RESUMEN AUTOBIOGRAFICO

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Candidato para el Grado de

Doctor en Ciencias con Especialidad en Morfología

Titulo de Tesis: OBTENCION Y CARACTERIZACION DE CELULAS DE *LEVADURA CON DISFUNCION PEROXISOMAL* INDUCIDA POR LA PEROXISOMICINA A_1

Area de Estudio: Morfología

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APENDICE A

Métodos de Cultivo de Levaduras

a) Composición de los Medios de Cultivo.

YPD *líquido: extracto de levaduras al 0.65 %*

peptona	2 %
glucosa	1 %

YPD sólido: extracto de levaduras al 0.65 %

peptona	2 %
glucosa	1 %
bacto agar	2 %

MM: medio mineral, cada litro contiene:

$(NH_4)_2SO_4$	2.5 g
$MgSO_4 \cdot 7H_20$	0.2 g
$NaH_2PO_4 \cdot H_2O$	1.5 g
Na_2HPO_4	0.35 g

Trazas minerales (solución de Vishniac) 0.5 mL;

solución madre:

EDTA	10 g
$ZnSO_4 \cdot 7H_2O$	4.4 g
$MnCl_2 \cdot 4H_2O$	1.01 g
$CoCl_2 \cdot 6H_2O$	0.32 g
$CuSO_2 \cdot 5H_2O$	0.315 g
$FeCl_3 \cdot 6H_2O$	20 mg
$(NH)_4Mo_7O_{24} \cdot 4H_2O$	0.22 g
$CaCl_2$	1.42 g
$FeSO_4 \cdot 7H_2O$	1 g

extracto de levaduras 0.25 g

vitaminas 1 mL; solución madre 1000X

Acido Pantoténico (Ca)	400 mg	(Merck)
Acido Fólico	2 mg	(Sigma)
Myo Inositol	200 mg	(Sigma)
Nicotinamida	400 mg	(Sigma)
Acido p-Aminobenzóico	200 mg	(Sigma)
Riboflavina	200 mg	(Sigma)
Tiamina	400 mg	(Sigma)
Piridoxina	400 mg	(Brocacef)

YNX líquido: medio mineral adicionado con uno de los siguientes substratos como fuentes de carbono y/o nitrógeno:

YND: glucosa 0.5 % (p/v)/ $(NH_4)_2SO_4$ 0.25 % (p/v)

YNM: metanol 0.5 % (v/v)/ $(NH_4)_2SO_4$ 0.25 % (p/v)

YNE: etanol 0.5 % (v/v)/ $(NH_4)_2SO_4$ 0.25 % (p/v)

YNMe: glucosa 0.5 % (p/v)/ metilamina 0.25 % (p/v)

YNG: glicerol 0.5 %(v/v)/ $(NH_4)_2SO_4$ 0.25 % (p/v)

Para los medios YNX sólidos se adicionó bacto agar al 2 %.

Para los cultivos de *Hansenula polymorpha leu 1-1*, se adicionó L-leucina (Merck)a una concentración final de 30 mg/L.

Para el análisis de complementación se utilizaron los siguientes medios: EM; extracto de malta 6 % y agar al 2 %, YND o YNM con y sin uracilo o leucina (medios selectivos).

Medio de Cultivo Quimiostático.

Cada litro contiene:

$(NH_4)_2SO_4$	50 g
$MgSO_4 \cdot 7H_2O$	4 g
$NaH_2PO_4 \cdot H_2O$	20 g
Na_2HPO_4 H	0.35 g
extracto de levaduras	10 g
trazas minerales	10 mL
vitaminas:	10 mL solución madre 1000X:

	Biotina	1 mg
	Tiamina	30 mg
	Amortiguador de fosfatos 10 mM	10 mL
glucosa		0.25 % (p/v), solución madre 25 %
Metanol		0.3 % (v/v), solución madre 50 %

b) Condiciones de Esterilización de los Medios de Cultivo.

medios para cultivos líquidos: 1atm de presión durante15 min.. en una olla de presión (Presto).

medios para cultivo quimiostático: 1atm de presión durante 45 min. en un autoclave (van Rietschoten en Houwen).

Para preparar las soluciones madre el metanol y etanol, así como las vitaminas se esterilizaron por filtración, y las soluciones madre de glucosa, glicerol y metilamina, por autoclave.

c) Determinación del Crecimiento Exponencial.

Una población de células genéticamente idénticas, dispersadas homogéneamente en un medio líquido apropiado para su crecimiento, toman nutrientes y los procesan mediante procesos de transporte y reacciones enzimáticas con la producción de calor, productos de deshecho, compuestos de alta energía, producción de unidades micro- y macromoleculares para la formación de estructuras celulares. Esto permite a la célula crecer, dividirse y multiplicarse[80].

La tasa o velocidad de este proceso se calcula al medir el cambio de la densidad de población con respecto al tiempo:

$$v = dx/dt$$

Si se divide la velocidad instantánea por la densidad de población instantánea, se obtiene la velocidad de crecimiento específico de ese momento:

$$m_g = (1/x)/(dx/dt)$$

Si el medio mantiene constantes las propiedades químicas y físicas, la velocidad de crecimiento específico no varía con el tiempo, entonces se incrementan los constituyentes de la biomasa con la misma velocidad. Estas condiciones de crecimiento de la población se denomina "crecimiento balanceado y exponencial".

$$m_g x = dx/dt$$

integrando, se obtiene la conocida expresión para el incremento de población durante el crecimiento exponencial:

$$x_t = x_0 \cdot e^{m_g t}$$

Tomando los logaritmos de ambos lados se linealiza la ecuación:

$$\log x_t = \log x_0 + m_g t$$

Si se grafican los logaritmos de los valores obtenidos de la densidad de población contra el tiempo, se obtiene una serie de puntos por los cuales se puede trazar una línea recta cuya pendiente, después de dividirse por el $\log_e$, es un estimado de la velocidad de crecimiento específico.

La relación entre el crecimiento específico y la concentración de un nutriente esencial que limita la velocidad de crecimiento esta dada por la ecuación:

$$m_g = YQS$$

en donde Y representa el factor de "producción", es decir la relación entre la biomasa formada y el nutriente limitante consumido, S es una función de la concentración del nutriente en el medio y Q representan factores definidos por el estado fisiológico de la población. Es decir que cualquier cambio en la velocidad de crecimiento estará influenciada por alteraciones en cualquiera de estos factores.

d) Control de Calidad de los Cultivos.

Para detectar posibles contaminaciones en los cultivos realizados, se tomaron alícuotas de cada uno de ellos y se colocaron en laminillas para observar al microscopio de luz. La detección de cualquier tipo de contaminante causó el cese y eliminación del cultivo.

APENDICE B

Técnicas de Inclusión en Resina Epóxica para Microscopia Electrónica de Transmisión.

1. Se examinó la muestra al microscopía de luz para verificar si no había contaminación.
2. La muestra se colocó en un tubo eppendorf, se centrifugó, decantó y se lavó con agua corriente.
3. Se fijó con KMnO4 al 1.5 % durante 20 a 40 min. a temperatura ambiente.
4. Se centrifugó, se lavó con agua corriente, o hasta que el sobrenadante fue incoloro.
5. Se contrastó con acetato de uranilo al 1 % por una hora o durante toda la noche.
6. Se deshidrató en etanol: Método rápido de deshidratación en horno de microondas.
   1- 50 % v/v por 5 min.
   2- 70 % v/v por 5 min.
   3- 96 % v/v por 5 min.
   4- 100 % v/v por 5 min.
   5- 100 % v/v por 10 min.
7. Se aclaró en óxido de propileno por 15 min.
8. Se preincluyó en mezclas 1:1 y 3:1 de epon:oxido de propileno por 20 min. cada uno en horno de microondas o una hora a temperatura ambiente.
9. Se incluyó en resina epóxica pura por 20 min. (dos veces) en horno de microondas (o una hora a temperatura ambiente).
10. Las muestras incluidas en cápsulas de plástico se colocaron en una estufa durante toda la noche a 60°C para su polimerización.

11. Se hicieron cortes finos con una cuchilla de diamante en un ultramicrotomo LKB.
12. Los cortes se montaron en rejillas de cobre, se observaron y fotografiaron al microscopio electrónico Phillips EM 300.

Obtención de Esferoplastos y Técnica de Inclusión para Microscopia Electrónica.

a) Obtención de esferoplastos.

1- Un volumen de células equivalente a 20 DO por mL se centrifugó y lavó con agua y posteriormente con amortiguador sorbitol 3 M:

sorbitol	3 M
amortiguador de fosfatos	50 mM pH 7.5
$MgCl_2$	1 mM
EDTA	1 mM

2- Se resuspendió y se incubó por 10 min. a temperatura ambiente en amortiguador A:

sorbitol	3 M
β-mercaptoetanol	0.2 M

3- Se centrifugó, se lavó con amortiguador de sorbitol 3 M.

4- Se resuspendió y se incubó durante 30 a 60 min. a 37°C en amortiguador B pre calentado:

amortiguador	A
zymoliasa	1 mg/mL

5- Se observó al microscopio de luz la formación de esferoplastos.

6- El proceso se detuvo al colocar la muestra en hielo.

b) *Técnica de Inclusión para Microscopia Electrónica* (para Esferoplastos).

1- Los esferoplastos se fijaron con una gota de glutaraldehído al 50 % en solución C: cacodilato de sodio al 0.1M pH 6.
2- La muestra se centrifugó por 5-10 min., se decantó el sobrenadante y se lavó con amortiguador sorbitol 3 M.
3- Se centrifugó, resuspendió y se incubó en glutaraldehído al 6 % en solución D: cacodilato de sodio pH 7.2 a 4°C, por una hora.
4- Se lavó con solución D.
5- Se post-fijó con mezcla 1:1 de tetraóxido de osmio:dicromato de potasio por una hora a temperatura ambiente.
6- Se lavó con agua destilada.
7- Se contrastó en bloque con acetato de uranilo durante toda la noche.
8- Se deshidrató e incluyó por el método ya descrito.

APENDICE C

Los resultados obtenidos en este trabajo de tesis doctoral, se incluyeron en un artículo que ha sido aceptado para su publicación en la revista Toxicon.

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Dear Dr Zapata

Manuscript **number 97 150**
R.V. Zapata, V.T. Gonzalez, J.S. Saavedra, A.P. Lopez, K.B. Rechinger, I.Keizer-Gunnink, J.A.K.W. Kiel and M. Veenhuis
PEROXISOMICINE A1 (PLANT TOXIN-514) AFFECTS NORMAL PEROXISOME ASSEMBLY IN THE YEAST HANSENULA POLYMORPHA

I am pleased to tell you that your paper has been accepted for *Toxicon*. I have sent it to the publishers, and you should get proofs in due course.

Yours sincerely

Alan Harvey

Peroxisomicine A1 (plant toxin-514) affects normal peroxisome assembly in the yeast *Hansenula polymorpha*.

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ABSTRACT.

Previously we demonstrated that peroxisomicine A1 (T-514), a plant toxin isolated from *Karwinskia* species, has a deteriorating effect on the integrity of peroxisomes of methylotrophic yeasts. Here we describe two strains of *Hansenula polymorpha*, affected in the normal utilization of methanol as sole source of carbon and energy due to peroxisomicine A1 treatment. The two strains isolated (L17 and RV31) grew poorly on methanol, apparently due to malfunctioning of their peroxisomes. Moreover, the cells displayed a high peroxisome turnover rate. We argue that the peroxisomicine A1 induced phenotype of both strains is due to a genomic mutation. Strain L17 was functionally complemented after transformation with a *H. polymorpha* genomic library. The complementing 2.8 kb DNA fragment did not contain a well-defined ORF and led us to speculate that it may contain regulatory sequences that, when present in multiple copies in the cell, result in a change of expression of specific genes, thus causing restoration of normal methylotrophic growth.

1. INTRODUCTION

T-514 is a plant toxin, isolated from plants of the genus *Karwinskia* (Fig. 1)(DREYER *et al.*, 1975, WAKSMAN *et al.*, 1989). In mammals it causes severe damage to the lung, kidney and liver, where it induces the formation of large intracellular fat deposits and necrosis (BERMUDEZ *et al.*, 1986). Toxicological studies demonstrated a selective toxicity of T-514 on various human tumor cell lines *in vitro* (PIÑEYRO *et al.*, 1994). Preliminary morphological observations suggested that one of the initial effects upon experimental intoxication of rats and monkeys included a significant decrease of the number of peroxisomes present in their hepatocytes (SEPULVEDA, unpublished results). The importance of functional peroxisomes for man is probably best exemplified by the discovery of a group of fatal inherited human diseases (peroxisome deficiency syndromes, e.g. Zellweger syndrome (WANDERS *et al.*, 1988, LAZAROW *et al.*, 1989, BOSCH *et al.*, 1992).

As a first approach to unravel the mechanism of action of T-514, SEPULVEDA *et al.* (1992) initiated a series of experiments designed to evaluate the effect of this compound on peroxisomes of methylotrophic yeasts. It was shown that administration of sub-lethal concentrations of T-514 to cultures of *Hansenula polymorpha* or *Candida boidinii* caused an irreversible and selective damage of the peroxisomal membrane resulting in malfunctioning of the organelle and leakage of a portion of the major matrix proteins in the cytosol (SEPULVEDA *et al.*, 1992). Based on this specific effect of T-514

on peroxisomes, the drug is now designated peroxisomicine A1.

During the past few years a series of peroxisome-deficient yeast mutants (*pex* mutants; BOSCH *et al.*, 1992) has been isolated from various yeasts. In case of *H. polymorpha* peroxisome-deficiency is invariably associated with the impairment of the cells to grow on methanol as sole source of carbon and energy (methanol-utilization defective: Mut$^-$ phenotype) (CREEG *et al.*, 1990, VEENHUIS *et al.*, 1992, TITORENKO *et al.*, 1993). The possibility to isolate yeast cells which are defective in peroxisome function due to treatment of cells with peroxisomicine A1 could be of considerable value in research on toxin/peroxisome interactions and, related to this, probably also on peroxisome function. *H. polymorpha* is a feasible model organism for such studies because of the extensive knowledge on the biochemistry/physiology and ultrastructure of the organism (VEENHUIS *et al.*, 1992), combined with its easy accessibility to classical and advanced molecular genetics (FABER *et al.*, 1994).

In the present contribution we describe the isolation and initial characterization of two strains of *H. polymorpha*, defective in normal growth on methanol due to the exposure of WT cells to sub-lethal concentrations of peroxisomicine A1. The possible impact of these studies on the use of methylotrophic yeasts as model organisms in toxicology is discussed.

2. MATERIAL AND METHODS

2.1. Microorganisms and growth conditions

Wild type (WT) *Hansenula polymorpha* CBS 4732, *H. polymorpha leu1-1* (GLEESON *et al.*, 1979), *H. polymorpha* RV31 (this work) and *H. polymorpha leu1-1* L17 (this work) were grown in batch cultures in mineral media at 37°C (VEENHUIS *et al.*, 1979, VAN DER KLEI *et al.*, 1991). As carbon sources either 0.5 % glucose, 0.5 % glycerol, 0.5 % ethanol or 0.5 % methanol were used and as nitrogen source either 0.25 % ammonium sulphate or 0.25 % methylamine. Leucine was added to a final concentration of 30 mg L^{-1}. Growth was monitored by measuring the optical density of the cultures at 660 nm in a Vitatron colorimeter (A_{660}). In addition, the strains were grown in carbon-limited continuous cultures at 37°C in mineral medium (VAN DER KLEI *et al.*, 1991) supplemented with either 0.25 % glucose or 0.25 % glucose/0.3 % methanol at a dilution rate (D) of 0.1 h^{-1}.

2.2. Peroxisomicine treatment of intact cells

The strains were pregrown to the mid-exponential growth phase (A_{660} = 1.5 - 1.8) in batch cultures supplemented with 0.5 % glucose and subsequently shifted into fresh 0.5 % methanol-containing media at an A_{660} of 0.1. After 2 hours of induction the cells were concentrated (by centrifugation) in fresh methanol-media to an A_{660} of approximately 1.0 and peroxisomicine A1 was administrated at a concentration of 5 µg ml^{-1}. Samples were taken at 1 or 2 h after addition of peroxisomicine A1 and analyzed for cell

viability, the capacity to grow on methanol and their subcellular morphology, using untreated cells as control. Growth was initially tested on 2% Bacto agar plates containing 0.67 % YNB (yeast nitrogen base without amino acids) and 0.5 % glucose (YND) or 0.5 % methanol (YNM). For viability tests peroxisomicine A1 treated cells were spread on YND plates. To identify methanol-utilization-defective (Mut$^-$) strains, cells were spread on YND plates at a dilution that produced approximately 500 colonies per plate; after 3 days of incubation at 37°C the YND plates were replica-plated onto YNM plates; after another 3 days of incubation at 37°C, colonies which failed to grow on methanol were isolated and further analyzed.

2.3. Preparation of crude extracts and biochemical methods

Crude extracts were prepared as described before (VAN DIJKEN *et al.*, 1976). Alcohol oxidase (AO; EC 1.1.3.13) was assayed as described by VERDUYN *et al.* (1984) and catalase (EC 1.11.1.6) by the method of LÜCK (1963). Protein concentrations were determined according to BRADFORD (1976) using bovine serum albumin as standard. Residual growth substrates in the culture fluid were determined after removal of intact cells by centrifugation in an Eppendorf microfuge, maximal speed, 5 min. Methanol was determined by gas chromatography (LAANBROEK *et al.*, 1982), and glucose by the glucose oxidase-peroxidase method (Boehringer, Mannheim, FRG).

2.4. Cloning and sequence analysis

To enable cloning of the L17 gene by functional complementation, mutant

L17 was transformed by electroporation with a *H. polymorpha* genomic library in vector pHRP2. Transformants were screened for the ability to grow at normal rate on methanol-containing plates. From one positive transformant, a plasmid pHRP2 with an insert of 2.8 kb could be isolated and retransformation to the *L17* mutant showed that indeed this plasmid could functionally complement the *L17* mutant. Double-stranded DNA sequencing of the complementing insert was performed using an ABI 313A automatic sequencer (Applied Biosystems Inc.) using the Taq Dye Deoxy Terminator Cycle Sequence Kit.

2.5. Electron microscopy

Whole cells were fixed with 1.5 % (w/v) $KMnO_4$ for 20 min at room temperature. The samples were poststained in 1 % (w/v) uranylacetate, dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

2.6. Immunocytochemistry

For immunocytochemistry, intact cells were fixed in 3 % (v/v) glutaraldehyde in 0.1 M sodiumcacodylate buffer, pH 7.2, for 30 min at 0°C dehydrated in a graded ethanol series and embedded in Lowicryl K4M. Immunolabelling was performed on ultrathin sections according to the method described by SLOT and GEUZE (1984) using specific antibodies against alcohol oxidase, catalase and dihydroxyacetone synthase. For labeling of the primary antibodies either 10 nm protein A-gold or 15 nm

gold labeled goat-anti-rabbit antibodies were used.

2.7. Miscellaneous

H. polymorpha NCYC 495 (*ade11 met6*), *H. polymorpha* RV31, a 5-fluoro-urotic-acid (5-FOA) resistant mutant derived from this strain (*H. polymorpha* RV31 *ura3*⁻) and *H. polymorpha* L17 were used for genetic analysis. Mating, complementation analysis, sporulation and random spore analysis were performed as described previously (CREGG *et al.*, 1990; TITORENKO *et al.*, 1993; GLEESON and SOUDBERY, 1988).

3. RESULTS.

3.1. Peroxisomicine A1 specifically affects peroxisome integrity of H. polymorpha cells.

Previous experiments suggested that the peroxisomicine A1 induced damage of peroxisomes in methanol-grown cells may be irreversible (SEPULVEDA *et al.*, 1992). For this reason we now studied the effect of the toxin in *H. polymorpha* WT cells which are known to contain generally only a single peroxisome, namely in cells pregrown in batch cultures to the mid-exponential growth phase on glucose and subsequently incubated in methanol-containing media for two hours (VEENHUIS *et al.*, 1979). We anticipated that damage of this single organelle might have severe consequences for the capacity of the cells to grow on methanol since the metabolism of this compound is strictly dependent on the presence of

intact organelles (VAN DER KLEI *et al.*, 1991). However, the growth experiments revealed that the viability of these freshly methanol-induced cells after peroxisomicine A1 treatment was similar as observed before in that concentrations >10 μg ml^{-1} were lethal whereas lower concentrations were sub-lethal in a dose/response related manner (for details see SEPULVEDA *et al.*, 1992).

Electron microscopical data revealed that treatment of cells, incubated in methanol for 2 h, with 5 μg ml^{-1} peroxisomicine A1 for 1-2 h specifically affected peroxisomes. As expected, the cells contained generally a single organelle, which frequently displayed ruptures of its surrounding membrane; other subcellular membranes, e.g. the cell membrane and surrounding membranes of nuclei, mitochondria or vacuoles were virtually unaffected (Fig. 2A). Similar results were obtained with the *H. polymorpha leu 1-1* auxotrophic strain (data not shown). We conclude from this that peroxisomicine A1, when administrated at sub-lethal doses, does affect the integrity of the peroxisomes in *H. polymorpha* but not their development.

3.2. Isolation and biochemical characterization of methanol- utilization-defective (Mut⁻) strains from peroxisomicine A1 treated cells.

To further investigate the reversibility of the peroxisomicine A1-induced damage of peroxisomes, cells were incubated in the presence of 5 μg ml^{-1} peroxisomicine A1 for 1-2 hours and subsequently analyzed for growth on glucose and methanol by means of the replica-plating technique described

in Material and Methods. These experiments resulted in the isolation of strains which were impaired to grow on methanol on solid agar plates, although at very low frequencies. Morphological analyses of cells, induced for 24 h on methanol, of the various strains obtained revealed that the cells were invariably affected in peroxisome size and/or number, ranging from cells containing large organelles of abnormal shape (*pss* phenotype; (TITORENKO *et al.*, 1992) to cells with few relatively small organelles (Fig. 2D).

Two strains, designated RV31 and L17(*leu1-1*), were selected for further analysis on the basis of their growth properties and peroxisome morphology. YND-grown colonies of both strains, replicated on YNM plates, failed to grow in a period of 5 days, after which growth started albeit very slow as compared to WT controls (Fig. 3). Subsequent studies in liquid cultures showed that the strains were able to grow on various carbon and nitrogen sources tested, including those that require peroxisome-borne enzyme activities for growth (Table 1; not shown for strain L17). However, the final optical density of the cultures always remained below the values observed for WT controls. Of all compounds tested, growth on methanol was most severely affected (Fig. 4). As is evident from this Figure, the rate of methanol utilization by RV31 cells is reduced compared to WT controls, although methanol is fully used up in both cultures. This is in agreement with the finding that AO activity is normally induced, although at a reduced levels compared to WT cells; in contrast, catalase activities were not affected (Table 2). However, comparison of the amounts of methanol used and the optical density of the culture, e.g. at a residual concentration

of approximately 0.25 % methanol (Fig. 4; A_{660} RV31 = ± 2, A_{660} WT = ± 3), clearly show that the conversion of the carbon source into biomass is much less efficient in the RV31 strain.

A reduced yield was also evident in glucose-limited chemostat cultures of both strains, to which methanol was added as a second growth substrate. The data obtained showed that the final yield of cultures of L17, measured as A_{660} at steady state conditions, was slightly reduced compared to WT controls (L17 A_{660} = 6.6, WT A_{660} = 7.0). In both the L17 and the WT cultures no residual glucose or methanol could be detected in the culture medium when the cultures had reached steady state conditions. Similar results were obtained with strain RV31. Also in chemostat-grown cells the specific AO activity remained significantly below the values normally found in WT cultures (AO activity L17 = 2.2 Units (mg. protein)$^{-1}$, WT = 8.0; VAN DER KLEI *et al.* 1991).

3.3. Ultrastructural analysis.

The morphological adaptations of L17 and RV31 cells, following a shift of cells from glucose to methanol-containing media, was examined by electron microscopy. The data, summarized in Fig. 5 show that in the initial 4 hours after the shift peroxisomes developed (Fig. 5A). Typically, the organelles showed ruptured peroxisomal membranes in ultrathin sections of $KMnO_4$ - fixed cells, while other organelles showed no obvious morphological deviations. Before this time no visible differences had ocurred in the cells.

As described previously, within the first 2 h after the change over the methanol, one small peroxisome per cell, surrounded by a single continuous membrane were found (VEENHUIS *et al.*, 1979). Immunocytochemical experiments on sections of these cells, using α-AO, α-CAT or α-DHAS, revealed that bulk of the labeling was concentrated on peroxisomal profiles; however, also a low but specific labeling was observed in the cytosol (Fig. 5C,D; not shown for DHAS). After approximately 8 h of incubation the initially formed organelles had reached their mature size and multiplied by fission. At this stage of cultivation also the first degrading peroxisomes were observed. Our data strongly suggested that in particular the larger, mature organelles were subject to degradation resulting in cells which generally contained only few, relatively small peroxisomes in conjunction with one or few organelles in various stages of degradation (Fig. 5B). After entering the stationairy growth phase (> 24 h of incubation) the cells of the culture gradually degenerated and subsequently died. Also in chemostat cultures of L17 and RV31 on glucose/methanol mixtures a high turnover rate of individual peroxisomes was observed (not shown). As a consequence, such cells contained a relatively low volume density of peroxisomes. This low volume density was in good agreement with the observed specific AO activities (see above).

3.4. Genetic analysis

In order to study whether the L17 and RV31 strains were in fact mutants affected in the same gene, genetic analysis was performed. Since mating of RV31 and L17*leu1-1* with the auxotrophic *H. polymorpha ade11 met6* strain failed, we generated a 5-fluoro-orotic-acid (5-FOA) resistant mutant

from RV31 (BOEKE *et al.*, 1984). The resulting *H. polymorpha* RV31 *ura3*⁻ strain was crossed with L17. In order to establish whether the eventual mutations in RV31 *ura3*⁻ and L17 *leu1-1* causing the *mut*⁻ phenothype were recessive or dominant, both mutants were crossed with their corresponding mutual isogenic strains carrying complementary markers, namely *ura3*⁻ and *leu1-1*, respectively. After crossings, all diploids normally grew on methanol at WT rates. Random spore analysis revealed that strains L17 and RV31 behave as recessive mutants which cannot complement each other.

The *H. polymorpha* L17 *leu1-1* strain was used for transformation with a *H. polymorpha* genomic library (GLEESON *et al.*, 1988). Transformation was carried out by the routine procedure, described before (FABER, *et al.*, 1994). Transformants were selected on YND plates, lacking leucine. After 3-4 days the transformants were replica-plated onto YNM-plates and selected for the restoration of growth on methanol as a sole source of carbon and energy at WT rates. One positive transformant was selected; plasmid DNA was isolated from this strain and amplified in *E. coli*. Restriction analysis of purified plasmid DNA from *E. coli* revealed a 2.8 kb *H. polymorpha* genomic fragment which, after retransformation in *H. polymorpha* L17, complemented the original growth defect of the strain on methanol. Electron microscopical analysis of the transformed *H. polymorpha* L17 strain revealed that the cells contained normal peroxisomes and did not display the typical peroxisome membrane ruptures and organelle turnover events of the L17 host strain during methylotrophic growth. The

complementing fragment was isolated as a *Nhe*I-*Sph*I fragment and cloned into the *Sma*I site of pBluescript II SK$^+$ using blunt-end cloning. Sequencing of the 2.8 kb fragment revealed no apparent open reading frames (ORFs); also, the sequence obtained did not show any significant homologies with known sequences in the databases.

4. Discussion.

We have isolated two strains of the methylotrophic yeast *Hansenula polymorpha* affected in the normal use of methanol as sole source of carbon and energy, which were obtained after treatment of whole cells with the plant toxin peroxisomicine A1. As shown before (SEPULVEDA *et al.*, 1992), Peroxisomicine A1 affects the integrity of the peroxisomal membrane when administrated at sub-lethal doses to methanol-grown cells of *H. polymorpha*. Our present result suggests that damage of the peroxisomal membrane of *H. polymorpha* by peroxisomicine A1 is a highly reproducible event and furthermore confirmed the suggestion that peroxisomes may indeed represent the initial target organelle of the toxin-514, which is therefore now designated peroxisomicine A1. Likely, the first target of the toxin is one (or more) specific peroxin(s), essential for peroxisome integrity. Possible target molecules are HpPex3p (BAERENDS *et al.*, 1996), an integral membrane protein essential for the membrane biosynthesis and integrity, or HpPex6p, a AAA ATPase essential for peroxisome biogenesis and associated with the peroxisomal membrane (KIEL *et al.*, submitted).

The two strains isolated (RV31 and L17) are clearly affected in peroxisome function. This was convincingly demonstrated by the findings that i) the enzymes essential for methanol catabolism were present and ii) the biomass in batch cultures of both strains was significantly decreased compared to WT controls while methanol was fully consumed by the cells. However, methanol can normally be used as additional energy source in glucose-limited cultures and then results in an increase in biomass which is largely comparable to identically grown WT cultures, indicating that the cytosolic dissimilation pathway is apparently efficiently operative. Our data therefore led us to conclude that growth of strains RV31 and L17 in batch cultures on methanol as the sole carbon and energy source suffers from specific energetic disadvantages in particular in the catabolism of methanol. We speculate that these disadvantages are related to peroxisome dysfunction rather than to other defaults in methanol intermediary metabolism and are most likely related to hydrogen peroxide metabolism. As shown before (VAN DER KLEI *et al.*, 1991), decomposition of hydrogen peroxide in methanol-grown *H. polymorpha* other than by peroxisomal catalase is energetically disadvantageous and leads to reductions in biomass. In L17 and RV31 cells hydrogen peroxide leakage from damaged organelles can easily be envisaged thus explaining the negative growth effects.

At present we can only speculate on the nature of the mechanism which accounts for the functional complementation of strain L17 with the isolated

2.8 kb genomic fragment. Clearly, this fragment does not contain a well-defined open reading frame (ORF) and as a consequence it does not contain a complete gene encoding for a specific protein. A likely explanation for its complementing effect is that the DNA fragment contains regulatory sequences which either interfere with a specific supressor gene or, less likely, that it encodes for only part of the mutated protein and complements by a recombination event. If the fragment acts as a promoter it may bind to transcription factors resulting in a change in the expression of certain genes and thus in faster growth on methanol.

It is also not beyond doubt whether peroxisomicine A1 can act as a mutagenic agent. After peroxisomicine-treatment of WT cells only few constitutive Mut$^+$ colonies were picked up and these low frequencies could readily be interpreted as representing spontaneous mutations. However, the genomes of the *H. polymorpha* strains used are very stable and spontaneous mutations are extremely scarce under normal laboratory conditions. Moreover, at least one example is known in which a comparable drug (the cytostaticum adriamycin (KEYANI and KEYANI, 1980) has a strong effect on peroxisome proliferation in yeast apparently due to a mutation of the proliferation machinery. Therefore, a mutagenic effect on the yeast by the toxin might be possible. In both strains isolated, it resulted in an irreversible slow growth on methanol after removal of the toxin. A possible explanation for this is that the mutation caused overexpression (or suppression) of a specific gene which resulted in restoration of normal growth of the strain on methanol. The yeast multidrug resistant phenotype due to overexpression of certain genes has been firmly documented (BALZI

and GOFFEAU, 1995); the *Saccharomyces cerevisiae PDR1, PDR3, PDR7* and *PDR9* genes encode transcription regulators that control the expression of the gene *PDR5* which encodes a membrane protein of the ATP-binding-cassette protein family, functioning as a drug extrusion pump (SWARTZMAN *et al.*, 1996). The possibility to use *H. polymorpha* as a model organism for toxicology, related to peroxisome function, is currently explored.

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Table 1. Optical densities of *H. polymorpha* wild type (WT) cells and RV31 cells, grown in batch cultures supplemented with various carbon and nitrogen sources.

Strain	Growth conditions	Optical density (A_{660})
WT	glucose/ammonium sulphate	5.5±0.1
RV31	glucose/ammonium sulphate	5.2±0.2*
WT	ethanol/ammonium sulphate	5.0±0.2
RV31	ethanol/ammonium sulphate	3.4±0.03*
WT	glycerol/ammonium sulphate	8.6±0.1
RV31	glycerol/ammonium sulphate	6.2±0.02*
WT	glucose/methylamine	4.3±0.1
RV31	glucose/methylamine	3.4±0.02*

Data are presented as final optical density of cultures in the stationairy growth phase. Each value is shown as mean of three determinations ± S.E.
*Significant difference from WT by Student's t-test ($p<0.05$).

Table 2. Activities of alcohol oxidase and catalase in *Hansenula polymorpha* RV31 cells after a shift of cells from glucose into methanol-containing media.

Time (h)	alcohol oxidase[§]		catalase	
	RV31 cells	WT cells	RV31 cells	WT cells
0	0	0	2.9±0.8[¶]	3.1±0.2
4	0.7±0.2	0.7±0.3	93±2.1	98±1.8
8	1.2±0.1	2.5±0.3	144±1.5	148±1.8
12	2.3±0.5	4.2±1.2	177±1.6	173±2.0
24	2.5±0.2	4.7±1.5	196±2.5	190±3.1

[§]Alcohol oxidase is expressed as Units. mg protein^{-1}, catalase as ΔE_{240}. min^{-1}. mg protein^{-1}.

[¶]Each value is shown as mean of three determinations ± S.E.

LEGENDS FOR FIGURES.

Electron micrographs are taken of $KMnO_4$-fixed cells unless otherwise stated. Abbreviations: N, nucleus; M, mitochondrion; P, peroxisome; V, vacuole. The bar represents 0.5 μm.

Fig. 1. Structure of toxin 514 (peroxisomicine A1).

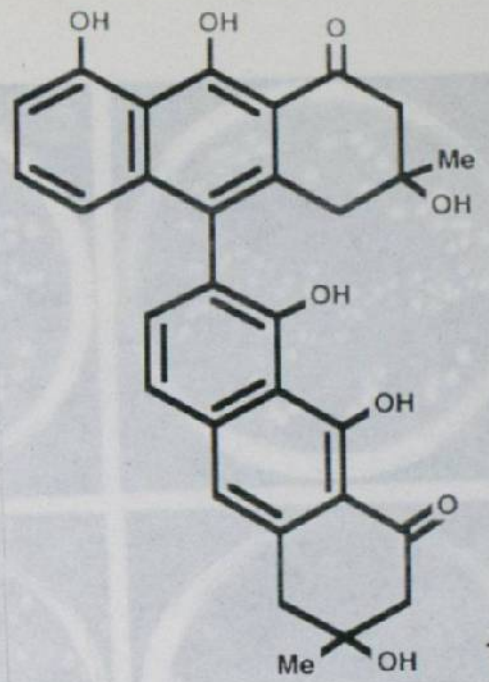
Fig. 2. Ultrathin section of $KMnO_4$-fixed cells of *H. polymorpha*, grown in batch culture on 0.5 % methanol for 4 h (Fig. 2A) and 12 h (Fig. 2B). After subsequent incubatioon with 5 $\mu g\ ml^{-1}$ peroxisomicine A1, the typical damage of the peroxisomal membrane is observed (Fig. 2C; arrow). Fig. 2D shows the relatively large peroxisomes of irregular shape observed in other incubated strains after peroxisomicine A1 treatment.

Fig. 3. Replica-plating technique showing methanol-utilization defective colonies isolated after treatment of *Hansenula polymorpha* WT cells with peroxisomicine A1. The plates were incubated for 5 days at 37°C. Upper panel: WT controls A: glucose, B: methanol: lower panel RV31 cells (C: glucose, D: methanol).

Fig. 4. Growth (▲) and metanol utilization (Δ) of *H. polymorpha* wild type and RV31 cells (O growth; ● methanol concentration) after a shift of cells from glucose into fresh cultures, supplemented with 0.5 % methanol. Growth is expressed as optical density at 660 nm (A_{660}), methanol

concentrations as percentages in the culture medium. Each value is the mean of three determinations.

Fig. 5. Ultrathin section of strain RV31, grown in batch culture on 0.5 % methanol for 4 h, showing development of a peroxisome with a ruptured peroxisomal membrane (Fig. 5A; arrow). Compare also Fig. 2A/C for datails. Fig. 5B shows the initial stage of peroxisome degradation, namely uptake of the organelle (*) in the vacuole. Fig. 5C,D: immunocytochemical experiments on ultrathin sections of methanol-induced RV31 cells, labeled with specific polyclonal antibodies against alcohol oxidase (Fig. 5C) and catalase (Fig. 5D). In both experiments most of the labeling is observed on peroxisomal profiles while a minor labeling is evident on the cytoplasm (Lowicryl sections; α-AO or α-CAT and goat-α-rabbit/gold, uranyl acetate).



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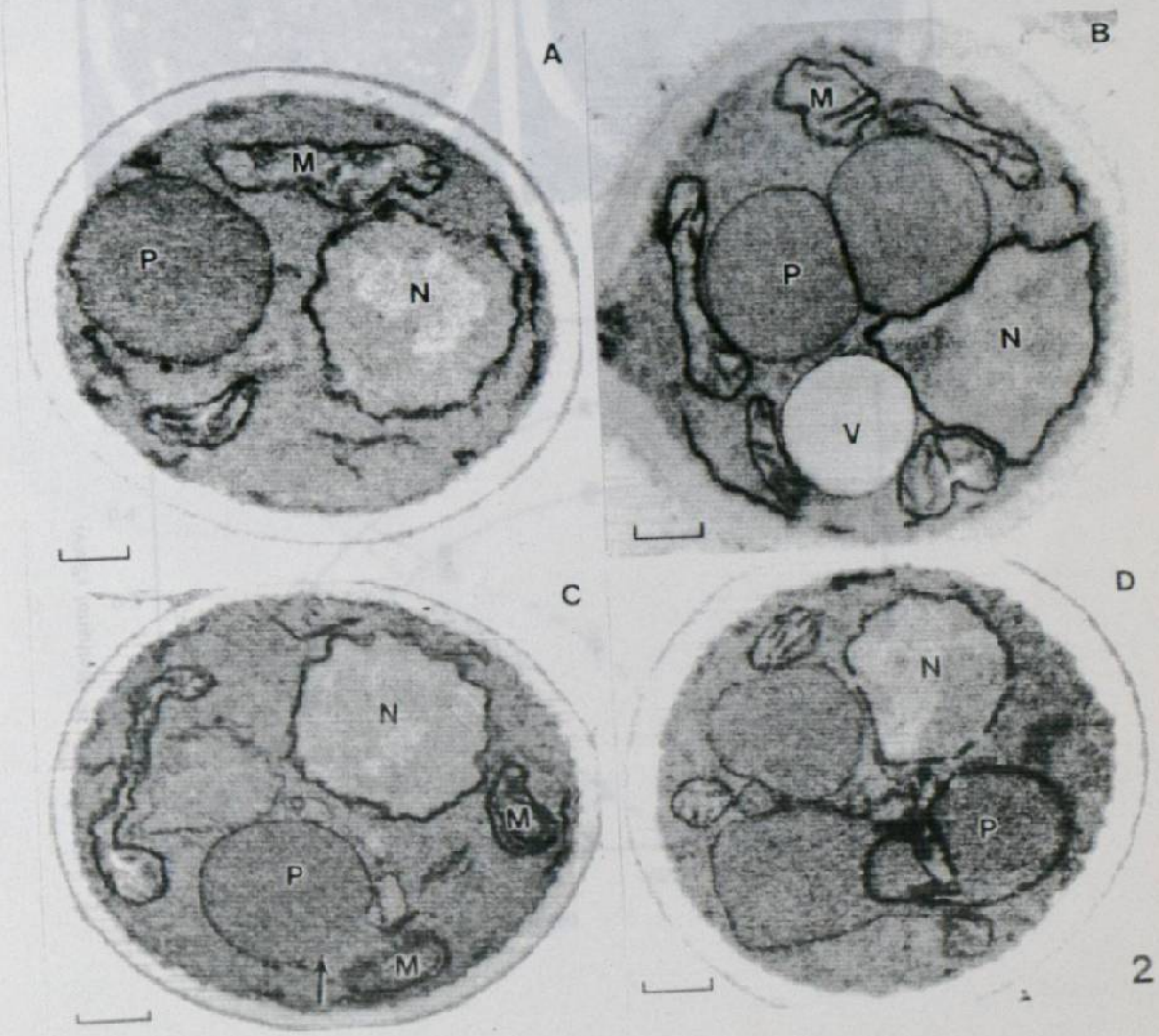

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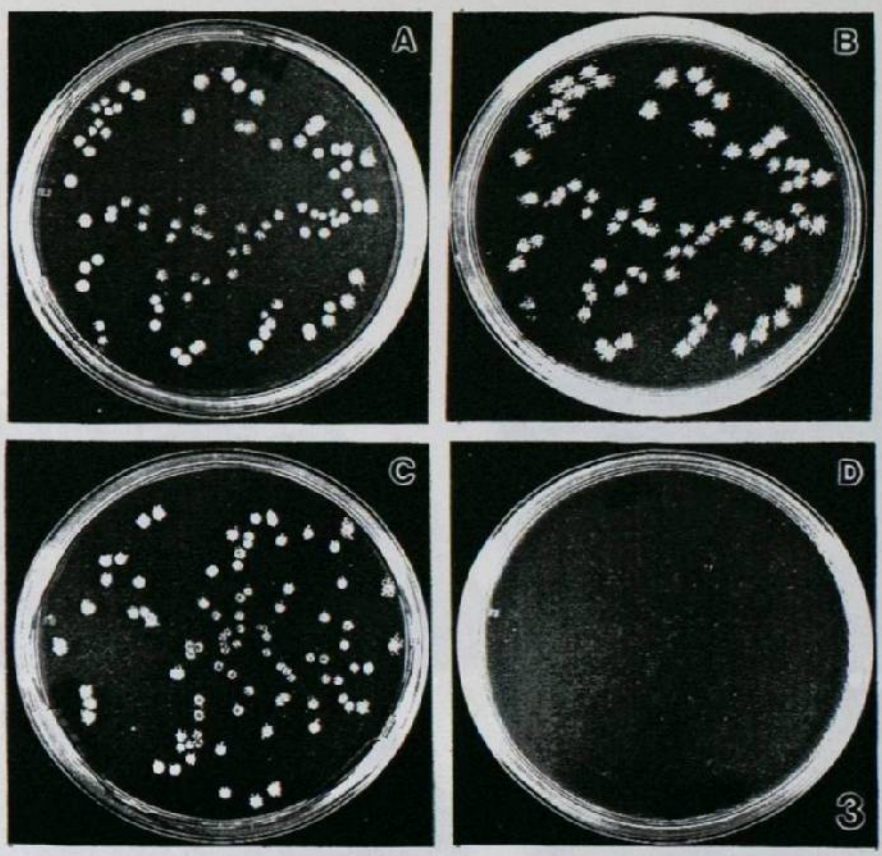
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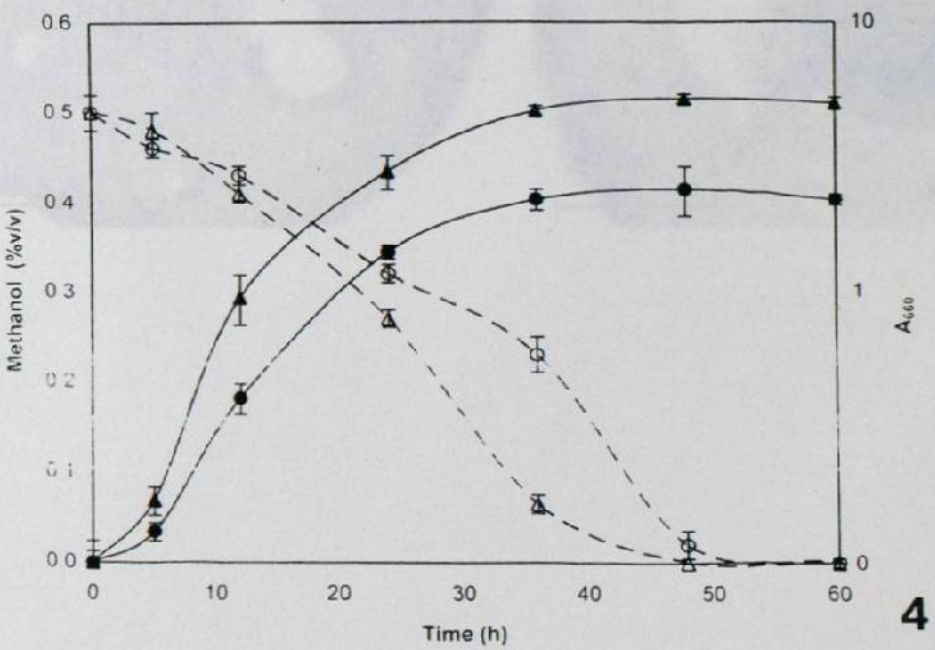
0.6
0.5
0.4
0.3
0.2
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A_{660}
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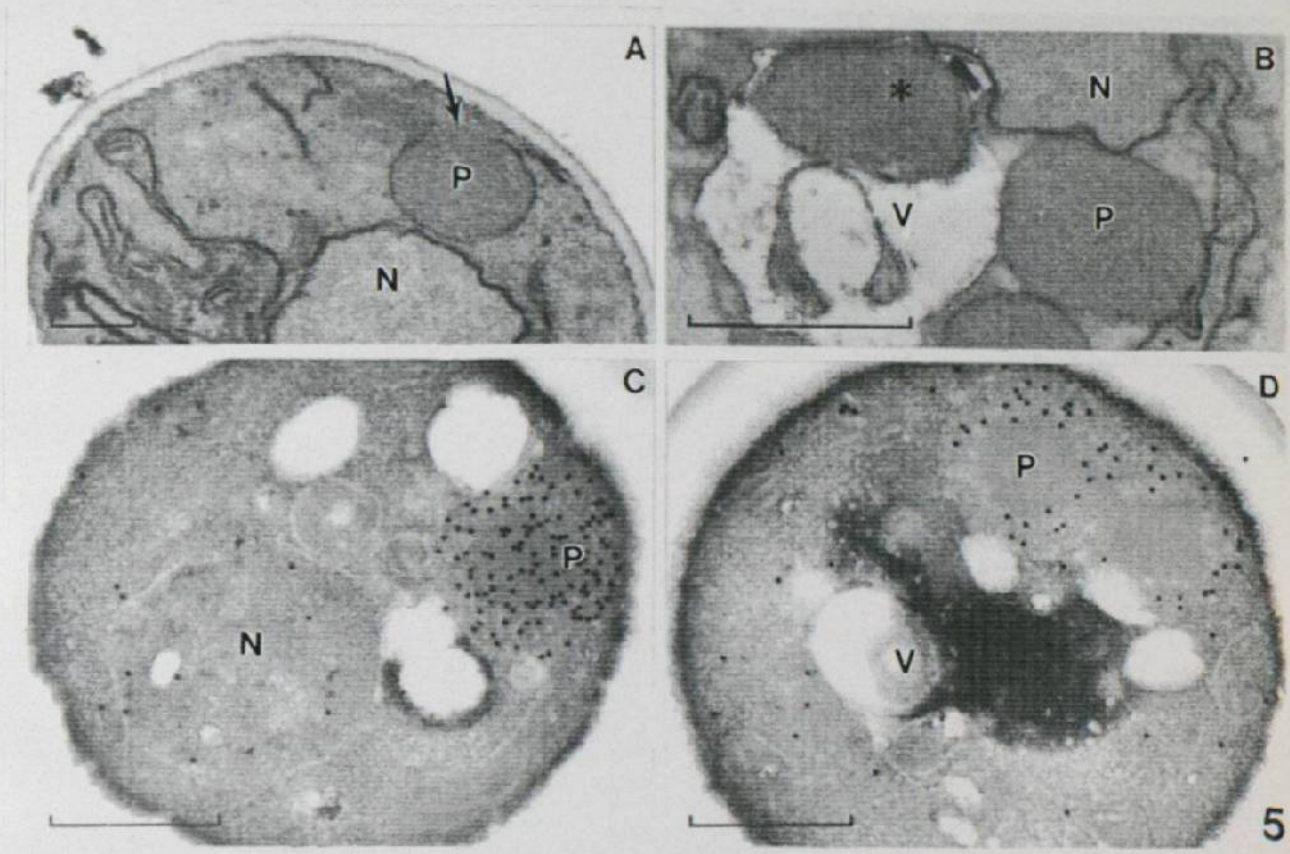
50
60
Time (h)

4



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