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*conference paper*

# Nutrition and Ultrastructure of the Mutants of Methylotrophic Yeasts Defective in Biogenesis and Degradation of Peroxisomes

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*Summary*

*In addition to methanol, methylotrophic yeasts are capable of utilizing multicarbon substrates. Some key reactions of methanol, ethanol and fatty acid metabolism are located in specific organelles, microbodies (depending on enzymatic content they are termed more specifically as peroxisomes or glyoxysomes) whereas utilization of other multicarbon substrates, e.g. glucose, does not require participation of peroxisomes. Depending on metabolic demands, peroxisomes can grow, propagate or degrade.*

*Numerous mutations leading to impairment of peroxisome biogenesis or altered regulation of this process are known. The mutations of methylotrophic yeasts Hansenula polymorpha and Pichia methanolica have been isolated which permit constitutive peroxisome biogenesis in the medium with glucose or ethanol and appear to be the regulatory mutations. Other mutations block biogenesis of peroxisomes even in the media with methanol and oleate. The methods for positive selection of the mutants of methylotrophic yeast Pichia pastoris defective in peroxisome biogenesis have been developed and the corresponding mutants have been characterized. The mutations leading to defect in peroxisome degradation of methylotrophic yeasts P. methanolica and P. pastoris have also been isolated. The corresponding mutations block peroxisome degradation after shift of methanol-grown cells to ethanol and/or glucose media due to the defects in vacuolar-dependent autophagy process.*

*Keywords:* methylotrophic yeasts, ultrastructure, peroxisomes, biogenesis, autophagy

## Introduction

Peroxisomes are ubiquitous for all eukaryotic cell organelles containing at least one $H_2O_2$-producing oxidase and catalase *(1,2)*. Peroxisomes of methylotrophic yeasts contain key enzymes of methanol oxidation and assimilation; mutations leading to the absence of peroxisomes block methanol metabolism *(3–5)*. Peroxisomes constitute only a small volume of the cells growing in glucose medium: methanol induces their growth and proliferation *(2,6)*. Under conditions favouring maximal induction of peroxisome proliferation, these organelles can occupy up to 80% of cell volume *(7)*. Under conditions, such as addition of glucose or ethanol to methanol-grown cells, massive peroxisome degradation occurs via a process termed autophagy *(8)*. In this process, peroxisomes are selectively engulfed by vacuoles and subsequently degraded by vacuolar proteinases.

Peroxisome biogenesis and degradation can be studied using mutants impaired in this process. Mutants defective in biogenesis of peroxisomes have been isolated in methylotrophic yeasts using methods of negative selection *(2)*. Such mutants can be selected by their inability to utilize methanol or methanol and oleate. Up to now, near 14 complementation groups of the mutants defective in peroxisome biogenesis (new designation: *pex* mutants) have been isolated and characterized in the methylotrophic yeasts *Pichia pastoris* and *Hansenula poly-*

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*morpha (9)*. The identified *PEX* genes mainly code integral membrane proteins which are involved in peroxisomal protein import as well as biogenesis, proliferation and inheritance of the organelle. Only few mutants of methylotrophic yeasts defective in selective peroxisome degradation (*pdg* mutants) are isolated and characterized *(10,11)*.

The primary aim of the present study was to develop the methods for isolation of the mutants of methylotrophic yeasts defective in peroxisome biogenesis *(per* or *pex)* including regulation of this process in peroxisome degradation (*pdg*) and to study ultrastructure and other properties of the mutants.

## Materials and Methods

*Organisms, growth conditions, mutant isolation.*The following strains were used as the wild-type strains and parental strains for mutant isolation: *Hansenula polymorpha* AS8 (*leu10*) and AS38 (*met6*) supplied by Dr. P. Sudbery (Sheffield University, UK); *Pichia pastoris* GS115 (*his4*) and GS190 (*arg4*) supplied by Dr. J.M. Cregg (Oregon Graduate Institute, Portland, USA); *Pichia methanolica* MH4 supplied by Dr. I.I. Tolstorukov (Institute of Genetics and Selections of Industrial Microorganisms, Moscow, Russia). The conditions of yeast cultivation and the composition of the media used were as previously described *(12,13)*. Concentration of carbon sources was 1%. The mutants were induced by UV light (10% cell survival). Mutant hybridization and random spore analysis were conducted as described before *(13–15)*.

Construction of the vectors which express green fluorescent protein (GFP) in *P. pastoris* under methanol-regulated promoters (O.V. Stasyk, J.M. Cregg, unpublished) was carried out using red-shifted GFP variant on plasmid pEGFP-C3 (Clontech Laboratories, Inc.). To direct GFP to the peroxisomes, peroxisomal targeting PTS1 signal AKL was introduced to the C-terminus of GFP gene using two synthetic oligonucleotides KR315 and KR316. The oligonucleotides were annealed and cloned in unique SAlI/HindIII sites of pEGFP-C3 yielding plasmid pOGP. GFP-PTS1 fragment was amplified by PCR with primers SO1 and SO2, both with EcoRI flanking site of *P. pastoris HIS4* based vectors pHIL-A1 and pK312 downstream of *P. pastoris AOX1* and *PER3* promoters creating plasmids pOAGP and pOPGP, respectively. These plasmids were linearized with SalI and integrated into genome of *P. pastoris* wild-type and *pdg his4* auxotrophic strains.

*Biochemical methods.* Enzyme activity levels were determined in cell-free extracts obtained by disruption method using glass beads in a planetary vibrator at 1000 rpm at 4 °C for 6 min. Cell debris were removed by centrifugation at 13 000 *g* at 4 °C for 20 min. The supernatant was used as the cell-free extract. Activity of alcohol oxidase was determined by the rate of hydrogen peroxide formation from methanol as recorded by the oxidation of o-dianizidine in the presence of horseradish peroxidase *(12)*. Catalase was determined spectrophotometrically *(16)*.

Differential centrifugation of gently disrupted spheroplasts was performed as described before *(5)*.

*Electron and fluorescent microscopy.* For electron microscopy whole washed cells were fixed in a 1.5% aqueous solution of $KMnO_4$ for 20 min at room temperature followed by post-fixation with 1% $OsO_4$ in cacodylate buffer for 90 min at 0 °C *(17)*. The samples were dehydrated by mixing with successive solutions containing increasing concentrations of ethanol with a final incubation in acetone. Samples were embedded in Epon 812. Ultrathin sections were sliced in an ultramicrotome UMTP-6 (Sumy, Ukraine) contrasted with lead citrate and examined with an electron microscope UEMV-100B (Sumy, Ukraine) at 75 kV. The final magnification of the micrographs was 10 000.

Electron microscopic cytochemistry was conducted on spheroplasts isolated by cell treatment with zymolyaze 5 000 *(18)*. Spheroplasts were fixed in 6% glutaraldehyde as described *(19)*. Localization of alcohol oxidase was determined by reaction with 3,3'-diaminobenzidine *(20)* and that of malate synthase by reaction with ferricyanide *(21)*.

Fluorescence microscopy was conducted with microscope »Laboval 2a-fl« (Carl Zeiss Jena, Germany). Cells expressing green fluorescence protein (GFP) were cultivated overnight on solid medium with methanol and were directly used for microscopy. Fluorescence staining of cells with fluorogenic dye dihydrotetramethyl rosamine (DTR) was conducted as follows: Fresh solution of dye in dimethyl sulfoxide (3 mg/mL) was diluted in 50 mM K-phosphate buffer, pH = 7.5. Suspension of methanol-grown cells for microscopy was prepared directly in dye solution.

## Results

### *Mutants constitutively producing peroxisomes and hybrid microbodies, glyoxyperoxisomes*

Glucose and ethanol strongly repress biosynthesis of peroxisomal enzymes and biogenesis of peroxisomes *(6)*. Some mutants of *H. polymorpha* and *P. methanolica* defective in catabolite repression of peroxisomal enzyme synthesis in the media with glucose or ethanol have been described *(12,13,22)*. Still, they were unable to produce peroxisomal enzymes in the medium without methanol. We have isolated the mutants of *H. polymorpha* resistant to 2-deoxyglucose (80–150 μg/mL) during growth in glucose medium. All the mutations appeared to be recessive and fell in the same gene designated as *GCR1*. It was found that these mutants were defective in glucose catabolite repression of both peroxisomal and cytosolic enzymes of methanol metabolism (data not shown). Peroxisomal enzymes alcohol oxidase and catalase were produced constitutively whereas synthesis of the cytosolic enzymes in the medium with glucose demanded methanol. Similarly to the peroxisomal enzymes, peroxisomes were formed constitutively in *gcr1* mutants during cultivation in glucose medium. They were numerous and large in size (Fig. 1) and few and small in number in glucose-grown wild-type cells (data not shown). This is the first example of massive peroxisome proliferation in the glucose-grown cells of methylotrophic yeast. Repression of methanol metabolizing enzymes and perox-

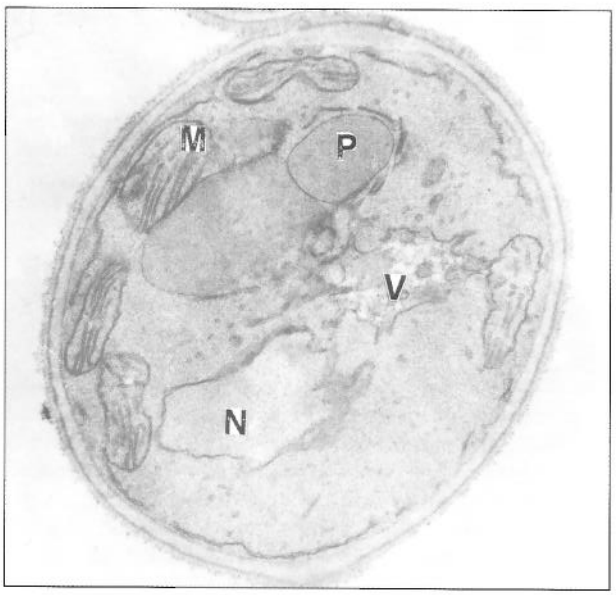


Fig. 1. Electron microscopic observation of the cells of *H. polymorpha gcr1* mutant from mid-log phase in the medium with glucose (1%). M, mitochondrion, N, nucleus, P, peroxisome, V, vacuole.

isome biogenesis in ethanol medium were not impaired in *gcr1* mutants.

Regulatory mutants of another methylotrophic yeast species, *P. methanolica*, are known which are defective in catabolite repression of peroxisomal enzyme synthesis in the media with glucose or, alternatively, ethanol *(12,23–29)*. The mutants *ecr1* and *adh1* defective in ethanol repression of peroxisomal enzymes alcohol oxidase and catalase are of especial interest. In the *ecr1* mutants, contrary to the wild-type strain, methanol repressed synthesis of glyoxysomal enzymes of two-carbon metabolism, isocitrate lyase and malate synthase, whereas *adh1* mutants simultaneously produced peroxisomal (alcohol oxidase, catalase) and glyoxysomal (isocitrate lyase, malate synthase) enzymes *(25–27)*. It was not known whether cells of the regulatory *adh1* mutant simultaneously contain two types of microbodies (peroxisomes, glyoxysomes) or, alternatively, hybrid microbodies, glyoxyperoxisomes. Electron microscopic cytochemical study of methanol-grown cells of the mutant *adh1* displayed positive reactions of the microbodies both for key peroxisomal enzyme alcohol oxidase and key glyoxysomal enzyme malate synthase (Fig. 2A,B, see also: *(19)*). Thus, the regulatory *adh1* mutation permits constitutive appearence of peroxisomal and glyoxysomal enzymes in the hybrid microbodies, glyoxyperoxisomes, during cell cultivation in the medium with ethanol.

### *The mutants defective in peroxisome biogenesis*

Previously the mutants defective in peroxisome biogenesis (*per, pas* or *pex* mutants) were isolated in methylotrophic yeasts only by laborious and time consuming methods of negative selection. The mutants were selected on the basis of their inability to utilize methanol in *H. polymorpha (3)* or simultaneously methanol and oleate in *P. pastoris (5)*. We have developed several new

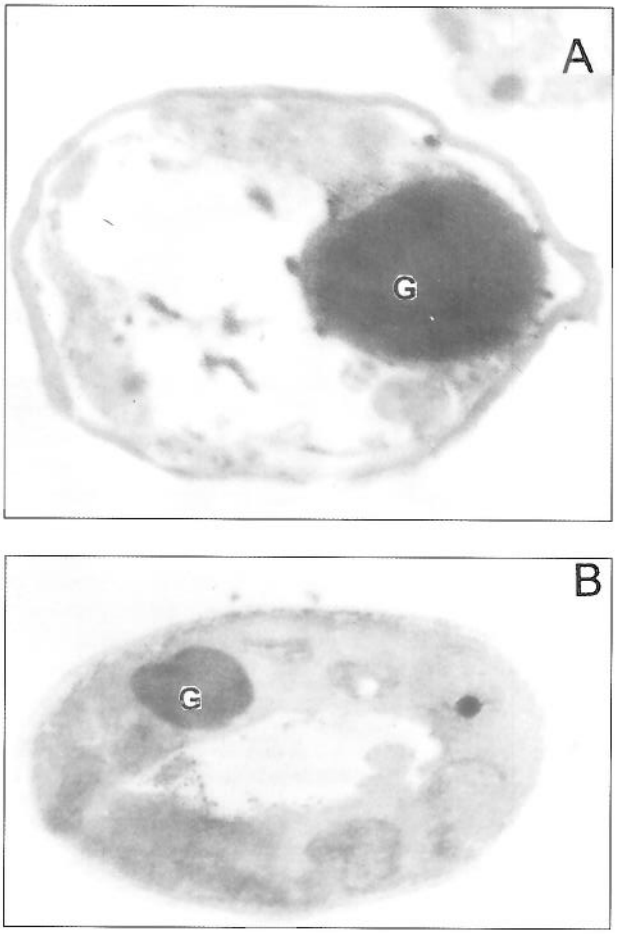


Fig. 2. Electron microscopic observation of spheroplast slices of *P. methanolica adh1* mutant grown in the medium with ethanol (1%). (A) Reaction for alcohol oxidase. (B) Reaction for malate synthase. G, glyoxyperoxisome.

approaches for positive selection of *P. pastoris* mutants defective in peroxisome biogenesis. The rationale of the schemes is based on the fact that the cells of the methanol-induced *per* mutants contain little or no activity for peroxisomal alcohol oxidase. Such low activity is thought to be a direct consequence of the absence of peroxisomes in *per* mutants. One scheme is based on the fact that alcohol oxidase has a rather broad substrate specificity and can oxidize allyl alcohol to very toxic aldehyde, acrolein, which kills the cells. Mutants possessing low alcohol oxidase due to defects in peroxisome biogenesis would produce little of acrolein in the medium with allyl alcohol and non-repressible carbon source (0.2% sorbitol) and thus would survive. Another scheme is based on our observation that *per* mutants are more resistant to high concentrations of methanol (12–14%) than the wild-type strains apparently because they do not produce as much of toxic alcohol oxidase products, formaldehyde and hydrogen peroxide. As the modifcation of this scheme, *fld* mutant of *P. pastoris* defective in formaldehyde dehydrogenase was used as the parental strain for selection. Defect of this enzyme leads to elevated susceptibility of the cells to methanol as formaldehyde, produced in alcohol oxidase reaction, cannot be efficiently

Table 1. New methods for positive selection of peroxisome defective *(per)* mutants of *Pichia pastoris*. Mth$^-$, unable to utilize methanol; Mth$^-$, Ole$^-$, unable to utilize both methanol and oleate.

| Selective agent | Concentration | Parental strain | Resistant mutants tested | Mth$^-$ | Mth$^-$, Ole$^-$ | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total | Lost (reverted, sterile etc) | Analyzed | Frequency of Mth$^-$, Ole$^-$ | New groups of *per* mutants |
| Allyl alcohol | 0.5 mM | GS190 | 145 | 11 | 2 | 0 | 2 | 1.4% | not found |
| Methanol | 10–13% | GS190 | 250 | 42 | 20 | 12 | 8 | 3.2% | 9 |
| | | GS115 | 90 | 30 | 12 | 8 | 4 | 4.4% | 9 |
| | 2% | *fld* | 1890 | 0 | 69 | 22 | 47 | 2.5% | 9 |
| | 10–13% | | 210 | 0 | 107 | 15 | 92 | 43.8% | 9,10,11,12,13 |

detoxified in *fld* mutant *(30)*. Thus, this selection scheme included wild-type strain or *fld* mutant as the parental strain, methanol (12–14%) as the selective agent and sorbitol (0.2%) as alternative non-repressive carbon source. Resistant to allyl alcohol or high concentration of methanol mutants were analyzed for growth on methanol and/or oleate as sole carbon source. The methanol and oleate non-utilizing mutants were further used for genetic and biochemical analysis. Using developed methods, more than 150 *per* mutants have been isolated (Table 1). All the mutants appeared to be recessive and fell into 15 complementation groups (Table 2). In some cases, determination of the intracellular localization of selected peroxisomal enzymes in the presumable *per* mutants was performed on homogenized spheroplasts prepared from methanol and oleate induced cells (Table 3). In the wild-type cells a large portion of peroxisomal catalase was found in the pellet fraction whereas in the mutant *per1* (used as a control strain) and S16 (presumable *per12*) catalase was found primarily in the supernatant, indicating mislocalization of the enzyme to cytosole. Methanol and oleate induced cells of peroxisome defective mutants did not possess visible peroxisomes (data not shown).

Table 2. Distribution of methanol and oleate non-utilizable (presumable *per*) mutants of *Pichia pastoris* into complementation groups

| Complementation group | Number of mutants | Parental strains for selection |
|---|---|---|
| *per1* | 20 | GS190, *fld* |
| *per2* | 9 | GS190, *fld* |
| *per3* | 10 | *fld* |
| *per4* | 4 | *fld* |
| *per5* | 9 | *fld* |
| *per6* | 4 | GS190, *fld* |
| *per7* | 3 | *fld* |
| *per8* | 16 | GS190, GS115, *fld* |
| *pas2* | 13 | GS190, *fld* |
| *pas6* | 8 | GS190, *fld* |
| *per9** | 17 | GS190, *fld* |
| *per10** | 2 | *fld* |
| *per11** | 3 | *fld* |
| *per12** | 2 | *fld* |
| *per13** | 1 | *fld* |

* – new complementation groups

## *The mutants defective in peroxisome degradation*

Study of peroxisome degradation was conducted on two model systems: *P. methanolica* and *P. pastoris*. *P. methanolica* is the best studied species of methylotrophic yeasts using the methods of classic genetics *(13)*. Formerly, a large collection of ethanol non-utilizing mutants of this species was isolated. The monogenic mutations leading to deficiency of two specific enzymes of ethanol utilization, acetyl-CoA synthetase (*acs1, acs2, acs3*) and isocitrate lyase (*icl1*), have been shown to result in impairment of peroxisomal enzyme (alcohol oxidase, catalase) by degradative inactivation in the medium with ethanol *(31)*. Since irreversible catabolite inactivation of peroxisomal enzymes is due to the autophagic degradation, it follows that the above mentioned mutants are defective in this process.

It was found that the shifting of washed methanol-grown cells of the wild-type strain to ethanol medium

Table 3. Activity and location of peroximal alcohol oxidase and catalase in the methanol- and oleate-induced cells of *Pichia pastoris* wild-type strain (GS115) and two peroxisomal mutants *(per1, per12)*. Specific activity in post-20 000 organelle supernatant (S) and pellets (P) differential centrifugation of homogenized protoplasts. Enzyme activities are expressed in U/mg of protein.

Methanol-induced cells

| Enzyme | wild type | | | *per1* | | | *per12* | | |
|---|---|---|---|---|---|---|---|---|---|
| | S | P | P/S ratio | S | P | P/S ratio | S | P | P/S ratio |
| Catalase | 300 | 270 | 0.9 | 300 | 25 | 0.08 | 407 | 75 | 0.18 |
| Alcohol oxidase | 0.83 | 0.97 | 1.2 | 0.02 | 0.005 | 0.25 | 0.07 | 0.02 | 0.29 |

Oleate-induced cells

| Enzyme | wild type | | | *per1* | | | *per12* | | |
|---|---|---|---|---|---|---|---|---|---|
| | S | P | P/S ratio | S | P | P/S ratio | S | P | P/S ratio |
| Catalase | 772 | 1160 | 1.5 | 360 | 21 | 0.06 | 330 | 24 | 0.07 |
| Alcohol oxidase | 0.01 | 0.05 | 5.0 | 0.02 | 0.002 | 0.1 | 0.02 | 0.03 | 1.5 |

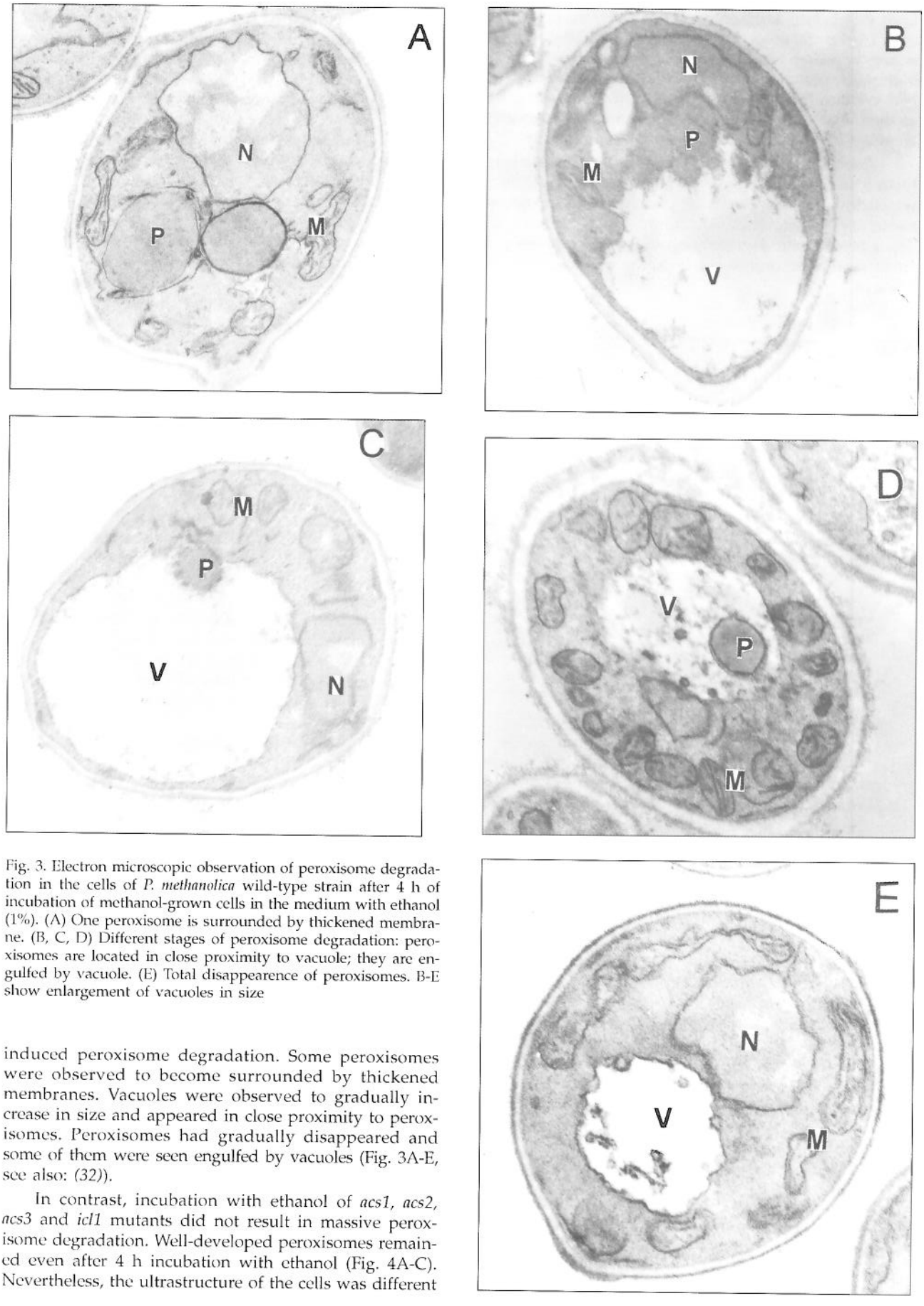


Fig. 3. Electron microscopic observation of peroxisome degradation in the cells of *P. methanolica* wild-type strain after 4 h of incubation of methanol-grown cells in the medium with ethanol (1%). (A) One peroxisome is surrounded by thickened membrane. (B, C, D) Different stages of peroxisome degradation: peroxisomes are located in close proximity to vacuole; they are engulfed by vacuole. (E) Total disappearence of peroxisomes. B-E show enlargement of vacuoles in size

induced peroxisome degradation. Some peroxisomes were observed to become surrounded by thickened membranes. Vacuoles were observed to gradually increase in size and appeared in close proximity to peroxisomes. Peroxisomes had gradually disappeared and some of them were seen engulfed by vacuoles (Fig. 3A-E, see also: *(32)*).

In contrast, incubation with ethanol of *acs1, acs2, acs3* and *icl1* mutants did not result in massive peroxisome degradation. Well-developed peroxisomes remained even after 4 h incubation with ethanol (Fig. 4A-C). Nevertheless, the ultrastructure of the cells was different

from that before addition of ethanol. The fractional volume of the vacuoles had increased and some peroxisomes appeared to be surrounded with thickened membranes. Occasionally, tight association of vacuoles and peroxisomes was observed. However, peroxisomes remained intact and morphologically normal. Peroxisomes were not observed inside vacuoles of the mutants.

We hypothesized that *acs1-3* and *icl1* mutants are defective in the conversion of ethanol to true effector which initiates the events of peroxisome degradation, namely, glyoxylic acid *(31,32)*. Obviously, glucose induces peroxisome degradation using a quite distinct effector as this process is not impaired in *acs1-3* and *icl1* mutants.

In another yeast model *P. pastoris* we have developed an original method for screening of peroxisome degradation defective (*pdg*) mutants based on a direct colony assay for activity of the peroxisomal alcohol oxidase which is known to be proteolytically degraded together with other constituents of peroxisomes after addition of glucose or ethanol to methanol-grown cells. After mutagenesis, methanol-grown colonies were replica plated onto media supplemented with 2% glucose or 2% ethanol, incubated for 8–10 h and after that alcohol oxidase activity was examined by overlaying the colonies with reaction mixture for enzyme assay and digitonin (1 mg/mL) as permeabilizing agent. Isolated mutants were subsequently analyzed in liquid cultures for *pdg* phenotype and used for genetic analysis (dominance test and complementation analysis). One mutation appeared to be dominant and the others were recessive. Genetic analysis of 10 mutants revealed six different genes (*PDG1-PDG6)*. The mutants were characterized by a strong defect in the rate of peroxisomal enzyme inactivation and were unable to degrade peroxisomes after the shift of methanol-grown cells to glucose or ethanol-containing media (data not shown). The isolated mutants will be used for isolation and characterization of the corresponding genes and their products.

As a convenient tool for further investigation of peroxisome biogenesis and autophagy, the *P. pastoris* vectors have been constructed that direct the expression of GFP fused to a type 1 peroxisomal targeting signal PTS1 (O.V. Stasyk and J.M. Cregg, unpublished). Methanol-grown wild-type cells showed strong fluorescence of peroxisomes (Fig. 5A) whereas glucose-grown cells did not fluoresce. In methanol-grown cells of *pdg1* mutant fluorescence was more diffuse than in wild-type cells (Fig. 5B),

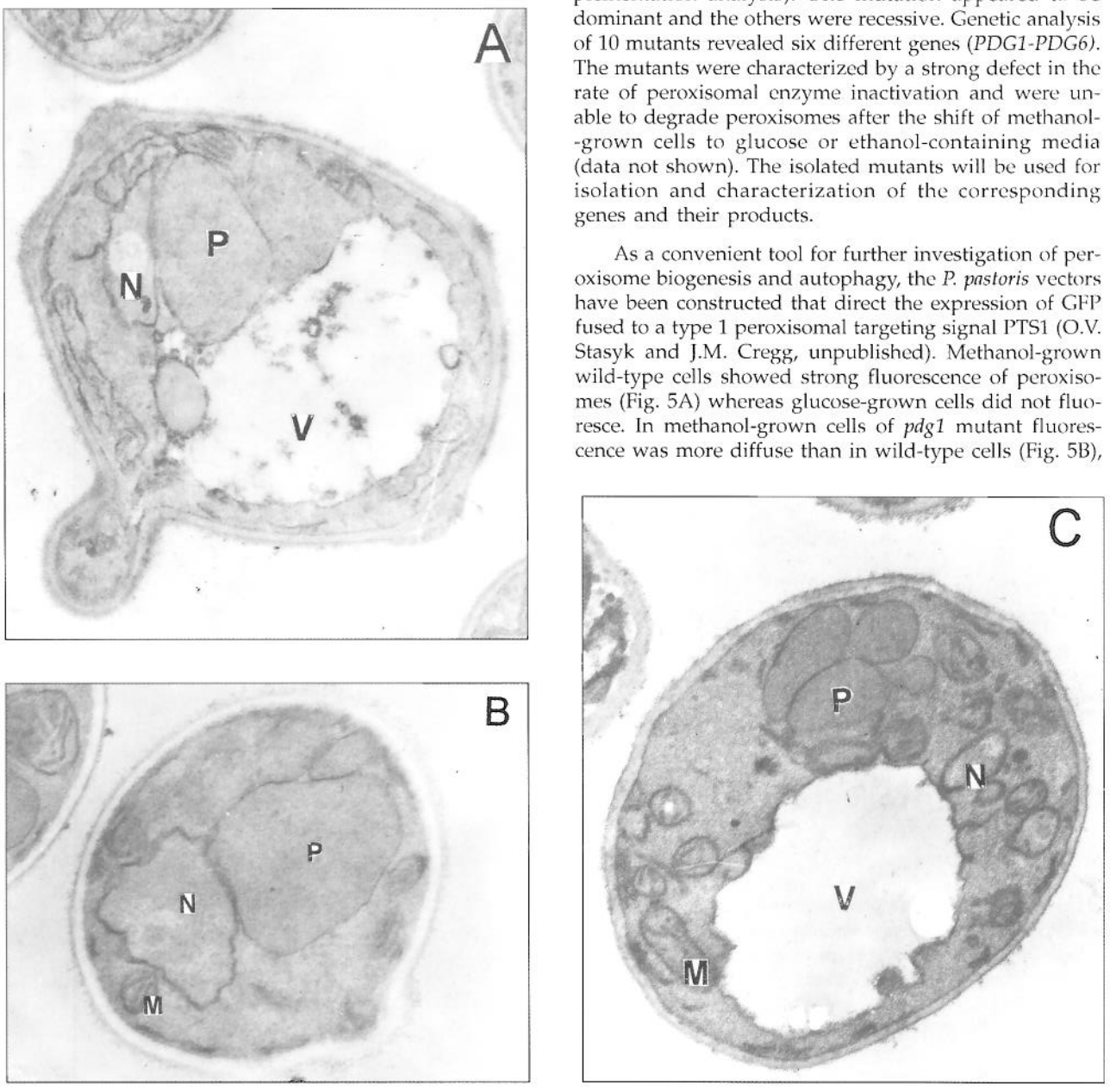


Fig. 4. Electron microscopic observation of cellular ultrastructure of the (A) *acs2*, (B) *acs3* and (C) *icl1* mutants after 4 h of methanol-grown cells incubation with ethanol (1%). (A, C) Peroxisome clusters are located near vacuole enlarged in size. (B) Peroxisome is surrounded by thickened membrane

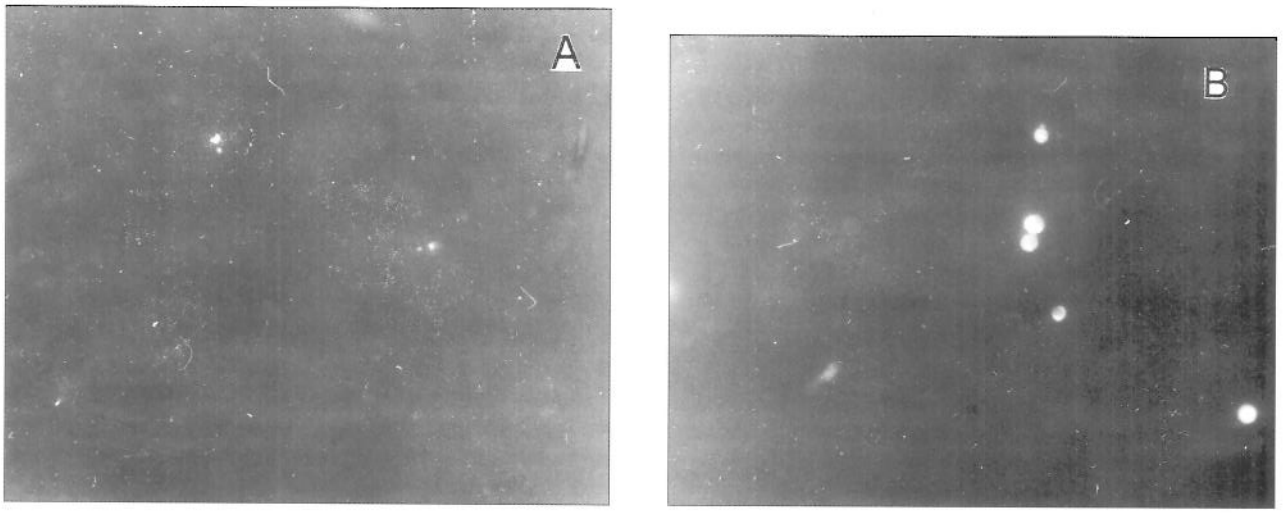


Fig. 5. Fluorescence of the GFP-PTS1 expressing methanol-grown cells of *P. pastoris* wild-type strain (A) and *pdg1* mutant (B)

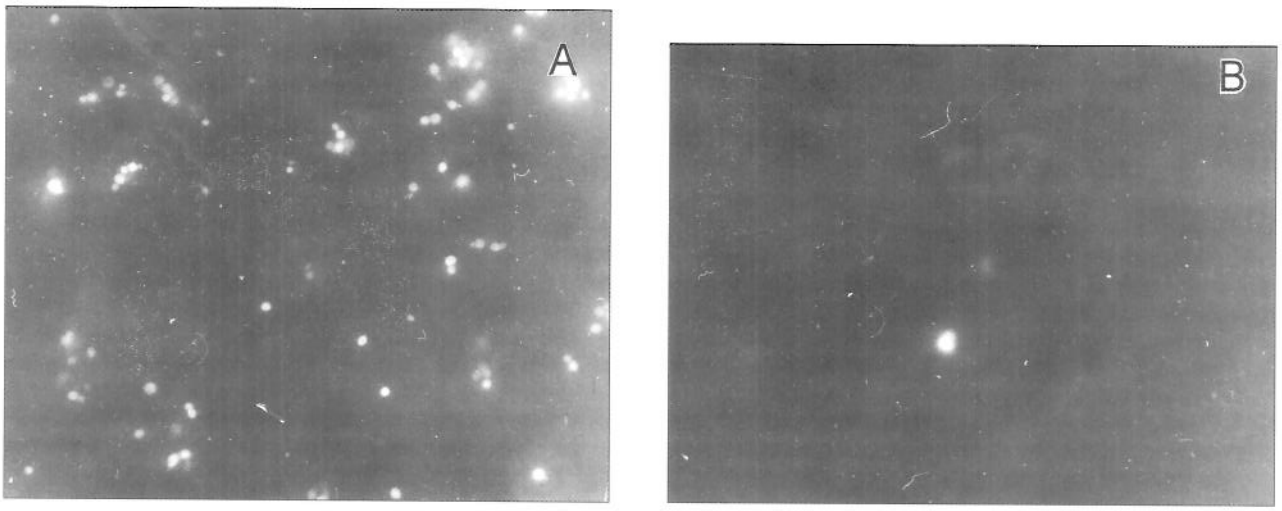


Fig. 6. Fluorescence of the methanol-grown *P. pastoris* (A) and *H. polymorpha* (B) wild-type strains in the medium with fluorogenic dye DTR

apparently, due to partial defect in localization of PTS1 peroxisomal proteins.

It was found that the methanol-grown wild-type cells of *P. pastoris* and *H. polymorpha* (Fig. 6) fluoresce also after their incubation on the microscope slides with fluorogenic dye, DTR. It is known that DTR is shifted to fluorescent compound after peroxidase oxidation *(33)*. Glucose-grown cells did not fluoresce even after addition of methanol to the DTR solution. Methanol-induced cells of *per* mutants were also unable to fluoresce (data not shown). Thus, use of fluorogenic dyes can be of interest for rapid identification of *per* mutants.

*Acknowledgements*

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# Ishrana i stanična ultrastruktura mutanata metilotrofnih kvasaca nesposobnih za biogenezu i degradaciju peroksisoma


### *Sažetak*

*Metilotrofni kvasci, osim metanola, sposobni su koristiti kao supstrate i druge spojeve s više ugljika. Neke ključne reakcije metabolizma metanola, etanola i masnih kiselina provode se u specifičnim organelama (ovisno o sadržaju enzima nazivaju se peroksisomi ili glioksisomi). Kada se koriste drugi supstrati s više ugljika (npr. glukoza), peroksisomi u tome ne sudjeluju. Ovisno o metaboličkim potrebama, peroksisomi mogu rasti, razmnožavati se ili degradirati.*

*Poznate su mnoge mutacije koje dovode do pogoršanja biogeneze peroksisoma ili do promjene regulacije tog procesa. U radu su izolirane mutacije kvasaca Hansenula polymorpha i Pichia methanolica koje dopuštaju biogenezu peroksisoma u podlozi s glukozom ili etanolom te izgleda da su regulacijske mutacije. Druge mutacije zaustavljaju biogenezu peroksisoma čak i u podlogama s metanolom ili oleatom. Razrađen je postupak za pozitivnu selekciju mutanata kvasca Pichia pastoris nesposobnog za biogenezu peroksisoma, a pritom su okarakterizirani odgovarajući mutanti. Također su izolirani mutanti u kojima je došlo do defekta u degradaciji peroksisoma metilotrofnih kvasaca P. methanolica i P. pastoris. Odgovarajuće mutacije sprječavaju degradaciju peroksisoma nakon prijenosa stanica koje su rasle na metanolu u podlogu s etanolom i/ili glukozom, zbog pogreške u vakuolarno ovisnom procesu autofagije.*