine (TEMED)	GibcoBRL
tricholoroacetic acid (TCA)	Sigma
tris(hydroxymethyl)aminomethane	Roche
trisodium citrate	BDH
Triton X-100	BDH
tryptone	DIFCO
Tween 20 (polyoxyethylene 20-sorbitan	Fisher

monolaurate)	
Tween 40 (polyoxyethylenesorbitan monopalmitate)	Sigma
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	Vector Biosystems
xylene cyanole FF	Sigma
yeast extract	DIFCO
yeast nitrogen base (YNB) without amino acids	DIFCO

2.1.2 Other Materials

amylose resin	NEB
BIOMAX MR scientific imaging film	Kodak
glass beads, acid-washed	Sigma
nitrocellulose	Amersham-Parmacia, Bio-Rad
Saran Wrap	
Whatman paper	Fisher
X-OMAT XK-1 film	Kodak

2.1.3 Enzymes

calf intestinal alkaline phosphatase (CIP)	NEB
DNA ligase, T4	NEB
DNA polymerase, T4	NEB
DNA polymerase I, Klenow fragment	NEB
dNTP's	NEB
restriction endonucleases	NEB
RNase A	Roche
Zymolyase 100-T	ICN

2.1.4 Kits

ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit	PE Applied Biosystems
DNA UltraFast Cleavage and Deprotection Kit	Beckman
ECL Direct Nucleic Acid Labelling and Detection Systems	Amersham-Pharmacia
ECL Western Blotting Detection Reagents	Amersham-Pharmacia
QIAprep Spin Miniprep Kit	Qiagen

QIAquick Gel Extraction Kit	Qiagen
Random Primed DNA Labeling Kit	Roche
Ready-to-Go PCR Beads	Amersham-Pharmacia

2.1.5 Plasmids

2.1.5.1 Escherichia coli Vectors

pGEM5Zf(+)	Promega
pGEM7Zf(+)	Promega
pMAL-c2	NEB

2.1.5.2 Yarrowia lipolytica/Escherichia coli Shuttle Vectors

pINA445	Dr. Claude Gaillardin (INRA, Thiverval-Grignon, France) (Nuttley *et al.*, 1993)
pSU (University	Mr. Arjuna Thiagarajah of Alberta, Canada)

2.1.6 Antibodies

2.1.6.1 Commercial Antibodies

anti-guinea pig IgG (from donkey), fluorescein-conjugated	Jackson
anti-guinea pig IgG (from donkey), rhodamine-conjugated	Jackson
anti-guinea pig IgG (from goat), horseradish peroxidase-conjugated	Sigma
anti-rabbit IgG (from donkey), horseradish peroxidase-conjugated	Amersham-Pharmacia
anti-rabbit IgG (from donkey), rhodamine-conjugated	Jackson

anti-*Saccharomyces cerevisiae* glucose-6-phosphate dehydrogenase (from rabbit) — Sigma

2.1.6.2 Laboratory-Produced Antibodies

Antibodies to the peptide NH_2-CRYHLKPLQSKL-COOH conjugated to keyhole limpet hemocyanin were raised in rabbit as described (Aitchison *et al.*, 1992). Antisera to *Y. lipolytica* 3-ketoacyl-CoA thiolase, Pex2p, and Pex16p were raised in guinea pig as described (Eitzen *et al.*, 1996, 1997). Antiserum to *Y. lipolytica* isocitrate lyase (ICL) was raised in rabbit as described (Eitzen *et al.*, 1996). Antiserum to acyl-CoA oxidase (AOX), raised in rabbit, was a kind gift of Dr. Jean-Marc Nicaud (INRA, Thiverval-Grignon, France). The raising of antiserum against a Pex19p-maltose binding protein fusion protein is described in section **2.9**.

2.1.7 Buffers and Other Solutions

A list of all buffers and other solutions used in this study is provided in Table 2.1.

2.2 DNA Manipulation and Detection

2.2.1 Molecular Cloning

All molecular cloning was performed essentially as described (Maniatis *et al.*, 1982; Ausubel *et al.*, 1989). Restriction endonucleases and other modifying enzymes were used as per the manufacturers' instructions. Bovine serum albumin (BSA) was added to digestion reactions as needed. 5' overhangs were made blunt

Table 2.1. Solutions used in this study

Solution	Composition[a]
breakage buffer	2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0[b]
buffer H	1 M sorbitol, 5 mM MES, pH 5.5, 1 mM KCl, 0.5M EDTA, 0.1% (v/v) ethanol[c]
column buffer	20mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA[d]
denaturing solution	0.5 M NaOH, 1.5 M NaCl[e]
destain	45% (v/v) methanol, 10% (v/v) glacial acetic acid[f]
DNA loading buffer	40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanole FF[g]
elution buffer	0.2 M Tris-acetate, pH 7.4, 1% SDS, 10 mM DTT[h]
glass bead lysis buffer	20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 10 mM KCl[i]
mounting medium	4% N-propyl gallate, 70.48% glycerol, 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4, pH 7.4[j]
neutralizing solution	1.5 M Tris-HCl, pH 8.0, 1.5 M NaCl[e]
running buffer	50 mM Tris-acetate, pH 7.4, 0.1% SDS[h]
SDS-PAGE running buffer	19 mM glycine, 25 mM Tris, 1% SDS[k]
SDS-PAGE sample buffer	10% glycerol (v/v), 84 mM Tris-HCl, pH 6.8, 1% SDS, 0.0025% bromophenol blue, 10 mM DTT[k]
solution B	100 mM KH_2PO_4/K_2HPO_4, pH 7.5, 1.2 M sorbitol[l]
SSC	0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0[g]

TBE	89 mM Tris, 89 mM boric acid, 2 mM EDTA[g]
TBST	20 mM Tris, 150 mM NaCl, 0.5% Tween 20[m]
TE	10 mM Tris-HCl, pH 8.0, 1 mM EDTA[g]
transfer buffer	20 mM Tris, 156 mM glycine, 20% (v/v) methanol[n]
zymolyase buffer	0.5 M KCl, 5 mM MOPS, pH 7.2, 10 mM Na_2SO_3

[a]All percentages are weight per volume (w/v) unless otherwise noted.
[b]Ausubel *et al.* (1989)
[c]Szilard *et al.* (1995)
[d]Modification of buffer suggested for use by NEB.
[e]Amersham-Pharmacia
[f]Modification of Ausubel *et al.* (1989).
[g]Maniatis *et al.* (1982)
[h]Harlow and Lane (1988)
[i]Eitzen *et al.* (1997)
[j]Modification of Pringle *et al.* (1991).
[k]Modification of Laemmli (1970).
[l]Pringle *et al.* (1991)
[m]Huynh *et al.* (1985)
[n]Towbin *et al.* (1979); Burnette (1981)

with the Klenow fragment of DNA polymerase I. 3' overhangs were made blunt with T4 DNA polymerase. dNTPs were added as needed. Calf intestinal alkaline phosphatase (CIP) was used to dephosphorylate 5' ends. DNA fragments were ligated together with T4 DNA ligase.

DNA fragments were run through agarose gels (for diagnostics) or GTG agarose gels (for purification) (1% agarose in TBE) (see Table 2.1) supplemented with 50 μL/L ethidium bromide. Samples were loaded in DNA loading buffer (see Table 2.1), and gels were run in TBE supplemented with 50 μL/L ethidium bromide. 1kb ladder (GibcoBRL) was used for standard mobility markers. DNA-ethidium bromide complexes were visualized using an ultra-violet transilluminator (Photodyne, Model 3-3006). DNA fragments were extracted from gels using the QIAquick Gel Extraction Kit (Qiagen).

2.2.2 Polymerase Chain Reaction (PCR)

2.2.2.1 Oligonucleotides

All oligonucleotides were synthesized by an Oligo 1000M Synthesizer (Beckman). Oligonucleotides were cleaved from the columns by the DNA UltraFast Cleavage and Deprotection Kit (Beckman) and diluted and stored in H_2O at -20°C. See Table 2.2 for a list of all oligonucleotides. Oligonucleotides MUT35-Pr1 through MUT35-Pr10 were used to sequence the *MUT35* open reading frame (ORF) from the smallest complementing fragment of the *mut35*

Table 2.2. Oligonucleotides used in this study

Name	Sequence
MUT35-Pr1	5'-TCATTTCATTAGCAGGGC-3'
MUT35-Pr2	5'-CTTAAACAAGGCGGCTA-3'
MUT35-Pr3	5'-CGGTACACTAAGACAGT-3'
MUT35-Pr4	5'-GCCAACAATGTCCTGC-3'
MUT35-Pr5	5'-GAAGGAGCGAGACCG-3'
MUT35-Pr6	5'-GAGCTCCCAGGAGGC-3'
MUT35-Pr7	5'-ACGAGTTCGACGAGCA-3'
MUT35-Pr8	5'-GCTGGTAACGTTGTTGA-3'
MUT35-Pr9	5'-TCAACAACGTTACCAGC-3'
MUT35-Pr10	5'-GAGACTGAGTCGTCTC-3'
Pex19-Pr1	5'-TAGAATTCATGTCACACGAAGAAGATCTTG-3'
Pex19-Pr2	5'-TAAAGCTTTCACTCGGGCATGTTCTCGGGC-3'
pMAL-Pr	5'-GCCGCCAGCGGTCG-3'
Pex19-Pr11	5'-CCTTGATTTCCATCGTG-3'
Pex19-CNQQ-*Mfe*I 3'	5'-TATCGCCGGCAATTGTTACTCGGGCATGTTCTCGG-3'[a]
Pex19C321S-*Mfe*I 3'	5'-TATCGCTAGCAATTGTTACTGCTGGTTGGACTCGGG-3'

[a]Stop codon positions are underlined.

mutation. Pex19-Pr1 and Pex19-Pr2 were used to amplify the *PEX19* ORF minus the nucleotides encoding the carboxyl-terminal 4 amino acids and the stop codon. An *Eco*RI site was engineered immediately 5' of the initiating ATG codon, and a *Hin*dIII site was engineered immediately 3' of the terminal 3' codon. Primers pMAL-Pr, MUT35-Pr1, MUT35-Pr5, MUT35-Pr7, and MUT35-Pr9 were used for sequencing to confirm the integrity of the *PEX19*-CNQQ⇒pMAL-c2 ligation product. Pex19-Pr1 was also used in conjunction with Pex19-CNQQ-*Mfe*I 3' and Pex19C321S-*Mfe*I 3' to amplify *PEX19* ORFs lacking the nucleotides encoding the 4 carboxyl-terminal amino acids and with a cysteine→serine mutation at position 321, respectively. Both of these engineered ORFs had *Mfe*I restriction sites immediately 3' to the stop codons. Pex19-Pr11 was used along with MUT35-Pr1, MUT35-Pr5, MUT35-Pr7, and MUT35-Pr9 to sequence the *PEX19*-CNQQ⇒pINA445 and *PEX19*C321S⇒pINA445 ligation products. A Beckman DU640 Spectrophotometer was used to quantitate DNA by measuring absorbance at $\lambda = 260$ nm.

2.2.2.2 Sequencing PCR Products

4 pmol of primer was added to 4 μL of miniprepped DNA and 8 μL of BigDye Terminator RR Mix (PE Applied Biosystems), and the mixture was brought to 20 μL with H_2O. Using a Stratagene Robocycler 40, reaction conditions were as follows: 1 cycle of 96°C for 2 min and 25 cycles of 96°C for 46 sec, 50°C for 51 sec, and 60°C for 4 min 10 sec, followed by storage at 6°C. 80 μL of 75% (v/v) isopropanol was added, and the reaction was allowed to sit for

20 min before being spun in a microcentrifuge on maximum speed for 20 min. The pellet was washed twice with 75% isopropanol, followed by 5 min centrifugations. The pellet was then dried in a vacuum dessicator for 5 min, immersed in 15 μL of template supression reagent (TSR) (PE Applied Biosystems), and heated at 95°C for 2 min. The solution was rapidly cooled in ice-water and subjected to sequence analysis by an ABI Prism 310 Genetic Analyzer automated sequencer (PE Applied Biosystems).

2.2.2.3 Amplification by PCR

DNA amplification was effected through the use of Ready-to-Go PCR Beads (Amersham-Pharmacia). 20 pmol of each primer and 4 μL of miniprepped DNA were used. Reaction conditions were as follows: 1 cycle of 94°C for 2 min, 25 to 30 cycles of 94°C for 1 min, 5°C below the lowest primer melting temperature for 1 min, and 72°C for 1 min per kilobase pair length of the template, and 1 cycle of 72°C for 7 min and 6°C for 1 min. PCR products were purified by running them through GTG agarose gels and extracting the relevant DNA using the QIAquick Gel Extraction Kit (Qiagen). DNA was stored at -20°C.

2.2.3 Southern Blotting

2.2.3.1 Labelling of DNA Probes

DNA probes were labelled using the ECL Direct Nucleic Acid Labelling and Detection Systems kit (Amersham-Pharmacia).

2.2.3.2 Southern Blotting

Genomic DNA was isolated from putative *Y. lipolytica E122* and *22301-3* knock-out cells (see section ***2.3.2***) and was digested with *Apa*I overnight. The digests were run through 0.75% agarose gels, which were then subjected to ultra-violet light for 7 min to nick the DNA. The gels were then incubated with gentle shaking in denaturing solution (see Table 2.1) for 30 min and neutralizing solution (see Table 2.1) for 30 min. DNA was transferred by capillary action overnight to nitrocellulose that had been hydrated and equilibrated in 5 X SSC (see Table 2.1) (Ausubel *et al.*, 1989 modified by Eitzen *et al.*, 1997). The DNA was then cross-linked to the nitrocellulose by exposure to ultra-violet light (twice at 120 000 $\mu J/cm^2$, $\lambda = 254$ nm; UV Stratalinker 1800, Stratagene). The blots were prehybridized, hybridized, washed, and exposed to X-OMAT XK-1 flim (Kodak) as per the ECL Direct Nucleic Acid Labelling and Detection Systems kit (Amersham-Pharmacia).

2.3 DNA Isolation from Microorganisms

2.3.1 Plasmid Recovery from Escherichia coli

2 mL LB (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 100 μg of ampicillin per mL (see section ***2.6.1***) were innoculated with *E. coli* DH5α cells containing the plasmid of interest and incubated for approximately 16 h at 37°C in a shaking water bath. For an 8 h period of growth, TYP (1.6% tryptone, 1.6%

yeast extract, 0.5% NaCl, 0.25% K_2HPO_4) containing 100 μg of ampicillin per mL (see section *2.6.1*) was used. Cells were collected by centrifugation at 16 000 X *g* for 2 min. Minipreps were made using the QIAprep Spin Miniprep Kit (Qiagen). Plasmids were stored at -20°C in elution buffer.

2.3.2 Isolation of Genomic DNA from Yarrowia lipolytica

Isolation of *Y. lipolytica* genomic DNA was performed essentially as described by Eitzen *et al.* (1995). 10 mL of YPD (1% yeast extract, 2% peptone, 2% glucose) (see section *2.6.2.1*) were innoculated with the strain of interest and incubated in a rotating wheel at 30°C for approximately 16 h. Cells were collected by centrifugation in a clinical centrifuge, and cells were resuspended in YPBO (0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1% Brij 35, 1% oleic acid) (see section *2.6.2.1*) to yield a culture with an optical density of approximately 2 at λ = 600 nm after 8 h of growth in a rotating wheel at 30°C. Cells were collected by centrifugation, washed twice with H_2O, resuspended in 500 μL of breakage buffer (see Table 2.1), and collected in a microcentrifuge tube. The cells were then resuspended in 200 μL of breakage buffer, an equal volume of acid-washed glass beads (Sigma), and 200 μL of phenol:chloroform (1:1). Mixtures were vortexed for 3 min at 4°C, and 200 μL of TE (see Table 2.1) was added. The mixtures were then spun in a microcentrifuge for 5 min at room temperature. The aqueous layers were removed and washed twice with phenol:chloroform (1:1) and once with chloroform. Nucleic acids were precipitated by adding 1 mL of absolute ethanol and mixing, and were collected

by centrifugation for 5 min. Pellets were washed with 1 mL of 70% (v/v) ethanol and then dried in a vacuum dessicator. Pellets were dissolved in TE containing 20 μg of RNase A per mL and incubated for 2 to 3 h at 37°C. DNA was stored at -20°C in TE containing 20 μg of RNase A per mL.

2.4 Protein Manipulation and Detection

2.4.1 Trichloroacetic Acid (TCA) Precipitation

0.25 X volume of TCA was added to samples to precipitate proteins. After a 15 min incubation on ice, samples were spun in a microcentrifuge for 15 min at 4°C. Pellets were suspended in chilled 80% (v/v) acetone, incubated on ice for 15 min, and spun in a microcentrifuge for 15 min at 4°C. Pellets were then dried in a vacuum dessicator and stored at -20°C.

2.4.2 Quantitation

Protein was quantitated using the Bio-Rad protein dye assay, essentially a modification of the method of Bradford (1976). Absorbance at $\lambda = 595$ nm was measured by a Beckman DU640 spectrophotometer. BSA was used as a standard.

2.4.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were dissolved in SDS-PAGE sample buffer (see Table 2.1) and immersed in boiling water for 5 min. Samples were concentrated through 3% stacking gels and resolved through 10% or 15% SDS-PAGE gels (Laemmli,

1970) (30:0.8 acrylamide:*N,N'*-methylene-bis-acrylamide, 370 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.1% [v/v] TEMED, 0.042% ammonium persulfate) run at no more than 200 V in SDS-PAGE running buffer (see Table 2.1). Prestained protein markers (NEB) were used for molecular weight standards.

2.4.4 Protein Detection

2.4.4.1 Coomassie Staining

Gels were stained with Coomassie Brilliant Blue R-250 (0.25% in destain) (see Table 2.1) and destained with destain to the desired resolution. Alternatively, gels were stained with 0.05% Coomassie Brilliant Blue R-250 in H_2O and destained with H_2O to the desired resolution. Gels were then dried in a gel dryer (Bio-Rad Model 583) at 80°C for less than 2 h between two sheets of Scientific Imaging Film (Kodak).

2.4.4.2 Silver Staining

Silver staining was performed as described by Blum *et al.* (1987) with a few modifications. Proteins were fixed in gels by incubation in 50% methanol/10% acetic acid for 30 min and 50% methanol for 15 min. Gels were washed with H_2O seven times for 3 min each and then incubated in 0.02% sodium thiosulfate for 1 min followed by two 1 min washes in H_2O. Gels were stained with 0.1% silver nitrate for 25 min at 4°C, washed twice with H_2O for 1 min, and developed to the desired resolution in 2% sodium carbonate containing 0.0148%

formaldehyde. Developing was stopped by incubation with 1.4% EDTA for 10 min followed by two 1 min washes in H_2O. Gels were stored in 20% (v/v) glycerol.

2.4.4.3 Western Blotting

Proteins were transferred to nitrocellulose using a semi-dry blotting apparatus (Model ET-20, Tyler Research Instruments) (Kyhse-Andersen, 1984) at 60 mA per minigel for 1 h in transfer buffer (see Table 2.1). Blots were stained with 0.1% Ponceau S red in 1% TCA for 15 min and destained with H_2O to the desired resolution for visual confirmation of transfer. Blots were blocked in 1% skim milk in TBST (see Table 2.1) (blocking reagent) for 1 h, incubated with primary antibody in blocking reagent for 1 h, washed twice with blocking reagent for 20 min, incubated with secondary antibody in blocking reagent for 1 h, washed twice with blocking reagent for 20 min, and washed twice with TBST for 5 min. Secondary antibodies were visualized using the ECL Western Blotting Detection Reagents (Amersham-Pharmacia) and exposure to X-OMAT XK-1 film (Kodak). All antibodies used are listed in Table 2.3 with their respective dilutions.

2.4.5 Enzymatic Assays

Table 2.3. Antibodies and their dilutions used for Western blotting

Antibody	Dilution
rabbit anti-SKL	1:5000
guinea pig anti-THI[a]	1:50 000
rabbit anti-AOX	1:5000
rabbit anti-ICL	1:5000
guinea pig anti-Pex2p	1:3000
guinea pig anti-Pex16p	1:3000
guinea pig anti-Pex19p	1:5000
HRP-conjugated donkey anti-rabbit IgG	1:30 000
HRP-conjugated goat anti-guinea pig IgG	1:30 000

[a]Abbreviations used: AOX, acyl-CoA oxidase; HRP, horseradish peroxidase; ICL, isocitrate lyase; IgG, immunoglobulin G; Pex, peroxin; THI, 3-ketoacyl-CoA thiolase.

2.4.5.1 Catalase

Various subcellular fractions were tested for the presence of catalase, a peroxisomal matrix enzyme (Lück, 1963; Szilard *et al.*, 1995). Aliquots of sample fractions were diluted in 50 mM potassium phosphate buffer, pH 7.5, to which 50 µL of 0.3% H_2O_2 were added. The change in absorbance at $\lambda = 240$ nm is directly proportional to the concentration of catalase in the aliquot.

2.4.5.2 Fumarase

Various subcellular fractions were tested for the presence of fumarase, a mitochondrial marker enzyme (Smith *et al.*, 1997). Aliquots of sample fractions were diluted in 50 mM potassium phosphate buffer, pH 7.5, to which 150 µL of 100 mM L-malic acid were added. The change in absorbance at $\lambda = 240$ nm is directly proportional to the concentration of fumarase in the aliquot.

2.5 Protein Isolation from Microorganisms

Protease inhibitors (PINS) were added to various solutions to prevent protein degradation. See Table 2.4 for a list of protease inhibitors and their concentrations of use.

2.5.1 Isolation of Fusion Proteins from Escherichia coli

1 L aliquots of RMG (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 100 µg ampicillin per mL (see section ***2.6.1***) were innoculated with overnight cultures of *E. coli* cells containing the *PEX19*-CNQQ⇒pMAL-c2

Table 2.4. Protease inhibitors (PINS) used in this study

Protease Inhibitor	Concentration
antipain dihydrochloride	2.5 μg/mL
aprotinin	1.4 μg/mL
benzamidine hydrochloride	0.5 mM
leupeptin	1 μg/mL
phenylmethylsulfonylfluoride (PMSF)	1 mM
sodium fluoride	5 mM

vector grown in 10 mL RMG containing 100 μg ampicillin per mL. Cultures were grown to an optical density of approximately 0.5 at λ = 600 nm, at which time IPTG was added to 1 mM. Incubation was continued for 2 h at which time cells were collected by centrifugation in a JA10 rotor (Beckman) at 4000 X *g* for 20 min, resuspended in 50 mL of column buffer (see Table 2.1) containing PINS and 1 mM DTT, and frozen at -20°C overnight. Cells were thawed, sonicated with a Branson Sonifier 250 at power level 3 and 30% duty cycle three times for 30 sec each, and centrifuged in a JA10 rotor (Beckman) at 9000 X *g* for 30 min at 4°C. The supernatant was added to amylose resin (NEB) that had been swollen with column buffer containing PINS and 1 mM DTT for approximately 1 h and protein was allowed to bind to the column for 1 h. The resin was loaded onto the column, drained, and washed twice with column buffer. Protein bound to the column was eluted with 10 mM maltose. 500 μL fractions were collected. 10 μL of flow-through, each wash, and each fraction were run on SDS-PAGE gels, which were then stained with Coomassie blue (see section *2.4.4.1*) to visualize protein. The fractions containing the fusion protein were pooled and stored at -20°C.

2.5.2 Isolation of Total Protein from Yarrowia lipolytica

Isolation of total protein from *Y. lipolytica* was performed essentially as described by Szilard *et al.* (1995). 10 mL of YPD (see section *2.6.2.1*) was innoculated with *Y. lipolytica* cells and incubated in a rotating wheel at 30°C for approximately 16 h. Cells were then collected by centrifugation in a clinical

centrifuge, resuspended in 10 mL of YPBO (see section *2.6.2.1*), and incubated in a wheel at 30°C for 8 h. Cells were then collected by centrifugation, washed three times with H_2O, and resuspended in 200 µL of cold glass bead lysis buffer (see Table 2.1) containing PINS and 1 mM DTT. Glass beads were added to the level of the bottom of the meniscus, and the cells were vortexed on high speed five times for 1 min each at 4°C with 1 min periods on ice between each vortexing. 200 µL of lysis buffer was added to the lysates, which were then spun in a microcentrifuge at 16 000 X *g* for 1 min at 4°C. The supernatants were removed and spun in a microcentrifuge at 16 000 X *g* for 20 min at 4°C. The supernatants were removed and stored at -20°C.

2.6 Growth of Microorganisms: Strains and Culture Conditions

Aliquots of all strains, including those strains carrying plasmids, were flash frozen in 50% glycerol in a dry ice-ethanol bath and stored at -80°C. All percentages listed in sections *2.6.1* and *2.6.2* are weight per volume (w/v) unless otherwise stated.

2.6.1 Escherichia coli

DH5α cells were grown in liquid LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl), in liquid TYP medium (1.6% tryptone, 1.6% yeast extract, 0.5% NaCl, 0.25% K_2HPO_4), or on LB agar plates at 37°C. Ampicillin was added to 100 µg/mL for selection of cultures containing plasmids. BL21 (DE3) cells were maintained on LB plates or LB plates containing 100 µg/mL ampicillin but

were grown in RMG medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose) containing 100 μg ampicillin per mL. Strains were maintained on 1.8% agar plates at 4°C.

2.6.2 Yarrowia lipolytica

2.6.2.1 Culture Propagation

See Table 2.5 for a list of all *Y. lipolytica* strains used in this study. Strains not carrying plasmids were grown in liquid YPD (1% yeast extract, 2% peptone, 2% glucose) or on YPD agar plates at 30°C. Peroxisome proliferation was induced by growth in YPBO (0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1% Brij 35, 1% oleic acid) at 30°C. Strains carrying plasmids were grown in liquid YNBD (0.67% yeast nitrogen base without amino acids, complete supplement mixture [CSM] minus the appropriate amino acids at twice the manufacturer's recommended concentration, 2% glucose) or on YNA agar plates (0.67% yeast nitrogen base without amino acids, 2% sodium acetate) at 30°C. Peroxisome proliferation was induced by growth in liquid YNO (0.67% yeast nitrogen base without amino acids, 2 X CSM minus the appropriate amino acids, 0.05% Tween 40, 1% oleic acid) at 30°C. Assays for growth on oleic acid were carried out on YNO agar plates containing the appropriate amino acids. All strains were maintained on 1.8% agar plates at 4°C. Strains to be transformed were initially grown in liquid YEPA (1% yeast extract, 2% peptone, 2% sodium acetate).

Table 2.5. *Y. lipolytica* strains used in this study

Strain[a]	Genotype
E122	*MATA ura3-302 leu2-270 lys8-11*
22301-3	*MATB ura3-302 leu2-270 his1*
MUT35[b]	*MATA ura3-302 leu2-270 lys8-11 mut35*
MUT35-c	*MATA ura3-302 leu2-270 lys8-11 p35CF(LEU2)*
pex19KOA	*MATA ura3-302 leu2-270 lys8-11 PEX19::URA3*
pex19KOB	*MATB ura3-302 leu2-270 his1 PEX19::URA3*
DWTA/WTB	*MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ +/his1*
DWTA/KOB	*MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ +/his1 +/PEX19::URA3*
D19-1/WTB	*MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ +/his1 pex19-1/+*
DKOA/WTB	*MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ +/his1 PEX19::URA3/+*
DKOA/KOB	*MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ +/his1 PEX19::URA3/PEX19::URA3*

[a]Strains *E122* and *22301-3* were gifts of C. Gaillardin (Thiverval-Grignon, France). All others were made in this study.
[b]*MUT35* was subsequently renamed *pex19-1* after sequencing the complementing fragment's ORF. The corresponding gene was renamed *PEX19*.

2.6.2.2 Mating of Haploid Strains

In order to create diploid strains, *MATA* strains were crossed with *MATB* strains in the following manner, essentially as described by Nuttley *et al.* (1993). Cells were streaked onto YPD plates and incubated at 30°C for 2 days. Cells from these plates were then streaked onto presporulation medium (PSM) plates (0.5% yeast extract, 0.5% ammonium sulfate, 0.2% KH_2PO_4, 2% glucose) and incubated at 30°C overnight. Strains to be mated were then streaked on top of one another on yeast mating medium (YM) plates (0.5% peptone, 0.3% yeast extract, 0.3% malt extract) and incubated at room temperature for 4 days. Diploids were selected on the basis of amino acid prototrophy on YNBD plates incubated at 30°C for 3 or 4 days. Diploids were rescued onto YPD plates and were analyzed for their ability to grow on YNO plates.

2.7 Mutagenesis of *Yarrowia lipolytica* and Isolation of the *PEX19* Gene

2.7.1 Mutagenesis of Yarrowia lipolytica

Mutagenesis was performed by Rachel Szilard as described (Szilard, 2000). Essentially, *E122* cells were grown in YPD and washed and resuspended in 0.1 M citrate buffer, pH 5.5. The mutagen 1-methyl-3-nitro-1-nitrosoguanidine (NTG) was added to a concentration of 25 µg/mL. The cells were incubated at room temperature for 33 min. Cells were then grown on YPD plates at 30°C and

replica plated onto YNA and YNO plates to screen for the ole⁻ phenotype (see section *1.5.2*).

2.7.2 Screening of the Yarrowia lipolytica Genomic DNA Library

Screening was essentially as described by Nuttley *et al.* (1993) and Szilard (2000). Ole⁻ *Y. lipolytica* cells were transformed with a genomic DNA library (see section *2.8.2.1*) cloned into the vector pINA445, and colonies were isolated that had been rescued from the ole⁻ phenotype. Plasmid DNA was isolated (Szilard, 2000) and introduced by electroporation into *E. coli* (Szilard, 2000). Plasmids isolated from *E. coli* were re-transformed into *MUT35* cells to confirm that complementation was linked to the introduction of the plasmid.

2.7.3 Sequencing of the PEX19 Gene

The complementing fragment was mapped by restriction enzyme digestion. Various fragments were excised from the complementing fragment, religated into pINA445, and transformed into *MUT35* cells. The smallest complementing fragment was sequenced by Dr. Richard Rachubinski using primers MUT35-Pr1 through MUT35-Pr10 (see section *2.2.2.1* and Table 2.2).

2.8 Transformation of Microorganisms

2.8.1 Transformation of Escherichia coli

Transformation of *E. coli* cells was essentially as described by Ausubel *et al.* (1989).

2.8.1.1 DH5α Subcloning Efficiency Cells

0.5 μL of miniprepped DNA was added to 25 μL aliquots of DH5α cells. After 30 min on ice, the cells were heat shocked at 37°C for 2 min, placed on ice for 2 min, spread onto LB plates containing 100 μg ampicillin per mL, and incubated at 37°C. For transformation of ligation reaction products, 2 μL of product DNA was added to the cells. After 30 min on ice, the cells were heat shocked at 37°C, placed on ice for 2 min, suspended in 1 mL of LB, and incubated in a 37°C shaking water bath for 45 min. Cells were then collected by centrifugation, resuspended in a minimum volume of LB, spread onto LB plates containing 100 μg/mL ampicillin, and incubated at 37°C.

2.8.1.2 BL21 (DE3) Cells

1 μL of miniprepped DNA was added to 20 μL of BL21 (DE3) cells. The cells were then electroporated with a Cell-Porator (GibcoBRL) at 395 V and 4 kΩ. The cells were rescued onto LB plates containing 100 μg ampicillin per mL and incubated at 37°C.

2.8.2 Transformation of Yarrowia lipolytica

Transformation of *Y. lipolytica* was essentially as described by Nuttley *et al.* (1993).

2.8.2.1 Transformation with Autonomously Replicating Plasmids

10 mL of YEPA was innoculated with *Y. lipolytica* cells and grown for approximately 16 h in a rotating wheel at 30°C. The entire cultures were subcultured into 40 mL of YEPA and grown to an optical density of approximately 1 at λ = 600 nm. Cells were collected by centrifugation, resuspended in 45 mL of TE (pH 7.5) (see Table 2.1) and 5 mL of 1 M lithium acetate, and incubated for 30 min at 30°C and 250 rpm, at which time 1 mL of 1 M DTT was added. Incubation was continued at 100 rpm for 15 min. Cells were collected, washed once with room-temperature H_2O, once with ice-cold H_2O, and once with ice-cold 1 M sorbitol, and resuspended in a minimum of sorbitol. 1 µL of miniprepped DNA was added to 20 µL of cells. The cells were subjected to electroporation with a Cell-Porator (GibcoBRL) at 250 V and 16 kΩ and rescued into 100 µL of ice-cold 1 M sorbitol. The cells were then spread onto YNA plates lacking the appropriate amino acids and incubated at 30°C.

2.8.2.2 Replacement of the PEX19 ORF of E122 and 22301-3 Cells with URA3 by Integrative Transformation

Nucleotides 16 to 1025 of the *PEX19* ORF and terminator regions were removed from a population of pGEM7Zf(+) vectors containing inserts of

approximately 4 kbp containing the *PEX19* gene by restriction digestion with *Bgl*II and *Mfe*I. The ends of the vectors with the remaining pieces of genomic DNA were made blunt, dephosphorylated, and ligated together with the *URA3* gene excised from the vector pSU with *Sal*I and made blunt. The *URA3* gene flanked by the genomic DNA was then excised from pGEM7Zf(+) with *Apa*I, and the linear fragment was transformed into *E122* and *22301-3* cells by electroporation as in section *2.8.2.1*. The cells were spread onto YNA plates lacking uracil to select for uracil prototrophs. Uracil prototrophs were then replica plated onto YNO to select for cells unable to utilize oleic acid as a carbon source (ole$^-$ phenotype). Southern blots were performed (see section *2.2.3*) with probes to the *PEX19* ORF, the *URA3* gene, and the approximately 4 kbp genomic complementing fragment to confirm respectively the absence of the *PEX19* ORF, the presence of the *URA3* gene, and the shift in mobility of the respective *Apa*I fragment, as the *URA3* gene is approximately 500bp longer than the *PEX19* ORF that it replaced. The knock-out and mutant strains were mated with wild-type strains and with each other to confirm that a suppressor gene had not been knocked out (see section *2.6.2.2*).

2.9 Generation of Anti-Pex19p Antibodies

Pex19p-maltose binding protein (MBP) fusion products were isolated as in section *2.5.1*. All relevant fractions eluted from the amylose resin column were pooled and analyzed on 10% SDS-PAGE gels. Protein was visualized by staining with Coomassie blue in H_2O (see section *2.4.4.1*). The bands of interest were

excised, broken into small pieces, and placed in dialysis tubing in elution buffer (see Table 2.1) (approximately 10 mL per gram of gel). The tubes were placed in running buffer (see Table 2.1) in between two chambers of a DNA gel box (Hoefer). The protein was eluted for 3 h at 100 V at 4°C. The elution buffer was replaced, and the elution was continued overnight at 50 V at 4°C. The combined elution buffer fractions were dialysed against four changes of 50 mM ammonium bicarbonate. The solution containing the protein was then frozen at -80°C and lyophilized. The protein was dissolved in a minimum of H_2O and quantitated.

A rabbit and two guinea pigs were immunized three times as recommended by Harlow and Lane (1988) by the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. Bleeds were taken before the first immunization and two weeks after each immunization. Animals were sacrificed two weeks after the third injection. Protein was mixed with Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for subsequent injections. A single band running at $M_r \simeq 48$ kDa was detected by each of the antibodies when Western blotting was performed on glass bead lysates of *E122* cells (see section ***2.5.2***). These bands were not detected on Western blots of glass bead lysates of *pex19-1* or *pex19KO* cells, indicating the specificity of the polyclonal antibodies.

2.10 Microscopy

2.10.1 Electron Microscopy

Electron microscopy was performed essentially as described by Goodman *et al.* (1990). 10 mL of YPD was innoculated with *Y. lipolytica* cells and incubated in a rotating wheel at 30°C for approximately 16 h. Cells were collected by centrifugation in a clinical centrifuge, resuspended in 10 mL of YPBO, and incubated for a further 8 h. Cells were then fixed in 1.5% $KMnO_4$ for 20 min at room temperature (kindly done by Honey Chan, University of Alberta). After dehydration in a series of graded ethanol, samples were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and observed with a Phillips 410 electron microscope.

2.10.2 Immunofluorescence Microscopy

Immunofluorescence microscopy was performed essentially as described by Szilard *et al.* (1995). 10 mL of YPD was innoculated with *Y. lipolytica* cells and incubated in a rotating wheel at 30°C for approximately 16 h. Cells were collected by centrifugation in a clinical centrifuge, resuspended in 10 mL of YPBO, and incubated for a further 8 h. Formaldehyde was added to cell cultures to 3.7% for 30 min at room temperature with occasional mixing. Cells were washed with and resuspended in solution B (see Table 2.1). Cells were incubated with 2 μg/mL of β-mercaptoethanol and 80 μg/mL of zymolyase 100-T (ICN) at 30°C for 45 min. Spheroplasts were placed on slides containing poly-L-lysine (Sigma) and permeabilized by placing in chilled methanol for 6 min followed by chilled acetone for 30 sec. Spheroplasts were incubated with 1% skim milk in

TBST (see Table 2.1) (blocking reagent) for 1 h. Spheroplasts were then incubated with primary antibody in blocking reagent for 1 h, washed ten times with blocking reagent, incubated with secondary antibody in blocking reagent for 1 h, washed ten times with blocking reagent, and washed three times with TBST, before being placed under mounting medium (see Table 2.1) and viewed with an Olympus BX50 fluorescence microscope.

A list of antibodies used for immunofluorescence and the concentrations at which they are used is given as Table 2.6. Pex19p antiserum was pre-cleaned by incubation overnight at 4°C with *pex19KO* spheroplasts that had been prepared as if for immunofluorescence.

2.11 Subcellular Fractionation and Organelle Isolation

2.11.1 Subcellular Fractionation

Subcellular fractionation was performed essentially as described by Szilard *et al.* (1995). 10 mL of YPD was innoculated with *Y. lipolytica* cells and incubated in a rotating wheel at 30°C for approximately 16 h. Sufficient cells were subcultured in 250 mL of YPD to reach an optical density of approximately 2 at λ = 600 nm after 16 h incubation at 250 rpm. At this time cells were collected by centrifugation and entirely resuspended in 1 L of YPBO (mutant cells) or cells were added in sufficient quantity to reach an optical density of approximately 2 at λ = 600 nm after 8h incubation to 1 L of YPBO (*E122* cells).

Table 2.6. Antibodies and their dilutions used for immunofluorescence

Antibody	Concentration
rabbit anti-SKL	1:100
guinea pig anti-THI[a]	1:1000
rabbit anti-AOX	1:500
rabbit anti-ICL	1:500
rabbit anti-Pex19p	1:50[b]
donkey anti-guinea pig IgG, fluorescein-conjugated	1:200
donkey anti-guinea pig IgG, rhodamine-conjugated	1:200
donkey anti-rabbit IgG, rhodamine-conjugated	1:200

[a]Abbreviations used: AOX, acyl-CoA oxidase; ICL, isocitrate lyase; IgG, immunoglobulin G; Pex, peroxin; THI, 3-ketoacyl-CoA thiolase.
[b]Concentration after pre-absorption (see section ***2.10.2***).

Cultures were incubated for 8 h at 180 rpm. Cells were then collected by centrifugation, washed three times with H_2O, resuspended in 4 mL of zymolyase buffer (see Table 2.1) per gram of cells containing approximately 2 mg of zymolyase 100-T per gram wet weight of cells, PINS, and 1 mM DTT, and incubated for 30 min at 30°C and 100 rpm. Spheroplasts were collected by centrifugation in a JA10 rotor (Beckman) at 2500 X *g* for 8 min at 4°C. Spheroplasts were resuspended in 3 mL buffer H (see Table 2.1) per gram wet weight of cells containing PINS and 1 mM DTT and homogenized with 10 strokes of a Cole-Parmer homogenizer at 34%. The homogenates were centrifuged in a JS13.1 rotor (Beckman) at 1000 X *g* for 10 min at 4°C to yield a post-nuclear supernatant (PNS) fraction plus debris. The PNS fraction was centrifuged in a JS13.1 rotor (Beckman) at 20 000 X *g* for 30 min at 4°C to yield supernatant (20KgS) and pellet (20KgP) fractions. The 20KgS fraction was centrifuged in a TLA120.2 rotor (Beckman) at 200 000 X *g* for 30 min to yield supernatant (200KgS) and pellet (200KgP) fractions. 20KgP and 200KgP fractions were resuspended in buffer H containing PINS and 1 mM DTT.

2.11.2 Isolation of Peroxisomes by Isopycnic Sucrose Density Gradient Centrifugation

Peroxisomes were isolated essentially as described by Titorenko *et al.* (1996). 20KgP fractions were resuspended in buffer H containing PINS 1 mM DTT and loaded onto discontinuous sucrose gradients. Sucrose gradients were composed of a 7 mL layer of 53% (w/w) sucrose under a 14 mL layer of 42%

(w/w) sucrose under a 7 mL layer of 35% (w/w) sucrose under a 4 mL layer of 25% (w/w) sucrose. All sucrose solutions were made in buffer H. Gradients were spun in a VTi50 rotor (Beckman) at 32 000 rpm for 1 h 15 min at 4°C. 2 mL fractions were collected from the bottom of the gradients.

2.11.2.1 Isolation of Peroxisomal Membrane Proteins

Duplicate aliquots of gradient fraction 4 (the peak peroxisomal fraction, as determined by catalase assay and Western blotting with various peroxisomal antibodies) of *E122* and *pex19KO* sucrose gradients containing 40 μg of protein were diluted with buffer H to twice the volume. The peroxisomes were then pelleted by centrifugation in a TLA120.2 rotor (Beckman) at 200 000 X *g* for 30 min at 4°C. One aliquot was resuspended in SDS-PAGE sample buffer (see Table 2.1) and placed in boiling water for 5 min. The other aliquot was resuspended in chilled 0.1 M sodium carbonate (pH 11) and incubated on ice for 1 h with occasional pipetting to mix. The sodium carbonate solution was then layered on top of a 0.3 M sucrose cushion and centrifuged in a TLA120.2 rotor (Beckman) at 200 000 X *g* for 30 min at 4°C. The pellet containing PMPs was dissolved in SDS-PAGE sample buffer and placed in boiling water for 5 min. The proteins remaining in the sodium carbonate buffer (the matrix and peripherally associated membrane proteins) were precipitated by addition of TCA (see section *2.4.1*), dissolved in SDS-PAGE sample buffer, and placed in boiling water for 5 min. Equal amounts of total peroxisomal protein were analyzed on SDS-PAGE gels, which were then stained with silver (see section *2.4.4.2*).

2.12 Farnesylation and Pex19p Function

In order to determine the importance of the farnesylation consensus sequence to the function of Pex19p, two mutants of *PEX19* were constructed to yield versions of Pex19p that could not be farnesylated. The first mutant was made by amplifying the *PEX19* ORF by PCR using primers Pex19-Pr1 and Pex19-CNQQ-*Mfe*I 3' (see section *2.2.2.3*). The amplified product was then digested with *Bgl*II and *Mfe*I and ligated into the smallest complementing fragment in pGEM-5Zf(+) which had also been cut with *Bgl*II and *Mfe*I. The resulting construct was a *PEX19* gene lacking the nucleotides coding for the carboxyl-terminal 4 amino acids, as well as the first 49 nucleotides of the terminator region. The whole gene was liberated by digestion with *Nco*I and ligated into pINA445 cut with *Nco*I. The second version was created in a similar manner, except that Pex19C321S-*Mfe*I 3' was used instead of Pex19-CNQQ-*Mfe*I 3' to yield a version of the *PEX19* gene that coded for a protein with a cysteine→serine mutation at position 321. This mutation effectively nullifies any chance of farnesylation. These two constructs, as well as the original smallest complementing fragment and the empty pINA445 vector, were transformed into *pex19KO* cells (see section *2.8.2.1*) and growth on YNO plates was observed to check for rescue of the ole$^-$ phenotype. See Figure 2.1 for a schematic of the constructs.

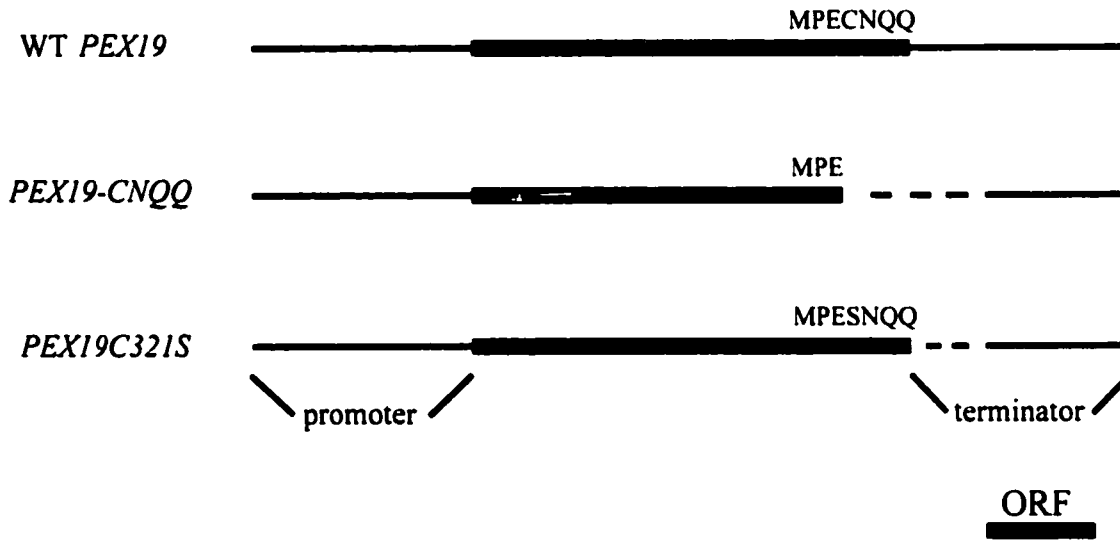


Figure 2.1 Constructs encoding mutant farnesylation motifs in Pex19p. The letters above the ORFs represent amino acids coded for. The dashed line indicates sequence missing relative to the wild-type gene.

3.0 Results

3.1 Isolation and Characterization of the *PEX19* Gene

The *MUT35* strain (see Table 2.5) was identified from among randomly mutagenized *Y. lipolytica E122* cells (wild-type cells) by its inability to grow on media containing oleic acid as the sole carbon source (ole$^-$ phenotype) (see sections ***2.7.1*** and ***2.7.2***). Subsequent morphological and biochemical analyses (data presented below) suggested that this inability was due to a defect in peroxisome biogenesis. *MUT35* cells were transformed with a *Y. lipolytica* genomic library subcloned into the yeast expression vector pINA445 (Nuttley *et al.*, 1993). Plasmid DNA was isolated from those cells that had regained the ability to utilize oleic acid as a carbon source (those that had regained the ole$^-$ phenotype). *E. coli* cells were transformed with this DNA to recover the plasmid containing the library fragment. The fragment was mapped using restriction endonuclease digestion (Figure 3.1), and various restriction fragments were subcloned into pINA445. The smallest insert that rescued the ole$^+$ phenotype of *MUT35* was sequenced in both directions. It contained an open reading frame (ORF) of 972 base pairs (bp) encoding a protein of 324 amino acids (Figure 3.2) and a predicted molecular mass of 34 822 Da.

A BLAST search was performed to determine if any orthologues had been documented. The search revealed that the sequenced ORF coded for a protein with homology to the peroxin Pex19p of various organisms (see Table 3.1). Accordingly, the *MUT35* gene was renamed *PEX19*, and the *MUT35* strain was

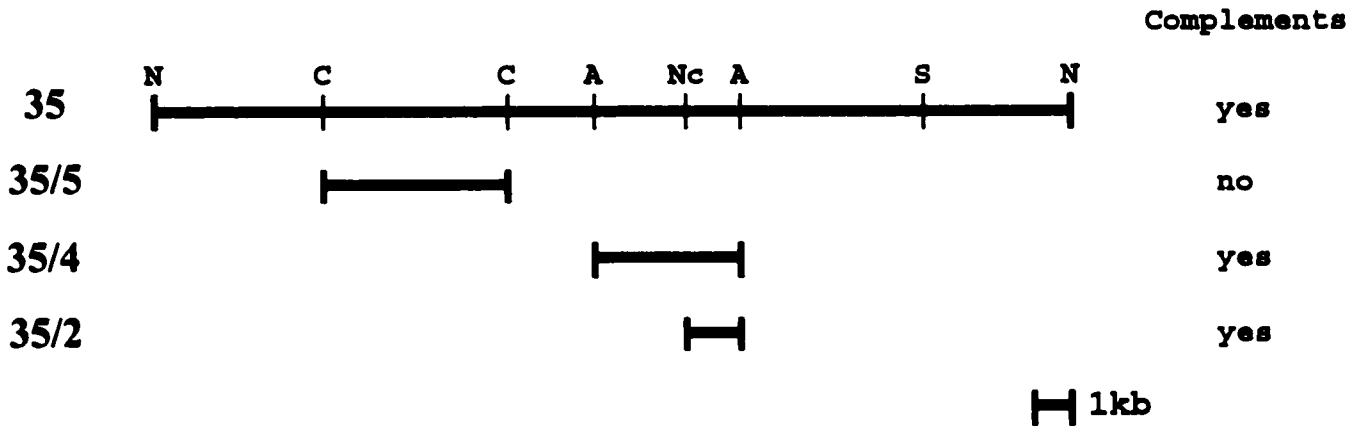


Figure 3.1 Restriction map of the *MUT35* complementing fragment. The *Y. lipolytica* library insert subcloned into the vector pINA445 isolated from *MUT35* cells that rescued the ole⁻ phenotype is represented by a straight line (35). Various restriction endonuclease sites are indicated (A = *Apa*I, C = *Cla*I, N = *Nde*I, Nc = *Nco*I, S = *Sph*I). 35/5, 35/4, and 35/2 fragments were subcloned into pINA445 and introduced into *MUT35* cells to find the smallest complementing fragment.

Figure 3.2 Sequence of the *MUT35* open reading frame (ORF) and its protein product. Nucleotides are numbered starting from the A of the ATG start codon. The deduced amino acid sequence is printed below the ORF. The one-letter code for amino acids is used. The farnesylation sequence is printed in bold. The highlighted sequence is an in-frame stop codon before the start codon of the ORF.

```
-250   5'-ATAGAAGCCAGAAGAGGAGGAAGTGGAGGAAGAATTTTCCATTGAA
-204 TATTCCGTGGTATTAATTACCACTACTTCATTAATTACACCTGTCTCACCT
-153 CCTTGGTCCAAATCTAACCACTCAGACGGCTGATGATCATAGACTCCCCCT
-102 GCACACCCCACCTTGTGAAAGAAACTAAGTTGACCAACACCTTGATTTCCA
 -51 TCGTGTTCGCTTCTTAACCACACCCCCACACAGTTCACACCCACAAAAACA
   1 ATG TCA CAC GAA GAA GAT CTT GAT GAC CTC GAT GAC TTT
       M   S   H   E   E   D   L   D   D   L   D   D   F    13
  40 CTC GAC GAG TTC GAC GAG CAG GTG CTG AGC AAG CCT CCT
       L   D   E   F   D   E   Q   V   L   S   K   P   P    26
  79 GGA GCT CAG AAG GAT GCT ACC CCT ACT ACA TCT ACT GCT
       G   A   Q   K   D   A   T   P   T   T   S   T   A    39
 118 CCT ACC ACT GCC GAG GCT AAG CCT GAT GCC ACT AAG AAG
       P   T   T   A   E   A   K   P   D   A   T   K   K    52
 157 AGC ACC GAA ACT TCC GGG ACC GAT TCA AAG ACT GAG GGA
       S   T   E   T   S   G   T   D   S   K   T   E   G    65
 196 GCC GAT ACT GCT GAC AAG AAT GCG GCC ACT GAC TCT GCG
       A   D   T   A   D   K   N   A   A   T   D   S   A    78
 235 GAG GCC GGT GCG GAG AAG GTT TCT CTG CCC AAC CTC GAG
       E   A   G   A   E   K   V   S   L   P   N   L   E    91
 274 GAT CAG CTC GCT GGG CTC AAG ATG GAC GAC TTC CTT AAG
       D   Q   L   A   G   L   K   M   D   D   F   L   K   104
 313 GAC ATC GAG GCA GAC CCC GAG TCC AAG GCC CAG TTT GAG
       D   I   E   A   D   P   E   S   K   A   Q   F   E   117
 352 TCT CTG CTC AAG GAG ATC AAC AAC GTT ACC AGC GCC ACC
       S   L   L   K   E   I   N   N   V   T   S   A   T   130
 391 GCA TCC GAA AAG GCC CAG CAG CCC AAG TCA TTC AAA GAG
       A   S   E   K   A   Q   Q   P   K   S   F   K   E   143
 430 ACC ATT TCC GCC ACT GCT GAC CGT TTG AAC CAG AGT AAC
       T   I   S   A   T   A   D   R   L   N   Q   S   N   156
 469 CAG GAG ATG GGG GAC ATG CCC CTT GGT GAC GAC ATG CTC
       Q   E   M   G   D   M   P   L   G   D   D   M   L   169
 508 GCT GGC CTC ATG GAG CAG CTG TCG GGT GCT GGA GGC TTT
       A   G   L   M   E   Q   L   S   G   A   G   G   F   182
 547 GGA GAG GGC GGG GAA GGC GAC TTT GGA GAT ATG TTA GGA
       G   E   G   G   E   G   D   F   G   D   M   L   G   195
 586 GGA ATC ATG CGT CAG CTG GCC TCC AAG GAG GTT CTG TAC
       G   I   M   R   Q   L   A   S   K   E   V   L   Y   208
 625 CAG CCG TTG AAG GAG ATG CAC GAC AAC TAC CCT AAG TGG
       Q   P   L   K   E   M   H   D   N   Y   P   K   W   221
 664 TGG GAC GAG CAC GGA TCC AAG GTG ACC GAG GAG AAG GAG
       W   D   E   H   G   S   K   V   T   E   E   K   E   234
 703 CGA GAC CGA CTT AAG CTG CAG CAG GAC ATT GTT GGC AAA
       R   D   R   L   K   L   Q   Q   D   I   V   G   K   247
 742 ATC TGT GCC AAG TTT GAG GAC CCC TCT TAC TCT GAC GAC
       I   C   A   K   F   E   D   P   S   Y   S   D   D   260
 781 TCC GAA GCC GAC CGA GCC GTC ATC ACC CAG CTG ATG GAC
       S   E   A   D   R   A   V   I   T   Q   L   M   D   273
 820 GAG ATG CAG GAG ACC GGT GCT CCT CCG GAT GAG ATC ATG
       E   M   Q   E   T   G   A   P   P   D   E   I   M   286
 859 TCC AAT GTT GCC GAC GGT AGC ATT CCT GGC GGT CTC GAT
       S   N   V   A   D   G   S   I   P   G   G   L   D   299
 897 GGT CTT GGA CTG GGA GGT CTT GGT GGC GGT AAG ATG CCC
       G   L   G   L   G   G   L   G   G   G   K   M   P   312
 936 GAG ATG CCC GAG AAC ATG CCC GAG TGC AAC CAG CAG TAA
       E   M   P   E   N   M   P   E   C   N   Q   Q  STOP 324
 975 GTGATAGATTATATAGGCGAGTGATGAAGGACACGAGACTGAGTCGTCTCA
1026 ATTGATGTTTATATTTTATATCTACTGTAGCAACGAGACCGAAATGATTGT
1077 ACCAGCAACGGTACACTAAGACAGTAGCCGCCTTGTTTAAGTAATAGGCTC
1128 ACCGTTTTCTAATTAAGCAGAACAATATTTATTGTGCACACGAAGAAGTAC
1179 AGTAGTGTGGGTGTGTAAATGTGTGTTGCTTCAGAGGTAAAGCCAATCCTC
1230 CGACAATAGCCAATATCTTCATTGTATCTGCCCTTTCAACTTCAAGCGCTG
1281 TGTTCACAGACTCATATGATAATATCTTGTGTGTCTCTTTGCCATGCCGTA
1332 GACACAACTCAAATGTCCCACTACACCCAGATACGTTATCAACCTTAGCTT
1383 CGTATAATCACGACGACACCATGG-3'
```

Table 3.1. Putative Orthologues of *Y. lipolytica* Pex19p

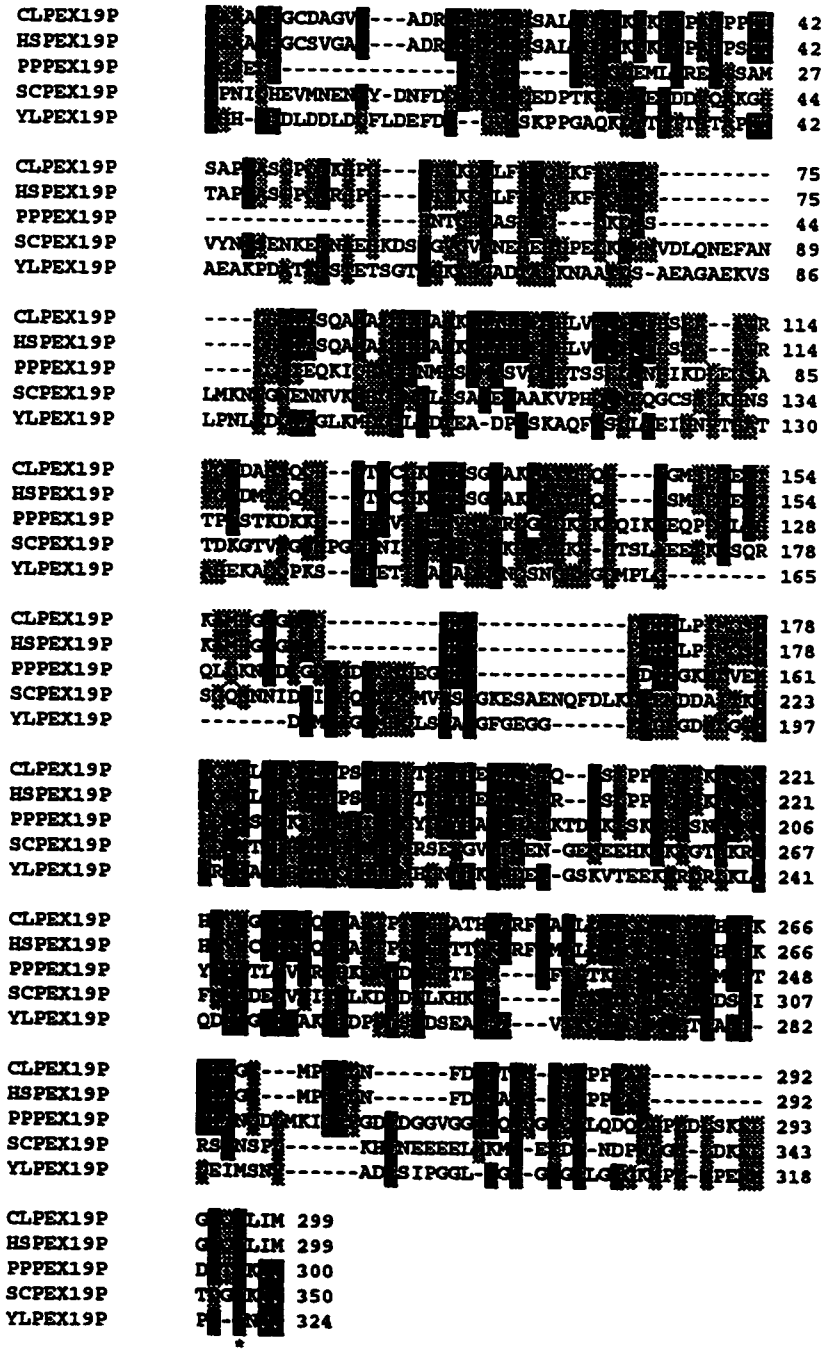
Organism	% Identity with *Y. lipolytica* Pex19p
CHO[a]	26%
Homo sapiens	24%
Saccharomyces cerevisiae	24%
Caenorhabditis elegans	38%[b]
Schizosaccharomyces pombe	33%[b]

[a]Chinese hamster ovary cells.
[b]Identities to hypothetical proteins.

renamed *pex19-1*. A protein sequence alignment of *Y. lipolytica* Pex19p with Pex19p of CHO cells, *Homo sapiens*, *P. pastoris*, and *S. cerevisiae* is presented in Figure 3.3. One amino acid motif that is conserved among all putative orthologues is a farnesylation consensus sequence at the extreme carboxyl-terminus. The putatively farnesylated cysteine is amino acid 321 in *Y. lipolytica*, and is marked with an asterisk in Figure 3.3.

PEX19 knockout strains of mating type A (*E122*; *pex19KOA*) and mating type B (*22301-3*; *pex19KOB*) were made as described (see section *2.8.2.2*) (see Table 2.5 for genotypes). These strains were unable to grow on media containing oleic acid as the sole carbon source and displayed similar morphological and biochemical features to *pex19-1* cells (see below). *pex19KOA* cells were mated to *22301-3* cells to yield strain *DKOA/WTB* and to *pex19KOB* cells to yield strain *DKOA/KOB*. *DKOA/WTB* cells grew on oleic acid-containing medium, whereas *DKOA/KOB* cells did not. *pex19-1* cells were mated to *22301-3* cells to yield strain *D19-1/WTB* and to *pex19KOB* cells to yield strain *D19-1/KOB*. *D19-1/WTB* cells grew on oleic acid-containing medium, whereas *D19-1/KOB* cells did not. *E122* cells were mated to *pex19KOB* cells to yield strain *DWTA/KOB*, which grew on oleic acid-containing medium. *E122* cells and *22301-3* cells were mated to yield strain *DWTA/WTB* as a positive control for growth. The results of these assays revealed the recessive nature of the *pex19-1* mutation and the *PEX19* gene deletions and revealed that at least one intact copy of *PEX19* is required for growth on oleic acid-containing media. They also confirmed that the authentic *PEX19* gene had been knocked out in the respective knockout strains.

Figure 3.3 Sequence alignment of *Yarrowia lipolytica* Pex19p with Pex19p of other organisms. The sequence of *Y. lipolytica* Pex19p was aligned with the sequences of Pex19p in CHO cells (CL), *Homo sapiens* (HS), *Pichia pastoris* (PP), and *Saccharomyces cerevisiae* (SC). Amino acids were aligned with the use of the ClustalW program (EMBL, Heidelberg, Germany). Conserved residues in three or more proteins are highlighted in black; similar residues in three or more proteins are highlighted in grey. Similarity rules: G = A = S; A = V; V = I = L = M; I = L = M = F = Y= W; K = R = H; D = E = Q = N; and S = T = Q = N. Dashes represent gaps in sequence similarity. The cysteine of the conserved farnesylation consensus sequence is marked with an asterisk.


CLPEX19P
HSPEX19P
PPPEX19P
SCPEX19P
YLPEX19P

3.2 *pex19-1* and *pex19KO* Cells Contain Peroxisome-Like Structures but Mislocalize Most Matrix Proteins and a Lumenal Peripheral Membrane Protein to the Cytosol

In electron micrographs of *E122* cells grown in oleic acid-containing medium for 8 h peroxisomes appear as single membrane-bound vesicular structures approximately 0.2 μm to 0.5 μm in diameter, with a homogeneous granular matrix (Figure 3.4A, arrows). Electron micrographs of *pex19-1* and *pex19KO* cells grown in oleic acid-containing medium for 8 h revealed the presence of normal-looking peroxisome-like structures (Figure 3.4B and C, arrows).

Immunofluorescence was performed on all three strains to determine how matrix protein import was affected by *PEX19* mutation and deletion. In *E122* cells, indirect immunofluorescence microscopy performed with primary antibodies to SKL-containing proteins (SKL), 3-ketoacyl-CoA thiolase (THI), acyl-CoA oxidase (AOX), and isocitrate lyase (ICL), all matrix proteins localized exclusively to peroxisomes, displayed punctate patterns of fluorescence typical of wild-type peroxisomes (Figure 3.5). In contrast, the same antibodies displayed a diffuse homogeneous staining pattern typical of proteins localized to the cytosol in both *pex19-1* and *pex19KO* cells. Immunofluorescence studies performed with the same antibodies in *pex19KO* cells that had been transformed with the smallest complementing fragment from the *Y. lipolytica* genomic library in the yeast expression vector pINA445 (35/2; Figure 3.1) revealed punctate patterns typical

Figure 3.4 Ultrastructure of YPBO-grown *E122*, *pex19-1*, and *pex19KO* cells. Cells were grown for 16 h in YPD and were then transferred to YPBO and grown for 8 h. Cells were then fixed in 1.5% $KMnO_4$ and processed for electron microscopy. Arrows indicate peroxisomes (in *E122* cells) or peroxisome-like structures (in *pex19-1* and *pex19KO* cells). Bar = 1 μm.

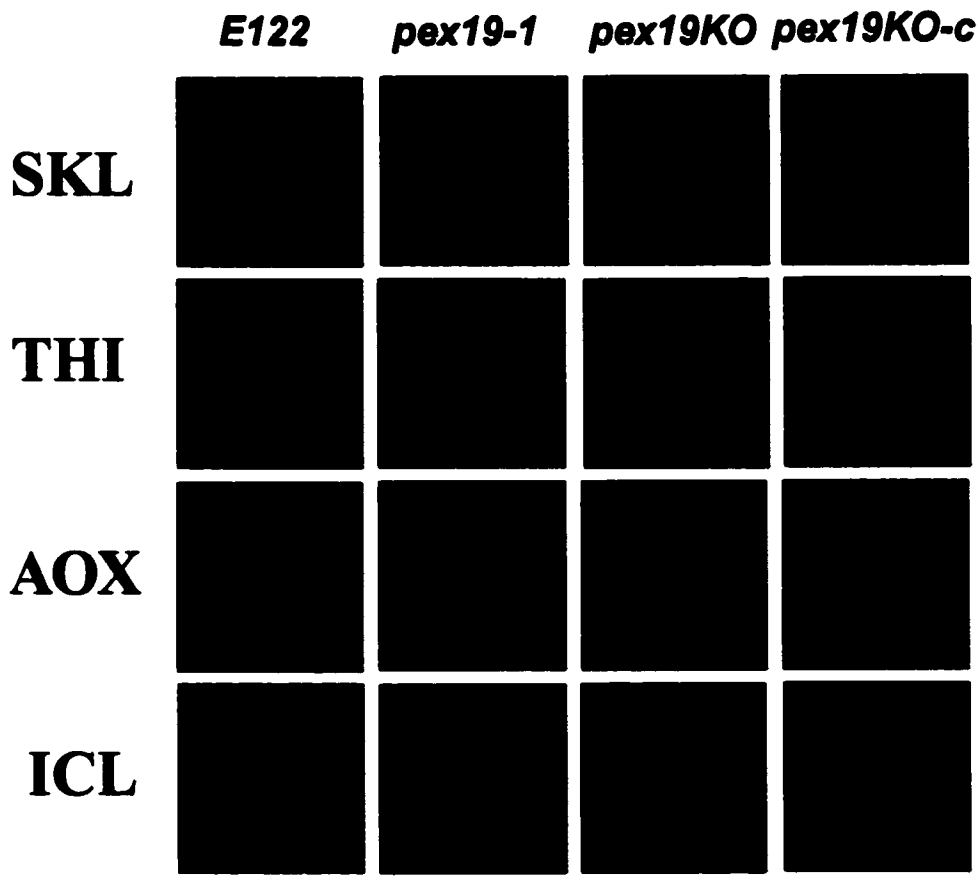


Figure 3.5 *pex19-1* and *pex19KO* cells mislocalize matrix proteins to the cytosol. *E122*, *pex19-1*, and *pex19KO* cells were grown for 16 h in YPD and were then transferred to YPBO and grown for 8 h. *pex19KO-c* cells (*pex19KO* cells transformed with 35/2) were grown for 16 h in YNBD and were then transferred to YNO and grown for 8 h. Cells were then fixed with 3.7% formaldehyde and the cell walls were digested with zymolyase 100-T. The resultant spheroplasts were permeabilized by immersion in chilled methanol followed by immersion in chilled acetone. Rabbit primary antibodies to SKL-containing proteins, AOX, and ICL, and guinea pig primary antibodies to THI were detected with rhodamine-conjugated secondary antibodies. THI, thiolase; AOX, acyl-CoA oxidase; ICL, isocitrate lyase.

of wild type cells (Figure 3.5), indicating the ability of this gene to rescue the import of these matrix proteins.

E122, *pex19-1*, and *pex19KO* cells were subjected to subcellular fractionation to yield first a postnuclear supernatant (PNS) fraction, which was then centrifuged at 20 000 X *g* to give pellet and supernatant fractions (20KgP and 20KgS, respectively; see section ***2.11.1***). The 20KgP fraction of wild-type cells is enriched for peroxisomes and mitochondria, whereas the 20KgS fraction is enriched for cytosol. As expected, the 20KgP of *E122* cells was found to be enriched in SKL-containing proteins (PTS1-containing proteins), thiolase (a PTS2-containing protein), AOX (a protein containing neither PTS1 nor PTS2), and ICL (a PTS1-containing protein). In agreement with the immunofluorescence data, the vast majority of these proteins in *pex19-1* and *pex19KO* cells were found in the cytosolic fraction (20KgS; Figure 3.6). However, trace amounts of these marker proteins were also found to be associated with the 20KgP.

The 20KgS and 20 KgP of *E122* and *pex19KO* cells were also assayed for the presence of Pex16p, a lumenal peroxisomal peripheral membrane protein (Figure 3.7). Pex16p seemed to be mislocalized to the 20KgS in *pex19KO* cells compared with its distribution primarily in the 20KgP in *E122* cells.

3.3 *pex19-1* and *pex19KO* Cells Contain Structures of Comparable Density to Wild-Type Peroxisomes but with Different Protein Components

The 20KgP fractions of each strain were subjected to isopycnic sucrose density gradient centrifugation (see section ***2.11.2***) to separate pelletable

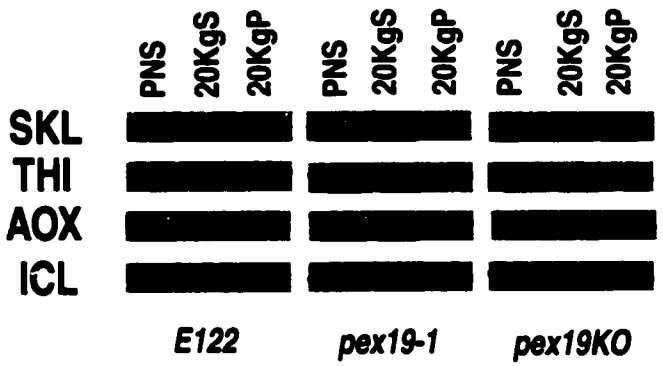


Figure 3.6 Most matrix proteins are mislocalized to the cytosol in *pex19-1* and *pex19KO* cells. *E122*, *pex19-1*, and *pex19KO* cells were grown for 16 h in YPD and then were transferred to YPBO and grown for 8 h. Cell walls were digested with zymolyase 100-T. The resultant spheroplasts were homogenized and centrifuged at 1000 X *g* to give a post-nuclear supernatant (PNS). The PNS was centrifuged at 20 000 X *g*, to yield pellet (20KgP) and supernatant (20KgS) fractions. Equal volumes of each fraction were analyzed on SDS-PAGE gels and transferred to nitrocellulose. Blots were probed with rabbit primary antibodies to SKL-containing proteins, AOX, and ICL, and guinea pig primary antibodies to THI. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies. THI, thiolase; AOX, acyl-CoA oxidase; ICL, isocitrate lyase.

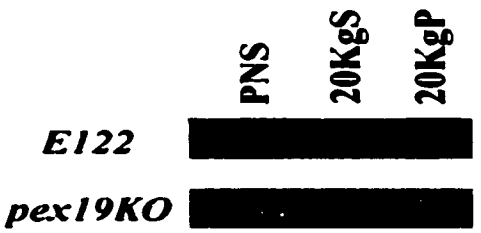


Figure 3.7 *pex19KO* cells mislocalize Pex16p to the cytosol. Subcellular fractions were isolated, analyzed by SDS-PAGE, and transferred to nitrocellulose as described in the legend to Figure 3.6. Blots were probed with guinea pig primary antibodies to Pex16p. Horseradish peroxidase-conjugated secondary antibodies were used to detect primary antibodies.

structures from one another. Blots of gradients of *E122* cells revealed the expected patterns of peroxisomal marker proteins (Figure 3.8A), with peaks in fractions 3 to 5. Blots of gradients of *pex19-1* and *pex19KO* cells revealed the presence of structures containing peroxisomal proteins of similar density to wild-type peroxisomes, as well as structures of lower density, in primarily fractions 10 to 14. Catalase assays yielded similar results (Figure 3.8B). Fumarase assays revealed that, as expected, mitochondria in all strains were isolated mostly in fractions 9 and 10.

Total protein profiles of wild-type peroxisomes and the peroxisome-like structures of *pex19KO* cells were obtained by pelleting the structures from fractions 4 of the respective sucrose gradients (see section *2.11.2.1*). The profile of total protein is shown as a silver stain of an SDS-PAGE gel in Figure 3.9A. Figure 3.9B compares the profiles of proteins that are extractable from these pellets with a high pH buffer (supernatant) and those that are not extractable (pellet). These profiles indicate differences in total protein content, and that these differences are mainly due to differences in matrix protein content, with the membrane protein profiles being similar.

3.4 Pex19p is Associated with Peroxisomes in *E122* Cells and Pex19p Expression Is Not Significantly Increased in Response to Growth in Oleic Acid-Containing Medium

E122 cells were grown for 9 h in oleic acid-containing medium. Aliquots were removed every hour, and total protein lysates were made. Proteins were

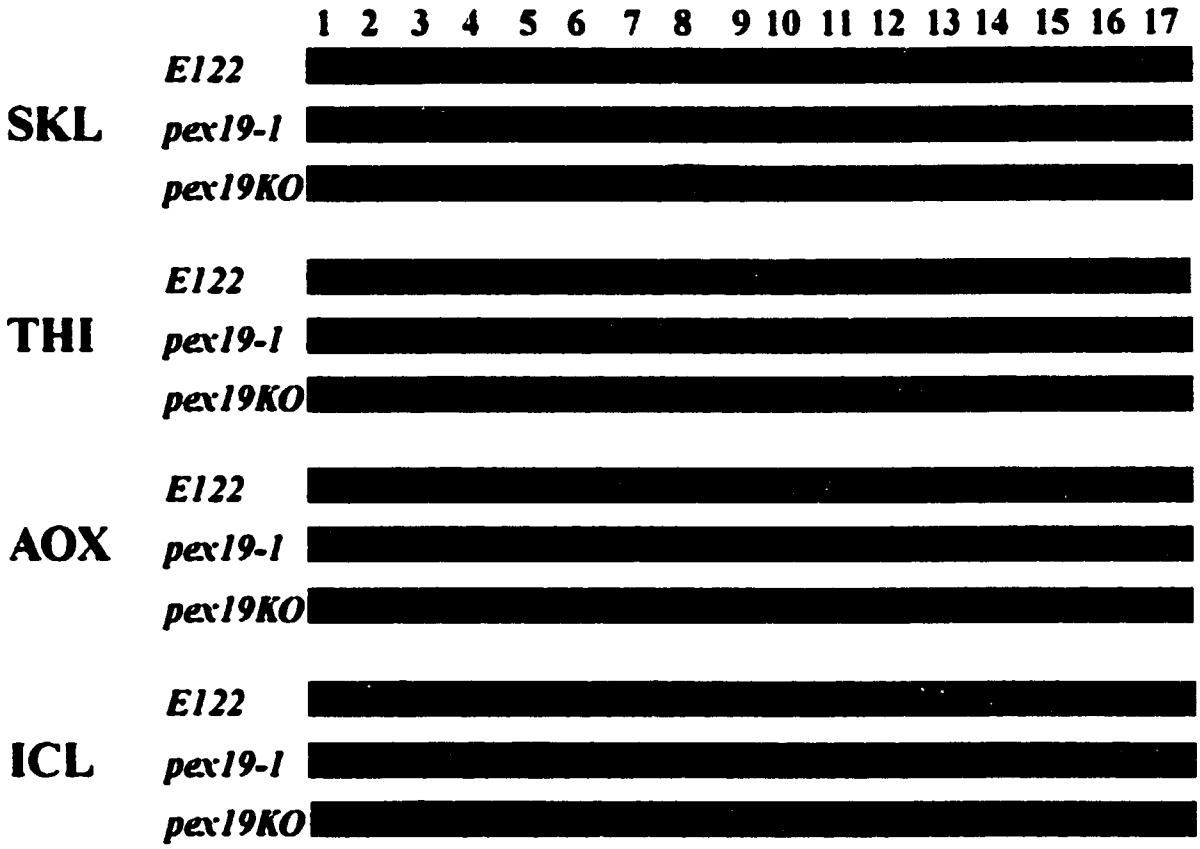
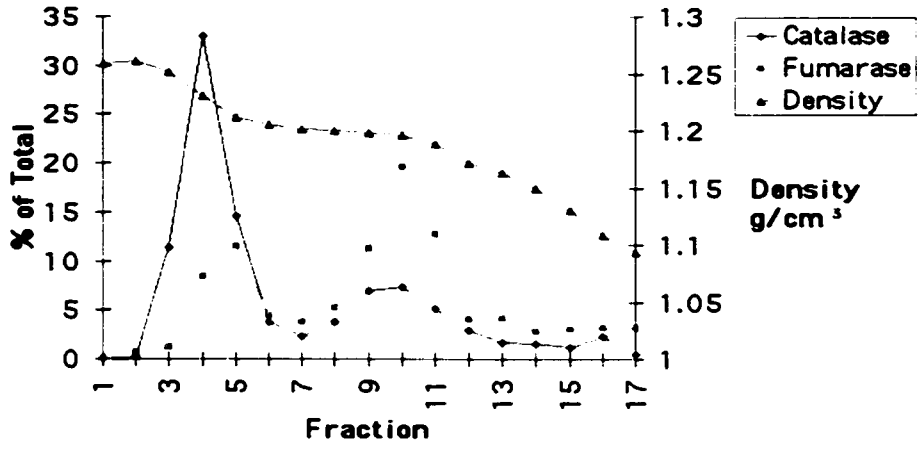


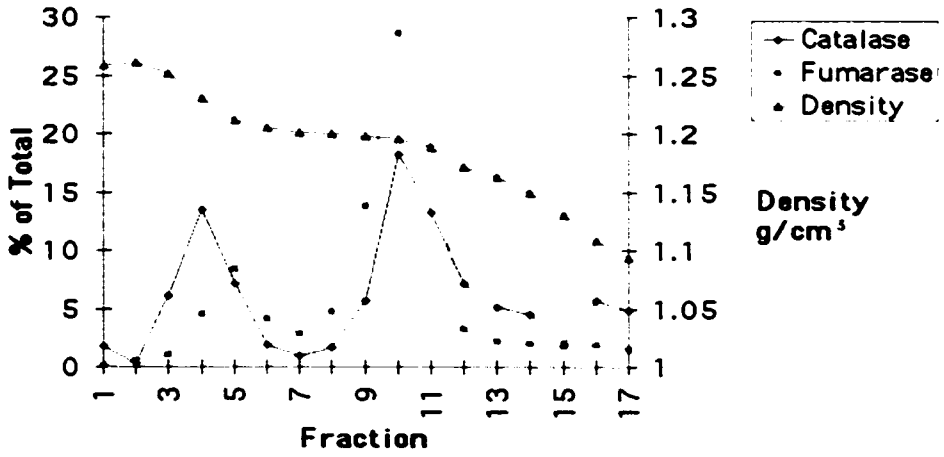
Figure 3.8 Peroxisomal matrix proteins can be detected in vesicular material of the same density as wild-type peroxisomes. The 20KgP fractions of the wild-type strain *E122*, the original mutant strain *pex19-1*, and the *PEX19* knock-out strain *pex19KO* were resuspended in buffer H (1 M sorbitol, 5 mM MES, pH 5.5, 1 mM KCl, 0.5 mM EDTA, 0.1% [v/v] ethanol) and centrifuged through a discontinuous sucrose gradient. Seventeen 2 mL fractions were collected from the bottom of the tube. (A) Equal volumes of each fraction were analyzed by SDS-PAGE and transferred to nitrocellulose. Blots were probed as in Figure 3.6. THI, thiolase; AOX, acyl-CoA oxidase; ICL, isocitrate lyase. (B) Fractions were assayed for catalase and fumarase activities (see section *2.4.5*). Results are the mean percent of activity from three independent experiments done in triplicate.

B

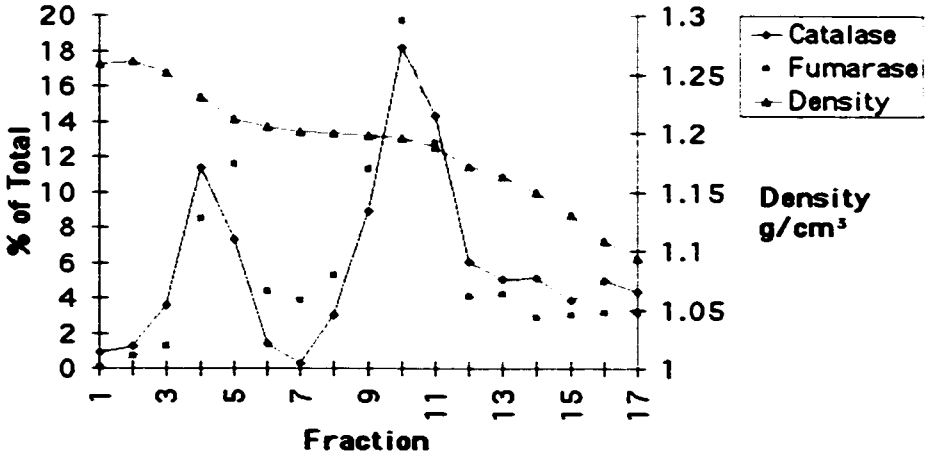
E122



pex19-1



pex19KO



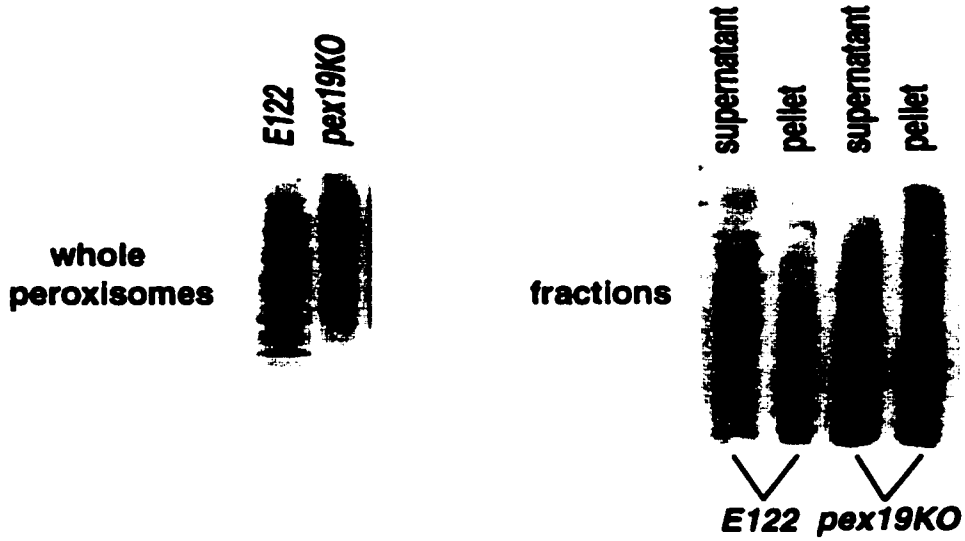


Figure 3.9 Protein profiles of fractions 4 of *E122* and *pex19KO* sucrose density gradients are slightly different. Fractions 4 of *E122* and *pex19KO* sucrose density gradients (the peak peroxisomal fraction; see Figure 3.8) were diluted with buffer H to halve the sucrose concentration. Peroxisomes or peroxisome-like structures were collected by centrifugation at 200 000 X *g* for 30 min at 4°C. (A) 5 μg of *E122* peroxisomes and 3 μg of *pex19KO* peroxisome-like structures were resuspended in SDS-PAGE sample buffer and analyzed on a 10% SDS-PAGE gel. The gel was stained with silver nitrate. (B) 10 μg of each 200Kg pellet was resuspended in ten volumes of 0.1 M $NaCO_3$ (pH 11), and the resuspension was incubated on ice for 1 h with occasional mixing. The membrane-associated material was collected by centrifugation through a 0.3 M sucrose cushion at 200 000 X *g*. The pellet was dissolved in SDS-PAGE sample buffer. The proteins in the supernatant were precipitated with TCA, washed with 80% acetone, and dissolved in SDS-PAGE sample buffer. The proteins were analyzed on a 10% SDS-PAGE gel and stained with silver nitrate.

separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with various antibodies to determine whether or not Pex19p levels increase when oleic acid is the only carbon source available (Figure 3.10A), as is the case with several peroxins (Erdmann and Blobel, 1995; Szilard *et al.*, 1995; Titorenko *et al.*, 1998; Brown *et al.*, 2000). The levels of thiolase and Pex2p both increased upon induction with oleic acid, as expected. The levels of glucose-6-phosphate dehydrogenase did not increase, also as expected, since this enzyme is not related to fatty acid metabolism or peroxisome biogenesis. The levels of Pex19p expression seemed to increase slightly over this time period.

Blots of subcellular fractions of *E122* cells were probed with anti-Pex19p antibodies (Figure 3.10B). These blots revealed that the majority of Pex19p associated with structures that pellet at 20 000 X *g* and that the remaining Pex19p associated with structures that pellet at 200 000 X *g*.

To determine whether or not some of these structures were peroxisomes, *E122* cells were doubly labelled with antibodies to thiolase and to Pex19p in immunofluorescence studies (Figure 3.10C). These studies revealed an almost exact colocalization of these two types of proteins, indicating that Pex19p is mostly associated with peroxisomes.

3.5 Farnesylation of Pex19p May Not Be Required for Pex19p Function

Pex19p has a farnesylation consensus sequence at its carboxyl-terminus (Figure 3.3). To determine whether or not this site is important to Pex19p

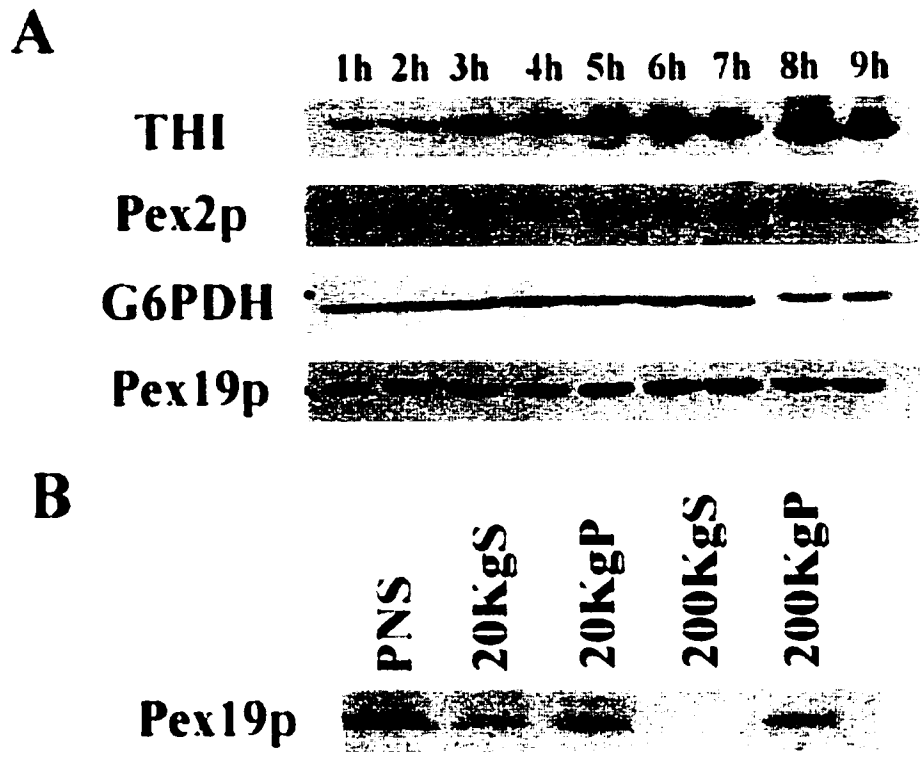
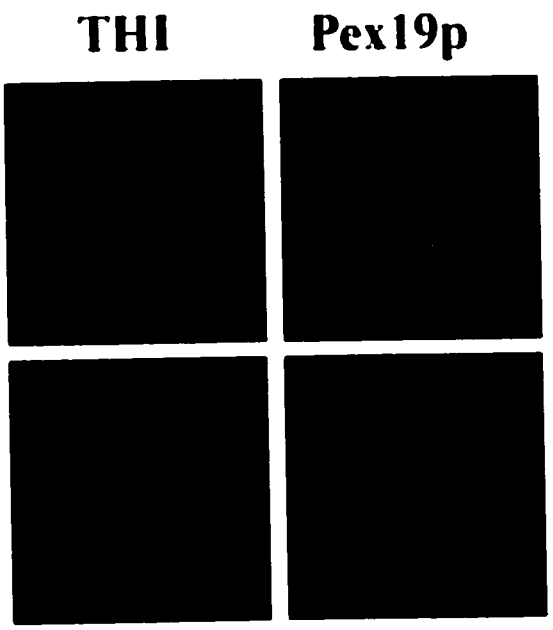


Figure 3.10 Pex19p expression is not significantly induced by growth in oleic acid-containing medium and Pex19p is localized to peroxisomes. (A) *E122* cells were grown in YPD for 16 h and then were transferred to YPBO. Cells were removed every hour from 0 h to 9 h and total cellular protein was recovered by lysis of cells with glass beads, followed by TCA precipitation. Equal amounts of protein were analyzed by SDS-PAGE and transferred to nitrocellulose. Blots were probed with antibodies to Pex2p, thiolase (THI), glucose-6-phosphate dehydrogenase (G6PDH), and Pex19p. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies. (B) Subcellular fractions of *E122* cells were collected, analyzed by SDS-PAGE, and transferred to nitrocellulose, as described in the legend to Figure 3.6, except that the 20KgS fraction was centrifuged at 200 000 X *g* to yield pellet (200KgP) and supernatant (200KgS) fractions. Blots were probed with guinea pig antibodies to Pex19p. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies. (C) *E122* cells were prepared for immunofluorescence as described in the legend to Figure 3.5. Cells were doubly labelled with guinea pig antibodies to thiolase (THI) and rabbit antibodies to Pex19p. Primary antibodies were detected with rhodamine-conjugated anti-guinea pig and fluorescein-conjugated anti-rabbit secondary antibodies.

C



function, various *PEX19* constructs were made that were designed to abolish the ability of Pex19p to be farnesylated. Constructs were made and cloned into the yeast expression vector pINA445 that coded for Pex19p variants that either lacked the 4 carboxyl-terminal amino acids (-CNQQ) or contained a cysteine→serine mutation at position 321 (C321S) (see section **2.12**). *pex19KO* cells were transformed with empty vector, the original smallest complementing fragment (35/2), and the engineered constructs. Growth on oleic acid-containing medium was restored to the three strains transformed with the original smallest complementing fragment and the two engineered constructs, but not the with empty vector (Figure 3.11A). However, immunoblotting equal amounts of total cellular protein from each of these strains indicated that Pex19p levels in the transformed strains were 5 to 10 times higher than in *E122* cells (Figure 3.11B). Overexpression of non-farnesylated protein has been shown to compensate for a lack of farnesylation of Pex19p in *S. cerevisae* (Götte *et al.*, 1998).

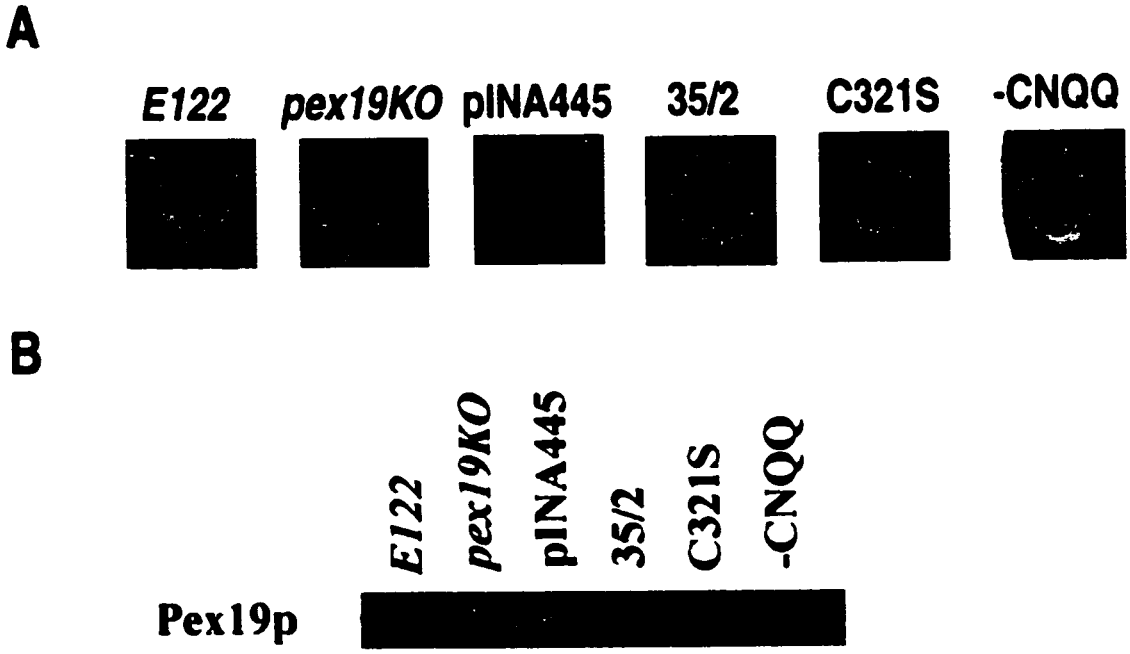


Figure 3.11. Pex19p variants mutated at the putative farnesylation site restore growth on oleic acid-containing medium to *pex19KO* cells. (A) Growth of wild-type and mutant strains of *Y. lipolytica* on oleic acid-containing medium. (B) *E122* and *pex19KO* cells were grown for 16 h in YPD and then were transferred to YPBO and grown for 8 h. *pex19KO* cells expressing variants of *PEX19* (see section **2.12**) were grown for 16 h in YNBD and then were transferred to YNO and grown for 8 h. Cells were lysed with glass beads, and protein was isolated by TCA precipitation. Equal amounts of protein were analyzed by SDS-PAGE and transferred to nitrocellulose. Blots were probed with guinea pig antibodies to Pex19p. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies. pINA445, empty vector; 35/2, original complementing fragment; C321S, *PEX19* expressing a Pex19p variant with a serine in place of the cysteine at position 321 (farnesylation incompetent); -CNQQ, *PEX19* expressing a Pex19p variant lacking the carboxyl-terminal 4 amino acids (farnesylation incompetent).

4.0 Discussion and Summary

4.1 *Yarrowia lipolytica PEX19* and Its Protein Product, Pex19p

The *PEX19* gene of *Y. lipolytica* encodes a protein of 324 amino acids and of a predicted molecular mass of 34 822 Da. Pex19p expression is not significantly increased when cells are shifted to growth on oleic acid-containing medium from glucose-containing medium, unlike other peroxins characterized to date (Erdmann and Blobel, 1995; Szilard *et al.*, 1995; Titorenko *et al.*, 1998; Brown *et al.*, 2000). Polyclonal antibodies raised against Pex19p recognized a single protein of approximately 48 kDa weight on Western blots of total protein extract from wild-type cells but not from *pex19-1* or *pex19KO* cells. This difference between the predicted and observed mobilities is also observed for Pex19p in other organisms (Götte *et al.*, 1998; Matsuzono *et al.*, 1999; Snyder *et al.*, 1999a).

Pex19p has been found to be a mainly cytosolic protein (Snyder *et al.*, 1999a) that has some association with the outer surface of the peroxisomal membrane (James *et al.*, 1994; Götte *et al.*, 1998). In contrast with these results, we have found that most of the Pex19p in *Y. lipolytica* cells is associated with material pelleted at 20 000 X *g* (20KgP), and the remainder is associated with material pelleted at 200 000 X *g*, which is predicted to consist of smaller vesicular elements, such as putative peroxisomal precursors (Titorenko *et al.*, 2000). Double labelling immunofluorescence studies have indicated that the majority of Pex19p colocalizes to punctate structures containing thiolase, which is found

exclusively in peroxisomes in wild-type cells. Together, these data support the hypothesis that the majority of Pex19p is associated with peroxisomes.

4.2 Farnesylation of Pex19p

All putative orthologues of Pex19p contain a farneslyation consensus sequence at their carboxyl-termini. Addition of a C_{15} farnesyl moiety to the cysteine located at the fourth position from the carboxyl-terminus occurs post-translationally. The first step involves addition of the farnesyl moiety. Cleavage of the 3 carboxyl-terminal amino acids is followed by the methylation of the now carboxyl-terminal cysteine. Farnesyl moieties are predicted to promote protein-membrane interactions through their hydrophobicity (Omer and Gibbs, 1994). Human, CHO, and *S. cerevisiae* putative Pex19p orthologues have all been demonstrated to be farnesylated (James *et al.*, 1994; Götte *et al.*, 1998; Matsuzono *et al.*, 1999). However, *P. pastoris* Pex19p is not farnesylated (Snyder *et al.*, 1999a). Because farnesylation is a post-translational modification, farnesylated proteins are often observed on Western blots as a doublet, one species running at a slightly reduced mobility to the other due cleavage of its carboxyl-terminal 3 amino acids during modification. Western blots of *Y. lipolytica* Pex19p alway revealed one species, which suggests that Pex19p is not farnesylated.

In *S. cerevisiae* farnesylation has been shown to be necessary for the proper functioning of Pex19p, although nonfarnesylated Pex19p constructs expressed at near wild-type levels were able only to partially rescue mutant

phenotypes. We find evidence that abolition of the farnesylation site of *Y. lipolytica* Pex19p seems to have no effect on the ability of the protein to carry out normal function. Two constructs of Pex19p, one lacking the 4 carboxyl-terminal amino acids, and one with a cysteine→serine mutation, both farnesylation incompetent, rescue the ole⁻ phenotype of *pex19KO* cells, as does the original smallest complementing fragment of *MUT35* (35/2). However, as we have not demonstratively shown that Pex19p is not farnesylated, this could mean that either *Y. lipolytica* Pex19p is not farnesylated and has found some other way to become associated with the peroxisomal membrane, or that it is normally farnesylated, but the ability of the nonfarnesylated Pex19p constructs to rescue the mutant phenotype is due to their overexpression in the system, as is the case for a massively overexpressed Pex19p construct in *S. cerevisiae* (Götte *et al.*, 1998). Further experiments are needed to clarify the situation.

4.3 *Yarrowia lipolytica pex19* Mutants Contain Structures Morphologically and Biochemically Similar to Peroxisomes

Y. lipolytica pex19 mutants mislocalize almost all peroxisomal matrix proteins to the cytosol, although some do remain associated with pelletable structures. *S. cerevisiae* and *P. pastoris pex19* mutants also show defects in the import of matrix proteins (Götte *et al.*, 1998; Snyder *et al.*, 1999a). However, electron micrographs of *Y. lipolytica pex19* mutant cells reveal the presence of single membrane-bound organelles with a homogeneous dense matrix that are approximately the same size and shape as wild-type peroxisomes. No such

structures are found in *S. cerevisiae pex19* mutants (Götte *et al.*, 1998), and only small vesicular and tubular structures are found in *P. pastoris pex19* mutants (Snyder *et al.*, 1999a; Hettema *et al.*, 2000).

The peroxisome-like structures of *Y. lipolytica* were isolated by sucrose density gradient centrifugation. Some were found to be of the same density as wild-type peroxisomes, and some were of a much lower density. Qualitatively, these isolated structures contain the normal complement of peroxisomal matrix proteins. Extraction experiments revealed similar, yet distinct, matrix and membrane protein profiles of these structures as compared to wild-type peroxisomes. Although under the electron microscope the peroxisome-like structures seemed to be present in numbers comparable to those of wild-type peroxisomes, total protein isolated at the wild-type density of sucrose gradients was much less in mutant cells than in wild-type cells. It seems, then, that *Y. lipolytica pex19* mutants are still able to form peroxisome-like structures, some of which are of a wild type density, although they contain a somewhat different complement of total protein, and some of which are of a density less than that of wild type. Subcellular fractionation experiments also reveal that the intraperoxisomal peripheral membrane protein Pex16p is mislocalized to the cytosol in *pex19* mutants.

4.4 A Role for Pex19p in Membrane Protein Stability

Y. lipolytica Pex19p appears to be important for the normal biogenesis of peroxisomes. Cells lacking Pex19p are still able to develop structures that are

morphologically similar to peroxisomes, but the inability of these cells to use oleic acid as a carbon source indicates that either peroxisomal integrity or the β-oxidation pathway of fatty acids is compromised in some way. The mislocalization of matrix proteins to the cytosol suggests that peroxisomal integrity is compromised. Some of the peroxisome-like structures in *pex19* mutants contain the normal complement of matrix and/or membrane proteins, and some reach wild-type densities, although the majority of structures are much less dense than wild-type peroxisomes and contain much less protein per volume. Some of each type of matrix protein studied is imported, although the vast majority of each is found to be localized to the cytosol. Also, membrane extraction experiments revealed similar yet distinct protein profiles of both matrix and membrane proteins between wild-type peroxisomes and mutant structures. It appears, then, that *pex19* mutants are almost able to form functional peroxisomes, but that a critical step in this process is impaired.

It has been suggested that Pex19p is a membrane protein receptor, recognizing membrane proteins after they are synthesized in the cytosol on polyribosomes and recruiting them to the peroxisomal membrane, most likely with the help of other proteins (Sacksteder *et al.*, 2000). Pex19p has been shown to interact with the domains of PMPs that also are required for targeting these proteins to the peroxisomal membrane. However, these domains are not minimal requirements for targeting, and therefore the actual amino acids required for targeting and for Pex19p binding may actually be different. In fact, it has been demonstrated for several PMPs in *P. pastoris* that the targeting sequences and the

Pex19p binding domains are separable (Snyder *et al.*, 2000). This has led to the proposal that Pex19p may perform a chaperone-like function (Snyder *et al.*, 2000). In this way, Pex19p would perform an important role in stabilizing the interaction between a putative membrane protein receptor and the PMP, or the interactions of various proteins at the translocation/insertion step at the peroxisomal membrane. Indeed, it has been shown in *S. cerevisiae* that Pex19p plays a role in PMP stability (Hettema *et al.*, 2000). A distribution that is partly cytosolic and partly peroxisomal has lent credence to this theory. However, it has also been shown that Pex19p does not interact with newly synthesized PMPs in the cytosol, and so is probably not involved in a receptor protein/PMP complex in the cytosol (Snyder *et al.*, 2000), but rather probably interacts with PMPs already present within the peroxisomal membrane.

Our studies of *Y. lipolytica* Pex19p are consistent with this hypothesis. It appears that in the absence of Pex19p, PMPs are still able to perform functions relating to the biogenesis and proliferation of peroxisomes. To perform such functions, PMPs must be inserted into the peroxisomal membrane. Therefore, Pex19p is probably not the PMP receptor molecule. The mislocalization of Pex16p (a peripheral membrane protein) and the dramatic reduction in Pex2p levels (an integral membrane protein) lends credence to the hypothesis that Pex19p is important to the stability of PMPs. It is interesting to note that it has recently been shown that Pex2p interacts with Pex19p (Snyder *et al.*, 2000). The primary location of Pex19p at the peroxisomal membrane does not support the proposition that it forms complexes with PMPs and other proteins in the cytosol,

but is consistent with a role involving binding of PMPs at the peroxisomal membrane. It has been postulated that Pex19p may play a role in co-ordinating certain functions of PMPs by stabilizing interactions between PMPs within the peroxisomal membrane (Snyder *et al.*, 2000). The fact that peroxisome-like structures are present, but that oleic acid cannot be utilized as a carbon source, indicates that these structures are only partially functional. The lack of complete function may be due to the lack of co-ordination of PMP activity by Pex19p. The decreased levels of matrix proteins present may be a secondary effect due to the improper functioning of the matrix protein translocation machinery, or of certain other PMPs needed for its proper functioning. Oleic acid could then not be utilized due to the lack of matrix proteins required for its metabolism.

4.5 Future Directions

Several questions about the nature and function of *Y. lipolytica* Pex19p remain to be answered. In several other species, many proteins have been found to interact with Pex19p. These interactions may or may not exist in *Y. lipolytica*. If the role of *Y. lipolytica* Pex19p is similar to its role in other organisms, one would expect several such interactions to become known through yeast two-hybrid screens and immunoprecipitation experiments.

It is also not known whether or not *Y. lipolytica* Pex19p is farnesylated. This question could be addressed by radiolabelling farnesyl moieties or farnesyl precursors and introducing them into yeast cells to determine if they are added to Pex19p. An *in vitro* approach may also be taken. Pex19 protein synthesized in

bacteria or in a *Y. lipolytica* strain lacking farnesyltransferase activity may be mixed with wild-type cytosol in the presence of radiolabelled farnesyl moieties.

On a more general scale, the role of Pex19p has yet to be determined. More experiments must be performed to characterize the nature of its interactions with PMPs. Where do these interactions take place within the cell? Do all these interactions serve the same purpose? Does Pex19p play a role in transport, docking, insertion, and/or stability of PMPs? Continued research with several different organisms will reveal the answers to these questions.

On an even more general scale, more peroxins and other peroxisomal proteins will continue to be discovered and their functions characterized in an effort to reveal the mechanism(s) behind peroxisome biogenesis and function. It is intriguing what the recent revival of the interest in the role of the ER will uncover. It is expected that continued research into peroxisome biogenesis will lead to greater understanding of PBDs and of the cell in general.

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