Instituto Juan March
de Estudios e Investigaciones

69 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Co-sponsored by

FEBS FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES

Advanced course on

Biochemistry and Molecular Biology of Non-Conventional Yeasts

Organized by

C. Gancedo

J. M. Siverio

J. M. Cregg

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The course summarized in this publication took place on the 7th through the 19th of July 1997, at the Instituto de Investigaciones Biomédicas (CSIC), Facultad de Medicina, UAM, Madrid.

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INSTRUCTORS

James M. CREGG

Carmen Lisset FLORES

Carlos GANCEDO

Celedonio GONZÁLEZ

Germán PERDOMO

Cristina RODRÍGUEZ

José M. SIVERIO

Oscar ZARAGOZA

LECTURERS

Gerold BARTH

James M. CREGG

Angel DOMÍNGUEZ

Hiroshi FUKUHARA

Claude GAILLARDIN

Carlos GANCEDO

Concha GIL

Cornellis P. HOLLENBERG

Jan A.K.W. KIEL

Cecilia LEÃO

Isabel LÓPEZ-CALDERÓN

Sergio MORENO

César NOMBELA

José PONTÓN

Rafael SENTANDREU

José M. SIVERIO

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INTRODUCTION

C. Gancedo, J.M. Siverio and J.M. Cregg

Introduction

This booklet present materials from the Advanced Course of Biochemistry and Molecular Biology of Non-conventional Yeasts held in Madrid in July 1997 and sponsored by FEBS and the Instituto Juan March de Estudios e Investigaciones. We have included a series of Protocols for the manipulation of non-conventional yeasts as well as the Outlines of Lectures presented in the Course.

It is clear that the choice of protocols is biassed by the experience or preferences of the authors. However we have tried to present a variety of techniques that show the versatility and usefulness of these organisms. We hope that at the end of the course the students will have a new feeling towards the increasing number of non-conventional yeasts used in research.

The protocols included are the ones that are being currently used in our laboratories or in those of colleagues with which we have close connections. This does not mean that they are the best available. Any suggestion for improvement will be always welcome.

We thank the colleagues who have helped us by sending materials, by reading parts of the manual or by helping in a particular manipulation. Our thanks to Singer Instruments for providing micromanipulators .

The organizers thank the sponsoring organizations, FEBS and Instituto Juan March de Estudios e Investigaciones, for their financial help and for the easy relation with them along all the steps of the preparation of the Course. Thanks are also due to the institutions to which the instructors and organizers belong for allowing them to dedicate time to this course.

We hope that the time and effort dedicated to the Course will be useful for people trying to work with non-coventional yeasts.

C. Gancedo, J.M. Siverio and J.M. Cregg

Madrid, July 1997

PART I

PRACTICALS

Obtention of auxotrophic mutants in *Hansenula polymorpha*

Aim:
Obtention and preliminary characterization of auxotrophic mutants in *Hansenula polymorpha* .

INTRODUCTION

Mutants are important tools for basic and applied research. Due to the low frequency of appearance of spontaneous mutations, it is usually necessary to use a mutagenic treatment to increase this frequency. Different mutagenic agents, UV light, ethyl methane sulfonate, nitrosoguanidine etc. may be used. Mutagenesis not only induces non-lethal mutations but also kills cells, likely due to mutations in essential genes. The frequency of mutation in a certain experimental condition is the fraction of mutants showing a determined phenotype among the surviving population after mutagenic treatment. Survival is determined by plating cells in a medium permissive for the majority of the population.

OBTENTION AND PHENOTYPIC CHARACTERIZATION OF AUXOTROPHIC MUTANTS

In this particular experiment we will isolate auxotrophic mutants using ethyl methane sulfonate (EMS). EMS is an alkylating agent causing mainly single base pair substitutions. The frequency of auxotrophic mutants should be high enough to allow detection without an enrichment step. Further, the nutritional requirements of the mutants will be determined and a complementation analysis will be performed. The standard protocol described here involves treatment with EMS of a wild type haploid strain, at pH 7, optimal for its mutagenic action. The reaction is stopped by adding sodium thiosulfate, which neutralizes EMS action effectively without significantly damaging the cells. Auxotrophic mutants are selected by their inability to grow in a medium without certain requirements.

A further step will be the characterization of the nutritional requirements of the auxotrophic mutants. All the auxotrophs are plated on YPD forming a pattern. Once they are grown, they are transferred to a set of 9 media containing the following amino acids and bases:

	1	2	3	4	5
6	ade	guanine	cys	met	ura
7	his	leu	ileu	val	lys
8	phe	tyr	trp	thr	pro
9	gln	ser	ala	asp	arg

The different growth of a mutant in the different media allows the unambiguous characterization of 20 different auxotrophic phenotypes. For instance, a His- strain will only grow on media 1 and 7. The set is designed to minimize the work needed for its preparation.

GENOTYPIC CHARACTERIZATION OF MUTANTS.

After isolation and phenotypic characterization of a mutant, the next step is the study of its genotype since mutations in diiferent genes might produce teh same phenotype.

To define the genotype causing a mutant phenotype it is first necessary to determine the number of genes affected. This is done by meiotic analysis. Mutants whose phenptype is determined by a single gene are usually retained for further work.

COMPLEMENTATION ANALYSIS

The dominance or recessivity of a mutation is determined by crosses with the wild type. If the diploid maintains the same phenotype as the wild type it means that the mutation is recessive. On the contrary if the diploid presents the same phenotype as the mutant this means that the mutation is dominant. Generally, auxotrophic mutations are recessive.

In the case of recessive mutations, crosses between strains with the same phenotype are used to determine the complementation groups. If two mutants with the same phenotype are crossed and the wild type phenotype is observed in the diploid, this usually indicates that the mutations are in different genes; if the mutant phenotype is observed, the mutations affect the same gene.

The number of complementation groups gives an idea about the number of genes involved in the production of a certain phenotype. *H.polymorpha* mates efficiently with itself due to the switching a-α on malt extract medium. Therefore, it is easy to carry out crosses in such a way that mutants with the same phenotype are crossed in all pairwise combinations on a single plate and screened for prototrophic growth on selective media.

PRACTICAL

Day 1
Streak *H. polymorpha* NCYC 495 strain on a YPD plate and incubate at 37° C overnight.

Day 2
With a loop collect the cells from the plate and suspend them in 2 ml of 0.1 M sodium phosphate pH 7. Determine optical density at 660 nm and prepare 0.5 ml of a suspension with an optical density of 1 (this is a thick turbid suspension). To determine initial cell viability make sequential dilutions 10^{-2}, 10^{-4}, 10^{-5} and 10^{-6} in water. Plate 0.1 ml of the 10^{-5} and 10^{-6} dilutions on a YPD plate. Incubate at 37° C for 2 days.
(**WARNING**: EMS is a potent carcinogenic, handle with care, use gloves and wash your hands)
Add 30 µl of EMS to the initial cell suspension and vortex . Incubate for one hour at 30° C with occasional stirring. After this time add 5 ml of 5% sodium thiosulfate to quench the effect of EMS. Dilute 10^{-2} and 10^{-3} in water. Spread 0.1 ml of each dilution on YPD plates. Spread 5 plates per dilution.

Day 4
Count colonies in the plates corresponding to the initial and the mutagenized culture.
Calculate percentage of viability after mutagenesis.
Replicaplate plates with mutagenised cells to SD and YPD plates. (Replica plate five plates with a cell number of 200-500)

Day 5
Compare the growth on SD and YPD plates of the replicated colonies. Colonies whose replica fail to grow are auxotrophic mutants. Determine the frequency of auxotrophic mutants obtained wiht respect to the survivors. With toothpicks, take the putative auxotrophic colonies, make patterns on YPD plates using the provided grid. Incubate the plates for 1 day at 37°C.
Complementation groups.- Instructors will provide recessive leucine and uracil mutants streaked on a YPD plate.
Stamp the plate on a velvet placed on a replica block.
Lift the plate. Turn the plate 90 ° and stamp again on the same velvet. Crosses between strains will take place (See figure 1).

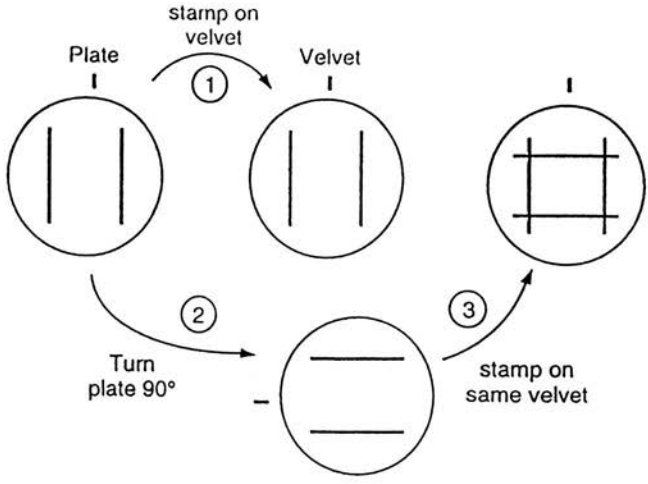

Figure 1

Day 6

Replica plate the master plate containing the mutants obtained (YPD plate of Day 5) to a set of SD plates containing several amino acids and bases numbered 1 to 9. As control use also SD and SC plates.

Complementation groups.- Replica plate the crosses on ME to a SC(-ura) plate or a SC(-leu) plateto check the resultant phenotype.

Day 7

Determine the auxotrophies of each mutant.

Day 9

Determine the complementation groups.

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Gleeson, M.A and Sudbery,P.E. (1988). The methylotrophic yeasts. Yeast, 4, 1-15.

Gleeson, M.A and Sudbery,P.E. (1988). Genetic analysis in the methylotrophic yeast *Hansenula polymorpha*. Yeast, 4, 293-303.

Strains and culture media used in the practical OBTENTION OF AUXOTROPHIC MUTANTS OF *Hansenula polymorpha*

Strain

H. polymorpha NCYC 495

Media

(Solid media have 1.5 % agar)

ME (mating and sporulation plates): 2 % malt extract .

YPD : 1%yeast extract ,2% bacto peptone, 2%, glucose.

SD (Synthetic minimal media) : 0.67 %yeast nitrogen base without amino acids, 2% glucose.

SC (Synthetic complete medium): 0.67 % yeast nitrogen base without amino acids,2 % glucose and all amino acids and bases (see table 1 for concentrations of requirements).

The media to characterize the auxotrophic mutants are based on SD medium supplemented with the amino acid and bases indicated in the introduction (SD1 corresponds to column 1, SD2 to column 2 etc). The concentration of the requirements indicated in the following table are taken, with modifications, from Sherman (1991).

Requirement	Final concentration (µg/ml)
Adenine (hemisulfate salt)	40
Guanine	40
Uracil	20
L-Alanine	100
L-Arginine (HCl)	20
L-Aspartic acid	100
L-Cysteine	20
L-Glutamic acid (monosodium salt)	100
L-Histidine	20
L-Isoleucine	60
L-Leucine	60
L-Lysine (mono-HCl)	30
L-Methionine	20
L-Phenylalanine	50
L-Proline	100
L-Serine	375
L-Threonine	200
L-Tryptophan	40
L-Tyrosine	30
L-Valine	150

Tetrad analysis of *Schizosaccharomyces pombe*

Aim:

To learn basic skills of classical *Schizosaccharomyces pombe* genetics

Introduction

Schizosaccharomyces pombe is a yeast that divides by fission, not by budding like most yeasts. It exists in two different mating types named h^+ and h^-. The usual state of *S.pombe* is the haploid one; heterozygous h^+/h^- diploids are unstable unless special precautions are taken. However in many experimental situations it is necessary to use diploid strains, for example to determine if a new mutation is recessive or dominant or to analyze if a mutation segregates as a single character or not.

There are several ways to maintain diploids: one is to use mutants that are unable to enter meiosis and sporulate. It is clear that these diploids cannot be used to study segregation of mutations. Another possibility is to use parental strains with complementing auxotrophies and maintain them in selective medium. Usually two mutations that are close in the chromosome are utilized to avoid recombination between them. Two mutant alleles of the *ade6* locus (*ade6-M210 and ade6-M216)* are widely used. Also growth on yeast extract medium inhibits sporulation and diploids can be maintained for some days. However as a rule diploids should be prepared anew when needed . Sporulation of diploids shall be always checked.

After sporulation tetrad analysis may be carried out. Tetrad analysis is the most powerful method for most genetic analyses. If a mutation is monogenic two of the spores of an ascus will exhibit the phenotype and the two others not (for more details refer to the lectures on Classical Genetics). Sporulation is induced in *S. pombe* in a medium rich in glucose and poor in nitrogen sources (a difference with *S. cerevisiae* where glucose inhibits sporulation). Asci will lyse spontaneously when they are placed in an adequate medium (another difference with *S. cerevisiae* where tetrad analysis is preceeded by a digestion of the ascus wall with appropriate lytic enzymes).

Practical:

In this practical we will isolate *S. pombe* diploids, sporulate them and micromanipulate the spores. (Due to time constraints the sporulated diploids will not be the ones prepared by the students but will be provided by the instructors).

Formation of diploids: Since actively growing cells perform better in crosses, it is important to set up fresh inocula the night before crossing.

We will use in this experiment two haploid strains carrying the mutations *ade6-M210* and *ade6-M216* and will select diploids in a minimal medium without adenine.

Day 1
Streak parent strains on rich medium (YEGlu+ supplements) and incubate at 30 ºC overnight

Day 2.
Mix with a sterile toothpick both parent strains on rich medium (YEGlu+ supplements). Incubate at 25-30ºC. (Avoid higher temperatures as *S. pombe* will not mate over 32ºC).

Day 3.
From the mating mixture streak to isolate individual colonies on a plate of minimal medium with all the requirements except adenine. Incubate at 30ºC for three days.

Day 6.
Take one colony, suspend it in 100 µl water and spread on a plate with the same medium to have abundant biomass. Incubate as before.

Day 8
Take a loopful of cells and streak on sporulation medium. Incubate at 30 ºC.

Day 11
Check the appearance of spores under the microscope.
Proceed to micromanipulate. Streak the diploid once along a line marked on the bottom of a plate. Place the plate in a microscope fitted with a micromanipulator and move individual asci to fixed positions in a vertical line (see figure). (Instructors will show the use of the micromanipulator). Try to place about 10 asci per plate .
Incubate the plate at 37ºC for 4 hours. Place the plate in the same position in the micromanipulator and find the asci. Once found check that they have lysed and released all four spores. Move each spore with the needle to a different position along the horizontal line so that all spores from one ascus remain in the same line (see figure).
Incubate three days at 30ºC. The spores should have grown into colonies .

Note: The calendar is given to micromanipulate the spores obtained from the diploids isolated by the students. However this will not be possible due to the time schedule. Instead the instructors will provide sporulated diploids and micromanipulation will be done in the appropriate course days.

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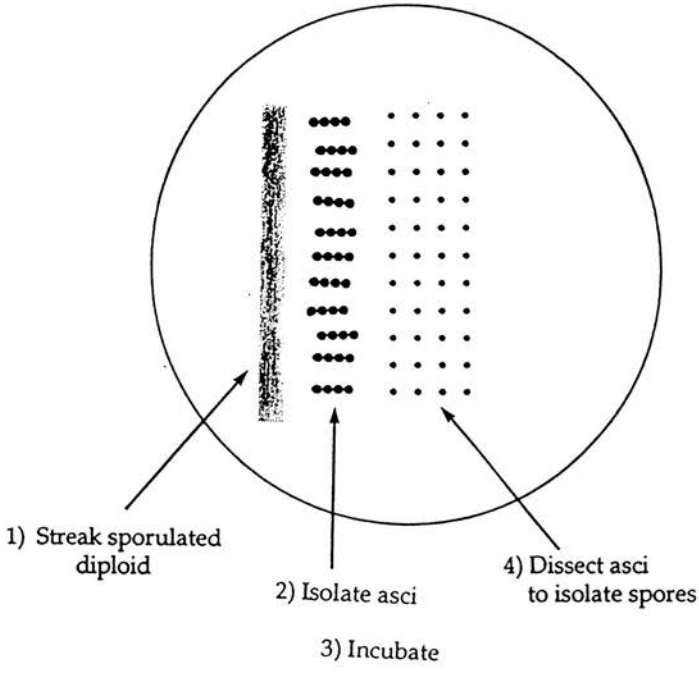

Diagram showing the process of micromanipulation in *S. pombe*.- First, a streak with the sporulated diploid is done on a plate with the adequate medium. Then, asci are moved with the needle of the micromanipulator and placed in an ordered array. After appropriate incubation the asci would have liberated the spores that are separated and placed orderly near the position where the initial ascus was placed

Strains and culture media used in the practical
TETRAD ANALYSIS OF *Schizosaccharomyces pombe*

Strain

S. pombe h⁻ ade6-M210 ura4D18 leu1-32 his3 d2
S. pombe h⁺ade6-M216 ura4D18 leu1-32 his3 d2

Media

(Solid media have 1.5 % agar)

Rich medium: YEGlu (with supplements).
2% glucose, 1% yeast extract. Heat sterilise. Add 100 µg/ml of the appropriate requirements filter sterilized.

Minimal medium: FYNBGlu
2% Glucose, YNB (see below), Mineral salts, Vitamins. Requirements at 100 µg/ml .

Sporulation medium: 1% glucose, 0.1 % KH_2PO_4. Add vitamins and the adequate requirements at 100 µg/ml .

YNB 10X: (For 400 ml) 20 g yeast nitrogen base w/o ammonium and aminoacids, 5% ammonium sulfate.

Mineral salts 50X: (For 100 ml). Sodium acetate 5 g, KCl 5 g, citric acid 5 mg . Sterilise by filtration.
Vitamins 1000X : Biotin 2 mg/l,Calcium pantotenate 200 mg/l, nicotinic acid 2 g/l, m-inositol 2 g/l. Sterilise by filtration.

The killer system in *Kluyveromyces lactis.*

Aim:

To detect the killer activity of certain *Kluyveromyces* strains and to isolate linear killer plasmids

INTRODUCTION

Certain strains of yeasts secrete a toxin that is lethal for other strains. These strains are called killer strains and are themselves immune agaist the toxin they produce. The killer phenotype has been extensively studied in *Saccharomyces cerevisiae* and with certain detail in *Kluyveromyces lactis*. In this last organism the killer phenotype is determined by the presence of two particular doubled stranded DNA plasmids termed pGKL1 and pGKL2. This is a difference with *S. cerevisiae* where the plasmids are double stranded RNA. These plasmids were first found in *K. lactis* strains IFO1267 (NRRL Y1140,CBS 2359). The two plasmids are present simultaneously. Plasmid pGKL1 encodes the killer toxin protein to which most of the *K. lactis* strains are sensible. Plasmid pGKL2 is necessary for maintenance of plasmid pGKL1.
Killer activity is easily visualized on plates. When spotted over a lawn of sensitive cells the killer strains produce a halo of inhibition of growth. The toxin of *K.lactis* kills sensitive cells of various yeast species.

PRACTICAL

Killer assay.
Day 1.
In this experiment two *K. lactis* strains will be tested for their killer activity.
You will receive:
Liquid cultures on YPD of yeasts belonging to different genera.
One plate with the strains of *K. lactis* to be tested for their killer activity .
Plates of YPD.
Procedure:
- Spread evenly 0.2 ml of each liquid culture on YPgalactose plates and allow to dry at 30° C for two to three hours.

- With a sterile toothpick take from the *K. lactis* plate a small quantity of the strain to be tested for its killer phenotype and suspend it in 200 µl water. Dilute this suspension 10, 50 and 100 times in water (20 µl to 200 µl , 10 µl to 500 µl, 10 µl to 1000µl) . Drop 5µl of each diluton separatedly on the plates inoculated previously.
- Incubate at 30ºC. Check the plates for growth the succesive days and score the results .

Detection of linear killer plasmids in colony lisates of *K. lactis* .

Day1.

In this experiment we will try to detect killer plasmids of *K. lactis* on mini-lysates of different colonies. You will receive two plates with isolated colonies of two different strains of *K. lactis*.

With sterile toothpicks take colonies from each plate and suspend each colony in 20 µl of 5XTE (label them to recognize from which plate they originated).

Add 3µl of the Zymolyase solution vortex very briefly and incubate at 37º C for an hour.

After this time add 2µl SDS solution and 5µl of proteinase K solution, vortex and incubate at 60-65º C for one hour.

Add 5µl of stop solution and centrifuge 1 minute in a microcentrifuge at maximal speed.

Carefully remove 20µl of supernatant without removing the pellet.

Load 15 µl in a 0.8% agarose gel and allow the electrophoresis to proceed.

Visualize the gel under UV light. The linear plasmids shall give characteristic sharp thin bands. Identify the corresponding strains.

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Wesolowski-Louvel M., Breunig K.D. and Fukuhara H. in Nonconventional Yeasts in Biotechnology. A handbook pp139-202 (1996) Ed by K. Wolf. Springer Verlag.

Strains and culture media used in the practical
THE KILLER SYSTEM IN *Kluyveromyces lactis*

Strains

Kluyveromyces lactis 2359/152 *Matα metA1* K^+

Kluyveromyces lactis 2360/7 *Matα lysA1*

Media

(Solid media have 1.5 % agar)

YP galactose: 1% yeast extract, 2%peptone, 2%galactose, adjusted at ph4.5 with 50 mM sodium phosphate.

Solutions

5XTE :(50 mM Tris-HCl,5 mM EDTA pH7.5)

Zymolyase 20T (Kirin Breweries, Tokyo): Stock solution 1mg/ml stored frozen in small fractions, do not freeze and defreeze)

5% SDS (sodium dodecylsulfate).

Proteinase K (Boehringer): Stock solution 2mg/ml stored frozen in small fractions, do not freeze and defreeze.

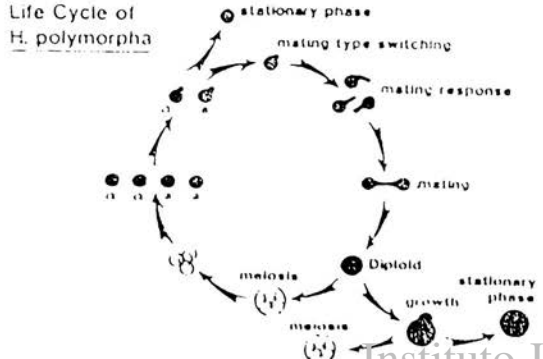
Stop solution : This is the usual electrophoresis stop solution (0,125 M EDTA, 25% glycerol, 0.12% Bromophenol blue, 2% N-lauryl sarcosine and 2 mg/ml RNAse).

Life Cycle of *Hansenula polymorpha*

Aim:
To construct diploids of *Hansenula polymorpha*/ To observe conjugation tubes/ To sporulate diploids/ To perform random spore analysis/ Eventually to try tetrad dissection

INTRODUCTION

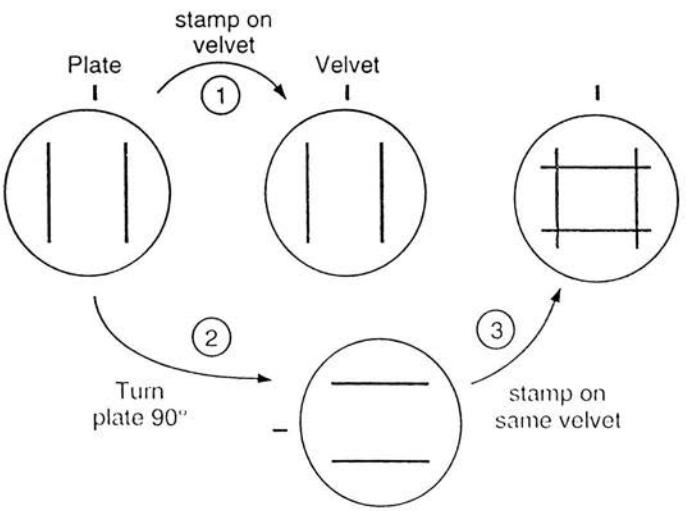
Hansenula polymorpha is an homothallic yeast that can exist in the diploid or haploid state. Mating takes place only between cells of opposite mating types (designated a and α). The high frequency of mating type switch leads to mixed populations of α and a cells. Since mating switch is repressed during growth in rich media, an actively growing culture remains usually in the haploid state. Upon depletion of the medium or transfer to a restrictive one, mating switch occurs, conjugation tubes develop, mating takes place and zygotes are formed. The zygote can be stably maintained in a diploid vegetative state if it is allowed to grow in a rich medium. However, if conditions do not permit the zygote to develop vegetatively, meiosis and spore formation will occur. Sporulation of a diploid yields an ascus with two to four haploid hat-shaped spores which may germinate to produce haploid cells.
In the laboratory diploids are forced to sporulate by incubation on malt extract medium (ME) at 28°C. Spores are usually seen after 4-5 days incubation. The appearance of a pink-red color in the medium indicates normally a high proportion of sporulation. Sporulated cultures can be stored at 4°C up to 2 weeks without apreciably loss of viability.



PRACTICAL

Day 1
Streak *H.polymorpha* strains 1) *leu1-1 ura3 YNR1* and 2) *leu1-1 ura3 ynr1::URA3* on a YPD plate. Incubate overnight at 37°C.

Day 2
Crosses.-
Stamp the plate on a velvet placed on a replica block.
Lift the plate. Turn the plate 90 ° and stamp again on the same velvet.
Replica plate the stamped cells on a ME plate. (Bear in mind that crosses between the same strain, i.e 1 and 1 , or 2 and 2 will also occur and diploid formation in these crosses can be observed under the microscope. However, selection of these diploids cannot be carried out in selective medium since the diploid possesses the same phenotype as the parental haploids).
After 8 hours observe under the microscope the appearance of conjugational tubes and the formation of zygotes.
Incubate the cells 1 day at 30°C.



Day 3
Observe under the microscope the cells in the cross area and outside it. Small and large cells along with some four spores asci can be observed in both cases. The ones observed in the area outside the cross arise as a consequence of the high frequence of a to α switching (This switching also occurs in the cross area). Big cells colonies correspond to diploids and form dull white colonies while small cells derive from haploids and form glossy white colonies.
Diploid selection-. Replicaplate the ME plate on a SDN+leu plate. The strain *leu1-1 ura3 ynr1::URA3* does not grow in nitrate and the *leu1-1 ura3 YNR1* does not grow in the absence of uracil, while the diploids grow in the medium used. Incubate plates 3 days a 37°C

Day 6
Sporulation.
Usually the diploids are repurified after isolation in selective medium to have a fair amount of biomass and to eliminate any haploid contamination. However, due to the tight time schedule, instructors will provide purified diploids.
Make a heavy streak of the provided diploids on ME medium and incubate a 28° C. Spores should appear in 4-5 days.
Follow the appearance of spores under the microscope after 4-5 days of incubation at 28° C. High frequency of sporulation is indicated by the development of a pink-red color in the medium.

Day 9
Observe the spores under the microscope.

Random spore analysis
(Day 1)
The success of this method depends on an effective selection for the haploid spores against the non-sporulated diploids. It has been observed that vegetative cells are more sensitive than spores to exposure to diethyl ether. When a mixed population of vegetative cells and spores is exposed to ether vapours, the unsporulated cells die and only the spores survive (although a proportion of these will also be killed).
A sporulated culture and a culture of vegetative diploids will be provided
Prepare suspensions of sporulated and non-sporulated diploids in 5 ml of sterile distilled water. Dilute them to reach an OD_{660} of about 1. Make 10^{-5} and 10^{-6} dilutions of each suspension and plate 0.1 ml of each on YPD plates

(**WARNING**: Ether is highly flammable. Keep away from sources of ignition).
Take 1 ml of each suspension of OD_{660} of about 1, add 1 ml of diethyl ether to each of them, seal the tube and incubate at room temperature with occasional stirring . Take samples of 100 µl at 10 min intervals along 1 hour and plate on a YPD plate. At a survival rate of 0.1 % or less it can be assumed that all colonies originate from spores.

(Day 3)
Compare survival rate of the sporulated and the vegetative cultures after ether treatment. If all the vegetative diploids are killed after the diethyl ether treatment it can be assumed that any unsporulated diploid in the sporulated culture will have also been killed.
Transfer 100 colonies with sterile toothpicks from a time treatment giving a survival rate lesser than 0.1 % to SDA(+leu+ura) SDA(+leu) and SDN(+leu+ura) plates using the provided grid.

(Day 5)
Score the distribution of the*URA3* marker.

[**Tetrad dissection**. Tetrad dissection is not easy in *Hansenula*. The spores remain attached to each other by some thread like structure making isolation of single spores very difficult (P. Sudbery, personal communication). If time allows, we may take profit from the Singer micromanipulator and try to dissect some tetrads. If tetrad dissection is possible the procedure will be explained in the lab.]

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Gleeson M.A and Sudbery,P.E. (1988). Genetic analysis in the methylotrophic yeast *Hansenula polymorpha*. Yeast, 4, 293-303.

Strains and culture media used in the practical
LIFE CYCLE OF THE YEAST *Hansenula polymorpha*

Strains
H. polymorpha leu1-1 ura3 YNR1
H. polymorpha leu1-1 ura3 ynr1::URA3 (incapable of growing in nitrate) Both strains derived from *H. polymorpha NCYC 495* .
Diploid *H. polymorpha leu1-1 ura3 YNR1 / leu1-1 ura3 ynr1::URA3* (*YNR1* nitrate reductase encoding gene)

Media
(Solid media have 1.5 % agar)
YPD: 1% yeast extract , 2% peptone, 2% glucose.
ME: malt extract 2% .
SDN+leu: 0.17% yeast nitrogen base without amino acids and ammonium sulphate,1mM sodium nitrate, 2% glucose supplemented with 7 mg/l leucine .
SDN+leu+ura: 0.17% yeast nitrogen base without amino acids and ammonium sulphate, 1 mM sodium nitrate, 2% glucose supplemented with 7 mg/l leucine and uracil.
SDA+leu+ura: 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 1 mM ammonium sulfate, glucose 2% supplemented with 7 mg/l leucine and uracil.
SDA+leu: 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 1 mM ammonium sulfate, glucose 2% supplemented with 7 mg/l leucine.

Transformation by electroporation of *Hansenula polymorpha*

Aim:

Transformation of *H. polymorpha* by replicative plasmids using the *Saccharomyces cerevisiae LEU2* gene as *ARS* and the homologous *HARS1*.
Gene disruption of *YNR1* gene encoding nitrate reductase using two different approaches

Introduction

Electroporation is a physical process that transiently creates pores in the membranes of cells allowing them to the take up macromolecules like DNA or proteins. Following closure of the pores the material taken remains in the cells. Critical parameters in the process are the field strength and the time constant. The field strength is the initial voltage set across the electrodes in the cuvette. The time constant is the time necessary for the initial peak voltage to decay to about 37%. Both parameters need to be optimized for each cell type and are not directly transferable from apparatus to apparatus. The cells used in electroporation are rendered competent by the treatment described below:
Overnight cultures of *H. polymorpha* grown in YPD medium at 37° C are diluted 100x in 200 ml of fresh, prewarmed, YPD medium and grown to an optical density (OD) at 660 nm of 1.2-1.5 (about $9x10^7$ cells/ml).Cells are harvested by centrifugation at 4° C at 3000xg for 10 min. and resuspended in 40 ml of 50 mM potassium phosphate pH 7.5, 25 mM dithiotreitol and incubated for 15 min. at 37°C. After this step the cells have to be maintained on ice and the buffers used have to be at 4° C. Subsequently, the cells are washed twice with elecropermeabilization buffer (270 mM sucrose, 10 mM Tris-HCl pH 7.5, 1 mM MgCl2); first with 200 ml, then with 100 ml. Finally the cells are resuspended in 1 ml of the same cold buffer to give approximately 2×10^{10} cells/ml. At this stage the cells are ready to be transformed, 60 µl of the cells suspension are enough to perform the transformation.

TRANSFORMATION WITH REPLICATIVE PLASMIDS

The autonomous replication sequence *HARS1* from *H. polymorpha* and the *LEU2* gene from*S. cerevisiae* allow unstable plasmid replication with a loss of 99% after 10 generations on non selective medium. The 2μ DNA of *S. cerevisiae* does not replicate in *H. polymorpha*. Two autonomous replication sequences from *H. polymorpha HARS1* and *HARS2* have been cloned and support 30-40 plasmids copy per cell. All the replicative vectors are mitotically unstable on non-selective medium. However, after several generations in selective medium these plasmids tend to form tandem multimers with up to 100 copies with high mitotic stability probably due to the integration into the genome

GENE DISRUPTION

Gene disruption can be performed in *H. polymorpha* in the same way as that reported for *S. cerevisiae*; however, the high rate of non-homologous recombination in *H.polymorpha* leads to a very low frequency of targeted integration. There are two ways to disrupt one gene in the genome: the one step gene disruption or the internal fragment disruption (Figure 1) .

The one step gene disruption uses a piece of DNA in which the gene has been disrupted with a selectable marker. The marker is flanked by DNA regions homologous to the chromosomal DNA locus to which the interruption is targeted. The efficiency of the disruption depends on length of the homologous regions flanking the DNA used to disrupt the original gene. In this practical the dependence of the targeted integration frequency with the length of the homologous regions flanking the selective marker will be tested. One step gene disruption results in a genetically stable disruption since no direct repeats are left flanking the insertion site (Figure 1, I) .

An alternative method to disrupt a gene is to use a gene internal fragment cloned into an integrative plasmid. The homologous recombination between the internal fragment in the plasmid and the chromosome creates a disruption because, after integration, the two copies of the gene flanking the plasmid sequence are not full length, one is truncated at the 5' end and the other at the 3' region (Figure 1, II).

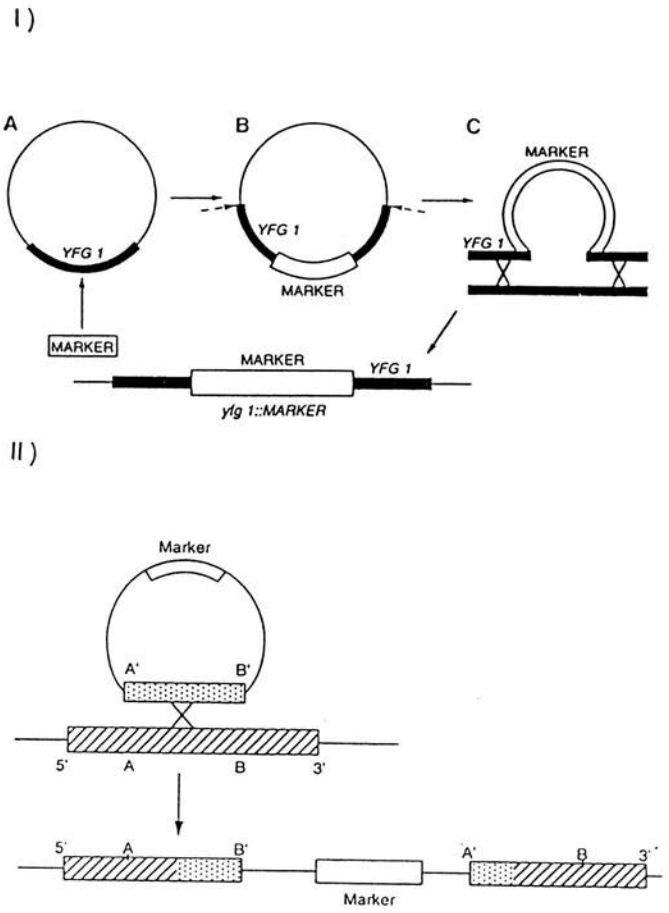

Figure 1.- Gene disruption.
I) One step gene disruption: A) A selectable marker is cloned within the gene *YFG1* to be disrupted . B) The disrupted gene is excised from the plasmid. C) The liberated fragment is used to transform yeast. The homologous ends recombine with the chromosome producing a chromosomal gene replacement (D).
II) Internal fragment disruption. An internal fragment of the gene to be disrupted (dotted box) is cloned into an integative plasmid carrying a selectable marker. Homologous recombination with the corresponding chromosomal locus results in a duplication of the gene with mutated 5´ and 3´ ends none of which is functional.

PRACTICAL

Electroporation (The procedure is the same in all cases considered in the practical).

Pre-chill electroporation cuvettes (2 mm electrode distance) on ice.

Mix 60 µl of competent cells with the adequate amount of DNA in a volume no higher than 5 µl. (The DNA must be free of salts)

Transfer the mixture to the cuvette and tap to the bottom, dry the cuvette externally with a cellulose tissue and apply the electric pulse. (Each electroporator has its own parameters that need to be established for the specific needs. For an Electro Cell Manipulator 600 (ECM600) from Biotechnologies and Experimental Research Inc. the conditions are 7.5 KV/cm 50 µF and 129 ohms , that result in a pulse length of about 5 msec).

After the electric pulse add immediately 1 ml of YPD medium at room temperature to the cuvette, transfer the mix to an Eppendorf tube and incubate for 1 hr at 37ºC without shaking

Centrifuge 20 sec at top speed in a microcentrifuge. Wash the cells once with 1 ml of the same medium as that in which they will be plated and resuspended then in 100 µl of that medium.

Plate the suspension on adequate selective plates. Incubate at 37ºC and look for the appearance of transformants.(usually 3-4 days).

TRANSFORMATION WITH A REPLICATIVE PLASMID

Plasmids pET1 carrying the *LEU2* gene from *S.cerevisiae* and pXYZ carrying the *H. polymorpha HARS1* will be used (see figure 2). Each group will perform a transformation using a certain amount of DNA from each plasmid as shown in the table below (Controls without DNA are marked in the table as no)

Group	1	2	3	4	5	6	7	8
DNA(µg)								
Plasmid pET1	no	0.001	0.01	0.1	1	0.01	0.1	no
Plasmid pXYZ	1	0.1	0.1	no	0.001	1	0.01	1

In an Eppendorf tube pipette 60 µl of *H. polymorpha leu1-1,ura3* electrocompetent cells and add the appropriate plasmid DNA. Electroporate and proceed as described above. After the incubation in YPD the cells are suspended in SC(-leu).

Plate 200 µl of the transformations done without DNA, with 0.001 and 0.01 µg and 100 µl of the other ones on SC(-Leu) plates.

GENE DISRUPTION

We will disrupt the *YNR1* gene encoding nitrate reductase. This disruption produces cells unable to grow in nitrate. The one step gene disruption and the internal fragment disruption methods will be used.

ONE STEP GENE DISRUPTION

The *YNR1* gene was disrupted with the gene *URA3* from *H. polymorpha*. (See Figure 2, B). To asses the effect of the length of the region flanking the marker (*URA3*) on targeted replacements, a set of constructs with different lengths of homologous flanking region to the *YNR1* gene will be used.

Constructs	5´flanking region	3´flanking region
1	1014	999
2	499	509
3	199	224
4	95	99
5	49	49
6	24	24

Electrotransformation is carried out as described above using 1µg of DNA.

Groups will use the constructs as follows:

Group	1	2	3	4	5	6	7	8
Constructs	1/6	2/5	3/4	1/5	2/6	3/4	4/1	5/6

Transformants are selected on SC(-ura)

A number of transformants are replicaplated onto SDA+leu and SDN+leu The cells unable to grow on nitrate (SDN plate) but growing on ammonia (SDA plate) contain the *YNR1* gene disrupted. Further characterization of the disruption could be done by testing the nitrate reductase activity, Southern blot analysis or PCR on the disrupted *YNR1* gene locus. (This will not be done in the course).

INTERNAL FRAGMENT DISRUPTION

The plasmid pGP20 based in pBluescript (Stratagene) is used (Figure 2,C). pGP20 contains *ca.* 1.500 bp internal fragment corresponding to the central region of the *YNR1* gene and carries the *URA3* gene from *H. polymorpha* . The plasmid is linearized at the single *NsiI* site situated in the *YNR1* region and used to transform *H.polymorpha*.

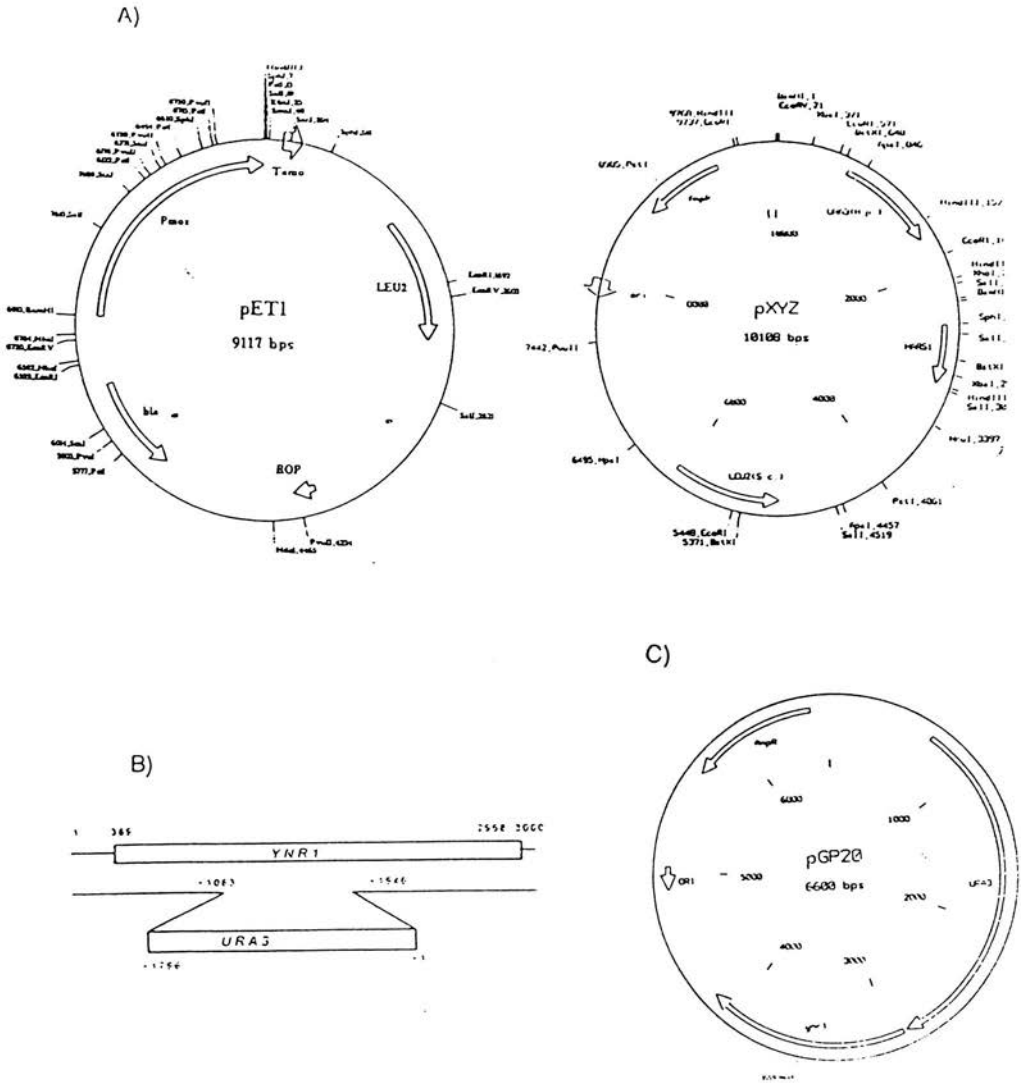

Figure 2.- A) Plasmid pET1 carries the *LEU2* gene from *S. cerevisiae* that has *ARS* function in *Hansenula* .Plasmid pXYZ carries *Hansenula HARS1*.
B) Coordinates of the disruption of the *YNR1* gene by the homologous *URA3* marker.
C) Plasmid pGP20 is an integrative plasmid that carries an internal fragment of the *YNR1* gene from *H. polymorpha*.

Electrotransformation is carried out as described using 1µg of DNA. To select transformants with the disruption of *YNR1*, the same procedure used in the one step gene disruption part will be used.

Day 1
Transformations with replicative plasmids pET1 and pXYZ and gene disruption (one step gene disruption with the different constructs and internal fragment disruption with plasmid pGP20).

Day 4
With toothpicks transfer 50 colonies corresponding to ONE STEP GENE DISRUPTION and INTERNAL FRAGMENT DISRUPTION to SDA(+leu) and SDN(+leu) containing ammonium and nitrate as sole nitrogen source respectively.

Day 6
Score the transformants obtained in the transformation with the replicative plasmids. Compare the frequency of transformation obtained depending on the type of ARS in the plasmid.
Score the number of transformants bearing the *YNR1* gene disrupted originated in the ONE STEP GENE DISRUPTION and INTERNAL FRAGMENT DISRUPTION transformations.

REFERENCES

Faber, N.C., Haima,P. Harder,W. Veenhuis, M. and Geert, AB. (1994). Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*. Current Genetics, 25, 305-310.
Rothstein, R. (1983) Targeting, Disruption, Replacement, and Allele Rescue : Integrative DNA Transformation in Yeast. Methods in Enzymology194, 281-301.
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Strains and culture media used in the practical
TRANSFORMATION BY ELECTROPORATION OF THE YEAST *Hansenula polymorpha*

Strains
Hansenula polymorpha leu1-1 ura3 derived from *H.polymorpha* NCYC 495.

Culture media
(Solid media have the same composition plus 1.5 % agar. To select the transformants bearing the disrupted *YNR1* gene a high quality agar is recommended))

YPD: 1% yeast extract, 2% peptone and 2% glucose
SC(-leu): synthetic complete medium: 0.67% yeast nitrogen base w/o amino acids, 2 % glucose and all the amino acids an bases except leucine .
SC(-ura): synthetic complete medium:0.67% yeast nitrogen base w/o aminoacids, 2 % glucose plus all the amino acids and bases except uracil .
SDA(+ leu): synthetic minimal medium plus ammonium sulfate as nitrogen source: 0.17% yeast nitrogen base w/o amino acids, 1mM ammonium sulfate as nitrogen source, 2% glucose plus leucine (7 mg/ml) .
SDN(+leu): synthetic minimal medium plus nitrate as nitrogen source: 0.17% yeast nitrogen base w/o acids, 1 mM sodium nitrate as nitrogen source, 2% glucose plus leucine (7 mg/ml) .

Disruption of the *LYS1* Gene Encoding Homocitrate Synthase in *Yarrowia lipolytica*.

Aim:

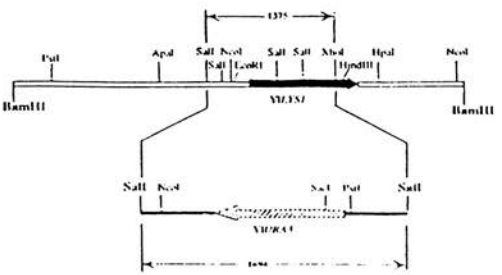
To demonstrate gene disruption in *Yarrowia lipolytica*

INTRODUCTION

Gene disruption in Y. lipolytica can be performed in the same way as in other yeasts. As indicated in the practical "Transformation by electroporation of the yeast *Hansenula polymorpha*" the one step gene disruption uses a piece of DNA in which the gene has been disrupted with a selectable marker. The marker is flanked by DNA regions homologous to the chromosomal DNA locus to which the interruption is targeted. In Y. lipolytica the best results are obtained when the homologous regions flanking the marker are about 500 to 1000 bp, however good results are obtained with one of them being 500 to 1000 and the other as shorter as 150 bp.

THE CONSTRUCT

The *LYS1* gene from *Y. lipolytica* was disrupted by insertion of the homologous *URA3* gene. The insertion replaces a 1375 bp fragment from of the *LYS1* gene ORF with a 1694 bp fragment containing the entire *URA3* gene. A digestion of the construct with *ApaI* and *BamHI* releases a disruption cassette consisting of the *URA3* gene flanked by regions of the *LYS 1* gene of 561 bp at its 5´ end and 1489 at its 3´end. This linear molecule will be used to transform *Y.lypolitica* P01a to uracil prototrophy.



PRACTICAL

Day 1

PREPARATION OF COMPETENT CELLS FOR TRANSFORMATION USING THE LITHIUM ACETATE/LITHIUM CHLORIDE METHOD.

(Will be done with the assistance of the instructors)

Inoculate in the morning one loop of cells in a 100 ml erlenmeyer flask containing 5 ml of YPD buffered to pH 4 with 50 mM citric acid-Na citrate. Shake at 28ºC. About 8 hours later determine the cell number in the culture by counting in a haemocytometer. Take an adequate amount of cells of the preculture to inoculate a 250 ml erlenmeyer flask containing 10 ml on YPD pH 4.0 to give an initial cell density of 10^5 cells/ml. Grow overnight at 28ºC with shaking.

Day 2

Harvest the culture next morning between $9x10^7$ and $1.5x10^8$ cells/ml. This cell density is critical for the succes of transformation!. When proceeding with an unknown strain, it is safe to inoculate three overnight cultures at $5x10^4$, $1x10^5$, and $2x10^5$ cells/ml and to harvest the one nearest the indicated values. Spin at room temperature at about 2000xg and wash twice the cells with 10 ml TE. Resuspend the cells at $5x10^7$ cells/ml in 0.1 M lithium acetate pH 6 and incubate 1 hour with gentle shaking at 28ºC. Spin and resuspend in one tenth of the volume (around $5x10^8$ cells/ml) in lithium acetate pH 6.0.

Storage of competent cells.-Competent cells can be stored for a week at 4ºC, spin and resuspend them in 0.1 M lithium acetate pH 6 at 10^8cells/ml before use.

[Long term conservation of competent cells is achieved by adding 25% glycerol (final concentration) to the cells and freezing at -80ºC. Thaw on ice when needed, and wash once with 1 ml 0.1 M lithium acetate pH 6 before use].

Transformation

The following manipulations are carried out on ice.

Pippette 5 µl of carrier DNA at the bottom of a 5 ml test tube Add 200 ng of transforming DNA in1-5 µl (restriction enzyme buffer usually does not interfere). Add 100 µl of competent cells, mix gently and incubate at 28ºC for 15 min. Add 0.7 ml of 40% PEG 4000 and incubate one hour at 28ºC in a rotary shaker set at about 250 rpm.

Heat shock the mix at 39ºC for 10 min. Add 1.2 ml of 0.1 M lithium acetate buffer pH 6.

Plate very gently 200 µl on SC (-ura)

Day 6
Transformants begin to appear after 2 days The fourth day the colonies are transferred with toothpicks to plates SC(-ura) and SC(-ura -lys). Those cells unable to grow in the absence of lysine contain the *LYS1* gene disrupted.

Some comments on the transformation procedure

pH of the culture.- In the range from pH 6.8 to 3.9 transformation frequency increases with decreasing pH values.
Cell density.- Transformation frequency is ten times higher with a cell density of $5x10^7$ cells/ml during the 1 hour treatment with 0.1 M Li-acetate pH 6
The number of transformants increases in a linear way with the number of cells mixed with the transforming DNA up to 10^8 cells/ml, then an abrupt drop is observed.
Effect of carrier DNA.-Transformation frequency shows a maximum for a carrier DNA size around 0.5 kb. The amount of carrier DNA should be at least 25 µg per tube but may be higher without inconvenience.
PEG treatment.- Stirring during the PEG treatment produces a 2 to 4 fold increase of transformation frequency.
Heat shock.- In our hands the best results was obtained with a treatment of 10 min at 39ºC. A 5 min treatment at 42ºC gave also good results.

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Barth, G. and Gaillardin C. (1996). *Yarrowia lipolytica.* In Non-conventional Yeasts in Biotechnology pp.313-380. Edited by K. Wolf. Berlin .Springer Verlag.
Perez-Campo, F.M, Nicaud, J-M, Gaillardin C. and Domínguez A. (1996). Cloning and sequence of the *LYS1* gene encoding homocitrate sysnthase in the yeast *Yarrowia lipolytica.* Yeast, 12, 1459-1469

Strains and culture media used in the practical
DISRUPTION OF THE *LYS1* GENE ENCODING HOMOCITRATE SYNTHASE IN *Yarrowia lipolytica*

Strains

Yarrowia lipolytica P01a *MatA, leu2-270, ura 3-3020*

Media :

YPD : 1% yeast extract, 1% peptone ,1% glucose, in 50 mM sodium citrate pH 4.
SC(-ura): 0.67% yeast nitrogen base, 1% glucose, supplemented with all aminoacids and bases at 50 mg/liter, except uracil
SC(-ura -lys): 0.67% yeast nitrogen base, 1% glucose, supplemented with all aminoacids and bases at 50 mg/liter, except uracil and lysine.

Reagents

TE: 10 mM Tris-HCl, 1mM EDTA pH 8

0.1 M Li-acetate pH 6 (adjusted with acetic acid)

Carrier DNA .- Salmon sperm DNA, 5 mg/ml in 50 mM Tris, 5 mM EDTA, pH8.0, sonicated to shear it in the 500 bp range.

40% PEG .- A recent solution of 40% PEG in 0.1 M lithium acetate, adjusted to pH 6.0 with acetic acid. (Old solutions become acidic and are not functional in the transformation procedure).

Expression of *Hansenula polymorpha* Nitrate Reductase under the control of the *MOX1* Promoter

AIM:

To study the effect of nitrogen sources on post-transcriptional regulation of nitrate reductase activity

INTRODUCTION

Nitrate reductase, encoded by the *YNR1* gene is the first enzyme in the nitrate assimilatory pathway in *H. polymorpha*. It catalyses the reduction of nitrate to nitrite. In *H. polymorpha* the expression of the *YNR1* gene is induced by nitrate and repressed by ammonia and other reduced nitrogen sources

The yeast *H. polymorpha* is able to use methanol as carbon source. The first step in methanol metabolism is catalyzed by methanol oxidase encoded by the gene *MOX1* . The promoter of this gene is one of the strongest promoters known in yeasts. The expression of the *MOX1* gene is repressed by glucose, derepressed by glycerol and induced by methanol Due to these features the *MOX1* promoter is used extensively to overexpress genes in *H. polymorpha.*

To express nitrate reductase under *MOX1* control, the ccoding region of the *YNR1* gene including 18 bp from the 5′ non coding region and 100 bp from the 3′ non coding region was cloned at the *Sma I* site of pET1 (see practical "Transformation by electroporation of the yeast *Hansenula polymorpha*") to obtain the plasmid pGPC16. To integrate this plasmid at the chromosomal *MOX1* locus, it was linearized at the *Stu I* site in the *MOX1* promoter before transformation into *H. polymorpha*. In this practical the *H. polymorpha* strain GP100 with genotype *PMOX1::pET1*(*PMOX1-YNR1_tAMO1*) *MOX1 ynr1::URA3* and a wild type strain will be used.(*tAMO1* indicates the terminator of the the gene *AMO1* encoding aminoacid oxidase) Expression of nitrate reductase will be examined in cells grown in YNB glucose and induced for 24 hours in the media indicated below

Groups	1	2	3	4	5	6	7	8
Strain	GP100id		id	id	id	id	WT	WT
Media	1	2	3	4	5	6	1	2

(see composition of media corresponding to each number at the end of the practical protocol)

PRACTICAL

Day 1
Grow overnight a culture of 300 ml of the GP100 strain and other of 100 ml ofthe wild type in YNB glucose ammonium at 37° C with shaking.(Will be done by the instructors).

Day2
Instructors determine OD_{660} nm of both cultures
Groups will receive a volume of the corresponding culture equal to $81/OD_{660}$.
Centrifuge the suspension 5 minutes and resuspend sediment in 15 ml of the appropriate medium (see group distribution above).
Determine OD_{660} of the new suspension (take 300µl to 3 ml of H_2O)
Take a sample of 3 ml and pour it in a tube containing 3 ml of cold distilled water (time=0) and centrifuge top speed for 5 min, discard the supernatant carefully and place the pelleted cells at -20 ºC.
Incubate the remaining suspension at 30 ºC with shaking.
Take a sample at 8 hours . Determine OD_{660} and proceed as before.

Day 3
-Take another sample at 24h Determine OD_{660} and proceed as before

Day X (The cells may be maintained frozen for extended periods of time)
Determine nitrate reductase activity as follows:
In a 5 ml glass tube add 200 µl of cold NRB1 buffer (see below) to about 100 mg of cells and 1 gram of 0.5 mm diameter glass beads. Vortex for about 90 s, maintain the tube for 1 min on ice, add 300 µl of cold NRB1 buffer and vortex again for 90 s. Pass the extract to an Eppendorf tube free of beads and centrifuge 5 min at top speed on a desk centrifuge. Pass the supernatant to an Eppendorf tube, keep it on ice and use it to measure nitrate reductase activity.

In 5 ml tubes pippete the following:

Tube	1	2
crude extract	100µl	200µl
NRB2 buffer	500µl	500µl
H_2O	300µl	200µl

Incubate the mixture 5 min at 30 ºC. Start the reaction by addition of 100 µl of 2 mM NADH and continue incubation for 15 min. The reaction is stopped with the reagents used to determine nitrite. Add 1 ml of reactive A ,N (naphtyl) ethylendiamine and 1 ml of reactive B (sulfanilamide).The nitrite reacts to form azo compounds and their concentration is determined at 540 nm. As reference for calibration use a solution of 1 ml of NRB2 plus 1 ml of reactive A and 1 ml of reactive B.
In a semi-quantitative determination of nitrate reductase activity, it will be assumed that the extracts have about the same protein concentration and the absorbance determined at 540 nm will be referred to OD 660 of the culture used (OD_{540}/OD_{660}).

References

Hansen, H. and Hollenberg, C.P. (1996). *Hansenula polymorpha* (*Pichia angusta*). In Non conventional yeasts in biotechnology pp.293-311. Edited by K. Wolf. Berlin:Springer Verlag.

Strains,culture media and reagents used in the practical
Expression of *Hansenula polymorpha* Nitrate Reductase under the control of the *MOX1* Promoter

Strains.
Hansenula polymorpha NCYC 495, wild type
Hansenula polymorpha, *PMOX1::pET1(PMOX1-YNR1_tAMO1)MOX1 ynr1::URA3*

Media
All media mentioned have 0.17% yeast nitrogen base
(Numbers refer to the table indicating the Group distribution).

Glucose ammonium: 2% glucose, 2mM NH_4Cl (Initial growth medium)
1.- 1 %Glycerol, 2 mM $NaNO_3$.
2.- 0.5% Methanol, 2 mM $NaNO_3$.
3.- 0.5% Methanol.
4.- 4% Glucose, 2 mM $NaNO_3$.
5.- 0.5% Methanol, 2mM NH_4Cl.
6.- 1 %Glycerol, 2mM NH_4Cl.

Reagents

NRB1:0.02 mM FAD,1mM EDTA, O.1 M potassium phosphate pH 7.4
NRB2: 40 mM $NaNO_3$,0.04 mM FAD,100 mM potassium phosphate pH 7.4
Reactive A: 0.02 % N(naphtyl) ethylendiamine.
Reactive B: 1% sulfanilamide in HCl 3 N.

EXPRESSION OF FOREIGN GENES IN *PICHIA PASTORIS*

Aims:

1. To construct strains of *P. pastoris* that secrete human serum albumin (HSA).
2. To analyze strains for HSA secretion by SDS–PAGE.
3. To analyze strains for intracellular expression of β-lactamase.
4. To identify potential HSA secretory strains by colony PCR.

Introduction:

Pichia pastoris is a yeast able to grow on methanol as a sole carbon and energy source. Growth on methanol requires the induction of numerous genes whose products are required for growth on this substrate. One of these genes, *AOX1*, encodes alcohol oxidase, the first enzyme in the methanol-utilization pathway. The *AOX1* promoter has been successfully used to direct expression of numerous foreign proteins in *P. pastoris*. In this practical, the *P. pastoris* expression system will be used to produce a secreted protein, HSA, and an intracellular protein, *Escherichia coli* β-lactamase.

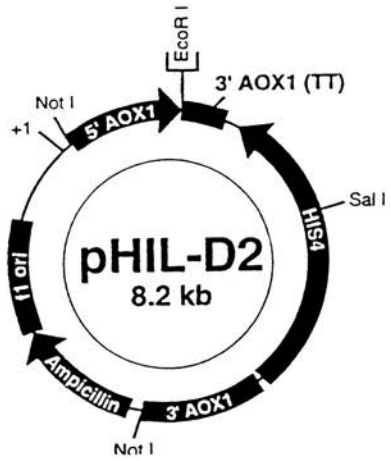
Experimental:

I. CONSTRUCTION OF HSA SECRETORY STRAINS OF *P. PASTORIS*

Day 1 A. Restriction digestion of pHSA413.

1. Digest ~2.0-μg samples of pHSA413 with 5–10 Units of *Sal*I and *Not*I in 100 μL of the appropriate buffer in a 1.5-mL minicentrifuge tube for 1 h at 37°C.

Vector: pHSA413 (*amp*^{r} *PHIS4* P_{AOX1}-*HSA*-t_{AOX1}) (pHILD2 with HSA cDNA inserted at *Eco*RI site)



5′ *AOX1* promoter fragment: bases 14–941
5′ *AOX1* primer site: bases 868–888
*Eco*Ri site: bases 956–961
3′ *AOX1* primer site: bases 1036–1056
3′ *AOX1* transcription termination (TT) fragment: bases 963–1295
HIS4 ORF: bases 4223–1689
3′ *AOX1* fragment: bases 4578–5334
Ampicillin resistance gene: bases 5686–6546
f1 origin of replication: bases 7043–6588
ColE1 origin: bases 7138–7757

Fig. 1. Map of *P. pastoris* vector pHIL-D2, parent vector of pHSA413.

2. Extract each sample with 100 μL of PCA; transfer top aqueous phase to a clean tube, and precipitate DNA with 200 μL of –20°C 100% ethanol. Centrifuge samples at full speed in a minicentrifuge for 15 min at 4°C, decant and air dry for 5 min, and redissolve DNA in 10 μL of TE buffer.
3. Examine 2-μL (~0.2 μg) samples of each cut DNA along with ~0.2 mg of uncut pHSA413 by electrophoresis through a 0.8% agarose gel.

Day 2 B. Electroporation

1. Mix ~0.5 μg of DNA sample in no more than 4 μL total volume of water

or TE buffer in a tube containing 40 µL of frozen or fresh competent cells. Hold mixture on ice for 5 min. Transfer sample to a 2-mm gap electroporation cuvette that has been held on ice for at least 5 min.

2. Pulse cells according to the parameters suggested for yeast by the manufacturer of the specific electroporation instrument being used (for Gene Pulser: 1,500 V, 25 µF, 200 Ω, 7,500 kV/cm, ~5 ms).
3. Immediately add 1 mL of cold 1 M sorbitol, keep one hour on ice and transfer the cuvette contents to a sterile 1.5 mL minicentrifuge tube.
4. Spread 100 µL on one YNB glucose plate. Concentrate the remainder of cells by centrifugation (~2 min at 10,000 rpm), and plate on another YNB glucose Incubate plates at 30°C until His^+ colonies appear (~3 days).

Day 7 C. Screening for Mut^S transformants

Cleavage of pHSA413 by *Not*I generates a DNA fragment containing, in order, (P_{AOX1}-*HSA*-t_{AOX1})-*PHIS4*-3′*AOX1*. Approximately 10% of His^+ cells transformed with this fragment will have undergone a gene replacement or gene "knock-out" event in which the fragment replaces the genomic *AOX1* gene. These transformants must rely on the transcriptionally weak *AOX2* gene for growth on methanol and, as a result, grow slowly on methanol (Mut^S phenotype). To identify gene replacement transformants, pick 50 to 100 colonies from transformation plates using sterile tooth picks or inoculation loop, and streak onto YNB methanol and YNB glucose plates. Incubate plates at 30°C for 2–3 days.

II. SECRETION OF HSA

Objective: Induce expression and secretion of HSA in liquid shake-flask culture and examine cultures samples for HSA by SDS-PAGE.

Procedures

A. Growth and induction of expression

1. Inoculate a single colony of each strain (wild type, GS-HSA #4141 into 100 mL of BMGY medium and grow overnight (15–18 h) with vigorous shaking at 30°C.

Day 1

2. In the morning, determine the OD_{600} and harvest 200 OD_{600} Units (volume × OD_{600}) by centrifugation. Decant supernatant and resuspend cell pellet in 10 mL of BMMY medium (starting OD_{600} ~20). Transfer cultures to a 100-mL sterile shake flask. Harvest 0.75 mL of each culture (t = 0 sample) and place the remainder of the cultures in a 30°C shaking incubator.

or TE buffer in a tube containing 40 µL of frozen or fresh competent cells. Hold mixture on ice for 5 min. Transfer sample to a 2-mm gap electroporation cuvette that has been held on ice for at least 5 min.

2. Pulse cells according to the parameters suggested for yeast by the manufacturer of the specific electroporation instrument being used (for Gene Pulser: 1,500 V, 25 µF, 200 Ω, 7,500 kV/cm, ~5 ms).
3. Immediately add 1 mL of cold 1 M sorbitol, and transfer the cuvette contents to a sterile 1.5-mL minicentrifuge tube.
4. Spread 100 µL on one YNB glucose plate. Concentrate the remainder of cells by centrifugation (~2 min at 10,000 rpm), and plate on another YND. Incubate plates at 30°C until His^+ colonies appear (~3 days).

Day 7 C. Screening for Mut^S transformants

Cleavage of pHSA413 by *Not*I generates a DNA fragment containing, in order, (P_{AOX1}-*HSA*-t_{AOX1})-*PHIS4*-3′*AOX1*. Approximately 10% of His^+ cells transformed with this fragment will have undergone a gene replacement or gene "knock-out" event in which the fragment replaces the genomic *AOX1* gene. These transformants must rely on the transcriptionally weak *AOX2* gene for growth on methanol and, as a result, grow slowly on methanol (Mut^S phenotype). To identify gene replacement transformants, pick 50 to 100 colonies from transformation plates using sterile tooth picks or inoculation loop, and streak onto YNB methanol and YNB glucose plates. Incubate plates at 30°C for 2–3 days.

II. SECRETION OF HSA

Objective: Induce expression and secretion of HSA in liquid shake-flask culture and examine cultures samples for HSA by SDS-PAGE.

Procedures

A. Growth and induction of expression

1. Inoculate a single colony of each strain (wild type, GS-HSA #4141 into 100 mL of BMGY medium and grow overnight (15–18 h) with vigorous shaking at 30°C.

Day 1 2. In the morning, determine the OD_{600} and harvest 200 OD_{600} Units (volume × OD_{600}) by centrifugation. Decant supernatant and resuspend cell pellet in 10 mL of BMMY medium (starting OD_{600} ~20). Transfer cultures to a 100-mL sterile shake flask. Harvest 0.75 mL of each culture (t = 0 sample) and place the remainder of the cultures in a 30°C shaking incubator.

Centrifuge sample in minicentrifuge for 5 min at maximum speed and transfer supernatant to a fresh tube, and store at –70°C (or –20°C) for later analysis.

Day 2+3 3. Add methanol to a final concentration of 0.5% every 24 h.

Day 1–4 4. At time points of t = 12, 24, 36 and 72 h, harvest 0.75 mL of each culture, and store supernatant in freezer as done for the t = 0 samples.

B. Analysis of culture broth samples by SDS-PAGE

Day 4

1. Mix 30 µL of each supernatant sample with 8 µL of loading buffer, and boil samples in a heating block for 5 min, and then centrifuge for 5 min.
2. Load 15 µL of each sample (along with protein molecular weight markers) into wells of SDS-PAGE gel using a Hamilton microliter syringe or other device.
3. Electrophores gels at 60 mA per gel (120 mA for two gels). Stop electrophoresis when tracking dye almost reaches the bottom of the gel (~45 min).
4. Remove the glass plate from the apparatus and place on a paper towel. Using a spatula, pry the plates apart. Place the gel in a micropipette tip box lid or other small container.
5. Stain gel with ~10 mL of Coomassie blue staining solution (enough to cover gel) for 1 h at room temperature with slow agitation.
6. Rinse gel with ~10 mL of destaining solution. Add 10 mL of additional destaining solution, and allow gel to destain for several hours at room temperature with slow agitation, changing destaining solution as needed. Store gel in water.
7. Examine gel for HSA (~66 kD).

Results

With GS-HSA #4141 strain, HSA should begin to appear by ~24 h and continue to increase through 72 h.

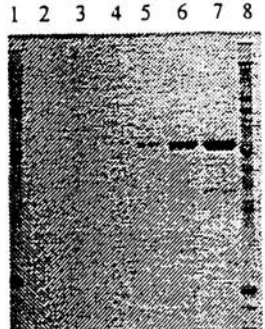


Fig. 2. SDS-PA gel of GS-HSA #4141 samples. Lanes 1 and 8 contain molecular weight markers. Lanes 2–7 contain broth samples harvested at 6, 12, 24, 36, 48, and 72 h after shift to methanol medium (BMMY).

III. INTRACELLULAR EXPRESSION OF β-LACTAMASE

Objective: Observe β-lactamase (β-lac) activity and protein in selected *P. pastoris* β-lac expression strains.

Procedures

A. Growth of β-lac expression strains

1. Inoculate wild-type, GS-HWO18 and GS-HWO19 strains into separate 50-mL cultures of YNB glucose, and grow overnight at 30°C with shaking.

Day 1

2. In the morning, determine the OD_{600} and harvest 80 OD_{600} units of each culture by centrifugation. Decant the supernatant and suspend each cell pellet in 2 mL of YNB medium (no carbon source).
3. Add 1 mL of cells to 150 mL of YNB glucose medium and 1 mL of cells to 150 mL of YNB methanol medium.
4. Determine the OD_{600} of each, and harvest 20 OD_{600} units of each by centrifugation (t = 0). Centrifuge samples and store pellets in minicentrifuge tubes at –70°C for later analysis.
5. Grow cultures at 30°C with shaking.

Day 1+2

6. At times of 8 and 24 h, determine the OD_{600}. Harvest 20 OD_{600} units of each culture, centrifuge, and store cell pellets at –70°C.

B. Preparation of cell-free extracts

Day 2

1. Resuspend cell pellets in 100 µL of ice-cold breaking buffer, and add an equal volume of glass beads to each.
2. Vortex the mixtures 7 times for 30–45 seconds at full speed. Incubate samples on ice for at least 1 min between vortexings.
3. Centrifuge samples for 15 min at full speed in a minicentrifuge at 4°C and keep supernatants (cell-free extracts).

C. Qualitative analysis of β-lac activity levels in extracts

1. For each cell-free extract, add 5 µL of extract to 193 µL of breaking buffer in a minicentrifuge tube. Include a control with 5 µL of breaking buffer instead of extract.
2. Start assay by adding 2 µL of PADAC substrate solution. Mix and incubate at 30°C. β-lac activity is indicated by a change of color from purple to yellow.

Results

All samples from strain GS-HWO19 should contain β-lac activity and protein, since P_{GAP} is a constitutive promoter expressed at high levels in both glucose and methanol. Only samples from methanol-grown cells of GS-HWO18 will contain β-lac, since P_{AOX1} is methanol-inducible but fully repressed by glucose.

IV. ANALYSIS OF TRANSFORMANTS BY COLONY PCR

Objective: Identify transformants that contain an HSA gene by the rapid colony PCR method.

Procedures

Day 1 1. For each sample, transfer ~1/2 colony to a minicentrifuge tube containing 1 mL of sterile water and vortex vigorously.

2. Determine OD_{600} of each sample, and dilute or concentrate to ~100,000 cells/5 µL (1 OD_{600} Unit = 5×10^7 cells).

3. For each sample, add 5 µL of cells to a minicentrifuge tube containing 29 µL water, 5 µL of 10× PCR buffer, 1 µL dNTP mix, and 5 µL of each primer. Include the following controls: GS-HSA #4141 without 5′ primer, GS-HSA #4141 without 3′ primer, GS-HSA #4141 without *Taq* polymerase, GS115, pHSA413 (100 ng/5 µL), pHIL-D2 (100 ng/5µL), no DNA or cells.

4. Place samples in PCR thermocycler and heat to 95°C for 5 min.

5. Add 0.25 µL of *Taq* polymerase to each sample, and overlay with 50 µL of mineral oil. Run the following program: cycle 1, 94°C for 2 min; cycles 2–25, 94°C for 2 min, 53°C for 1 min, 72°C for 3 min; cycle 36, 72°C for 5 min.

Day 2 6. Examine samples by subjecting 10 µL of each to agarose gel electrophoresis.

Results

The following PCR products should be observed: GS115, 2.2-kb *AOX1* fragment; pHIL-D2, 159-bp empty cassette fragment; GS-HSA #4141, 2.2-kb *AOX1* fragment and 1.9-kb *HSA* fragment. Mut^S strains should show only 1.9-kb *HSA* fragment. Note, in strains containing both AOX1 and HSA expression cassette, the 2.2-kb *AOX1* fragment may be underrepresented relative to the smaller *HSA* fragment or not visible at all.

Materials:

Yeast Strains and Vectors

Name	Genotype	Phenotype
Wild Type		His^+ Mut^+
GS115 *his4*		His^- Mut^+
GS-HSA #4136	*his4 aox1Δ::PHIS4* P_{AOX1}-*HSA*-t_{AOX1}	His^+ Mut^S
GS-HSA #4141	*his4::pHSA413* (P_{AOX1}-*HSA*-t_{AOX1})	His^+ Mut^+
GS-HWO18	*his4::pHWO18* (P_{AOX1}-*bla*-t_{AOX1})	His^+ Mut^+
GS-HWO19	*his4::pHWO19* (P_{GAP}-*bla*-t_{AOX1})	His^+ Mut^+
pHSA413	(amp^r *PHIS4* P_{AOX1}-*HSA*-t_{AOX1})	
pHIL-D2	(amp^r *PHIS4* P_{AOX1}-t_{AOX1})	

Culture Media

YPD medium: 1% yeast extract, 2% peptone, 2% dextrose (sterilize by autoclaving).

YNB glucose plates: 2% agar, 0.67% yeast nitrogen base (with NH_4SO_4), 2% dextrose (sterilize by autoclaving).

YNB methanol plates: 2% agar, 0.67% yeast nitrogen base (with NH_4SO_4), 0.5% methanol. (For 500 mL, add 10 g agar and 3.4 g yeast nitrogen base to 450 mL water and autoclave. Let cool in 50°C water bath for at least 1 h. Add 50 mL of 5% methanol and pour plates.)

YNB medium: 0.67% yeast nitrogen base (with ammonium sulfate) (no carbon source).

BMGY medium: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base (with ammonium sulfate), 1% glycerol.

BMMY medium: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base (with ammonium sulfate), 0.5% methanol.

[For 1 L of BMGY BMMY, dissolve 10 g of yeast extract and 20 g of peptone in 700 mL of water and autoclave. Prepare separate 10× stocks of the remaining ingredients (i.e., 1 M potassium phosphate, pH 6.0, 13.4% yeast nitrogen base, 10% glycerol, and 5% methanol). Autoclave potassium phosphate, yeast nitrogen base, and glycerol stocks. Prepare methanol stock by adding 100% methanol to bottle containing sterile water. Do not autoclave methanol solutions.]

Reagents

Restriction enzymes *Sal*I and *Not*I.

10× PCR buffer: 500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C) and 1% Triton X-100, 25 mM $MgCl_2$

100 mM dNTPs (25 mM each)

5′ *AOX1* primer (0.1 µg/µL) (Invitrogen, Carlsbad, CA)

3′ *AOX1* primer (0.1 µg/µL) (Invitrogen, Carlsbad, CA)

Taq polymerase (5 U/µL)

5× Loading buffer for SDS-PAGE samples: 25 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 10% SDS, 0.1% bromophenol blue, 30% glycerol. (5× Loading buffer can be stored at room temperature.)

Electrophoresis buffer for SDS-PAGE: 25 mM Tris, 250 mM glycine (electrophoresis grade), pH 8.3, 0.1% SDS (electrophoresis grade). (A 5× stock can be made by dissolving 15.1 g of Tris base and 94 g of glycine in 900 mL of water. Add 50 mL of 10% solution of SDS and adjust volume to 1 L with water.)

Coomassie blue stain: 0.25 g of Coomassie Brilliant Blue R250 in 90 mL of methanol:water (1:1 v/v) and 10 mL acetic acid. Filter the solution through a Whatman No. 1 filter to remove particulate matter.

Destaining solution: 90 mL of methanol:water (1:1 v/v) and 10 mL of acetic acid.

PADAC substrate: 0.6 mg/mL in 50% DMSO. (PADAC = 7-(thienyl-2-acetamido)-3-[2-(4-N, N-dimethylaminophenylazo)pyridinium-methyl]-3-cephem-4-carboxylic acid and is obtained from Calbiochem. The extinction coefficient at 569 nm is 44.403 $cm^{-1}M^{-1}$.

PART II

LECTURES

GENETICS AS A TOOL FOR THE MANIPULATION OF YEAST. FROM CLASSICAL TO POST-MODERN METHODS

Isabel L. Calderón
Department of Genetics
University of Sevilla. Spain

Genetics is a valuable system of experimentation that allows the *in vivo* exploration of biological processes by analyzing the consequences of new combination of genes and by relating the structure and organization of the genetic material, with its function. In classical genetics, this is done by exploiting the sexual cycle of the organisms and by mutagenesis.

Among the eukaryotes, the yeast *Saccharomyces cerevisiae* has one of the most facile genetics. This is specially true for what can be called "laboratory strains", which were deliberately engineered with this purpose (*S. geneticus*?). The biological features that contribute to its ease of use are: a stable haploid state, the possibility of constructing stable diploid strains by mating and the possibility of inducing meiosis and to analyze the products of single meiosis by tetrad dissection. Furthermore, a long history of modern yeast genetics has produced a large number of useful tools for the molecular biologist. In combination, classical and modern genetic methods have converted *Saccharomyces* in one of the most potent systems in biological research. Unfortunately, other yeast even brewing, baking and wine *S. cerevisiae* strains, despite their industrial or clinical interest, are either not so well suited for the genetic analysis or not so well studied. However, for those experts trying to develop genetics in these organisms, *Saccharomyces* can serve both as a guideline and inspiration.

In these two lectures we will explore the basics of classical genetic manipulation and analysis in *S. cerevisiae* and try to establish similarities and differences with other *Saccharomyces* and with the non-conventional yeasts. We will also present other techniques such as cytoduction, protoplast fusion and haploidization, that may allow the circumvention of the sexual cycle in those cases in which it is not so easy or just impossible to use. Finally we will see how molecular biology has revolutionized this field and how can we take advantage of all these techniques in the resolution of specific problems.

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Biochemistry and physiology of the yeast *Yarrowia lipolytica*

Yarrowia lipolytica is one of the yeasts which can be very easily isolated from foods like cheese and sausage or from substrates containing normal paraffins or polyalcohols. During the time as n-alkanes were cheap and abundant (in the mid 1960s) this yeast has been proved as a organism for single-cell protein projects and as a producer of organic acids like citric acid or 2-ketoglutaric acid.

This species was long time described as *Candida lipolytica* before a sexual state was identified in the late 1960s by Wickerham. The perfect form was reclassified as *Endomycopsis lipolytica* (Wickerham et al., 1970), later as *Saccharomycopsis lipolytica* (Yarrow, 1972), and finally as *Yarrowia lipolytica* (van der Walt and von Arx, 1980).

Y. lipolytica is a heterothallic yeast (mating types A, B) which forms ascospores in a separate ascus. It is naturally dimorphic. Several nutritional conditions are known which can preferentially induce the formation of yeast cell, pseudohyphae or true hyphae. Some genes has been identified which are included in this yeast-hyphae transition.

This yeast seems to be evolutionary far distant from most of the other ascomycetous yeasts which was shown by the unusual structure of rDNA genes, size of snRNA and 7SRNA as well as the low level of similarity of homologous genes to their counterparts.

The utilization of hydrocarbons, fatty acids, alcohols and acetate has been studied at physiological, biochemical, and genetical level. The overproduction of citric and isocitric acid, 2-ketoglutaric acid, and lysine was a long time in the centre of interest for several studies.

Y. lipolytica secretes some enzymes like proteases, phophatases, RNase, lipases, and esterases. Especially the secretion of alkaline protease is well studied, because this enzyme can be secreted in high amounts and its promoter and secretion signal sequence was used for overproduction and excretion of foreign proteins in this yeast.

Classical and Molecular Genetic Manipulation of *Pichia pastoris*

James M. Cregg, Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, P.O. Box 9000, Portland, OR 97291-1000

The methylotrophic yeast *Pichia pastoris* is an important system for the production of foreign proteins and for basic studies on the biogenesis and degradation of peroxisomes (1,2). A significant advantage of *P. pastoris* as an experimental system is the ability to readily bring to bear both classical and molecular genetic approaches to a research problem (3).

Although yeast molecular genetics has introduced new and exciting capabilities, classical genetics remains the approach of choice in many instances. These include: the generation of mutations in previously unidentified genes (mutagenesis); the removal of unwanted secondary mutations (backcrossing); the assignment of mutations to specific genes (complementation analysis); and the construction of strains with new combinations of mutant alleles. To comprehend classical strategies employed with *P. pastoris*, it is first necessary to understand basic features of the life cycle of this yeast. *P. pastoris* is an ascomycetous budding yeast that most commonly exists in a vegetative haploid state. Upon nitrogen limitation, mating occurs and diploid cells are formed. Since cells of the same strain can readily mate with each other, *P. pastoris* is by definition homothallic. However, it is probable that *P. pastoris* has more than one mating type that switches at high frequency and that mating occurs only between haploid cells of the opposite mating type. After mating, the resulting diploid products can be maintained in that state by shifting them to a standard vegetative growth medium. Alternatively, they can be made to proceed through meiosis and to the production of asci containing four haploid spores.

The key feature of the *P. pastoris* life cycle that permits genetic manipulation is its physiological regulation of mating. *P. pastoris* is most stable in its vegetative haploid state, a great advantage in the isolation and phenotypic characterization of mutants. [In wild-type homothallic strains of *Saccharomyces cerevisiae*, the reverse is true: haploid cells are unstable and rapidly mate to form diploids.] To cross *P. pastoris*, selected pairs of complementarily marked parental strains are mixed and subjected to nitrogen limitation for a time period sufficient to initiate mating. The strains are then shifted to a non-limiting medium supplemented with a combination of nutrients that select for growth of hybrid diploid strains and against the growth of the haploid parental strains and self-mated diploid strains. To initiate meiosis and sporulation, diploid strains are simply returned to a nitrogen-limited medium. Because *P. pastoris* spores are small and adhere to one another, tetrad dissection via micromanipulation is difficult. Therefore, spore products are analyzed using a random spore procedure.

The key to the molecular-genetic manipulation of any organism is the ability to introduce and maintain DNA sequences of interest. For *P. pastoris*, the fate of introduced DNAs is generally similar to those described for *S. cerevisiae* (4). Vectors can be maintained as autonomously replicating elements or integrated into the *P. pastoris* genome. Integration events occur primarily by homologous recombination between sequences shared by the transforming vector and *P.*

pastoris genome. Thus the controlled integration of vector sequences at pre-selected positions in the genome via yeast gene targeting and gene replacement (gene knock-out) strategies are readily performed in *P. pastoris*.

Four methods for introducing DNAs into *P. pastoris* have been described and vary with regard to convenience, transformation frequencies, and other characteristics. With any of the four transformation procedures, it is possible to introduce vectors as autonomous elements or to integrate them into the *P. pastoris* genome. The spheroplast generation-polyethylene glycol-$CaCl_2$ (spheroplast) method is the best characterized of the techniques and yields a high frequency of transformants (~10^5/mg) but is laborious and results in transformed colonies that must be recovered from agar embedding. The other three methods utilize intact or whole cells and are more convenient with transformants on the surface of agar plates which are easily picked or replica plated for further analysis. Of the whole-cell methods, electroporation yields transformants at frequencies comparable to those from spheroplasting and is the method of choice for most researchers. However, for laboratories that do not have access to an electroporation instrument, either of the other whole-cell procedures based on polyethylene glycol or alkali cations generates adequate numbers of transformants for most types of experiments and without the labor of spheroplasting.

All *P. pastoris* vectors are of the shuttle type, i.e., composed of sequences necessary to selectively grow and maintain them in either *Escherichia coli* or *P. pastoris* hosts. *P. pastoris* transformations most commonly involve an auxotrophic mutant host and vectors containing a complementary biosynthetic gene. Selectable markers include: (a) the *P. pastoris* or *S. cerevisiae* histidinol dehydrogenase genes (*PHIS4* and *SHIS4*) (1); (b) the argininosuccinate lyase genes from these yeasts (*PARG4* and *SARG4*) (2); and the *P. pastoris* orotidine-5'-phosphate decarboxylase gene (*URA3*). Recently, a dominant selectable marker based on the Zeocin resistance gene has been developed. Advantages of the Zeocin system relative to biosynthetic gene/auxotrophic host systems are that transformations are not limited to specific mutant host strains and that the Zeocin resistance gene is also the bacterial selectable marker gene, thus substantially reducing the size of shuttle vectors.

Autonomous replication of plasmids in *P. pastoris* requires the inclusion of a *P. pastoris*-specific autonomous replication sequence (PARS). PARSs will maintain vectors as circular elements at an average copy number of approximately 10 per cell. However, relative to *S. cerevisiae*, *P. pastoris* appears to be particularly recombinogenic and, even with a PARS, any vector that also contains more than approximately 0.5 kb of *P. pastoris* DNA will integrate into the *P. pastoris* genome at some point during the first ~100 generations after transformation. Thus, when cloning *P. pastoris* genes by functional complementation, it is important to recover the complementing vector from the yeast as soon as possible. Replication elements analogous to the *S. cerevisiae* 2μ circle plasmid or centromers have not been described for *P. pastoris*.

In *P. pastoris*, the frequency of gene replacement events is highly dependent on the length of terminal fragments responsible for proper targeting of replacement vectors. The frequency of

gene replacement events can be greater than 50% of the total transformant population when the targeting fragments are each greater than 1 kb but drops precipitously to less than 0.1% when their total length is less than 0.5 kb. Gene replacements have been performed with linear vectors that lack a selectable marker gene via co-transformation. This is an especially useful technique in situations where the number of genome manipulations to be performed is greater than the number of selectable markers available for the host strain. For co-transformations, *P. pastoris* cells are simply transformed with a mixture of two DNA vectors: an autonomously replicating vector that contains a selectable marker and an approximately 10-fold excess of a linear gene replacement vector. Transformants are selected for the presence of the marker gene phenotype and then screened for ones that also receive the non-selected gene replacement vector. Typically, less than 1% of the transformants will have undergone a gene replacement event with the non-selected vector, a frequency sufficient to identify co-transformants by the phenotype conferred by the replacement event. After identifying a proper co-transformant, the autonomous vector is cured from the strain by growing it in a non-selective medium.

As in other non-conventional yeasts, the number of genetic manipulations (e.g., gene replacements or gene knock-outs) that can be performed on a single strain is constrained by the limited number of selectable marker genes that are available. Since each new marker requires considerable effort to develop, a convenient means of regenerating previously used markers is sometimes useful. One general method takes advantage of the high frequency of homologous recombination events in diploid strains of *P. pastoris* undergoing meiosis (5). In addition to expected recombination events between genes and their homologues at the normal loci on the homologous chromosome, recombination events also occur between genes and homologous copies located at other (ectopic) sites in the genome. Thus, a wild-type *P. pastoris* marker gene inserted into the *P. pastoris* genome at an ectopic location as part of a gene knock-out construction can be meiotically stimulated to recombine with its mutant allele located at the normal locus. A frequent result of such events is an ectopic gene conversion in which the wild-type allele at the knock-out site is converted to its mutant allele. Spore products that harbor a mutant allele-containing knock-out construction are once again auxotrophic for the selectable marker gene and can be identified by a combination of random spore and Southern blot analyses.

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Gene expression in *Hansenula polymorpha*

Cornelis P. Hollenberg, Institut für Mikrobiologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstr.1, D-40225 Düsseldorf, Germany.

In recent years, a number of yeast species other than *Saccharomyces cerevisiae* have become accessible for molecular genetics and thereby for potential application in biotechnology (Hansen and Hollenberg, 1996). In this respect, the methylotrophic yeasts, *Hansenula polymorpha* and *Pichia stipitis*, have already been proven to offer significant advantages over *S. cerevisiae* for the production of certain heterologous proteins (Gellissen and Hollenberg, 1994; Gellissen and Melber, 1996). The methylotrophic yeasts share general pathways to assimilate a catabolized methanol. Growth on methanol is accompanied by a strong induction of peroxisomes and enzymes involved in methanol metabolism. The strong inducible promoters of the corresponding genes are used for the expression of heterologous genes.

To improve the use of these promoters we have analyzed in great detail the regulation of the *MOX* promoter of the gene encoding methanol oxidase. We have found methods to circumvent the tight glucose repression of this promoter. In *S. cerevisiae*, the *MOX* promoter can mediate a glucose repressible expression of a fused *lacZ* gene. This repression was mediated by *MOX-B*, a 240 bp promoter region which is also involved in catabolite repression in *H. polymorpha*. The negative regulation mediated by *MOX-B* was counteracted by Adr1p, a transcription factor which has been shown to be involved in the derepression of *ADH2* and, most remarkably, of genes encoding peroxisomal proteins (Pereira and Hollenberg, 1996). Details for the binding of Adr1p to the *MOX* promoter and its action will be discussed.

During *MOX* derepression, two different transcripts have been detected starting in the MOX promoter at -25 and -425, from which the smaller transcript accounts for the translation of methanol oxidase. Several small ORFs in the leader sequence of the larger transcript prevent efficient translation. A model for the function of the strong MOX promoter, involving the adr1p homologue and a coordinated switch between the two transcription points will be presented.

In a study of the nucleosome structure in the *MOX* promoter region, we found that the 4 nucleosomes analyzed are organized in families: they localize in alternative positions along a unique rotational phase, and the linker regions can be occupied by alternative nucleosomes. This organization underscores substantial freedom of choice by histone octamers when nucleating on a promoter region (Costanzo et al., 1995).

Finally, the suitability of *H. polymorpha* for application in biotechnology will be

demonstrated by the discussion of promising developments of pharmaceutical proteins such as the production of hirudin and hepatitis B, L- and S-antigens. Moreover the use of recombinant *H. polymorpha* for bioconversion will be presented.

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Genetic and molecular tools for the yeast *Yarrowia lipolytica*

The ascomycetous heterothallic yeast *Yarrowia lipolytica* is in some respects a true nonconventional yeast. Especially the development of several genetic and molecular tools made a lot of troubles and it always seems that this yeast would never do anything as expected. Nevertheless, after 25 years in the fight to tame this beast most of the genetic and molecular tools needed are available now.

Methods for induction of conjugation, sporulation and spore analysis (tetrad analysis, random spore analysis) were developed and improved. However, it took a long time to construct genetic improved laboratory strains by inbreeding. At present several inbred lines are available, which have satisfactory mating, sporulation and spore germination frequencies.

Several searches for natural plasmids in wild strains of this yeast were unsuccessfully. Artificial vectors containing chromosomal ARS elements have been constructed. However, contrary to other yeasts such vectors are only stable when a centromer region is also included. Therefore copy number of these elements exceed never more than 2 - 3. To get higher copy numbers integrative vectors have been developed which use rDNA genes or a long terminal repeat (zeta) of a retrotransposon as sites for multiple homologous integration. Copy numbers from 10 to 50 could be obtained with such vectors.

Several genes of *Y. lipolytica* were cloned and analysed. Some strong inducible vectors are used for expression of foreign genes. The secretion signal of the *XPR2* gene (encoding extracellular alkaline protease) has been successfully used for secretion of foreign proteins into the medium.

Common used molecular tools are well established, sometimes with modifications, for this yeast now.

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Candida albicans: biology, dimorphism and pathogenecity.

Candida albicans is a medically important diploid fungus with no known sexual cycle. It is widely in the nature as a commensal but also can produce infections that range from the superficial to the systemic. *C.albicans* is able to grow in at least two different morphological forms, either as a mycelium or as a yeast cell. Such a transition is induced by several environmental conditions and seems to be involved in the pathogenecity of these yeast. Other virulence factors have also been studied.

A. BIOLOGY

1. *Taxonomy*
2. *Distribution and epidemiology*
3. *The imperfect state*
4. *The ploidy*

B. DIMORPHISM

1. *Experimental conditions for germ-tube formation*
2. *Inducers of germ tube formation*
3. *Morphological mutants*
4. *Genes involved in the morphological transition*
5. *Signals and signalling pathways*

C. PATHOGENECITY

1. *Virulence factors:*
   - *a) Hyphal production*
   - *b) Proteinase Activity*
   - *c) Adherence*
   - *d) Variability*
2. *Host defense mechanisms*
3. *Animal models for experimental infection*

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Understanding *Candida albicans* at the molecular level

A. INTRODUCTION

1. *Relevance of C. albicans in antifungal research*
2. *The basic problem of* C. albicans *genetics: an historical overview*

B. CLASSICAL GENETICS

1. *Mutant isolation*
2. *Parasexual genetics*

C. *ALBICANS* KARYOTYPE VARIABILITY

1. *Towards the development of a chromosome map in* C. albicans
2. *Karyotype modifications: a role in varibility*

D. DEVELOPMENT OF A TRANSFORMATION SYSTEM IN *C. albicans*

1. Pecualiarities of C. albicans *transformation*
2. *Integrative transformation*
3. *Development of C. albicans ARS sequences: Isolation of fungal ARS sequences*
    a) *Structure, function and properties of* S. cerevisiae ARS *sequences*
    b) *Isolation of* C. albicans *ARS sequences*
4. *Some ARS-derived vectors for use in C. albicans*

E. GENERAL STRATEGIES FOR ISOLATION OF *C. albicans* GENES

1. *Isolation using* S. cerevisiae *or* C. albicans *as the genetic host*
2. *Isolation based on DNA sequence homology*
3. *Other strategies*

F. GENE DISRUPTION STRATEGIES

G. DEVELOPMENT OF A GENE REPORTER SYSTEM

H. FUTURE TRENDS: MAJOR DRAWBACKS IN *C. ALBICANS* GENETICS

I. CONCLUSIONS

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Regulation of the lactose/galactose pathway in *Kluyveromyces lactis*

Cornelis P. Hollenberg, Institut für Mikrobiologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstr.1, D-40225 Düsseldorf, Germany.

In yeast, the genes *GAL1* (galactokinase), *GAL7* (transferase), and *GAL10* (epimerase) belong to a family of coordinately regulated genes (the Gal regulon) which enable the cell to utilize galactose as a sole carbon source (review Johnston and Carlson, 1992). The regulation of the galactose metabolism is dependent on the functions of the activator protein Gal4p, the negative regulator Gal80p and the inducer protein Gal3p. In the absence of galactose, Gal4p is kept inactive by its binding to Gal80p. The presence of galactose leads to activation of Gal4p presumably without dissociation of the complex with Gal80p (Leuther and Johnston 1992) followed by transcription of the genes concerned. The Gal3p is required for normal induction.

The galactose regulons of the yeasts *Kluyveromyces lactis* and *Saccharomyces cerevisiae* are similarly organized and regulated. The comparative study of both systems has contributed much to the present understanding of this model induction system. The galactose regulon of the milk yeast *K. lactis* comprises additionally the *LAC4* gene encoding ß-galactosidase (Dickson and Markin 1980) and is also indicated as galactose/lactose regulon.

In *K. lactis*, the *GAL1* gene encoding galactokinase, an enzyme of the Leloir pathway, has been shown to play an important role in the induction of the galactose/lactose regulon (Meyer et al.,1991). In disruptants of Kl*GAL1* or specific regulatory mutants *(gal1r)*, all genes of the regulon are no longer inducible implicating a regulatory function of galactokinase. The fact that the Kl*GAL1* gene can complement a *S. cerevisiae gal3* mutation indicated that the regulatory function of Kl*GAL1* may be similar to that of *GAL3*. *K. lactis* lacks a gene homologous to *GAL3*; its function is exerted by *GAL1*. The *gal3* phenotype was shown to be suppressed also by the *S. cerevisiae GAL1* explaining why induced cells do not require the presence of Gal3p (Bhat and Hopper 1991).

The function of *GAL3* during induction has recently become more clear. Earlier models assumed that *GAL3* and also *GAL1* were required to convert galactose to the inducer or co-inducer. Recently, we have provided strong evidence showing that galactose itself is the genuine inducer (Cardinali et al. 1997). We have analyzed a *gal7* mutant of *K. lactis*, which lacks the galactotransferase activity and is able to express the genes of the Gal/Lac regulon semi-constitutively that means, also in absence of galactose. We found that this expression undergoes a strong induction during the stationary phase. The mutant *gal1-209*, which has a strongly reduced galactokinase activity, but retains its positive regulatory function, also shows a constitutive expression of ß-galactosidase, suggesting that galactose is the inducer. A *gal10* deletion in *gal7* or *gal1-209* mutants reduces the expression under wild-type level. The presence of the inducer could be demonstrated in both *gal7* crude extracts and culture medium by means of a bio-assay using the induction in *gal1-209* cells. A mutation in the transporter gene *LAC12* decreases the level of induction in *gal7* cells, indicating that galactose is partly released into the medium and then retransported into the cells. NMR analysis of crude extracts from *gal7* cells revealed the presence of 50 mM galactose. We concluded therefore that galactose is the inducer of the Gal/Lac regulon, and is produced via UDP-galactose through a yet unknown pathway.

The observation that the function of Gal1p required for induction is not dependent on galactokinase activity indicates that Gal3p and Gal1p have no enzymatic function for inducer synthesis but rather in response to the presence of galactose may indirectly or directly activate the Gal4p activator by overcoming Gal80p repression. Such a regulatory function is also

supported by the fact that cells overexpressing *GAL1* or *GAL3* show a constitutive expression of the galactose regulon (Bhat and Hopper 1992; Meyer et al. 1992). How Gal1p or Gal3p lead to activation of Gal4p has remained a matter of speculation. Recently, we and others were able to show that Gal1p and Gal3p can interact directly with Gal80p (Zenke et al. 1996; Suzuki-Fujimoto et al. 1996). We have biochemical and genetic evidence showing that Gal1p activates Gal4p by direct interaction with the Gal4p inhibitor Gal80p. Interaction requires galactose, adenosine triphosphate, and the regulatory function of Gal1p. These data indicate that Gal1p-Gal80p complex formation results in the inactivation of Gal80p, thereby transmitting the galactose signal to Gal4p.

Further data will be presented that underline the function of KlGal1p as a regulatory protein. We were able to generate a new class of *Klgal1* mutant alleles that still have kinase activity but lack the regulatory function. These mutations further support the concept of two separable functions of Gal1p: kinase activity and regulatory function.

A new class of *K. lactis GAL1* mutants, *gal1-m*, was isolated that have lost the derepression activity but have retained galactokinase activity indicating that both Gal1p activities are functionally independent. The mutations were localized to two different regions conserved between Gal3p, Gal1p and KlGal1p. Overexpressed, gal1-m alleles lead to constitutive expression of ß-galactosidase which could not be further induced by galactose. In another type of screen, a dominant mutation, *GAL1-d*, was isolated that leads to a high level of constitutive expression. The results from 2-hybrid and mutational analysis indicate that both the N-terminal and the C-terminal moiety of KlGal1p are involved in specific interaction with KlGal80p.

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1. **Physiology of the fission yeast *Schizosaccharomyces pombe.***

- General introduction to *S.pombe.*
- Life Cycle
- Cell Biology
- Classical genetics
- Molecular genetics

References:

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2. **Use of *S. pombe* as a genetic tool for cell cycle studies.**

Isolation of cell cycle mutants

Cloning cell cycle genes

Regulation of the cell cycle by cyclins and Cdk1

Cell cycle inhibitors

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Morphogenesis in *Candida albicans*: Mechanisms of Cell Wall Synthesis.

Sentandreu R. Department of Microbiology, Faculty of Pharmacy. University of Valencia.

The walls are responsible of the cell morphology and this morphology depends on the ambient conditions, at least, in dimorphic fungal cells. This observation suggests that a cascade of events lead to the final molecular architecture of the cell walls.

The most abundant components of the wall are β-glucans and mannoproteins with small amounts of chitin. We will focus our interest on the mannoproteins, their nature, localization and how are connected to other wall macromolecules. Information about this last aspect has been deduced by the procedures used in their solubilization and several type of linkages have been suggested.

Up to day only a few wall proteins have been cloned in *Saccharomyces cerevisiae* and some common structural characteristics are found in them.

Recently a mannoprotein specific of *Yarrowia lipolytica* mycelial cells has been cloned. This protein is linked to β-glucans as it is solubilized only following digestion with (1,3)- β-glucanase. The protein is localized in the outer surface of the tip of the growing mycelial cells and is found partially cryptic in sub-apical locations, suggesting that it participates directly in the mycelial cell wall architecture. Disruption of the gene that codifies this protein results in no phenotype.

It has been suggested that the wall proteins are incorporated into the growing structure through a GPI membrane intermediate. In addition a transglutaminase activity that may participate in the linkage between proteins has been detected in the domains of the wall of *Candida albicans*, *Y. lipolytica* and *S. cerevisiae*.

Topology of the macromolecules has been studied by controlled degradation of the walls by hydrolytic enzymes and chemicals, used of poly- and monoclonal antibodies and by electron and confocal microscopy. The results show that mannoproteins are distributed throughout the entire wall while chitin is accumulated in the inner part of the cell wall, and that glucans, which form a secondary layer on the outer surface below the mannoprotein external layer, are responsible for the mechanical strength of the wall. In addition, cytoplasmic proteins are often found in the external part of the wall and this fact seems a consequence of the cell lysis and not a result of a secretory process.

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Energy metabolism in non-conventional yeasts.

Carlos Gancedo. Instituto de Investigaciones Biomédicas. C.S.I.C. Arturo Duperier 4. 28029 Madrid Spain.

Energy metabolism has been very much studied in *Saccharomyces cerevisiae* due to its importance for the industry of fermentations and also for its basic interest. In fact our understanding of glycolysis is due in great part to the studies in *S. cerevisiae*. Also the relationship between energy metabolism and gene expression (catabolite repression) has been mainly studied in this yeast. There have not been much studies on this topic in non-conventional yeasts other than *Kluyveromyces* .
Genes encoding enzymes of the glycolytic pathway from some non-conventional yeasts have been cloned but no systematic studies of the pathway as a whole have been performed. Also studies on the mechanism of catabolite repression are scarce (again with the exception of *K. lactis* and a few data on *Schizosaccharomyces pombe).*
I will try to present in this lecture a series of data from different species to show where the field is at this moment.
The results obtained with *Saccharomyces* will be used as a reference, although this yeast has peculiarities in its energy metabolism that do not allow to extrapolate data freely to other yeasts.

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Advanced Course on Biochemistry and Molecular Biology of Non-conventional yeasts.
Madrid, 7-19 July 1997

Kluyveromyces lactis for experimental use: a short review and practical considerations

Hiroshi Fukuhara. Institut Curie, Secttion de Recherche, Bâtiment 110, Université Paris XI, Orsay 91405, France.
Phone: 33 1 6986 3063; Fax: 33 1 6986 9429; E-mail: fukuhara@curie.u-psud.fr.

Summary

In recent years, *Kluyveromyces lactis* has become a focus of attention in the study of heterologous gene expression. Secreted production of proteins by this yeast has been an object of intensive studies stimulated by industrial interest. This development owes much to the basic research on the genetics and biochemistry of this yeast which started in early 1960's.

My talk will include (i) the history of *K. lactis* research, (ii) the availability of strains, mutations and gene manipulation systems and (3) practical procedures for molecular genetics of *K. lactis*. Discussion will put emphasis on the comparison with what we know about *Saccharomyces cerevisiae*. Whilst *K. lactis* is close to *S. cerevisiae* at the level of induvidual genes, the difference of physiological properties and genomic organisation between the two species constitutes an interesting tool for studying evolution of regulatory networks.

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Kluyveromyces lactis (a review). Wésolowski-Louvel, M., Breunig, K. and Fukuhara, H. In: Non-conventional Yeasts in Biotechnology, a Handbook. K. Wolf (ed.) Springer Verlag, Berlin. 1996. p. 139-201.

Advanced Course on Biochemistry and Molecular Biology of Non-conventional yeasts.
Madrid, 7-19 July 1997.

Nature, frequency, and distribution of plamids in yeast species.

Hiroshi Fukuhara. Institut Curie, Secttion de Recherche, Bâtiment 110, Université Paris XI, Orsay 91405, France.
Phone: 33 1 6986 3063; Fax: 33 1 6986 9429; E-mail: fukuhara@curie.u-psud.fr.

Summary

Only a few plasmids have been studied in yeast. The best known are the double-stranded (ds) killer RNA, the 2μ circular DNA and the linear killer DNAs. They are all interesting because of their different life styles and original replicative devices.

What kind of plasmids are distributed in different species of yeast? A systematic survey conducted on about 2600 strains, including practically all of the known 600 species, has provided an overall view on yeast plasmids.

On average, one finds a plasmid in every ten strains. The most frequent are the dsRNA plasmids. These retrovirus-like molecules are largely disseminated among very diverse species.

The second largest group of plasmids is represented by small linear DNAs. They also are distributed over a large range of species. Many, but not all, seem to be replicating in the cytoplasm. This is a remarkable aspect of yeast linear DNA plasmids, as opposed to the linear plasmids of the filamentous fungi and plants which are always associated with mitochondria.

Circular DNA plasmids are the rarest. Their occurrence seems to be restricted within a few related species of yeast (the so-called ubiquinone 6 group - *Saccharomyces*, *Zygosaccharomyces*, *Kluyveromyces* and *Torulaspora*). All these circles have a genetic organization related to the 2μ DNA.

As a source of gene vectors, plasmids can be a particularly important instrument for gene expression studies in non-classical species on which formal genetics is usually unpractical. Available vector systems based on these plasmids will be discussed.

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Use of *Pichia pastoris* for the Production of Foreign Proteins

James M. Cregg, Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, P.O. Box 91000, Portland, OR 97291-1000

As systems for the production of foreign proteins, yeasts combined the growth and genetic manipulation advantages of *Escherichia coli* with the ability to perform important post-translational modifications such as proper folding, disulfide bridge formation and glycosylation (1). Relative to *Saccharomyces cerevisiae*, the methylotrophic yeast *Pichia pastoris* has two significant advantages as a host for the production of foreign proteins (2,3). The first is the promoter used to transcribe foreign genes; it is derived from the *P. pastoris* alcohol oxidase I gene (*AOX1*) and is ideally suited for this purpose. It is efficiently transcribed in cells exposed to methanol as the sole carbon source but is highly repressed under most other growth conditions. The second advantage is that *P. pastoris* does not have a tendency to ferment as does *S. cerevisiae*. A product of fermentation is ethanol which can rapidly build to toxic levels in high-density cultures. As a consequence of these advantages, *P. pastoris* expression strains are relatively easy to scale up from shake-flask cultures to large-volume fermenter cultures growing at cell densities of greater than 100 grams/liter, dry cell weight.

The structure of a typical *P. pastoris* expression vector, pPHIL-D2, is shown in Fig. 1 of the practical instructions (4). The vector is composed of the *AOX1* promoter and transcriptional terminator fragments, separated by a unique EcoRI site into which the foreign gene is inserted. The vector also contains the *P. pastoris* histidinol dehydrogenase gene (*HIS4*) for selection in *P. pastoris his4* hosts. To maximize the stability of *P. pastoris* production strains, expression vectors are integrated into the host genome. Two integration options are available. Vectors can be linearized by cutting at one of several unique restriction sites in either *HIS4* or *AOX1* 5' sequences to stimulate single crossover-type integration events. Alternatively, vectors can be cut to release the expression cassette on a DNA fragment flanked by *AOX1* terminal sequences which stimulate gene replacement events at *AOX1*. The resulting strains are deleted at *AOX1* which forces them to rely on the transcriptionally weak *AOX2* gene. These strains metabolize methanol at a reduced rate but sometimes express higher levels of foreign protein than wild-type hosts, especially in shake-flask cultures.

Recently, methods have been described to construct *P. pastoris* strains with multiple copies of a heterologous gene expression cassette. Three approaches toward obtaining multi-copy expression strains have evolved. The first two involve identifying multi-copy strains that exist naturally within transformed cell populations at a frequency of a few per cent. One approach is to directly screen strains for foreign protein levels or indirectly for those with multiple copies of the foreign gene. The second approach utilizes expression vectors that contain the *kan*^{r} gene which confers a resistance to G418 or the *Sh ble* gene which confers resistance to Zeocin. The level of resistance conferred by these vectors is approximately proportional to vector copy number. Thus, high-copy-number strains can be identified by high resistance to G418 or Zeocin.

The third approach is to introduce multiple expression cassette copies into a single vector prior to transformation using vectors designed for this purpose such as pAO815.

The *P. pastoris* expression system is particularly valued for its ability to secrete heterologous proteins. Since the organism secretes only very low levels of native proteins, the secreted product is often the major protein species in the medium. Thus, secretion serves as an effective purification step to separate the product from most other cellular components. Secretion requires an amino-terminal signal sequence to target the proteins into the secretory pathway. The most generally applicable secretion signal is the *S. cerevisiae* prepro alpha mating factor (α-MF prepro) leader sequence. Using the α-MF prepro leader, a number of foreign proteins have been secreted at high levels from *P. pastoris*.

The structure of carbohydrate added to secreted proteins is organism specific (5). In both mammals and yeasts, O-glycosylation involves the attachment of carbohydrates to polypeptides via the hydroxyl group of Ser and Thr. However, mammalian O-oligosaccharides are composed of N-acetylgalactosamine, galactose (Gal) and sialic acid (NeuAc), whereas yeast O-oligosaccharides are composed of one to four mannose (Man) residues. The number of mannose residues per chain, their manner of linkage and the frequency and specificity of O-glycosylation in *P. pastoris* have yet to be determined. N-glycosylation begins in the ER with the transfer of a lipid-linked oligosaccharide unit, $Glc_3Man_9GlcNAc_2$ (Glc = glucose; GlcNAc = N-acetylglucosamine), to asparagine at the recognition sequence Asn-X-Ser/Thr. This oligosaccharide unit is subsequently trimmed to $Man_8GlcNAc_2$. In the mammalian Golgi apparatus, a series of trimming and addition reactions generates oligosaccharides composed of either $Man_{5-6}GlcNAc_2$ (high-mannose type), a mixture of several different sugars (complex type), or a combination of both (hybrid type). In lower eukaryotes including *P. pastoris*, N-linked oligosaccharides are elongated in the Golgi apparatus through the addition of mannose outer chains. These chains vary in length but can be long, containing more than 50 mannose monomers. As a result, N-glycosylated proteins appear heterogeneous in size. Mammalian glycoproteins secreted from yeasts also receive the long mannose outer chains, a condition referred to as hyperglycosylated. These structures can be exceedingly antigenic when introduced into mammals and are rapidly cleared from the blood. As a result, the production of foreign glycoproteins in yeasts for use as human therapeutics is problematic.

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NITRATE ASSIMILATION IN YEASTS

José Manuel Siverio, Celedonio González, Nélida Brito, Julio Avila, MªDolores Pérez, Germán Perdomo, Pablo Lugo, Helena Lorenzo and Félix Machín

Departamento de Bioquimíca y Biología Molecular. Universidad de La Laguna E-38206 La Laguna, Tenerife, Canarias. E-mail :jsiverio@ull.es

Yeasts from genus *Hansenula*, *Candida*, *Pachysolen* and *Rodothorula* are able to use nitrate as single nitrogen source, while *Saccharomyces* and *Schizosaccharomyces* cannot. The yeast *Hansenula polymorpha* and *Hansenula anomala* are the objects of our studies on nitrate assimilation. Nitrate assimilation in yeast take place as in the rest of assimilatory organisms, nitrate is transported into the cell by a nitrate transporter and once nitrate enters the cell it is reduced to ammonium by the consecutive action of the nitrate reductase and nitrite reductase that reduce the nitrate to nitrite and this to ammonium respectively.

Nitrate is the main source of nitrogen in plants, the nitrogen assimilated as nitrate is even higher than that through N_2 fixation. However, nitrate is also a powerful contaminant of lakes, rivers and coasts. Therefore, a better knowledge of the nitrate utilisation by plant and micro-organism would contribute to a more rational use of this nitrogen source. The nitrate assimilation in yeast has been object of few studies, perhaps due to the fact that only the so called non-conventional yeasts use nitrate. However, the development of classical genetic and DNA technology in non-conventional yeasts has made it possible to tackle the study of the nitrate assimilation in yeasts.

In the *H. polymorpha* *YNT1*, *YNI1* and *YNR1* genes encoding the nitrate transporter, nitrate reductase and nitrite reductase respectively are clustered and independently transcribed. These genes are co-ordinately regulated being induced by nitrate and repressed by ammonia. Fusion of each of these gene promoters with *LacZ* show that in the presence of nitrate the high expression is driven by *YNR1* promoter followed by *YNI1* and *YNT1*. These results are consistent with the levels of the corresponding enzyme activities. Strains bearing null alleles of each of these genes show incapacity to grow in nitrate. Additionally to the structural genes two genes encoding transcriptional activators *GAL4* type have been isolated, mutation of these genes lead to cells unable to use nitrate as sole nitrogen source, although the growth is not handicapped in other nitrogen sources.

The yeast *H. anomala* unlike *H.polymorpha* presents the same preference by nitrate as amonium as nitrogen source. The isolation of genes involved in nitrate assimilation revealed an extraordinary complexity in comparison with *H. polymorpha*, up to three nitrate reductase and two nitrite reductase encoding genes have been isolated so far.

The isolation of *YNT1*, *YNI 1* and *YNR1* in *H. polymorpha* open a broad field of studies on nitrate assimilation. A t present we have focused our attention on the regulation of the pathway, studying as well the factors affecting protein and

activity levels as gene expression. To asses the effect on the nitrogen source on the levels of NR and NT proteins promoters of *YNR1* gene and *YNT1* have been substituted by MOX1(metanol oxidase) and ADH (alcohol dehydrogenase) promoters, under these conditions the nitrogen source should not affect the expression of the gene permitting us to study the effect of nitrogen sources on the proteins.

The null allele *ynr1 : :URA3* is being used as host to express plant nitrate reductase with two aims : 1.- To overexpress nitrate reductase and 2.- To perform structure function relationship on NR. Likewise, the null allele *ynt1 : :URA3* has been used successfully to verify the function of plant genes with homology with those isolated from filamentous fungi and yeast involved in the nitrate transport.

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Development of *Yarrowia lipolytica* as a model for analyzing dimorphism in lower eukaryotes.

Angel Domínguez. Departamento de Microbiología y Genética. Universidad de Salamanca. Spain. Phone, 34-23-294477. Fax, 34-23-222876. E-mail, ado@gugu.usal.es

Dimorphism in fungi can be defined as a reversible transition from the spherical or quasi- spherical (yeast form) mode of growth to a polarized (hyphal) mode. Most important systemic fungal pathogens are pleomorphic, growing as mycelia in the enviroment and in the yeast form in humans. *Yarrowia lipolytica* is a natural heterothallic dimorphic fungus that forms yeast cells and septate hypha. It has a stable haploid phase and procedures for mating and sporulation have been described. Integrative and autonomous replication systems have also been reported.

We designed a culture medium that permits growth in one of both phases (yeast or hypha) and found, in contrast to the results described for *Saccharomyces cerevisiae*, that *Y.lipolytica* haploid cells exhibit a bipolar budding pattern. Haploid daughters bud opposite the previous bud site (distal budding), while diploid mother cells bud bipolarly, i.e. either adjacent to or opposite the previous bud site.

We also set up procedures for obtaining monomorphic mutants (that only proliferate in the yeast form). Smooth colonies exhibited an unmottled and unwrinkled surface with no aerial mycelium and all the cells of the colony grew exclusively in the budding form. By complementation analysis, we found several complementation groups. After transformation of all the Fil$^-$ mutants with an integrative gene bank, we obtained stable transformants with a rough phenotype. One of them codes for a gene *YlHOY1* (*Yarrowia lipolytica* homeotic gene 1) showing homology with genes containing homeobox sequences.

It has also been described that deletion of *YlSEC14*, the structural gene for the major phosphatidylinositol/phosphatidylcholine transfer protein, blocks the yeast-hypha transition.

All these findings, together with the fact that *Y.lipolytica* is a non pathogenic organism and diverges strongly from both *S.cerevisiae* and *Schizosaccharomyces pombe* make this yeast an excellent model for dissecting the yeast-to-hypha transition in fungi at molecular level.

1st Lecture

C. Leão

The Genus *Zygosaccharomyces*: biochemical and molecular traits

Species of the yeast genus *Zygosaccharomyces* are of major importance to the food and beverage industries causing spoilage in several products. An overview will be given of the main physiological/biochemical and molecular tools available for the identification and characterisation of *Zygosaccharomyces* species. Particular emphasis will be given to the discrimination between the most important spoilage species *Z. bailii, Z. bisporus* and *Z. rouxii.* After a brief reference to the traditional yeast identification methods and their limitations for the discrimination among these species, different biomarkers recently developed, will be discussed: electrophoretic patterns of isoenzymes, total fatty acid profiles, and DNA polymorphisms. According to the fatty acid profiles the group containing *Z. bailii* and *Z. rouxii* strains could be distinguished by the presence of C18:2 and the absence of C18:3 fatty acids. The esterase and acid phosphatase isoenzyme profiles obtained in several strains of *Z. bailii* revealed a significant heterogeneity which may correspond to genetic diversity not detected by traditional methods used in yeast identification. The RAPD method was shown to be useful for the identification of strains at the species level, allowing to discriminate between *Z. bailii* and *Z. rouxii.* High resolution molecular genetic approaches that have been investigated to improve the identification and strain typing of *Zygosaccharomyces* species, will also be discussed (18S rRNA gene sequences and development of gene probes).

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2nd Lecture

C. Leão

***Zygosaccharomyces bailii* as a spoilage yeast in food and beverage: mechanisms of tolerance to acidic environments**

It is generally recognised that *Zygosaccharomyces bailii* is one of the most dangerous yeasts in food technology due to its high tolerance to acid preservatives and/or osmotic stresses. The main subject of this lecture will be on the mechanisms which may underlie the higher tolerance to acidic environments exhibited by that yeast species when compared with *Saccharomyces cerevisiae*, much less resistant to such environments. Emphasis will be given on: intracellular acidification, enzymatic glycolytic activities at lower pH, inhibition of mediated transport of nutrients, permeability of the plasma membrane to the undissociated acid, active extrusion of the preservative, sensitivity of respiration/fermentation of glucose to acids and ethanol. Based on the acquired knowledge, strategies for the design of selective and differential media for *Z. bailii*, will be discussed.

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Genetic approaches to the study of protein secretion in *Yarrowia lipolytica*.

C. Gaillardin
Laboratoire de Génétique cellulaire et moléculaire, Institut National Agronomique Paris-Grignon, Centre de Biotechnologies Agroindustrielles, 78850 Thiverval-Grignon, France.
Tel : 33-1-30-81-54-52 ; Fax : 33-1-30-81-54-57 ; mail : claude@cardere.grignon.inra.fr

Production of heterologous proteins with eukaryotic microbial hosts is an economically important goal which often turns out to be disappointingly inefficient. Non conventional yeasts often proved superior to *S. cerevisiae* for secreting specific foreign proteins, but there is no absolute best host. In all cases, adjusting the heterologous protein to the host, or the host to the protein, is still an empirical process of unpredictable outcome. Understanding of the critical components of the secretory apparatus and of their possible specificities may enable the construction of specifically engineered host strains in order to maximize the secretion of foreign proteins. Although bottlenecks may occur all along the secretory pathway, efficient crossing of the ER membrane and proper folding in the ER lumen are clearly critical events which may limit the overall flow through the pathway.

Why study protein translocation in *Yarrowia lipolytica*?

Most of the genetic studies on protein secretion in eukaryotes have so far been conducted with *S. cerevisiae* where many of the important genes involved in the process have been identified. *S. cerevisiae* may not however be ideally suited for this study, because it differs qualitatively in some aspects at least from higher eukaryotic cells. As described below, early events in protein translocation into the ER for example look somewhat different : although a SRP homolog and its cognate receptor were identified in *S. cerevisiae*, they are not essential for cell viability (Ogg et al. 1992).

We used as a model system the yeast *Yarrowia lipolytica* which secretes naturally very high levels of an alkaline extracellular protease (AEP) encoded by the *XPR2* gene. Extensive biochemical and genetic studies on AEP secretion have been conducted over the past years, thus making it by far the best studied example of a secreted extracellular protease in yeast (Ogrydziak, 1993). The system is particularly attractive because AEP is a highly inducible protein (up to 2% of total protein synthesis) which accounts for more than 80% of total protein secreted, and exhibits a fast transit rate ($t_{1/2}$ of 5 min) during which an elaborate processing occurs. As shown below, translocation of AEP into the ER appears to conform quite exactly to the mechanisms proposed for higher eukaryotes. Moreover, some secretory mutants exhibit much clearer phenotypes than in *S. cerevisiae*, thus facilitating genetic screens. Finally, sophisticated genetic approaches are easily conducted in *Y. lipolytica* to unravel the determinants of complex pathways.

In this conference, we will focus on the use of genetic approaches to identify new proteins involved in translocation and to approach their function.

Short overview of translocation in yeast and higher eukaryotic cells.

Secretion of eukaryotic proteins first involves crossing of the endoplasmic reticulum (ER)-membrane, as a result of two successive events: a targeting phase where secretory polypeptides have to be directed to secretion competent sites of the ER, and a translocation phase where the polypeptide is extruded through a proteinaceous pore (the translocon) into the ER lumen (Rapoport, 1992).

The targeting phase essentially prevents the polypeptide from reaching a conformation incompatible with translocation, until it hits the translocon. Two major mechanisms seem to coexist in order to achieve this goal, the preference for one or the other depending on the type of polypeptide and on the type of organism. In baker's yeast, chaperone-type proteins (Hsp70 family) retard folding of the secretory polypeptide by binding to it during translation or shortly after. Accordingly, most secretory proteins are (or can be) translocated posttranslationally. This is not the main pathway found in higher eukaryotes, where misfolding is mainly prevented by coupling translation and translocation, so that precursors of most secretory proteins are never found in the cytosol. The major device used here is the signal recognition particle (or SRP), which recognizes the signal peptide of a nascent secretory polypeptide as soon as it is extruded from the ribosome, binds to it and to the ribosome, and blocks further translation. The complex remains in a frozen state until it encounters an ER bound SRP-receptor, where SRP release is triggered, thus relieving translational arrest, in close vicinity to the translocon.

This initiates the second phase, where the ribosome, now anchored to the translocon, resumes protein synthesis and promotes the vectorial discharge of the polypeptide into the ER lumen.

Functionally identical components have been identified at this step in both mammalian and yeast cells : they include proteins which form part of the conducting channel and are tightly associated with translocating ribosomes (Sec61 complexes), transmembrane proteins which may assist Sec61p in translocating the precursor (Sec62p and Sec63p in yeast, TRAM in higher eukaryotic cells), and various proteins which help the proper folding of the polypeptide in the lumen of the ER (signal peptidase or SP, oligosaccharyl transferase or OST, peptidylprolyl isomerase or PPI, peptidyl disulfide isomerase or PDI...).

Whereas precocious folding of the precursor is clearly prevented during the targeting phase and is known to be detrimental for translocation both in vivo and in vitro, rapid folding in the lumen of the ER seems to be required in vivo, but much less in vitro. SP, OST, PDI, Sec62p and Sec63p, Kar2p/BiP are all dispensable for in vitro translocation of precursors, but are essential genes in vivo, where translation, maturation and folding occur simultaneously to translocation.

Folding, synthesis and secretion of AEP : mutated AEP forms mimic poorly secreted heterologous proteins.

A combination of biochemical and genetic approaches has led to the current view of the AEP secretion process. AEP is synthesised as a preproenzyme which is cotranslationally translocated into the ER. The 15 aminoacid signal peptide is cleaved during translocation of the nascent precursor, and an oligosaccharide core is immediately added to a single site (Thr_{125}) of the proregion. The first intermediate detected in the cell is thus a glycosylated, signal peptide processed 55kDa proenzyme, which is enzymatically inactive. This form travels to the Golgi apparatus where a dipeptidyl-aminopeptidase removes 9 X-Ala/X-Pro dipeptides located at the N-terminus. The resulting 52 kDa form is immediately processed by the Xpr6p endoprotease (an homologue of Kex2p in *S. cerevisiae*) which cleaves the precursor at the $LysArg_{157}$ site, thus releasing the 32 kDa mature enzyme and the 20 kDa proregion which are both secreted in the growth medium. Mutants defective in the *XPR6* gene secrete the 52 kDa precursor, which is still catalytically inactive. Proteolytic removal of the proregion triggers a conformational change of the mature part : whereas all preproenzyme forms appear proteinase K sensitive, mature AEP is resistant.

We generated mutant forms of AEP, e.g. *Del2* where the mature enzyme was directly abutted to the X-Ala/X-Pro dipeptide, or *DelG* which could not be glycosylated. Synthesis of these mutated constructs was strongly reduced, and none was secreted efficiently to the growth medium. They all accumulated inside the ER in a catalytically inactive and proteinase K sensitive form. Interestingly, coexpression of these deleted constructs with the wild type *XPR2* gene resulted in longer retention time of the wild type precursors and in a slight decrease of AEP made from the wild type copy, suggesting that mutant precursors were titrating factor(s) required for efficient synthesis and/or ER exit of the wild type precursor. These results suggested that the prodomain and other unknown factors were essential for the folding and transit of the precursors.

A direct demonstration of the role of the proregion in precursor synthesis and transit was obtained by expressing the AEP preprodomain in prodeleted strains. Whereas neither the prodomain alone nor the deleted precursors were secreted when expressed alone, coexpression of both restored secretory transit of each partner and enhanced synthesis of prodeleted precursors (Fabre et al. 1992).

These results show that the cell senses accumulation of misfolded precursors in the ER and blocks further synthesis when their amount reaches a certain level. Since accumulation of misfolded AEP precursors does not affect the overall secretion process or cell viability, the simplest hypothesis is that sensing occurs during folding of the nascent secretory polypeptide at a very early step, may be extraction from the translocation apparatus. The fact, as shown below, that AEP displays a very strong translational coupling with translocation, rendered this hypothesis very attractive.

AEP translocation is cotranslational and relies on the SRP-dependent pathway.

In higher eukaryotic cells, the SRP is a soluble ribonucleoprotein consisting of a 7SRNA backbone and of six polypeptide chains, Srp9,-14, -19, -54, -68, and -72. In vitro studies suggested that Srp9 and Srp14 were attached to the ends of the 7SRNA, an Srp68/72 complex to the center of the RNA, and an Srp19/54 complex to the loops present on each arm of the RNA. Current models on SRP functioning admit that each of these complexes is endowed with a specific function, respectively translational arrest, receptor recognition and signal peptide recognition.

The existence of SRP-like complexes in yeasts was demonstrated in *Y. lipolytica* and in *Schizosaccharomyces pombe* (Poritz et al. 1988), and later in *S. cerevisiae* (Hann and Walter, 1991). The 7SRNA isolated from *Y. lipolytica* and from *S. pombe* were shown to be remarkably similar to

higher eukaryotic 7SRNAs with respect to size, predicted secondary structure and binding to SRP polypeptides, although primary sequence conservation was very low.

Two highly similar genes encoding 7SRNA were isolated from *Y. lipolytica*, *SCR1* and *SCR2*, the double deletion of which appeared lethal (He et al. 1990). Using a plasmid shuffle strategy, conditional mutations in *scr1* and *scr2* were isolated, which resulted in a thermosensitive growth phenotype (Yaver et al. 1992, He et al. 1992). The mutations exhibited remarkably similar phenotypes : both were viable in an *scr1-scr2* double deletion background only when the mutant alleles were carried on a replicative plasmid (2-3 copies per cell), but were lethal when integrated as single copies in the genome. They resulted in a 60 to 70% reduction of AEP synthesis at the non permissive temperature, but the precursor was still secreted cotranslationnally and its further transit occurred at a normal rate. This demonstrated that the mutant SRP was still proficient in signal peptide recognition and translational arrest, but was partially defective in its interaction with the translocation apparatus, thus resulting in sustained translational arrest. These results provided clear evidence showing that wild type AEP secretion was strongly SRP-dependent and constituted the first direct indication that SRP performed translational arrest in vivo.

In order to identify partners of the 7SRNA and of the SRP, we devised several genetic screens. In the first one, we looked for secondary mutations which would enhance the thermosensitive phenotype displayed by *scr2-ts* mutants (synthetic lethals). In the second screen, suppressors of the conditional mutation *scr2-ts* were looked for.

Synthetic lethals identify a new soluble ER protein required for efficient translation and translocation of secretory proteins.

Several mutations aggravating the phenotype confered by a defective 7SRNA molecule were isolated. Two monogenic recessive mutations (*sls1* and *sls2*) were selected. In the presence of a wild type *SCR2* allele, they confered a thermosensitive phenotype and led to a reduction of the synthesis of AEP and of several secreted proteins. The wild type *SLS1* gene was isolated and identified a soluble protein carrying a consensus signal sequence at its N-terminus and a carboxy-terminal ER retention signal. A putative homolog was identified when the complete sequence of *S. cerevisiae* became available, but its role is still unknown. Antibodies were raised using a glutathione-transferase Sls1p fusion protein. They revealed a major 60 kDa protein in crude extracts of the wild type strain, which was shown to reside in a membrane-bound compartment by protease protection assays, likely the ER as evidenced by immunolocalization. Taken together, these results suggested that Sls1p was a major ER resident protein which is required for efficient synthesis of the AEP precursor.

Sls1p is not an essential gene at 28°C although AEP synthesis already appears markedly affected, but *sls1* deleted strains grow poorly at 34°C, suggesting that Sls1p might be important under (heat or secretory) stress conditions.The level of Sls1p indeed increases when misfolding of secretory precursors is induced (heat shock, tunicamycin treatment). We thus tested the effect of overexpressing Sls1p in a strain synthesizing an AEP precursor known to have a folding defect (*DelG* or *Del2* mutations, see above). Overexpressing Sls1p in these contexts severely enhanced the AEP secretion defective phenotype of the strains : strikingly this appeared to be due primarily to a synthesis shut-down which nearly suppressed accumulation of misfolded precursor in the ER. A likely explanation would be that Sls1p is actually required for allowing release of AEP precursors (and other secretory proteins) from the translocation machinery once their proper folding has been ascertained. In keeping with this hypothesis, Sls1p could be coimmunoprecipitated with Sec61p and with Kar2p (Boisramé et al. 1996).

Suppressors of 7SRNA mutations identify a new gene family.

Suppressors of the growth defect confered by the *scr2-ts* mutation were isolated. Among 40 suppressive events tested, 5 were shown to display a thermosensitive growth defect in the presence of a wild type *SCR2* copy. One of these mutation called *tsr1-1* led to a strong reduction of AEP synthesis at the non permissive temperature in an otherwise wild type context, thus mimicking the phenotype of the original *scr2-ts* mutation. Again, no cytoplasmic form of AEP could be detected, and whatever amount of precursor was translocated, its transit rate was not modified. The wild type allele was cloned by complementation, and shown to encode a new, essential protein of 461 aminoacids, with an N-terminal signal peptide, a 20 amino acid putative transmembrane domain, and a C-terminal RRXXR motif, characteristic of ER resident proteins. Using anti-Tsr1p antibodies, it could be directly shown that Tsr1p was a transmembrane protein

Five putative homologues of Tsr1p were detected in the databases : 4 in *S. cerevisiae*, one in *Hansenula polymorpha*. All proteins share a common structure : a signal sequence followed by a cysteine-rich domain, a serine and threonine rich domain, a linker domain of variable size, a transmembrane region, a conserved C-terminal region harbouring the RRXX motif. The (presumably lumenal) cysteine-rich domain is highly reminiscent of a family of scorpion neurotoxins and might be similarly structured by cysteine bonds; like these toxins, these domains might interact with membrane channels. Deletion of one of the *S. cerevisiae* homologue resulted in a thermosensitive growth phenotype and in a defect in the translocation of several secretory proteins.

Coimmunoprecipitation assays indicated that Tsr1p was interacting both with the the SRP (Srp19 and 7SRNA), the ribosomes (5.8SRNA), and possibly Kar2p. A highly speculative model currently tested would be that Tsr1p is involved in the coupling of translation /translocation and folding, probing on the lumenal side clearance of the translocon and controlling SRP-ribosome dissociation on the cytoplasmic side (Ben Mamoun et al. 1997 and submitted).

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Transcriptional control of secreted proteinases in *Yarrowia lipolytica* : transduction of an environmental pH signal.

C. Gaillardin
Laboratoire de Génétique cellulaire et moléculaire, Institut National Agronomique Paris-Grignon, Centre de Biotechnologies Agroindustrielles, 78850 Thiverval-Grignon, France.
Tel : 33-1-30-81-54-52 ; Fax : 33-1-30-81-54-57 ; mail : claude@cardere.grignon.inra.fr

The yeast *Yarrowia lipolytica* has been used for the large scale production of single cell proteins and organic acids like citric acid, for fatty acid bioconversion and, more recently, for the synthesis and secretion of heterologous proteins valuable for the pharmaceutical or agrofood industries. Improving some of these processes may obviously benefit from a better knowledge of the basic biology of this organism, for example protein secretion or control of gene expression. This talk will focus on recent developments in gene expression control, and more specifically on transcriptional effects of environmental pH, a topic poorly studied in yeasts in general until now, which receives increasing attention in filamentous fungi. Controlling gene expression by changing the pH of the ambient medium is obviously a technically appealing and cost effective procedure.

Why study transcriptional regulation of proteinases by environmental pH in *Yarrowia lipolytica*?

Secretion of aspecific proteolytic activities is a widespread characteristic of many yeast species. In a recent litterature survey of 110 yeast species, representing 31 genera, around 80% were found to secrete proteinases (33). Surprisingly little is known about the regulation of these extracellular proteinases, although it is widely admitted that they may significantly contribute to yeast ecological distribution, potential pathogenicity or importance in biotechnological applications. Since *S. cerevisiae* does not secrete any proteinase, most studies focused on two species, *Candida albicans* which secretes at least seven acidic aspartyl proteinases (Sap) possibly involved in pathogenicity, and *Yarrowia lipolytica* which secretes both an acidic aspartyl proteinase (AXP) and an alkaline seryl proteinase (AEP) depending on the pH of the growth medium (Young et al. 1996). The regulation of *C. albicans* and *Y. lipolytica* extracellular proteinases appear to be quite complex, but share some common elements. Their synthesis is strongly "repressed" by low-molecular-weight nitrogenous substrates (ammonia, glutamate, urea) and mildly sensitive to repression by preferred carbon sources such as glycerol or glucose, whereas they are strongly "induced" in media containing high molecular weight proteinaceous substrates. Besides carbon/nitrogen availability and presence of inducing proteins, the pH of the growth medium appears as a major determinant. As a rule, acidic proteinases progressively disappear when the pH is raised from 3.2 to neutral, and are replaced by AEP in *Y. lipolytica* or by specific Sap isoenzymes in *C. albicans*. Interestingly, very similar induction conditions have been reported for extracellular neutral and alkaline proteinases of *Aspergillus nidulans*, and may generally apply to many extracellular fungal proteinases.

The conservation at the molecular level, if any, of the underlying regulatory mechanisms is presently totally unknown.

Transcriptional control of the alkaline extracellular promoter in *Yarrowia lipolytica*.

For both historical and practical reasons, the promoter of the *XPR2* gene encoding the extracellular alkaline protease (AEP) has been widely used to direct heterologous protein production in *Yarrowia lipolytica*. The *XPR2* promoter is silent in early exponential phase and strongly induced in late exponential phase where it directs the synthesis of 2% of total mRNAs. Its regulation is however complex and poorly understood, a feature directly limiting its utilization : *XPR2* is induced in a growth phase dependent manner (when the yeast-mycelium transition occurs), in a specific pH window (pH6 to 8), and its full induction requires high amounts of peptones in the growth medium, which obviously complicates further purification of secreted proteins. We decided to construct variants of this promoter, or strains with improved genetic backgrounds, in order to get rid of some of these limitations without affecting the overall efficiency of this promoter.

Deletion analysis of the promoter evidenced two major UAS, one close to the TATA box (UAS2) and the other 700 bp upstream (UAS1), both of which are permanently bound by proteins, as shown by DMS footprints *in vivo*. Each UAS has a complex structure, and putative binding sites for

general transcription factors like Rap1, Abf1, Gcn4 and Car1, although the existence of such homologous factors has still to be proven in this yeast (Blanchin-Roland et al. 1994).

The role of each UAS was investigated using a *LEU2* TATA box as a reporter basal element. In this system, the upstream UAS drives a constitutive expression, while the downstream UAS seems to respond to several environmental factors, including pH, carbon and nitrogen availability. When several copies of the upstream UAS are tandemly inserted upstream from the *LEU2* TATA box, a strong constitutive expression is observed.

In view of the complexity of the *XPR2* promoter and of its regulation, we decided to isolate *trans*-acting regulatory mutants.

Identification of genes controlling response to environmental pH.

Trans-acting factors involved in transcriptional control of *XPR2* have been looked for using several genetic screens. Recessive mutations preventing *XPR2* derepression identified four unlinked genetic loci, *PAL1* to *PAL4*. These mutations have only marginal effects on the transcription of the acid extracellular protease (AXP).

A dominant multicopy suppressor of all four *PAL* mutations was isolated from a replicative library. It identified a (fortuitously truncated during cloning) homologue of the Zn-domain containing transcriptional factors Rim101p (controlling entry into meiosis in *S. cerevisiae*) and PacCp (controlling expression of pH sensitive genes in *A. nidulans*). The zinc finger domains of all three proteins are highly conserved and probably recognize the same binding site identified as GCAARG in *A. nidulans*. Such sites are present in the promoters of *AXP*, *RIM101* and *XPR2*. In this latter case, we know that they are bound by protein(s) in vivo, and that their deletion abolishes transcriptional activation of *XPR2* under all conditions. Deletion of *YlRIM101* abolishes *XPR2* expression under all conditions, but has no effect on *AXP* expression. The YlRim101p suppressor form is dominant over its wild type form (gain of function mutation) and encodes a C-terminally truncated version of YlRim101p, which apparently renders *XPR2* expression somewhat independent of external pH and completely independent of all *PAL* genes identified so far (Lambert et al. submitted).

These results are highly reminiscent of those obtained in *A. nidulans*, the best studied organism for transcriptional response to environmental pH. In this organism, PacC is present in a full length inactive form at acidic pH, where transcription of acidic genes takes place. At alkaline pH, PacC is proteolytically converted to a transcriptionnally active form which enters the nucleus and turns on alkaline genes while repressing acidic genes (Tilburn et al. 1995, Orejas et al. 1995). The pH signal is transmitted to PacC through a gene cascade involving the products of at least six *pal* genes, two of which have been recently cloned. Comparing the PacC and YlRim101p structure, we proposed a putative truncation site in YlRim101p : when this form was expressed, it rendered *XPR2* expression totally constitutive, irrespective of external pH and of the status of the *PAL* genes.

In a second screen, mutants able to express *XPR2* under acidic conditions were searched for (Cordero-Otero and Gaillardin, 1996). Two monogenic, dominant mutations (*RPH1-1* and *RPH2-5*) were identified. Although the mutants behave as if they were always growing under "alkaline" conditions, they are not detectably affected in their ability to regulate cytoplasmic pH in response to extracellular pH changes. *XPR2* expression in these contexts is still dependent on all other environmental conditions, showing that pH is an independent signal for *XPR2* regulation.

The cognate genes were recloned from the mutants as able to turn on *XPR2* in a wild type context. *RPH2-5* is actually a new *RIM101* allele, truncated by a stop codon at position 419. *RPH1-1* identified a NTF2 homologue, which is involved in nuclear import in higher eukaryotic cells (Pashal and Gerace, 1995). Immunolocalization of Rph1p indicated that it was both cytosolic and perinuclear, like its NTF2 counterpart. Epistasis studies suggest that *RPH1* acts downstream from *PAL* genes and upstream from *RIM101*. Deletion of *RPH1* results in phenotypes similar to those exhibited by a *rim101* deletion, and the dominant allele *RPH1-1* mimics the effects of truncated forms of *RIM101* irrespective of the pH. All our results are thus compatible with Rph1p being a downstream target of the *PAL* gene cascade, responding to environmental changes by triggering activation and/or import of the (truncated) transcriptional activator Rim101p. The fact that NTF2 was postulated on a structural basis to have enzymatic activity (Bullock et al. 1996) opens the exciting possibility that Rph1p/NTF2 may modify Rim101p so as to render it protease sensitive and permit its nuclear import (Cordero Otero et al., submitted).

Genes involved in the transduction of environmental pH signals are also involved in the control of mating, sporulation and morphogenesis.

The Rim101p homologue in *S. cerevisiae* was identified genetically as required for entry into meiosis (Su and Mitchell, 1993). We noticed that all *pal* mutations as well as *rim101* or *rph1* deletions strongly affected sporulation in homozygous diploids, but also resulted in very low mating frequency of the haploid strains. All the phenotypes could be reversed by expressing the truncated Rim101p suppressor forms, suggesting that mating and sporulation were «alkaline» processes in *Y. lipolytica*.

None of these mutations on the contrary affected the dimorphic transition (yeast to hyphae) characteristic of this species, except for the *rph1* deletion : strains harbouring this mutation exhibited a completely smooth colonial phenotype on plates, and failed to form hyphae in liquid. These results correlate with the fact that the dimorphic switch in this species is not strictly under pH control; they further indicate that signal from different pathways may be integrated through the *RPH1* gene product.

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Yeasts as pathogens

José Pontón San Emeterio

Fungi are eukaryotic organisms which can be found in different habitats in nature, always dependent on living or dead organic matter. The vast majority of the fungi grow as molds. About 1,000 species are able to grow as yeasts and only a small group of these yeasts are regularly associated with human disease. Relevant human yeast pathogens include members of the genus *Candida*, *Cryptococcus*, *Trichosporon*, *Malassezia* and *Rhodotorula*. Despite its small number, the yeasts and especially *C. albicans*, are the leading causes of life threatening fungal infections. As a result of the advances in modern medicine, other yeast species can occasionally infect immunosuppressed patients. Most yeasts are free-living in nature and are not dependent on humans or animals for their survival. However, there are some yeast species that can colonize human body surfaces to form part of the human body microbiotota. Infections, caused by yeasts found in natural habitats are acquired through inhalation, ingestion or traumatic implantation. The yeasts colonizing the body surfaces do not usually cause infections because they are in balance with the host's defensive mechanisms. However, these yeasts can develop an infection if the balance is changed. Yeast infections can be classified into three groups according to the initial site of infection: the superficial, the subcutaneous and the systemic mycoses. Due to the increasing importance of the yeast pathogens, diagnosis is an essential step and it depends on a combination of clinical observation and laboratory investigation. The number of antifungal drugs used for the treatment of patients with fungal diseases is very small. There are several families of antifungals and all members in each family share a common mechanism of action. Currently used antifungals include, the polyenes Amphotericin B and Nystatin, the synthetic pyrimidine 5-fluorocytosine and the azoles Fluconazole, Itraconazole and Ketoconazole.

PEROXISOME FUNCTION, BIOGENESIS AND TURNOVER IN THE YEAST *HANSENULA POLYMORPHA*

Marten Veenhuis, Jan A.K.W. Kiel and Ida J. van der Klei
Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), Biological Centre, Kerklaan 30, 9751NN Haren, The Netherlands

Introduction

Microbodies represent a class of ubiquitous and important cell organelles, characterized by a proteinaceous matrix surrounded by a single membrane. In contrast to their simple and uniform morphology, their physiological role is complex and highly variable, ranging from photorespiration in plant leaves to ether-lipid biosynthesis in mammalian cells. In yeasts microbody-bound enzymes are crucial for the metabolism of specific growth substrates. In spite of this functional diversity, the principles of peroxisome biogenesis are conserved throughout the eukaryotic kingdom.

During the last decade significant progress has been made in research on yeast peroxisomes. A major milestone was the discovery of growth conditions to induce peroxisomes in *Saccharomyces cerevisiae* (1987), which allowed to apply powerful molecular genetic techniques already available for bakers yeast at that time. Simultaneously, such techniques were developed for non-convential yeasts, whose peroxisomes were extensively studied since the early 1970s. These and other developments formed the foundation to design successful protocols to isolate the first peroxisome-deficient yeast mutants (*pex* mutants) in 1989 and to clone the corresponding genes (*PEX* genes). To date, yeasts have evolved into the model organisms of choice to study peroxisomes. Recent achievements in this research, in particular in the methylotrophic yeast *Hansenula polymorpha*, are addressed in this paper.

Induction of microbodies.

In yeasts, the induction and metabolic significance of microbodies (peroxisomes) is largely prescribed by environmental conditions. In *H. polymorpha* maximal peroxisomal induction is obtained during growth of cells in a methanol-limited chemostat; under these conditions the organelles may take up to 80% of the cytoplasmic volume; other substrates known to induce microbodies in this organism are ethanol, primary amines, D-amino acids and purines. Characteristic feature of these organelles is that they contain the key enzymes involved in the metabolism of the above carbon/nitrogen sources. The present knowledge on this topic now allows to precisely adjust both the level of microbody induction as well as their protein composition by specific manipulations in growth conditions.

Physiological functions of peroxisomes

Simplified, yeast peroxisomes can be typified as enzyme bags characterized by a very high protein content and the low surface to content ratio. The latter is probably related to the fact that yeast peroxisomes do not contain membrane bound enzymes. The protein content of peroxisomal membranes is relatively low. The typical smooth fracture faces of peroxiomal membranes also indicated the low abundance of large integral membrane proteins. Common themes in microbody metabolism in yeasts are hydrogen peroxide producing and decomposing enzymes (peroxisomes), the glyoxylate cycle (glyoxysomes) and the ß-oxidation pathway. The data obtained so far suggest that the general advantage for the cell to sequester these enzymes in microbodies is that it increases the efficiency of certain metabolic pathways.

The peroxisomal membrane as barrier

In vivo the peroxisomal membrane is not permeable to small solutes. The presence of a pH gradient across the membrane necessitates impermeability to protons. Studies on *H. polymorpha* showed that NAD(H) and glutathione also can not freely pass the peroxisomal membrane. Moreover, in bakers yeast acetyl-CoA may only cross the peroxisomal membrane after conversion into intermediates of the glyoxylate cycle or as a carnetine esther. However, this predicts the presence of several transporters in the peroxisomal membrane.

So far, only two peroxisomal transporter proteins have been identified in yeasts. PMP47 of *C. boidinii* is homologous to proteins belonging to the mitochondrial family of solute transporters. Disruption of the gene encoding PMP47 resulted in a specific import defect for DHAS protein, which accumulated as protein aggregates in the cytosol. Possibly, PMP47 transports molecules (ATP, the co-factor TPP?), required for proper activation and/or import of DHAS. Recently, in *S. cerevisiae* a peroxisomal protein has been identified which is a member of the ABC-transporters. It consists of a heterodimer of the gene products of *PXA1* and *PXA2* and is probably involved in transport of substrates for the ß-oxidation. Biochemical evidence exists for a calcium-regulated pore forming protein and for an H^+-ATPase in the peroxisomal membrane of *H. polymorpha*. Further analysis of these proteins awaits the cloning of the corresponding genes.

Peroxisome degradation

In *H. polymorpha*, peroxisomes are actively degraded after a shift of methanol-grown cells to a new environment in which the organelles have become redundant for growth. A similar phenomenon is observed for organelles which have become functionally inactive e.g. as a result of chemical stress. The degradation process is shown to be energy-dependent but independent of protein synthesis. In *H. polymorpha* peroxisomes invariably were degraded individually by means of an autophagic process. The process is characterized by three distinct steps: i) sequestation of the organelles destined for degradation from the cytosol by a number of membranous layers; ii) fusion of this compartment with -part of- the vacuole to acquire hydrolytic enzymes and iii) proteolytic degradation of the microbody contents by these vacuolar enzymes.

Degradation of individual peroxisomes is a rapid process and generally completed within 30 min. Interestingly, peroxisomal matrix enzymes present in the cytosol of *H. polymorpha pex* mutants are not degraded after exposure of the cells to glucose-excess conditions. This suggests that the signals, initiating peroxisome turnover, are not directed against the matrix proteins but instead, to the intact organelle.

Various *H. polymorpha* mutants affected in the selective degradation of peroxisomes (*pdd* mutants) have been identified. The mutants were screened by a direct colony colour assay, which allowed to monitor the decrease of alcohol oxidase activity as a result of selective inactivation. The collection of mutants obtained this way, all were impaired in either the first or the second step of the selective autophagy of peroxisomes. At present, we have identified five complementation groups, *PDD1* - *PDD5*. Mutations mapped in gene *PDD1* were affected in the initial step of peroxisome degradation We cloned the *H. polymorpha PDD1* gene by means of functional complementation of the mutant phenotype, using a *H. polymorpha* gene library. *PDD1* encodes a 116 kDa protein with high similarity to *S. cerevisiae* Vps34p, a protein shown to be involved in vacuolar protein sorting as well as endocytosis. Also *H. polymorpha* Pdd1p seems to play an essential role in the sorting of the vacuolar proteinase CPY to its target organelle, indicating that the protein is the true homologue of Vps34p. The protein is expressed constitutively, and localizes to peroxisomes as well as to vesicular structures. We propose a general role for Pdd1p/Vps34p in the tagging of membranous structures (vesicles, peroxisomes) that are destined for transport to endosomes en route to the vacuole for degradation.

Peroxisome-deficient mutants of H. polymorpha

In 1989 the first peroxisome-deficient mutants of *S. cerevisiae* (*pas*-mutants) and *H. polymorpha* (*per*-mutants) were isolated in a combined effort together with the groups of Kunau (Bochum; Germany) and Cregg, Oregon (USA). At present we have identified 28 complementation groups, namely 12 groups containing only constitutive *pex* mutants (renamed *PEX1-PEX12*) and 16 groups of conditional (ts) mutants.

Extensive complementation analysis of the 12 constitutive *pex* mutants revealed many cases of conditional non-complementation which were predominantly observed at lowered temperatures (cold sensitive non-complementation). These data strongly suggest the existence of functional and physical links between at least ten *PEX* gene products, essential for peroxisome biogenesis. At present, nine *H. polymorpha PEX* genes have been cloned and characterized.

Peroxisomal protein import.

In *H. polymorpha* the size of what we consider to represent 'mature' organelles is remarkably constant and predominantly prescribed by the prevailing growth conditions. We demonstrated that *in vivo* solely the few small organelles from the total peroxisomal population of the cells are capable to import newly synthesized matrix proteins. Our data unequivocally demonstrated that a heterogeneity exists between microbodies within one cell with respect to their capacity to incorporate newly synthesized proteins. This was not only true for *H.polymorpha*, but also for other yeasts e.g. *C. boidinii*.

The mechanisms which control the capacity to import proteins are not yet fully clear. Waterham at al. argued that Pex8p in involved in specifying the import capacity of individual peroxisomes but preliminary experiments indicated that also other *PEX* gene products play a role in this (e.g. Pex3p and Pex11p).Our results furthermore strongly suggested that the capacity of individual organelles to import proteins was correlated with the capacity to multiply (by fission). One possible explanation for this is that special regions on the peroxisomal membrane exist which mediate import ('import sites'); based on the observed functional links between different *PEX* genes, we speculate that different functions are concentrated in these regions (e.g. import, fission, protection against degradation) which are donated to the newly formed organelle during fission.

Peroxisomal proteins are encoded by nuclear genes and synthesized in the cytosol. The molecular mechanisms involved in sorting of these proteins do not share the typical features of other, extensively-studied protein translocation mechanisms (e.g. for mitochondria, ER, secretion in bacteria). The most striking example is the finding that proteins to be incorporated in the organelle do not necessarily have to be unfolded (see below). Moreover, all proteins involved in peroxisomal protein import identified so far are novel proteins, which have no homologous counterparts in other organelles. Hence, peroxisomal protein import seems to comprise novel and unique principles.

Matrix proteins are targeted by peroxisomal targeting signals (PTS), which are present within the primary sequence of the proteins, either at the extreme C-terminus (PTS1) or within the N-terminus (PTS2). So far, little is known on the sequences required for targeting of peroxisomal membrane proteins. As in other organisms, also in *H. polymorpha* most matrix proteins contain a PTS1. In this organism, variants of the PTS1 motif are found in the enzymes of methanol metabolism AO (-ARF), dihydroxyacetone synthase (-NKL) and catalase (-SKI). In *H. polymorpha*, the PTS1 import machinery is functional in fully repressed glucose-grown cells but strongly induced by methanol.

Only few *H. polymorpha* PTS2 proteins are known namely thiolase, amine oxidase, HpPex8 and Per6p. Faber et al. studied the PTS2 import machinery of *H. polymorpha* in detail and showed that this pathway was substrate-inducible.

The occurrence of different targeting signals for peroxisomal matrix proteins already predicted the presence of separate import machineries for PTS1 and PTS2 proteins. This was confirmed by the

isolation of mutant yeast strains, specifically impaired in import of PTS1 proteins (*PpPAS8, ScPAS10* and *HpPER3*, now called *PEX5*) or PTS2 proteins (*PEX7*). This function of the two receptor proteins is yet uncontroversial in that they bind to the corresponding PTS sequences. On the other hand, the exact location of the PTS receptors is still matter of debate. Different locations have been reported, ranging from completely cytosolic to a soluble peroxisomal matrix constituent. After delivery of their cargo, the receptors shuttle back to the cytosol.

In *H. polymorpha*, we believe that Pex5p has a dual location and is present in both the cytosol and the peroxisomal matrix. Based on this location we propose that Pex5p shuttles the protein to be imported into the organellar matrix, dissociates (probably mediated by the low internal pH of the organelle), eventually followed its assembly/activation and subsequent release of Pex5p from the matrix. In this shuttling mechanism of Pex5p, HpPex4p, a ubiquitin conjugating enzyme, is shown to play an essential role. Two mechanisms have been envisaged: 1) modification of a protein by ubiquitination is an essential step for shuttling Pex5p back to the cytosol or 2) Pex4p is essential to maintain functional import complexes. In this scenario Pex4p carries out the classical function of UBC proteins, namely tagging of proteins to be degraded and ubiquitinates non-functional protein import complexes or individual components of this complex, which are subsequently degraded (e.g. like degradation of non-functional SecY complexes by FtsH in *Escherichia coli).*

Recently two putative docking proteins, Pex13p and Pex14p , have been isolated with interact with the PTS1 (Pex13p) or both PTS1 and PTS2 (Pex14p). These data suggest that the PTS1 and PTS2 import machinery have common elements. On the other hand, both pathways are highly specific and cannot complement for each others functions.

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INSTRUCTORS

Course on

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF NON-CONVENTIONAL YEASTS

James M. Cregg	Department of Biochemistry & Molecular Biology, P.O. Box 9000, Portland, OR. 97291-1000 (USA).Oregon Graduate Institute of Science & Technology, Tel.: 1 503 690 12 17 Fax : 1 503 690 14 64 E-mail: cregg@admin.ogi.edu
Carmen Lisset Flores	Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 585 46 23 Fax : 34 1 585 45 87 E-mail: clflores@biomed.iib.uam.es
Carlos Gancedo	Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 585 46 20 Fax : 34 1 585 45 87 E-mail: cgancedo@biomed.iib.uam.es
Celedonio González	Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de La Laguna, 38206 La Laguna, Tenerife (Spain) Tel.: 34 22 60 37 92 Fax : 34 22 60 37 24 E-mail: cglez@ull.es
Germán Perdomo	Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de la Laguna, 38206 La Laguna, Tenerife (Spain). Tel.: 34 22 60 37 92 Fax : 34 22 60 37 24 E-mail: gperdomo@ull.es

Cristina Rodríguez

Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain).
Tel.: 34 1 585 46 23
Fax : 34 1 585 45 87
E-mail: crodriguez@biomed.iib.uam.es

José M. Siverio

Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de La Laguna, 38206 La Laguna, Tenerife (Spain)
Tel.: 34 22 60 37 92
Fax : 34 22 60 37 24
E-mail: jsiverio@ull.es

Oscar Zaragoza

Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain).
Tel.: 34 1 585 46 23
Fax : 34 1 585 45 87
E-mail: ozaragoza@biomed.iib.uam.es

LECTURERS

Course on

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Gerold Barth	Institute of Microbiology, University of Technology, Dresden, Mommsenstrasse 13, D-01062 Dresden (Germany) Tel.: 49 351 463 76 17 Fax : 49 351 463 77 15 E-mail: gbarth@rcs.urz.tu-dresden.de
James M. Cregg	(see list of instructors)
Angel Domínguez	Instituto de Microbiología Bioquímica, Departamento de Microbiología y Genética, Edificio Departamental, Campus Miguel de Unamuno 37007 Salamanca (Spain). Tel.: 34 23 29 46 77 Fax : 34 23 22 28 76 E-mail: ado@gugu.usal.es
Hiroshi Fukuhara	Institut Curie, Section de Recherche, Bâtiment 110, Université Paris XI, 91405 Orsay (France). Tel.: 33 1 69 86 30 63 Fax : 33 1 69 86 94 29 E-mail: fukuhara@curie.u-psud.fr
Claude Gaillardin	Genetique et Cellulaire Moleculaire Institut National Agronomique Paris Grignon, F-78850 Thiverval Grignon (France). Tel.: 33 1 30 81 54 52 Fax : 33 1 30 81 54 57 E-mail: claude@cardere.grignon.inra.fr
Carlos Gancedo	(see list of instructors)
Concha Gil	Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal, 28040 Madrid (Spain) Tel.: 34 1 394 17 48 Fax : 34 1 394 17 45 E-mail: gilmifar@eucmax.sim.ucm.es

Cornelis P. Hollenberg Institut für Mikrobiologie, Heinrich-Heine-Universität, Universitätsstr. 1, Gebäude 26.12.01, D-40225 Düsseldorf (Germany)
Tel.: 49 211 81 14 720
Fax : 49 211 81 15 370
E-mail: cornelis.hollenberg@uni-duesseldorf.de

Jan A.K.W. Kiel Department of Microbiology, University of Groningen, Biological Centre, Kerklaan 30, 9751 NN Haren (The Netherlands).
Tel.: 31 50 363 22 18
Fax : 31 50 363 21 54
E-mail: KielJAKW@biol.rug.nl

Cecilia Leão Departamento de Biología, Escola de Ciencias, Universidade do Minho, Campus de Gualtar, 4719 Braga (Portugal)
Tel.: 351 53 60 43 11
Fax : 351 53 60 43 19
E-mail: cleao@ci.uminho.pt

Isabel López-Calderón Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, 41080 Sevilla (Spain).
Tel.: 34 54 55 71 08
Fax : 34 54 55 71 04
E-mail: genilc@cica.es

Sergio Moreno Instituto de Microbiología Bioquímica, CSIC, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca (Spain).
Tel.: 34 23 12 15 89
Fax : 34 23 22 48 76
E-mail: smo@gugu.usal.es

César Nombela Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal, 28040 Madrid (Spain)
Tel.: 34 1 394 17 43
Fax : 34 1 394 17 45
E-mail: nommifar@eucmax.sim.ucm.es

José Pontón	Departamento de Inmunología, Microbiología y Parasitología, Facultad de Medicina y Odontología, Universidad del País Vasco, Apdo. 699, 48080 Bilbao (Spain). Fax.: 34 4 464 92 66 E-mail: oipposaj@lg.ehu.es
Rafael Sentandreu	Sección Departamental de Microbiología, Facultad de Farmacia, Universidad de Valencia, Avda. V. Andrés Estellés s/n, 46100 Burjassot, Valencia (Spain). Tel.: 34 6 386 42 99 Fax : 34 6 386 46 82 E-mail: rafael.sentandreu@uva.es
José M. Siverio	(see list of instructors)

PARTICIPANTS

Course on

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Lectures and Practicals

Judit Arenas-Licea	Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT (U.K.). Tel.: 44 171 380 70 33 Ext. 2234 Fax : 44 171 380 71 93 E-mail: judit@biochem.ucl.ac.uk
Manuel Becerra	Departamento de Bioloxía Celular e Molecular, Facultade de Ciencias. Universidade de A Coruña, Campus da Zapateira s/n, 15071 A Coruña (Spain). Tel.: 34 81 16 70 50 Ext. 2136 Fax : 34 81 16 70 65
Geraldine Butler	Department of Biochemistry, University College Dublin, Belfield, Dublin 4 (Ireland) Tel.: 353 1 706 15 53 Fax : 353 1 283 72 11 E-mail: gbutler@ucd.ie
Laura Capa	Glaxo Wellcome, Research Department, c/Severo Ochoa 2, 28760 Tres Cantos, Madrid (Spain). Tel.: 34 1 807 03 01 Fax : 34 1 807 03 10 E-mail: lc30720@ggr.co.uk
Francisco P. Chávez	Center for Genetic Engeneering and Biotechnology, Avda. 31 entre 158 y 190, PO Box 6162, Playa / Habana (Cuba) Tel.: 53 7 21 80 08 Fax : 53 7 21 80 70 E-mail: YeastLab@cigb.edu.cu
Inmaculada C. Cosano	Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense, Pza. Ramón y Cajal s/n, 28040 Madrid (Spain). Tel.: 34 1 394 17 44 Fax : 34 1 394 17 45

Ortansa Csutak	University of Bucharest, Faculty of Biology, Department of Microbial Genetics, Aleea Portocalilor No. 1-3, Bucharest 77206 (Romania). Tel.: 40 1 638 66 40 Fax : 40 1 312 10 08
Pau Ferrer	Departament d'Enginyeria Química, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona (Spain). Tel.: 34 3 581 10 18 Fax : 34 3 581 20 13 E-mail: ferrer@uab-eq.uab.es
José A. García-Lillo	Departamento de Microbiología, Facultad de Ciencias, Universidad de Extremadura, Avda. Elvas S/n, 06071 Badajoz (Spain). Tel.: 34 24 28 94 24 Fax : 34 24 28 94 28 E-mail: jagarcia@ba.unex.es
Susana García Sánchez	Departamento de Microbiología y Genética, Universidad de Salamanca, Edif. Departamental, Avda. Campo Charro s/n, 37007 Salamanca (Spain). Tel.: 34 23 29 46 77 Fax : 34 23 22 48 76 E-mail: tutanag@gugu.usal.es
Hernâni V. Gerós	Department of Biology, University of Minho, Campus de Gualtar, 4719 Braga Codex (Portugal). Tel.: 351 53 60 43 10 Fax : 351 53 67 89 80 E-mail: geros@ci.uminho.pt
Begoña Gorgojo	Instituto de Salud Carlos III, Centro Nacional de Microbiología, Unidad de Micología, Majadahonda, Madrid (Spain). Tel.: 34 1 509 79 61 Fax : 34 1 509 79 66
June Kopelowitz	Department of Clinical Microbiology and Infectious Diseases, Hadassah Medical School, The Hebrew University, 2nd Floor, 91120 Jerusalem (Israel) Tel.: 972 2 677 80 32 Fax : 972 2 643 44 34 E-mail: june@cc.huji.ac.il

Shwetal V. Mehta — Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400 005 (India).
Tel.: 91 22 215 29 71
Fax : 91 22 215 21 10
E-mail: shwetal@tifr.res.in

Rossella Menghini — Department of Cell and Development Biology, University of Rome "La Sapienza", P.le Aldo Moro 5, 00185 Roma (Italy).
Tel.: 39 499 12 259
Fax : 39 499 12 351
E-mail: bianchimic@axcasp.caspur.it

Valérie Meyrial — Heinrich-Heine-Universität, Institut für Mikrobiologie, Universitätstr.1, Geb. 26-12, 40225 Düsseldorf (Germany).
Tel.: 49 211 81 14 720
Fax : 49 211 81 15 370
E-mail:meyrial@rz.uni-duesseldorf.de

Daniela Mileto — Università degli Studi di Milano, Dipartimento di Fisiologia e Biochimica Generali, Sezione di Biochimica Comparata, Via Celoria 26, 20133 Milano (Italy).
Tel.: 39 2 706 44 801
Fax : 39 2 706 32 811

Elena B. Peskova — Institute of Biochemistry and Physiology of Microorganisms, RAS, Pushchino, Moscow region (Russia).
Tel.: 7 95 925 74 48
Fax : 7 95 923 36 02
E-mail: PESKOVA@ibpm.serpukhov.su

Catarina P. Prista — DBEB, Departamento de Botánica e Engenharia Biológica, Instituto Superior de Agronomía, Universidade Técnica de Lisboa, 1399 Lisboa (Portugal).
Tel.: 351 1 363 81 61
Fax : 351 1 363 50 31

Florian A. Salomons	Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), Biological Center, University of Groningen, Kerklaan 30, 9751 NN Haren (The Netherlands). Tel.: 31 50 363 21 50 Fax : 31 50 363 21 54 E-mail: F.A.Salomons@biol.rug.nl
Raffael Schaffrath	Harvard Medical School, BCMP Bldg. C1-214, 240 Longwood Ave., Boston, MA. 02115-5730 (USA). Tel.: 1 617 432 31 03 Fax : 1 617 432 25 29 E-mail: schaffra@bcmp.med.harvard.edu
Sabine Strahl-Bolsinger	Institute of Cell Biology and Plant Physiology, University of Regensburg, Regensburg (Germany). Tel.: 49 941 943 30 06 Fax : 49 941 943 33 52 E-mail: sabine.strahl-bolsinger@biologie.uni-regensburg.de
H. Markus Weiss	Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Straβe 7, 60528 Frankfurt/Main (Germany). Tel.: 49 69 96 76 93 54 Fax : 49 69 96 76 94 23 E-mail: Weiss@MPIBP-Frankfurt.MPG.DE
Igor Zeman	Department of Biochemistry, Faculty of Science, Comenius University, 84215 Bratislava (Slovakia) Tel.: 421 (0)7 796 540 Fax : 421 (0)7 729 064 E-mail: IZEMAN@fns.uniba.sk

Only Lectures

Joaquín Ariño	Departament de Bioquimica i Biologia Molecular, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona (Spain). Tel.: 34 3 581 21 82 Fax : 34 3 581 20 06 E-mail: J.ARINO@CC.UAB.ES

Susanne Gangl	Institut für Biochemie und Molekulare Zellbiologie, Universität Wien, Dr. Bohr-Gasse 9, A-1030 Wien (Austria) Tel.: 43 1 79 515 55 12 Fax : 43 1 79 952 72 E-mail: Sg@abc.univie.ac.at
Félix M. Machín	Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Avenida Astrofísico Fco. Sánchez s/n, 38206 La Laguna, Tenerife (Spain). Tel.: 34 22 60 37 92 Fax : 34 22 60 37 24
Antonio Martínez Ruiz	Departamento de Bioquímica y Biología, Facultad de C. Químicas, Molecular I, Universidad Complutense, 28040 Madrid (Spain). Tel.: 34 1 394 42 58 Fax : 34 1 394 41 59
José Ramos Ruiz	Departamento de Microbiología, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad de Córdoba, Avda. Menéndez Pidal s/n, 14080 Córdoba (Spain) Tel.: 34 57 21 85 21 Fax : 34 57 21 85 63

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