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Isolation and Characterization of Mutants Impaired in the Selective Degradation of Peroxisomes in the Yeast *Hansenula polymorpha*

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We have isolated a collection of peroxisome degradation-deficient (Pdd$^-$) mutants of the yeast *Hansenula polymorpha* which are impaired in the selective autophagy of alcohol oxidase-containing peroxisomes. Two genes, designated *PDD1* and *PDD2*, have been identified by complementation and linkage analyses. In both mutant strains, the glucose-induced proteolytic turnover of peroxisomes is fully prevented. The *pdd1* and *pdd2* mutant phenotypes were caused by recessive monogenic mutations. Mutations mapped in the *PDD1* gene appeared to affect the initial step of peroxisome degradation, namely, sequestration of the organelle to be degraded by membrane multilayers. Thus, Pdd1p may be involved in the initial signalling events which determine which peroxisome will be degraded. The product of the *PDD2* gene appeared to be essential for mediating the second step in selective peroxisome degradation, namely, fusion and subsequent uptake of the sequestered organelles into the vacuole. *pdd1* and *pdd2* mutations showed genetic interactions which suggested that the corresponding gene products may physically or functionally interact with each other.

In yeasts, peroxisomes develop in response to metabolic needs (23, 28). In *Hansenula polymorpha*, the organelles are strongly induced by methanol and, to a lesser extent, by a number of unusual nitrogen sources, including primary amines. The opposite may also occur: under certain conditions, peroxisomes may be actively degraded after a shift of cells to a new environment in which the organelles become redundant for growth (32).

At present, two conditions which lead to a rapid turnover of alcohol oxidase-containing peroxisomes in *H. polymorpha* are known, namely, a shift of cells from methylotrophic to nonmethylotrophic conditions (29, 32) and irreversible inactivation of the organellar function (21, 27).

The degradation process appeared to be energy dependent (29). The peroxisomes are degraded individually by a highly selective autophagic process, and the degradation process appeared to be identical under either of the above conditions. As an initial step, organelles to be degraded were sequestered from the cytosol by a number of membranous layers, closely surrounding the organelle, which most probably were derived from the endoplasmic reticulum; subsequently, hydrolytic enzymes required for the degradation of the microbody contents were supplied by fusion of part of the vacuole with the sequestered organelle (21, 27, 29, 32).

The degradation of peroxisomes in *H. polymorpha* is a rapid process; generally, the total turnover of a single organelle is accomplished within 20 to 45 min (29). However, not all organelles of the peroxisomal population present in one cell are affected: in particular, the large mature peroxisomes are rapidly degraded. These and other results (26) strongly suggest that the organelles destined for degradation are specifically tagged. Recently, we have obtained indirect evidence that the signals, initiating peroxisome turnover, are not directed against the matrix proteins but instead to the peroxisomal membrane. This was deduced from the fact that peroxisomal matrix enzymes present in the cytosol of *H. polymorpha per* mutants are not degraded after exposure of the cells to excess glucose conditions. Remarkably and in line with the above view, in *H. polymorpha pim* mutants (36), only the few small but intact peroxisomes are susceptible to proteolytic degradation whereas the cytosolic portion of the matrix proteins remained unaffected (26).

In order to get more insight into the molecular mechanisms of selective peroxisome degradation in *H. polymorpha*, we set out to generate mutants affected in this process (*pdd* mutants). This study describes the screening procedure for the isolation of such mutants and characterization of representatives of the first two *pdd* complementation groups which were obtained.

MATERIALS AND METHODS

Strains and growth conditions. Repeatedly backcrossed isogenic strains of *H. polymorpha* NCYC495 carrying the auxotrophic markers *ade11-1*, *met6-1*, *leu1-1*, and *ura3-1* (11) were used in all experiments.

Solid media used for mating and complementation analyses, sporulation of the hybrids, and random spore analysis have been described elsewhere (11). *H. polymorpha* strains were grown at indicated temperatures in YPD (1% [wt/vol] yeast extract, 2% [wt/vol] Bacto Peptone, 1% [wt/vol] glucose) or in mineral medium MM (31) supplemented with 0.5% (wt/vol) carbon source. For induction of peroxisome degradation, glucose (0.5%, wt/vol) or ethanol (0.5%, vol/vol) were added to batch cultures of *H. polymorpha* in the mid-exponential growth phase on methanol. Amino acids, adenine, and uracil were added to a final concentration of 50 μg/ml. For plates, 2% Bacto Agar (Difco) was used.

Mutant isolation. Chemical mutagenesis by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was performed as previously described (7). The mutagenized culture was spread on YPD plates at dilutions resulting in 100 to 300 colonies per plate. Peroxisome degradation-deficient mutants were screened by a plate colony assay, on the basis of visualization of alcohol oxidase activity, using 2,2'-azinodi-[3-ethylbenzthiazolim sulfonate (ABTS) as a substrate (34). For this purpose, the plates were incubated at 37°C for 2 days and the colonies formed were replica plated onto MM agar plates supplemented with 0.5% methanol (vol/vol). After 24 h of incubation at 37°C, colonies were replica plated onto two sets of MM agar plates, supplemented with either 1% (wt/vol) glucose or 1% (vol/vol) ethanol. After 6 h of incubation at 37°C, the plates were overlaid with 9 ml of the alcohol oxidase activity assay mixture, containing 50 mM Tris-HCl buffer (pH 8.0), 0.3% (wt/vol) agar, digitonin (1 mg/ml), ABTS (0.5 mg/ml), peroxidase (6 IU/ml), and 1% (vol/vol) methanol. The overlying assay mixture was allowed to set, and the plates were incubated at 37°C for up to 1 h. Colonies that stained purple, indicating the presence of high alcohol oxidase activity, were selected.

Electron microscopy. Whole cells were fixed in 1.5% (wt/vol) $KMnO_4$ for 20

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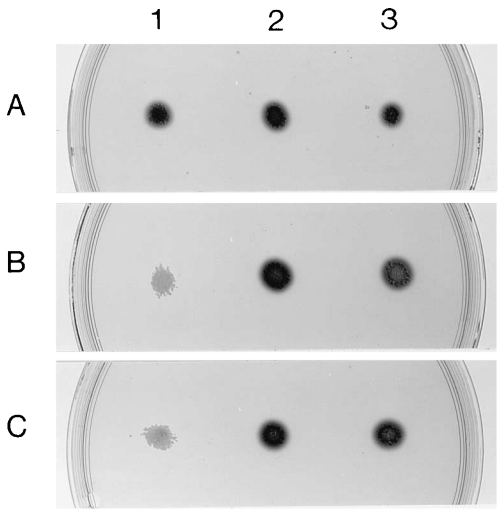

FIG. 1. Plate test assay for the identification of peroxisome degradation-deficient mutants on the basis of visualization of alcohol oxidase activity. Colonies of wild-type (lane 1), *pdd1-201* (lane 2), and *pdd2-1* (lane 3) cells were replica plated from plates containing methanol (A) to plates containing glucose (B) or ethanol (C), incubated for 6 h at 37°C, and subsequently overlaid with top agar containing the permeabilizing agent (digitonin) and the reaction mixture to demonstrate the oxidase activity. Staining of colonies 30 min after overlaying of top agar is represented.

min at room temperature. After dehydration in a graded ethanol series, the samples were embedded in Epon 812; ultrathin sections were cut with a diamond knife and examined with a Philips EM 300.

For immunochemistry, intact cells were fixed in either 3% (vol/vol) glutaraldehyde or 3% (vol/vol) formaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 90 min at 0°C, dehydrated in a graded ethanol series, and embedded in Unicryl. Immunolabelling was performed on ultrathin sections with specific antibodies against alcohol oxidase and gold-conjugated goat anti-rabbit antibodies by the method of Slot and Geuze (22).

Biochemical methods. Whole-cell lysates for immunoblotting analysis were prepared as described previously (12). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels (18). Transfer of proteins onto nitrocellulose after SDS-PAGE with a semidry electroblotter was done by the method of Kyhse-Andersen (17). For immunodecoration, polyclonal antibodies against alcohol oxidase, catalase, dihydroxyacetone synthase (36), or the tripeptide-SKL-COOH were used (13). Crude extracts for enzyme assays were prepared as described previously (36). Protein concentrations were determined as described previously (4), using bovine serum albumin as the standard. Enzyme activities of alcohol oxidase (34), catalase (19), and dihydroxyacetone synthase (5) were determined by established methods.

RESULTS

Mutant isolation. The mutant isolation procedure is based on earlier observations that the addition of glucose or ethanol to methanol-grown cells of *H. polymorpha* results in a rapid loss of peroxisomes, which is caused by selective degradation of the organelles (29, 32). Therefore, we developed a direct colony color assay on the basis of oxidation of ABTS (34; for details, see Materials and Methods), which allowed visualization of the activity of the major peroxisomal matrix protein, alcohol oxidase. Control experiments on methanol-grown wild-type cells indicated that the colonies treated this way turned purple within 15 to 30 min. The use of this method to monitor the decrease of alcohol oxidase activity as a result of selective inactivation was determined as follows. YPD-grown wild-type strain cells were spread onto YPD plates; colonies, developed after incubation for 2 days at 37°C, were replica plated onto MM agar plates with methanol. After 24 h of incubation at 37°C, colonies were replica plated onto MM agar plates containing either glucose or ethanol. After various incubation times (at 37°C) ranging from 1 to 24 h, the plates were overlaid with top agar containing alcohol oxidase activity assay mixture and incubated at 37°C. The results indicated that the staining intensity of the replicated colonies was directly related to the previous incubation time on plates containing glucose or ethanol. In methanol-grown wild-type control cells, staining of the colonies occurred within 15 to 30 min; this period increased to approximately 60 min for plates incubated for 1 to 3 h on plates containing glucose or ethanol. Six hours after replication on plates containing glucose or ethanol, appropriate staining of the colonies took 4 to 5 h after being overlaid with the top agar. Therefore, for the selection of peroxisome degradation-deficient mutants, we have chosen 6 h as an appropriate incubation time for the replicated colonies on plates containing glucose or ethanol. At prolonged incubation times (10, 16, or 24 h), significant growth of cells was observed, and thus, the dilution effect became dominant and hampered proper analysis.

By the above method, 43 clones were selected from about 32,000 independent mutagenized cells, still displaying a high alcohol oxidase activity 6 h after replication from plates containing methanol on plates containing glucose or ethanol. They were all intensely stained, like methanol-grown wild-type controls, within 15 to 30 min after the addition of the alcohol oxidase assay mixture onto the plates (Fig. 1). The presumed effect, namely, a failure of these mutants to lose alcohol oxidase activity, was confirmed by the analysis of crude extracts prepared from cells from batch cultures shifted from methanol to glucose (data not shown). Subsequently, seven mutants were selected and subjected to further detailed analysis.

Genetic analysis. The seven selected mutants were backcrossed three times. After confirmation of their mutant phenotype by Western blot (immunoblot) analysis using antibodies against alcohol oxidase, the backcrossed derivatives were crossed with the wild-type strain. All resulting diploids displayed the wild-type phenotype. Random spore analysis indicated that the mutant phenotypes segregated in a 1:1 pattern in all strains. Therefore, the defects in these mutants were caused by recessive monogenic mutations. After complementation analysis, the mutants were assigned to two complementation groups designated as *PDD1* and *PDD2* (peroxisome degradation-deficient mutants). Random spore analysis showed that these complementation groups corresponded to two nonlinked genes (Table 1); four mutations were mapped into the *PDD1*

TABLE 1. Data from random spore analysis of the hybrid *pdd1-201/PDD1 PDD2/pdd2-1*

Genotype	Inactivation[a]	Growth on methanol[b] at the following temp:			No. of segregants[c]
		30°C	37°C	43°C	
PDD1 PDD2	+	+	+	+	172
pdd1 PDD2	−	+	+/−	−	158
PDD1 pdd2	−	+	+	+/−	164
pdd1 pdd2	−	+/−	−	−	161

[a] Glucose- and ethanol-triggered inactivation of alcohol oxidase was scored as rapid inactivation (+) or no inactivation (−).

[b] Growth of segregants on MM medium supplemented with methanol at the indicated temperature was scored as follows: +, normal growth; +/−, slow growth; −, no growth.

[c] The number of meiotic segregants possessing the indicated genotype was scored by plate assay of alcohol oxidase activity developed for selection of Pdd$^-$ mutants and by complementation tests of *pdd* segregants with appropriate tester strains bearing *pdd* mutations of parental strains. According to the data of chi-square analysis which was carried out on the ratio of parental versus recombinant combinations of *PDD* alleles, the *PDD1* and *PDD2* genes showed no linkage to each other.

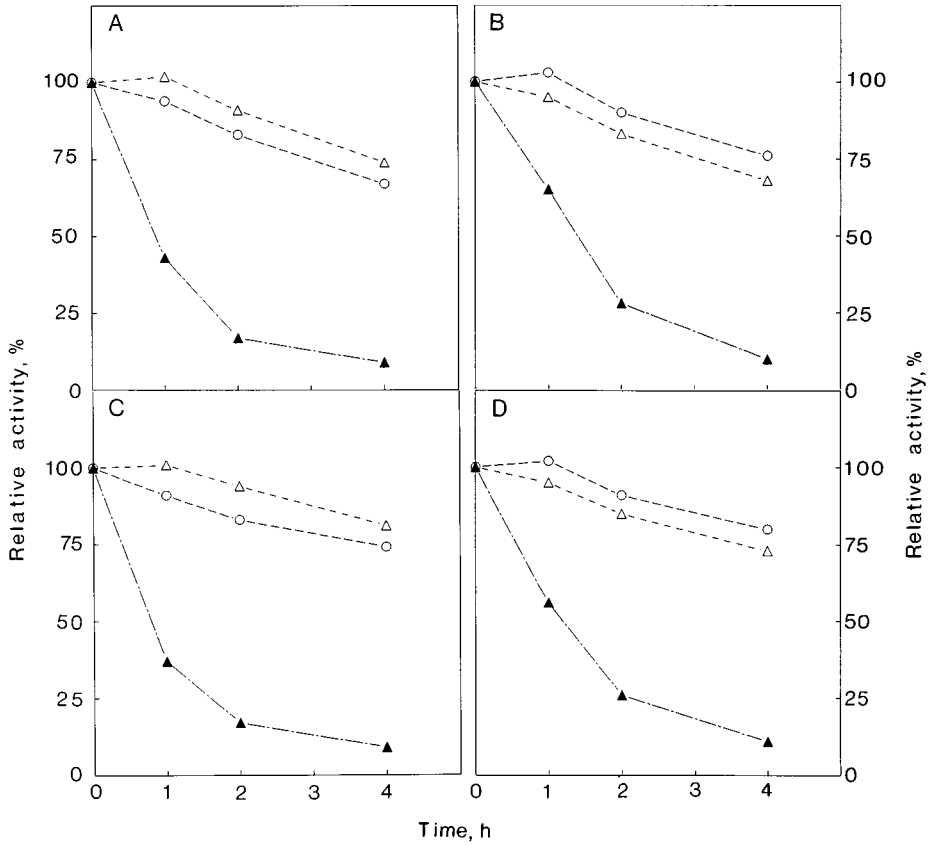

FIG. 2. Kinetics of changes in specific activities of various peroxisomal enzymes, determined in crude extracts from wild type (▲), *pdd1-201* (△), or *pdd2-1* (○) cells after the addition of glucose (A and C) or ethanol (B and D) to batch cultures in the exponential growth phase on methanol at 37°C. The decrease in specific activities is expressed as a percentage of the initial value. (A and B) alcohol oxidase activity; (C and D) dihydroxyacetone synthase activity.

gene, and three were mapped to the *PDD2* gene.

Biochemical and ultrastructural analyses. Two mutants, namely, *pdd1-201* and *pdd2-1*, were subjected to detailed biochemical and ultrastructural characterization. As expected, the addition of glucose or ethanol to cultures of wild-type cells in the exponential growth phase on methanol resulted in a rapid inactivation of alcohol oxidase and dihydroxyacetone synthase activity (Fig. 2), which was caused by degradation of the respective proteins (Fig. 3). Also, catalase (Fig. 3) and proteins recognized by specific antibodies against the C-terminal tripeptide SKL (13) were degraded (not shown). In contrast, mutants *pdd1-201* and *pdd2-1* were impaired in both glucose- and ethanol-induced inactivation and degradation of the above peroxisomal proteins (Fig. 2 and 3). Cytosolic proteins like formaldehyde dehydrogenase, alcohol dehydrogenase, or fructose-1,6-bisphosphatase were not inactivated or degraded in both wild-type and mutant cells (data not shown). After exposure of methanol-grown wild-type cells to excess glucose or ethanol conditions, rapid degradation of peroxisomes took place, initiated by sequestration of the organelles to be degraded from the cytosol and subsequent fusion of this compartment with the vacuole in which the organellar contents are degraded (three typical stages are shown in Fig. 4A to C); for a detailed morphological analysis of microbody turnover in *H. polymorpha*, see references 29 and 32. In both *pdd* mutants, peroxisome degradation was not observed; instead, the organelles remained virtually unaffected during prolonged incubation of cells in the presence of glucose or ethanol (Fig. 5). Mutant *pdd1-201* appeared to be impaired in the first morpho-

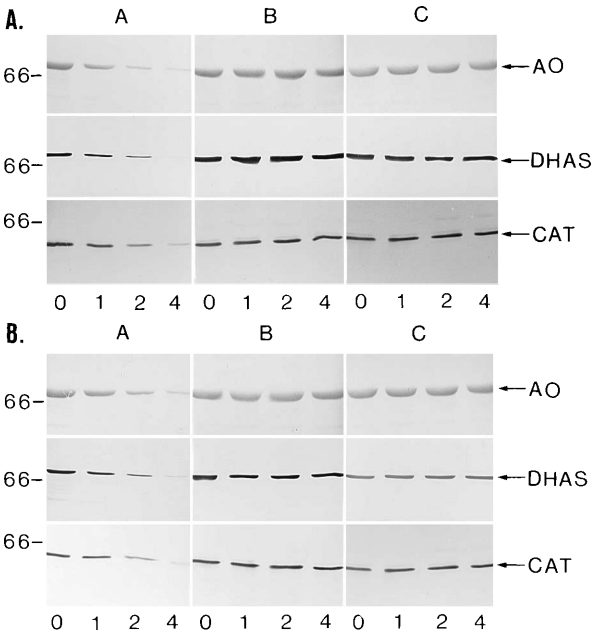

FIG. 3. Changes in protein levels of alcohol oxidase (AO), catalase (CAT), and dihydroxyacetone synthase (DHAS) in whole-cell lysates from wild-type (A), *pdd1-201* (B), and *pdd2-1* (C) cells, as determined by Western blotting, in the initial hours (indicated under the gels) after a shift of cells from methanol to glucose (A) or ethanol (B) as described in the legend to Fig. 2. Equal amounts of protein were loaded on the lanes.

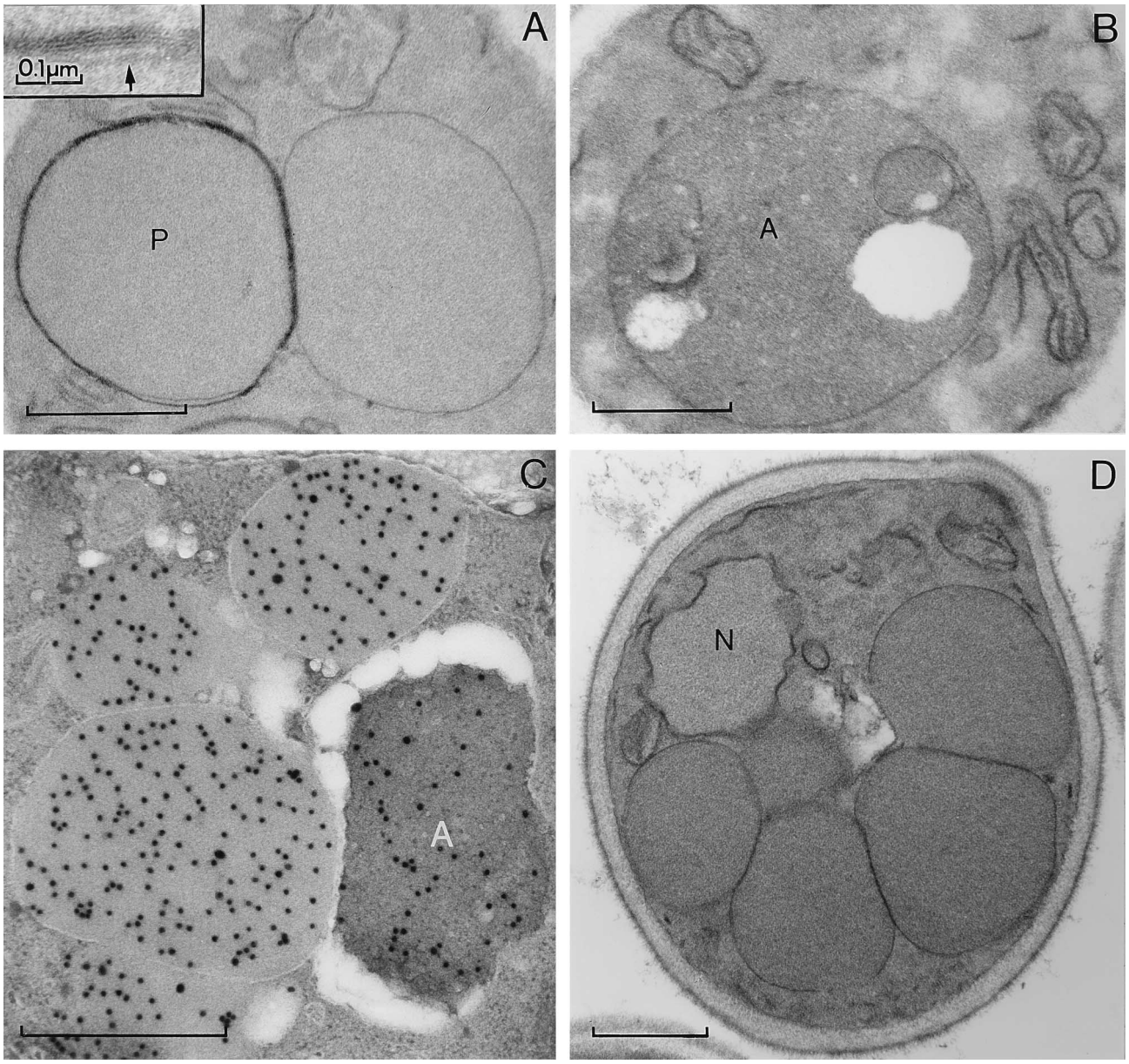

FIG. 4. Electron micrographs of three characteristic stages of peroxisome turnover in wild-type *H. polymorpha* after a shift of cells from methanol to glucose. The micrographs are of $KMnO_4$-fixed cells, unless otherwise stated. (A) Sequestration of a peroxisome (P) after 20 min of incubation on glucose (inset: high magnification to show the number of membranous layers; the arrow indicates the peroxisomal membrane). (B) Typical morphological appearance of a degrading peroxisome. (C) Immunocytochemically, these organelles are recognized by specific antibodies against alcohol oxidase. Cells were fixed with glutaraldehyde and uranyl acetate. (D) Overall morphology of a wild-type cell from the late exponential growth phase on methanol; several large peroxisomes are evident. Abbreviations: A, autophagic vacuole: M, mitochondrion; N, nucleus: P, peroxisome; V, vacuole. The unlabelled bars represent 0.5 μm.

logically distinguishable step of peroxisome degradation, namely, sequestration of the organelles from the cytosol by multilayer membranes; also, after prolonged incubation times (4 to 6 h), these membranes were never observed (Fig. 5A). In the *pdd2-1* mutant, sequestration took place but subsequent fusion with the vacuole was never observed (Fig. 5B and D). Immunocytochemically, we could not detect any alcohol oxidase protein in vacuoles, which was an obvious phenomenon in wild-type control cells (compare Fig. 5E and F with Fig. 4C). In both *pdd* mutants, alcohol oxidase protein remained confined to the peroxisomal matrix (Fig. 5E and F). This result confirmed the morphological observations, that peroxisome degradation was indeed fully inhibited in the two *pdd* mutants after a shift of cells from methanol to either glucose or ethanol.

Growth properties and gene interaction. Unexpectedly, both *pdd* mutations appeared to affect growth on methanol, but not on glucose, at elevated temperatures (43°C; Fig. 6). At 30°C, *pdd1-201* and *pdd2-1* mutants grew normally on methanol at wild-type rates both on plates and in liquid cultures. Random spore analysis showed that the Pdd^- and the temperature-sensitive (Ts^-) phenotypes cosegregated in the meiotic progeny of diploids, heterozygous for either the *pdd1* or *pdd2* mutation (approximately 600 colonies were tested for each mutant); this result indicated that the Ts^- effect was not

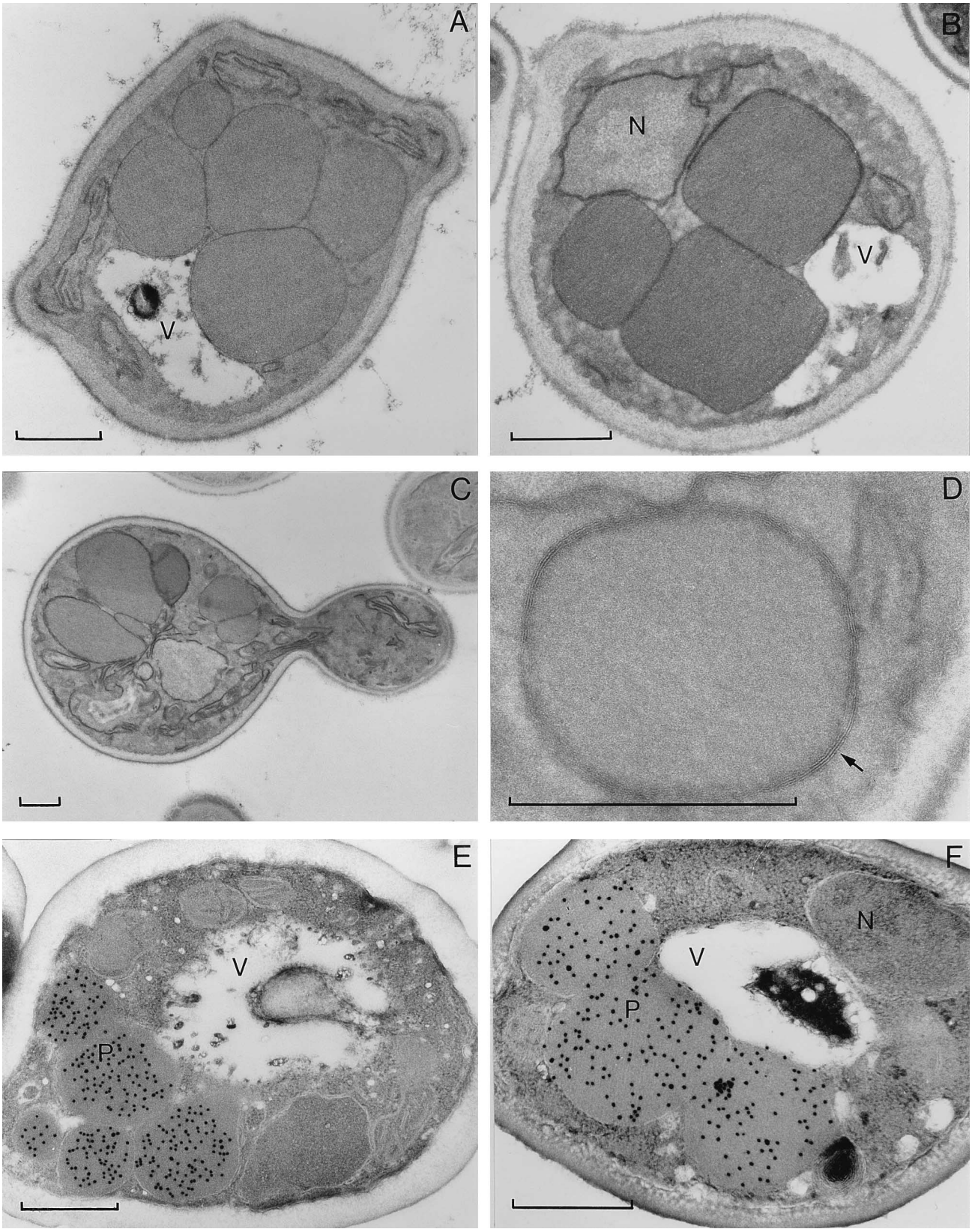

FIG. 5. Electron micrographs of *pdd1* and *pdd2* mutants from methanol-grown cells incubated in the presence of glucose or ethanol. The micrographs are of $KMnO_4$-fixed cells, unless otherwise stated. (A) *pdd1* mutant incubated for 2 h in ethanol; (B) *pdd2* mutant incubated for 2 h in glucose. The cells in panels A and B contain normal peroxisomes and vacuoles but lack autophagosomes. In panel B, one peroxisome is partially enveloped by membranous layers; a high magnification of a fully enveloped peroxisome (arrow) is depicted in panel D. (C) *pdd2* cell incubated for 4 h on ethanol. Partial enveloping of peroxisomes in the mother cell is evident: the developing bud typically lacks peroxisomes. Immunocytochemically, alcohol oxidase protein was confined to peroxisomes; with antibodies against alcohol oxidase, vacuolar labelling was never observed. (E) *pdd1* mutant incubated for 2 h in glucose; (F) *pdd2* mutant incubated for 2 h in ethanol; (E and F, cells fixed in glutaraldehyde and uranylacetate). Abbreviations: V, vacuole; N, nucleus; P, peroxisome. Bars, 0.5 μm.

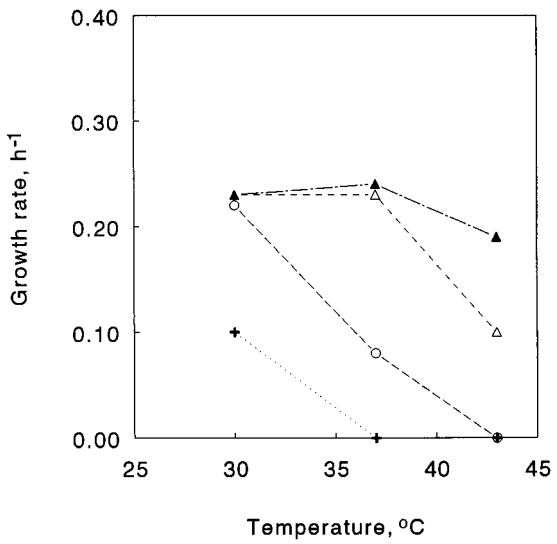

FIG. 6. Growth rates of wild-type strain (▲), *pdd1-201* (○), *pdd2-1* (△), and *pdd1-201 pdd2-1* (×) mutants on methanol at different temperatures.

caused by a hidden mutation but that indeed both mutant phenotypes are determined by single mutations in either *PDD1* or *PDD2*. It should be emphasized that both mutants showed a Pdd$^-$ phenotype at 30°C, thus under conditions of normal growth. Furthermore, the *pdd1-201 pdd2-1* double mutant grew more poorly on methanol than each of the single mutants. Growth of the double mutant on methanol was already reduced at 30°C and fully prevented at 37 and 43°C (Fig. 6). This synthetic enhancement, in which mutation *pdd1-201* exacerbates the severity of the *pdd2-1* mutation (or vice versa), suggests the existence of genetic interactions (for a recent review, see reference 14) between the *PDD1* and *PDD2* gene products.

DISCUSSION

This report describes the isolation and characterization of the first peroxisome degradation-deficient (*pdd*) mutants of *H. polymorpha*. By a newly developed screening procedure, a collection of *pdd* mutants impaired in glucose- and ethanol-induced degradation of peroxisomes has been isolated. On the basis of the known morphological sequence of events of peroxisome degradation in *H. polymorpha*, three different mutant phenotypes of selective peroxisome autophagy were expected. These mutants include (i) mutants with blocks in the sequestration of peroxisomes to be degraded from the cytosol, (ii) mutants with impaired fusion and subsequent uptake of sequestered peroxisomes in the vacuole, and (iii) mutants with affected vacuolar proteinases which may lead to the accumulation of the organelles in the vacuole.

From our collection of mutants, we have identified two genes, *PDD1* and *PDD2*, which are involved in the first two steps of peroxisome degradation in *H. polymorpha*. Mutations in the *PDD1* gene fully inhibited the initial sequestration of the peroxisomes destined for degradation by a number of membranous layers (29). Analysis of this class of *pdd* mutants may allow the identification of different components required to initiate the degradation pathway, for example, components involved in the signalling events, (specific) tagging of the organelle to be degraded, as well as components essential for recognition of the tagged peroxisomes to enable sequestration biogenesis.

In *pdd2* mutants, sequestration is not prevented but subsequent fusion of peroxisomes with the vacuole did not occur. Therefore, the *PDD2* gene product may play a crucial role in the second step of selective peroxisome degradation, namely, in uptake of the organelles to be degraded in the vacuole. The fact that *pdd1-201* and *pdd2-1* mutations showed synthetic enhancement of their Ts$^-$ growth defects on methanol suggests that *PDD1* and *PDD2* gene products can physically or functionally interact with each other. On the reasons for this Ts$^-$ growth defect, we can only speculate. One plausible explanation is that under normal physiological conditions, a low rate of turnover is essential for optimal growth; alternatively, the *PDD* genes may play a role in peroxisome assembly or function in that they interact with *H. polymorpha PER* genes (24, 28) e.g., in the control of peroxisome phospholipid incorporation.

To obtain further insights into the functions of *PDD1* and *PDD2* gene products, we are currently cloning the genes by functional complementation of the mutants with a *H. polymorpha* gene bank, using restoration of growth at 43°C as a selection criterion.

The isolated *H. polymorpha pdd* mutants represent the first mutants obtained which are impaired in the selective autophagy of eukaryotic organelles. In contrast to general (nonselective) autophagy, e.g., observed under nutrient deprivation and leading to the simultaneous degradation of different organelles and cytosolic components (2, 6, 8, 9, 16, 37, 38), selective autophagy is restricted to one type of organelle. For example, in mammalian cells the endoplasmic reticulum or peroxisomes are selectively degraded upon removal of their proliferators (3, 20). In the methylotrophic yeasts *H. polymorpha* and *Pichia pastoris*, selective degradation of peroxisomes occurs after a shift of cells from methylotrophic to nonmethylotrophic growth conditions or after irreversible inactivation of the organellar function (21, 25, 27, 29, 32). However, not all peroxisomes are degraded; in *H. polymorpha*, at least one or few small organelles escape degradation; these organelles subsequently serve as the target organelle for newly synthesized matrix proteins in the new growth environment (33).

The intriguing question of course is how the cell manages to specifically tag and recognize redundant peroxisomes for degradation. For the first time, such studies are now possible with the isolation of *H. polymorpha* mutants blocked in the initial steps of peroxisome turnover. The importance of studying the process of selective autophagy of peroxisomes is emphasized by the observation that in human Zellweger fibroblasts peroxisomes which are probably normal are synthesized but rapidly converted into autophagic vacuoles referred to as peroxisomal ghosts (1, 15).

H. polymorpha may represent a suitable model organism for studies on selective organelle autophagy in eukaryotes in general; not only is it readily accessible to genetic manipulation (10, 24), but also the extensive knowledge on the physiology, biochemistry, and morphological events of peroxisome degradation (30) favor its use for molecular studies on selective organelle turnover and thus may add to our understanding of the process of organelle homeostasis which ensures the adaptation of organelle assembly and degradation rates to environmental needs.

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