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Isolation and characterization of cold sensitive *pex6* mutant of the methylotrophic yeast *Hansenula polymorpha*

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A cold sensitive (cs) suppressor mutant was isolated from the H. polymorpha pex6 strain defective in peroxisome biogenesis. The restored cs pex6 growth on methanol at a permissive temperature was associated with the presence of morphologically normal peroxisomes. The enlarged peroxisomes present at restrictive temperature failed to support methylotrophic growth in the cs pex6 strain. The isolated mutation has no effect on the peroxisome degradation in H. polymorpha.

Introduction. Studies on the interrelationship between the processes of peroxisome biogenesis and degradation to identify genetic elements mutual for both mechanisms have been initiated recently [1]. Although oppositely directed, previous studies demonstrated the existence of such overlapping factors, i. e. the *H. polymorpha PEX14* gene product [2]. Besides Pex14p, the peroxisomal matrix protein import requires the action of two AAA ATPases, Pex1p and Pex6p. They form a complex of main importance for peroxisome biogenesis, and the mutations affecting this complex are the most common cause of the Zellweger syndrome in humans [3]. This work was carried out with a mutant of methylotrophic yeast *H. polymorpha* deficient in peroxisome biogenesis due to the mutation in *PEX6* gene. To elucidate, whether this peroxin is involved in the opposite process of peroxisome degradation, the temperature or cold sensitive (ts or cs) *pex6*-derivative mutants can be utilized. The aim of this work was to isolate and characterize these mutant strains.

Materials and Methods. *Strains, media and growth conditions. H. polymorpha* NCYC495, auxotrophic derivatives thereof [4] and *per5-C79* (*leu1.1*) [5] were used in this study. Yeast were grown at 28 or 37 °C in the YPD medium (1 % yeast extract, 1 % peptone and 1 % glucose) or selective mineral media (MM) with 0.05 % yeast extract [6], supplemented with 1 % glucose, 0.5 % methanol (MMM) or 0.5 % glycerol. Leucine (40 mg/l) was added to all MM. For solid media agar (2 %) was used. Sporulation/mating media and techniques were essentially as described in [4]. Cells were fixed and prepared for electron microscopy as in [7].

Conditional mutant isolation. To isolate ts and cs mutants the initial strain *pex6* was grown in liquid YPD, washed twice with distilled water, spread on the plates with MMM ($5 \cdot 10^7$ cells/plate) and UV-mutagenized for 60 s. Afterwards the plates were incubated for 4—5 days at 28 or 37 °C, and replica plated on the plates with MMM for identification of the mutants with conditional phenotypes at 28 and 37 °C.

Biochemical methods. Cell-free extracts for enzyme assays were prepared as in [8]. The protein concentration was determined according to [9] using bovine serum albumin as the standard. Alcohol oxydase (AOX) (EC 1.3.3.13) was assayed as in [6].

Results and Discussion. *Isolation of the cs pex6 mutant.* To identify other than *PEX14* genes possibly involved in both peroxisome biogenesis and degradation, we attempted to isolate UV-induced, condi-

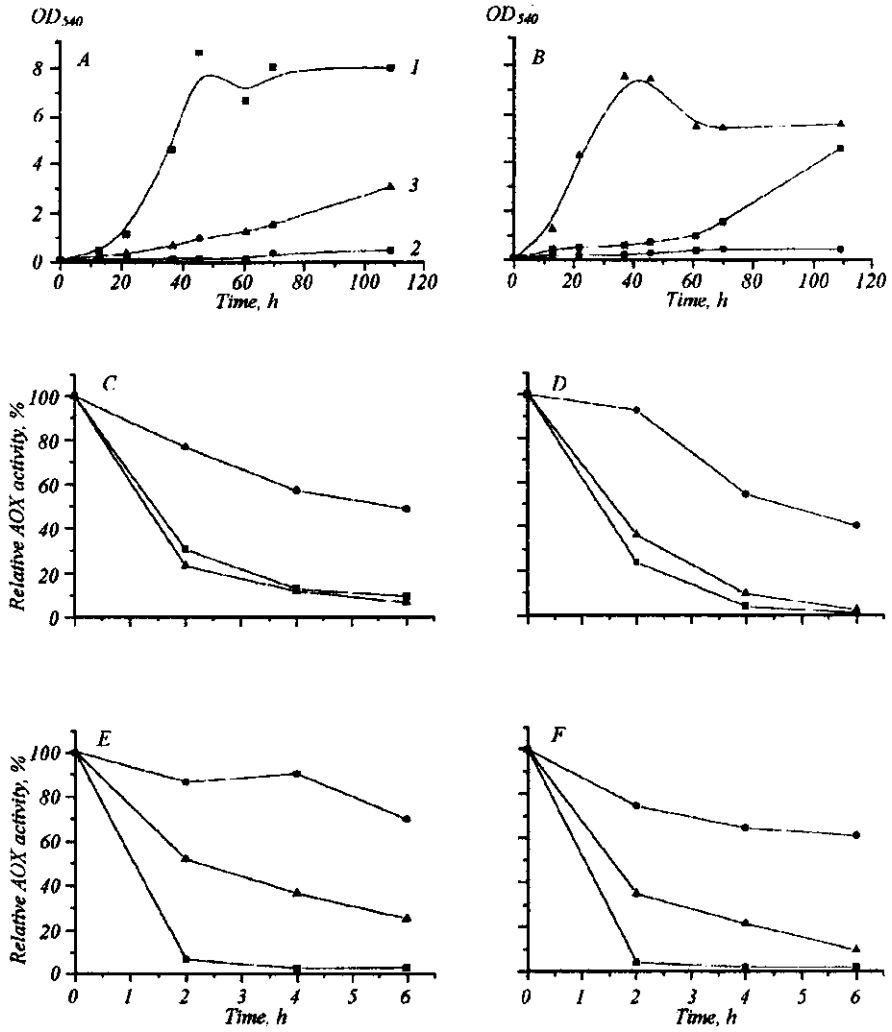

Fig. 1. Kinetics of growth in the methanol mineral medium at 28 °C (*A*) and 37 °C (*B*) and kinetics of alcohol oxidase (AOX) inactivation after shift of 17 h methanol-induced at 28 °C (*C*, *D*) and 37 °C (*E*, *F*) cells of *H. polymorpha* wild-type strain (*1*), *pex6* (*2*) and derivative cold sensitive *pex6* (*3*) mutants to glucose medium for 6 h incubation at 28 °C (*C*, *E*) and 37 °C (*D*, *F*)

tional (ts or cs) revertants from the *H. polymorpha* *pex6* mutant as an initial strain. The *pex6* cells were mutagenized, and the colonies with a restored ability to grow on MMM plates were selected. We were able to isolate only one cs clone with normal growth on methanol at 37 °C (permissive temperature) and very slow growth at 28 °C (restrictive temperature) among *pex6* derivatives. The cs *pex6* mutant exhibited the cs phenotype only in a methanol medium (Fig. 1, *A*, *B*), not in glucose, ethanol or glycerol media.

Characterization of the cs pex6 strain. To study the biogenesis of peroxisomes in the cs *pex6* mutant cells, the electron-microscopic analysis was carried out (Fig. 2). It revealed the restoration of the peroxisome biogenesis at both temperatures after 12 h induction by 0.5 % methanol. The wild-type peroxisomes induced by methanol in the cs *pex6* cells at the permissive temperature (Fig. 2, *F*), and the morphologically altered enlarged peroxisomes at the restrictive for methylotrophic growth temperature (Fig. 2, *E*) were observed. The intriguing question, why the cs *pex6* mutant cells with restored peroxisome biogenesis are unable to grow in MMM at 28 °C, is now under investigation.

The genetic analysis revealed that the corresponding cs mutation is most probably tightly linked with, or resides in the *PEX6* locus. The biochemical analysis of pexophagy at permissive and restrictive temperatures did not reveal considerable differences between the cs *pex6* cells and the wild-type strain (Fig. 1, *C*—*F*). Further biochemical and genetic analysis of the isolated conditional mutant is in

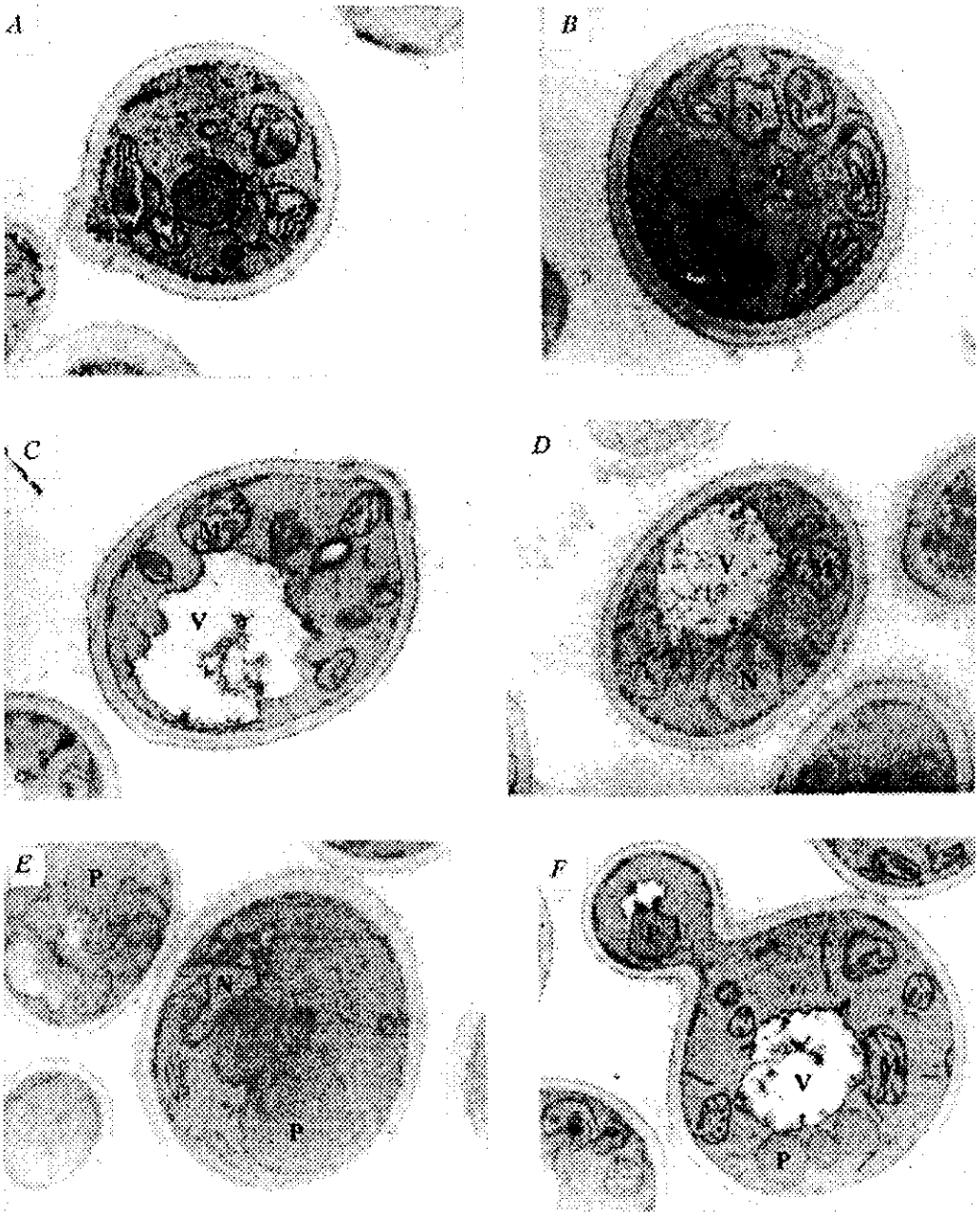

Fig. 2. Electron-microscopic images of *H. polymorpha* wild-type strain (*A*, *B*), pex6 (*C*, *D*) and derivative cold sensitive *pex6* (*E*, *F*) mutants induced in the methanol mineral medium for 12 h at 28 °C (*A*, *B*, *C*, *E*) and 37 °C (*D*, *F*). *M* — mitochondrion; *N* — nucleus; *P* — peroxisome; *V* — vacuole

progress. The nature of mutations in the utilized initial *pex6* and derivative cs mutants will be established by isolation of both alleles and sequencing but it seems that the cs *pex6* mutation has no effect on pexophagy in *H. polymorpha*. Together with the data on susceptibility of peroxisomal remnants in Δ*pex6* to the glucose-induced peroxisome degradation [1], it will be the second experimental evidence, that organelle development and turnover do not converge at Pex6p in *H. polymorpha*.

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Виділення та характеристика холодочутливого мутанта *pex6* метилотрофних дріжджів *Hansenula polymorpha*

Резюме

Виділено холодочутливий супресорний мутант зі штаму pex6 H. polymorpha з пошкодженим біогенезом пероксисом. Відновлення росту холодочутливого pex6 на метанолі при пермісивній температурі корелювало з наявністю морфологічно нормальних пероксисом. Збільшені у розмірах пероксисоми при рестриктивній температурі не здатні підтримувати метилотрофний ріст холодочутливого штаму pex6. Виділена мутація не впливає на деградацію пероксисом H. polymorpha.

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Выделение и характеристика холодочувствительного мутанта *pex6* метилотрофных дрожжей *Hansenula polymorpha*

Резюме

Выделен холодочувствительный супрессорный мутант на основе штамма pex6 H. polymorpha с поврежденным биогенезом пероксисом. Восстановление роста холодочувствительного pex6 на метаноле при пермиссивной температуре коррелировало с наличием морфологически нормальных пероксисом. Увеличенные в размерах пероксисомы при рестриктивной температуре не способны поддерживать метилотрофный рост холодочувствительного штамма pex6. Выделенная мутация не влияет на деградацию пероксисом H. polymorpha.

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