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Fundación Juan March

FEBS

Advanced Course

Biochemistry and Genetics of Yeast

Organized by

C. Gancedo
J. M. Gancedo
M. A. Delgado
I. L. Calderón

Fundación Juan March

Serie Universitaria

254

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INTRODUCTION

This manual contains protocols for a variety of genetic experiments which use ***Saccharomyces cerevisiae*** as biological material. The field is nowadays so vast and rapidly expanding that only a partial vision of it is possible. However we have tried to provide a collection of detailed protocols so that after completion of the course the participants shall be able to carry out the basic techniques used by the laboratories working in this area of modern biology.

The protocols provided are the product of an evolutionary process. With time there have been deletions, substitutions, insertions.....Therefore it is almost impossible to adscribe to an author the actual protocol. But this is the way of science; the work remains and the individual vanishes like raindrops falling into a flowing river.

We thank all persons and Institutions that have helped in different ways in the organization of this course. Particular thanks are due to **FEBS** and **Fundación Juan March** for their generous financial support, to the Instituto de Investigaciones Biomédicas C.S.I.C., Departamento de Genética de la Facultad de Biología de Sevilla, and Cruzcampo S.A., for allowing the organizers to dedicate their time to the course, to the Departamento de Bioquímica ,Facultad de Medicina UAM for the locals where the course takes place and to Andrés González (Fundación Juan March) for his warm support

We hope that the time and effort dedicated to the course will be useful for people working with yeast.

The organizers

Madrid and Sevilla , July 1990

EXPERIMENT A

ETHYLMETHANE SULFONATE INDUCED MUTAGENESIS

INTRODUCTION

Since spontaneous mutation frequencies of nuclear genes are low, yeast mutants are usually isolated by treating a culture with a mutagenic agent such as ultraviolet radiation, ethylmethane sulfonate (EMS), nitrous acid (NA) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). These mutagens induce mutations at a rate of 10^{-5} to 10^{-3} per gene, which is substantially higher than the spontaneous rate (10^{-6} to 10^{-8} per gene). Haploid yeast strains should be used if recessive mutations are desired; for dominant mutations, diploid strains may also be used.

In this experiment we will use EMS to induce mutants in yeast. Auxotrophic mutants will be isolated and characterized.

Ethylmetane sulfonate mutagenesis

EMS is an alkylating agent causing mainly single base pair substitutions. 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 thiosulphate, which neutralizes it effectively without damaging the cells.

When dealing with EMS mutagenesis, two precautions must be considered:

1) Like any other mutagen, EMS does not discriminate between yeast and human. If applied without care, it may be hazardous for our environment. Therefore, EMS handling must be absolutely clean and safe.

2) Yeast cells are more than just the gene we try to mutate. A powerful mutagenic treatment may induce unwanted additional mutations. Mutagenesis should be, therefore, as light as possible. It is also advisable to clean a newly isolated mutation from its mutagenized genetic background by at least two consecutive crosses with a wild type strain.

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Controls in mutagenesis experiments

Mutagens not only induce non-lethal mutations, but also have toxic effects. Viability (V) after treatment is determined by plating cells on complete medium (YPD) before and after the mutagenesis and comparing the number of colonies grown. It is expressed as the fraction of cells which survives the mutagenic treatment. For any given mutagen, V is proportional to the dose of the treatment (mutagen concentration, time of exposure, etc.), thus becoming a measure for it.

For any given V, some mutagens yield more mutants than others depending on their strength. Mutant frequency (MF) is expressed as the fraction of the surviving cells which has become mutant. It only applies to the particular type of mutant we are detecting. The MF represents an estimation of the mutation rate for a certain gene or genes.

A practical way of controlling a mutagenesis experiment is to determine V and MF, applying MF to an easy to score type of mutants (e.g. auxotrophs or resistant to canavanine). In this way we can check that the intensity of the treatment has not been too weak (low mutant yield), or too strong (high genetic background noise).

Isolation and identification of mutants

The need for a selective step in the isolation of mutants depends on mutation rate and the properties of the mutant phenotype. Imagination, resolution and luck are the principal ingredients to find the right selective protocol for a particular mutant.

In this experiment we will isolate auxotrophic mutants. Their frequency should be high enough to make a selective step unnecessary. The mutagenized culture is plated on YPD at a concentration which yields about 100 - 200 cells per plate. After these cells have grown into colonies, they are relica plated to minimal medium (SD). Newly generated auxotrophs are detected by their inability to grow on the replicas.

Phenotypic characterization of auxotrophic mutants

A further step in the experiment is the characterization of nutritional requirement of the auxotrophic mutants. All the auxotrophs are placed on a YPD plate 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	adenine	guanine	cysteine	methionine	uracil
#7	histidine	leucine	isoleucine	valine	lysine
#8	phenyl-alanine	tyrosine	trypto phan	threonine	proline
#9	glutamate	serine	alanine	aspartate	arginine

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

Genotypic characterization of mutants. Complementation analysis

After isolating and phenotypically characterizing a mutant, the next step would be to study its genotype. A clear distinction must be drawn between phenotype and genotype: the first describes a behaviour and has only an operational value; the second refers to inherited properties of genes, with a precise molecular meaning in terms of DNA sequence.

To define the genotype corresponding to a mutant phenotype it is first necessary to determine the number of genes affected. This is done by meiotic analysis. We usually look for mutant phenotypes determined by an alele of a single gene (monogenic).

Auxotrophic mutations are normally recessive. For a set of monogenic, recessive mutants with the same phenotype, e.g. His^-, the next step in genetic analysis would be to determine their complementation relationship. This is performed by constructing diploids carrying each pair of different mutations. Complementation of the

mutations results in a wild type diploid and indicates that they are located in different genes. The number of complementation groups gives information on the total number of genes involved in a certain function.

Strains

- Saccharomyces cerevisiae X2180-1A : MATa mal gal2 CUP1 mel SUC2

Experimental

Day1

Spot the strain on YPD. Incubate at 30°C overnight.

Day 2

EMS mutagenesis. Suspend cells in 2 ml of sterile sodium phosphate buffer (0.1 M, pH 7) to get a concentration of about 10^8 cells/ml. A 10^8 cells/ml suspension has a milky appearance, which has to be learned through experience. To determine the concentration, count a 10^{-2} dilution in the Thoma chamber.

Take an aliquot of the cell suspension and dilute sequentially 10^{-4}, 10^{-5} and 10^{-6} in sterile water (0.55 ml in 5 ml is a 10^{-1} dilution; 0.05 ml in 5 ml is a 10^{-2} dilution). Plate 0.1 ml of each dilution on a YPD plate. Spread 2 plates per dilution. Incubate at 28ºC for 4 or 5 days.

Put 1 ml of the cell suspension in a new tube, add 30 µl of EMS and disperse by gentle agitation. Incubate for 1 h 30°C with occasional shaking. Stop the EMS action by adding 0.55 ml of EMS suspension to 5 ml of sterile 5% sodium thiosulphate. Dilute to 10^{-2}, 10^{-3} and 10^{-4}. Plate 0.1 ml of each dilution on a YPD plate. Make 5 plates per dilution. Incubate at 28°C for 4 or 5 days.

Day 3

Estimate viability after mutagenesis (V) by counting colonies grown in non-mutagenized and mutagenized plates.

With toothpicks, take about 200 colonies from the mutagenized plates and make patterns on YPD plates (about 60 colonies per plate, as marked in the provided grid). Incubate for 1 to 2 days at 30°C.

Day 4

Replica plate the YPD patterns to SD plates.

Day 5

Compare growth in YPD and SD plates. Note the frequency of auxotrophes among total colonies.

Colonies whose replicas fail to grow on the SD plates are the auxotrophic mutants; transfer them with toothpicks to a pattern on a fresh YPD plate. Incubate overnight at 28ºC.

Day 6

Replica plate the master plate containing the mutants to a set of SD plates containing several amino acids and bases numbered #1 to #9. As controls, use also SD and C (complete) plates.

Day 7

Determine the auxotrophic phenotype of each mutant.

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EXPERIMENT B

RANDOM SPORE ANALYSIS

Meiosis and sporulation in Saccharomyces

The haploid genome of Saccharomyces has 16 chromosomes and a small chromosomal fragment. This high number, together with a high frequency of crossing over, offer plenty of possibilities for meiotic recombination between markers.

Only strains heterozygous for mating type, usually MATa/MATα diploids, can sporulate. Sporulation is a complex morphogenetic process which shows some sensitivity to temperature (sporulating cultures should be incubated at temperatures not higher than 25 ºC). Typical sporulating protocols start by growing cells in a pre-sporulation medium (GNA), very rich in sugar (10% glucose or sucrose) until well into the stationary phase. Biomass is then transferred to a sporulation medium (SPO), containing sodium or potasium acetate as the principal carbon source. Meiosis takes place in SPO after 5-15 days, resulting in the formation of asci with four haploid spores. Lytic enzymes can then be used to digest the ascal wall, liberate the spores and analyse their genotypes. The meiotic products can be studied as a population (*random spore analysis*) , or, alternatively, individual asci can be micromanipulated and isolated allowing the analysis of the products of a single meiosis event (*tetrad analysis*).

Yeast sporulation ressembles human pregnancy in that it requires certain time. The researcher is advised to be patient and wait for a good sporulating yield, with many four-spored, ripened asci.

Random (mass) spore isolation

After the lytic enzyme treatment, a vigorous shaking of the sporulating suspension would break up clumps and tetrads and liberate the spores which can then be plated out for single colonies. The success of the method depends on an effective selection for the haploid spores and against the non-sporulated diploids. A strong treatment with the lytic enzyme will (at least in theory) kill the diploids since their cell

wall is more sensitive than that of the spores. More effectively, haploids can be selected for by using genetic markers. For instance, if the diploid is heterozygous for a recessive resistance marker, e.g. can1/CAN1 (canavanine resistance) and the digested suspension plated on medium containing canavanine, only haploid *can1* spores will grow. In this course we will use another selection method based on the pink colour conferred to the colony by the *ade1* and *ade2* alleles.

Random spore analysis

Two important genetic results can be derived from random spore analysis:

1) Construction of strains. The random spore method is an easy and quick tool for the construction of strains with a certain combination of markers. It is the method of choice for a researcher frightened by tetrad micromanipulation.

2) Number of aleles responsible of a certain phenotype, e.g. His^-. A His^-/His^+ diploid is constructed and analyzed. If the His^-, recessive mutant phenotype, is determined by only one gene, e.g. HIS4, the spore segregation will be 1 His^-:1 His^+.

3) Linkage between genetic markers. If two genes are linked, the random spores analysis will show a significantly higher parental than non-parental combination of genotypes.

Even if the above considerations are true (and they are true in theory), random spore analysis is not an accurate genetic method. Unknown selective pressures may operate against certain genotypes. This makes impossible to warrant any genetic interpretation of the segregation data. Tetrad analysis (see **Experiment C**) is the only safe alternative. Random spore analysis should be used only when poor spore viability makes difficult tetrad analysis.

In this experiment we will use a random spore analysis protocol to determine linkage between different markers present in a diploid. In the first place we will isolate spores from a sporulating biomass. Determination of nutritional requeriments of the spores will be done by replica plating to an appropriate set of media.

STRAINS

- Sporulated diploid Saccharomyces cerevisiae D649 MATa/MATα MAL2/mal2 trp1/TRP1 pet6/PET6 ade2/ADE2 ADE1/ade1 lys2/LYS2 HIS4/his4 LEU2/leu2 THR4/thr4

EXPERIMENTAL

Day 1

Digestion of asci. Resuspend a loop of sporulated biomass in 1 ml of a 1:40 dilution of the lytic enzyme. Shake intensively. Incubate at room temperature with occasional shaking to digest most of the asci, which results in liberation of free spores (1 to 2h). Disperse the clumps and tetrads by sonicating the suspension for about 1 minute (until most cells and spores are isolated). To avoid excessive heating produced by the sonication , the tube has to be placed in ice after treatment for about 2 min.

Make 10^{-3} and 10^{-4} dilutions in sterile water. Plate 0.1 ml of each dilution on three YPD plates. Incubate at 30ºC.

Day 2

Pick about 40 pink colonies with sterile toothpicks and streak them making a pattern on a YPD plate (use the provided grid).

Day 3

Replica plate the pattern to the following media :

1. YPD
2. YPG
3. SD+ Trp+Lys+His+Leu+Thr
4. SD+Ade+ Lys+His+Leu+Thr
5. SD+Ade+Trp+ His+Leu+Thr
6. SD+Ade+Trp+Lys+His+Thr
7. SD+Ade+Trp+Lys+His+Leu
8. SD+Ade+Trp+Lys+His+Leu+Thr

Day 4

Score the segregation results. Try to establish linkage between markers analysing the segregation of each pair of markers.

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EXPERIMENT C

MEIOTIC MAPPING BY TETRAD ANALYSIS

Introduction

Tetrad analysis is the most important genetic tool in Saccharomyces. Provided simple laboratory facilities and some training, it becomes an easy task. Beginners tend to be afraid of the difficulties found in asci micromanipulation. We will try to do away with those feelings. In this Introduction, rather than discussing genetic principles which can be found in many textbooks, we emphasize the practical aspects of asci dissection and micromanipulation.

Micromanipulating yeast cells

Yeast cells, because of their size, shape and hardness, are easy to micromanipulate. Extensive use of micromanipulation is made not only in tetrad analysis. It is also widely used in the isolation of vegetative cells and zygotes. Basically, this technique consists in picking up a cell with a glass microneedle and then transferring it to a marked place on an agar surface. The equipment needed for successful micromanipulation consists of:

1) Microscope. The best suited microscopes are the "inverted" ones, in which focusing takes place by moving up and down the optical tube, rather than the stage. This allows a fixed vertical relationship between the micromanipulation chamber (or the Petri dish) and the microneedle. Ideal magnification is 100-200x, which may be obtained with a 10-20x objective and a 10x binocular. Objective and condenser should have long focal lenght to allow for the intercalation of the micromanipulation chamber without loses of optical resolution.

2) Micromanipulator. Many different models are available. All are good once you become used to them. Most important is the smoothness of the vertical movement, which picks up the cells.

3) Micromanipulation chamber. Not always needed; usually bricolaged. In certain equipments, it becomes necesary in order to adapt the agar surface where micromanipulation takes place, to the

microscope. Sometimes it is possible to micromanipulate directly on the surface of a YPD Petri dish.

4) Glass microneedle. The most important item of the equipment. It can be bought but hardly borrowed. It can easily made shaping a 2 to 3 mm diameter Pyrex glass rod in the small flame of an ordinary Bunsen burner. The end is broken off so that the tip has a diameter of 10 to 100 µm and a length of a few mm. It is most important that the microneedle has a perfectly flat end, without convexities, cavities or protrusions. This can be got by cutting the tip with a razor blade or with the edge of a cover slip, usually under binocular observation. This operation is the most crucial step in microneedle making; it is certainly time consuming and sometimes requires several attempts. Microneedles with seemingly flat ends should be checked under the microscope before testing for their micromanipulating performance. A good microneedle has to pick up and release cells easily.

It is worhtwhile to spend time in the making of microneedles; whereas a good microneedle makes micromanipulation a fruitful and relaxing job which ressembles knitting, not so good ones waste time and may lead to very frustrating experiences.

Dissecting tetrads

It goes through the following steps:

1) Sporulated biomass. (See Experiment B). Sporulation rate should reach a minimal level (at least 20% cells transformed to asci, more than 50% of which contain 4 spores). If not so, the microscopic searching for tetrads becomes very difficult and a random spore protocol is advised.

2) Digestion of asci . Put a drop of a dilution of the lytic enzyme of choice on a piece of parafilm paper placed in an empty Petri dish. Suspend a loop of sporulated biomass in the drop. The dilution factor of the enzyme depends on its particular source, but ussually ranges between 1:4 to 1:40; it has to be established by preliminar trials. Time of incubation of the asci is also variable and depends on the specific preparation. It is important to apply the right digestion treatment:

underdigestion makes difficult the dissection of the asci; overdigestion impairs spore viability.

3) Asci dissection. A loop of digested suspension is streaked on an agar surface (slab or Petri dish). Cell concentration is critical to allow the microneedle to pick up asci without being hindered by neighbouring cells. Four points, separated at least 5 mm between them and from the streak, are marked on the agar surface for each tetrad. A whole tetrad is picked up and transferred to the nearest of the 4 points, where the actual dissection takes place. The four spores are separated with the microneedle. Three of the spores are transferred to the 3 other marked points. The dissected tetrad is incubated to grow into colonies which are then characterized.

Applications of tetrad dissection

1) Construction of strains. Tetrad dissection allows the construction of a strain with novel combination of genetic markers. To be sure of its genotype, it is preferable that it descends from a wholly viable tetrad. All the products of this particular meiosis have to be known, giving an accurate internal control of the novel strain origin and composition.

2) Number of aleles responsible of a certain phenotype. The first step after isolating a mutant is to establish the number of genes that determine its phenotype. This is best done by analysing tetrads derived form the cross of the mutant by a wild type strain. A 2 mutant : 2 wild type segregation means single gene inheritance.

3) Linkage between genetic markers. When analysing tetrads derived from a hybrid which is heterozygous for two markers (e.g. AB / ab), three types of tetradsare obtained:

- Parental ditype (PD) AB : AB : ab : ab
- Non-parental ditype (NPD) Ab : Ab : aB : aB
- Tetratype (T): AB:Ab:aB:ab

The following frequency distributions are expected:

a) Independent assortment (unlinked genes)

1PD : 1NPD : 4T

b) Linked genes PD > NPD

c) Unlinked genes and at least one of them, unlinked to the centromere
1PD : 1NPD : 4T

d) Genes in different chromosomes but both linked to their respective centromere
1PD : 1NPD : <4T

4) Mapping. Tetrad analysis is the most accurate method to map linked genes in a chromosomes. Distance (d) in centimorgans is given by:

$$d = \frac{T + 6\,NPD}{PD + NPD + T} \times 50$$

STRAINS

-Sporulated diploid : D649 MATa/MAT α MAL2/mal2 trp1/TRP1 pet6/PET6 ade2/ADE2 ADE1/ade1 lys2/LYS2 HIS4/his4 LEU2/leu2 THR4/thr4

- Tester strains for the mating type:
  MMY1 MATa ura3-52 CyhR
  MMY2 MATa ura3-52 CyhR

EXPERIMENTAL

Tetrads will be dissected by the course participants during the first week, and subsequently analysed. In case of failure in the dissection, or low spore viability, tetrads dissected by the instructors will be provided. Proceed similarly to Experiment B. Determination of nutritional requeriments of the spores will be done by replica plating to an appropriate set of media. Mating type, which can be considered as just another genetic marker, will be established by using the cross-stamping method. This method is especially useful where the clones are known to contain auxotrophic requirements which can be utilized to detect the occurrence

of prototrophic diploids arising from mating with suitable tester strains. These strains, one a and one a, should carry an auxotrophy complementary to any marker present in the sporulating diploid.

Day 1
Prepare a YPD master plate containing the spore colonies, using the provided piece of paper (Note that this time we will make streaks rather than crosses)

Day 2
Replica plate the master plate to the following media:

1. YPD
2. YPG
3. SD+ Trp+Lys+His+Leu+Thr
4. SD+Ade+ Lys+His+Leu+Thr
5. SD+Ade+Trp+ His+Leu+Thr
6. SD+Ade+Trp+Lys+His+ Thr
7. SD+Ade+Trp+Lys+His+Leu
8. SD+Ade+Trp+Lys+His+Leu+Thr

To test the mating type, you will need the replicated YPD plate (plate n. 1), sterile wooden strips and a plate containing wide streaks of the tester strains, which will be provided. Remove a wooden strip from the container with sterile forceps and dip it lightly into the a tester strain, so that the whole edge is covered. Press the strip down, perpendicularly to the upper part of the first set of streaks on the YPD plate, as shown in the figure. Take other strips and repeat the process to cross stamp the upper part of all the sets of streaks. Now repeat the process cross-stamping the lower part of the streaks with the α tester strain. Incubate all plates at 28ºC.

Day 3
Replica plate the cross-stamped plate to SD and incubate at 28ºC.

Day 4

Score results (along with Experiment B). Try to establish linkage between markers and with centromeres, analysing the segregation of each pair of markers.

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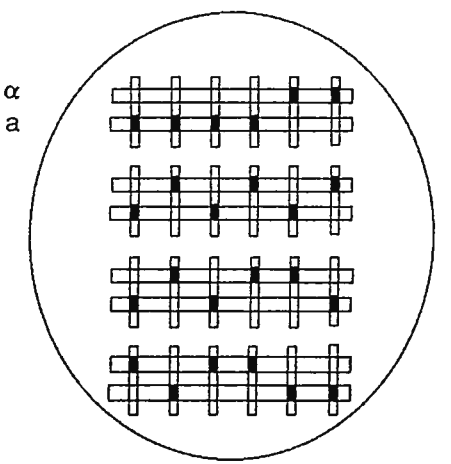
(Same as in Experiment B)



EXPERIMENT D

ISOLATION OF NONSENSE SUPRESSORS

INTRODUCTION

When a mutant, or a group of mutants, revert due to the appearance of a new mutation at a different locus, this new mutation is called a *suppressor*. Suppressors that act on any of the three nonsense codons UAA (ochre), UAG (amber), or UGA (umber), are called nonsense suppressors. In E. coli, the amber suppressor act on UAG (amber) mutants, the ochre suppressors act on both UAG (amber) and UAA (ochre) mutants, and the opal suppressors act on UGA mutants. In yeast there appear to be suppressors that act solely on each of the nonsense suppressors, and these are referred to, respectively, as UAA, UAG or UGA suppressors. Suppressors that can suppress all three nonsense codons are denoted as omnipotent. UAA, UAG, and possibly UGA suppressors are formed by the mutational alteration of the anticodons of tyrosine, leucine, and serine tRNAs; most, if not all, omnipotent suppressors arise by the mutational alteration of certain components of ribosomes, resulting in decreased fidelity of translation.

A convenient but sometimes ambiguous method for detecting and defining a suppressor mutant is by the action on a group of suppressible genes, such as the nine suppressible genes in the strain 3971-5B. Suppressor mutations can be isolated by plating the strain on media lacking one or more of the growth requirements. The pattern of suppression can be determined by testing the revertant strains on various media. The single-gene nature of suppression can be verified by analysis of crosses containing the suppressors.

Strain

Saccharomyces cerevisiae 3971-5B : MATa gal10 SUC mal trp1-1_a ura3 ura4_o met8-1_a ade5-7_a leu2_u lys1-1_o aro1D_a ilv1-1_o can1_o

The aro1D mutation causes simultaneous requirement for tyrosine, phenylalanine and tryptophan.

met8-1 , lys 1-1 and leu2 are amber, ochre and opal(umber) suppressible markers, respectively; trp1-1, ura4, ade5-7, aro1D, ilv1-1 and can1 are also suppressible markers

EXPERIMENTAL

Day 1

Inoculate 3 YPD plates with a lawn of the strain 3971-5B.

Day 2

Select for the reversion of markers by replica plating each YPD plate to one set of three plates containing the following media:

A) SD + trp + ura + ade + leu + lys + tyr + phe + ilv (-MET)
B) SD + trp + ura + met + ade + leu + tyr + phe + ilv (-LYS)
C) SD + trp + ura + met + ade +lys + tyr + phe + ilv (-LEU)

Day 3

Pick several colonies grown on each type of media and pattern them on YPD plates.

Day 4

Replica plate the master plate on media lacking single growth requirements and, as controls,on a medium containing all the requirements.

Day 5

Determine which markers are probably suppressed by the same type of suppressor by "correversion" of the different markers.

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EXPERIMENT E

MATING BETWEEN HOMOTHALLIC AND HETEROTHALLIC STRAINS

INTRODUCTION

In yeasts, there is generally a bipolar mating system. In S. cerevisiae , the mating-type alleles (MATa and MATα) perform a regulatory function controlling the phenotypic expression of the haploids a or α and of the diploid a/α. There are several copies of the mating type alleles in an haploid genome: one is expressed, the others are silent. They can however be activated either by mutation of controlling genes or by a transposition/gene conversion event. These phenomena result in a change of the mating type. The switching frequency is controlled by the locus HO. Laboratory strains are Ho^-; they only rarely change mating type and are called ***heterothallic***. Industrial strains are usually Ho^+; they change mating type as frequently as once per generation, and are called ***homothallic***. As a result, their haploid phase is only transient and most of them are isolated as diploids. This makes genetic analysis and construction of hybrids with these strains especially difficult.

Several methods have been developed for making hybrids involving industrial and laboratory yeast strains. In this experiment we will use one of them based on the dominant mutation CYH , which confers to the strain resistance to cycloheximide. We will induce sporulation and break up asci of a Cyh^s wine yeast strain. The spores will be crossed with a Cyh^r Aux^- laboratory strain. Hybrids will be selected by their ability to grow an a minimal medium plate + cycloheximide in which neither of the parents can.

STRAINS

-S. cerevisiae IFI182 (strain isolated from Jerez wine)
MATa /MATα HO /HO suc/ suc

-S. cerevisiae MMY1 (laboratory strain)
MATα ura3-52 Cyh^r

EXPERIMENTAL

Day 1

Digestion of asci.

Resuspend a loop of sporulated IFI 82 biomass in 1 ml of a 1:40 dilution of the lytic enzyme. Mix. Incubate at room temperature with occasional shaking to digest most of the asci, which results in liberation of free spores (1h). Disperse cells and spores vortexing intensively the suspension for 2 or 3 min. Spin suspension and resuspend cells in 0.5 ml of YPD. *Mating*

Make a suspension of MMY1 of about 10^7 to 10^8 cells/ml in liquid YPD. Mix the IFI182 and the MMY1 suspensions in a 1 to 3 proportion and allow mating by incubating the mixture at 28ºC for 3h to 4h. Make 10^{-3} and 10^{-4} dilutions in sterile water. Plate 0.1 ml of each dilution on three plates of SD + cycloheximide (5 mg/ml). As a control, make similar dilutions of the starting suspensions and plate on two SD +cyh plates. Incubate plates at 28ºC.

Day 2

Compare growth on the different plates.

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Cross, F, L.H. Hartwell, C. Jackson and J.B. Konopka (1988) Conjugation in Saccharomyces cerevisae Ann. Rev. Cell Biol. 4: 429-457.

EXPERIMENT F

THE SEXUAL CYCLE OF SACCHAROMYCES CEREVISIAE

In this experiment we observe the sexual cycle of S.cerevisiae and make use of it for different purposes. The sexual cycle in haploid S. cerevisiae strains follows an ordered sequence of events: haploid cells of opposite mating type MAT α and MAT a when mixed together commit themselves to the process of mating. This process has three visually observable stages : 1) Courtship, 2) Cell fusion, 3) Nuclear fusion and diploid budding. Courtship is the mating limiting step. Aerobic incubation and a rich medium enhance mating efficiency. Mixing cells with a toothpick on a YPD plate is a simple and effective mating protocol.

MAT α and MAT a produce pheromones which influence the cells of opposite mating type. One effect is to produce sexual agglutination. Under the effect of the sexual peptides clumps of 10 to 10000 cells form with intimate contact between cells. In these clumps pear shaped cells named ***shmoos***. Two shmoos of opposite mating type fuse and a binucleated zygote is formed. Nuclear fusion occurs immediately and the zygote starts to bud off **diploid** vegetative cells. A prominent advantage of Saccharomyces as a genetic system is the facility with which stable diploids are formed . Diploids have many genetic applications: dominance analysis, complementation tests, mitotic and meiotic recombination , mapping, gene dosage studies etc. Diploids may be differentiated by size, shape, budding patern and sporulating ability from their haploid counterparts.

In some very rare cases nuclear fusion fails . In this case ***heterokaryons*** are formed. They are very unstable as the component nuclei are often unable to migrate to the daughter bud (missegregation) or loose chromosomes (inactivation). Aś a result heterokaryons become quickly a mixture of ***heteroplasmons*** , strains which contain the nuclear genome of one of the parents in a cytoplasm that is a mixture of the cytoplasmic components of both . Heterokaryons have been used to transfer single chromosomes from one nucleus to another. A

mutation named *kar1-1*, has been described (Conde and Fink 1976) that is defective in nuclear fusion. The intercellular transfer of cytoplasmic replicons using heteroplasmon formation has been called ***cytoduction*** . Cytoduction mediated by *kar 1-1* is a very efficient process and finds many applications in extranuclear genetics. We will use it in this experiment to transfer mitochondria from a ρ^+ donor to a ρ^- cell. (kar1-1 mutants are leaky, *ca.* 10^{-2} of the binucleated zygotes fuse nuclei normally and become diploids).

Forced mating.- Only cells of opposite mating types mate. If we wish to mate cells sexually incompatible i.e. homosexual pairs a x a or α x α or pairs in which one of the partners is an a / α non-mater a forced mating protocol is used. The genotypes of the cells to be mated must be able to complement in one medium where none of the parents would grow. This selective pressure picks up the rare events where one of the cells has become phenotypically or genotypically a mater. An extreme form of forced mating is protoplast fusion. In this case the biological constraints of mating are bypassed in an often succesful chemical tour de force.

Sporulation.- Freshly isolated diploids are grown on a rich medium containing a high concentration of glucose (GNA) and then transferred to a poor one (SPO) . In these conditions meiosis and sporulation take place with the formation of four spored asci usually called ***tetrads.*** Tetrad analysis is a powerful genetic method of analysis that everyone trying to work seriously with yeast should master.

The following strains will be used along this assignement:

S.cerevisiae K5-5A : MAT α *his 4-d15, ade2-1, kar1-1, can1* , ρ^-.

S.cerevisiae D585 11C : MAT a, *lys 1*, ρ^+.

Day 1

Spot both strains on a YPD plate.

Day 2

The spots will have already grown. Make a mating mixture on the same YPD plate taking biomass from both spots with a sterile toothpick and mixing them together. Incubate at 30º C . After 3 to 5 hours shmoos and zygotes will be visible under the microsope. When

enough zygotes are formed (at least 10 % of the whole population) streak the mating mixture on plates of YPD, SG + His+Ade and SD. Incubate at 30 ºC

Day 3

Transfer several colonies from the SD plate to GNA plates using a toothpick. Try to streak a surface of about 1 cm^2 to get enough biomass. Incubate at 30 ºC

Day 4

Transfer the well grown biomass from the GNA plate to a SPO plate spreading it as a thin layer with a toothpick. Incubate at 30°C.

Day 5

Observe the streaks done on Day 2.

YPD plate: Parental colonies, diploids and heterokaryons are white on YPD, whereas His^- Ade^- ρ^+ heteroplasmons are red.

SG+ His+Ade plate : Only diploids , heterokaryons and His^- Ade^- ρ^+ heteroplasmons are expected to grow on this plate.

SD plate: Only diploids and heterokaryons are expected to grow on SD. Colony size of heterokaryons is very small compared to diploids , also their cell shape is different.

On three YPD plates streak with a toothpick representative colonies of the three groups (diploids, heterokaryons and His^- Ade^- ρ^+ heteroplasmons) using the grid provided to make a pattern. Include the parentals as a control. Incubate at 30 ºC.

Day 6

Replicaplate the three patterns to SD, SD+His+Ade, SD+Lys and YPG plates. This set of media allows the distinction between parentals, *ade 2-1* ρ^+ heteroplasmons , diploids and heterokaryons.

Day 7

Observe the biomass of the SPO plate under the microscope.

Score the results of the plates replicated on Day 6.

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Conde J. and Fink G.R., A mutant of Saccharomyces cerevisiae defective for nuclear fusion. Proc. Natl Acad.Sci. (USA), 69,2846-2849 (1976).

(The isolation and properties of *kar1-1* mutants)

Dutcher S.K. Internuclear transfer of genetic information in kar1-1/KAR1 heterokaryons in Saccharomyces cerevisiae. Mol. Cel.Biol., 1,245-253 (1981)

(Description of transfer of single chromosomes from a donor to a recipient nucleus.)

Nilsson-Tillgren T.C., Gjermansen M.C., Kiellandt-Brandt J.G., Petersen L. and Holmberg S. Genetic differences between Saccharomyces cerevisiae and Saccharomyces carlsbergensis. Analysis of chromosome III by single chromosome transfer. Carlsberg Res. Comm., 46,65-76 (1981).

(Transfer and substitution of single chromosomes between nuclei of two Saccharomyces species)

EXPERIMENT G

FORMATION OF RESPIRATORY-DEFICIENT *PETITES* BY ETHIDIUM BROMIDE TREATMENT

Petite mutants (ρ^- mutants), are respiratory deficient mutants that arise spontaneously with a relatively high frequency in cultures of normal strains of S.cerevisiae. They were called so due to their smaller colonial size in certain media . The frequency of appearance of *petites* may be increased by a variety of treatments. Ethidium bromide is particularly effective for this purpose. It interferes with replication of yeast mitochondrial DNA inducing its complete loss. An easy and effective method to obtain *petites* using ethidium bromide is shown in this experiment.

CAUTION : Ethidium bromide is highly carcinogenic. Handle with care. Report immediately any spill !.

Strains provided:

Saccharomyces cerevisiae K5-5A

MATα his4-Δ15 ade2-1 kar1-1 can1 ρ+

Day 1

A solution of 10 mg/ml of ethidium bromide will be provided . Take 5 µl of this solution and place them in the center of a YPD plate. Immediately take a loop of fresh yeast biomass and extend it along a diameter of the YPD plate crossing the ethidium bromide droplet. Incubate at 30 ºC.

Day 2

Observe the plate. A growth inhibition zone around the ethidium bromide droplet will be visible. Take a loop of biomass from the border of the inhibition zone and streak it in zig-zag on a YPD plate. Incubate at 30ºC.

Day 3

Make a pattern on a YPD plate with colonies grown on the zig-zag.

Day 4

Replicaplate to YPG and YPD plates.

Day 5

Evaluate the results.

(Petites may be easily distinguished by colony size on plates of YP + 0.1% glucose +2% glycerol. After three to four days growth on this medium normal cells are much bigger than the *petites* since these can only use the limiting amount of glucose for growth).

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O´ Connor R.M., McArthur C.R. & Clark-Walker G.D., J. Bacteriol., 126, 959- 968 (1976). Respiratory-deficient mutants of Torulopsis glabrata, a yeast with circular mitochondrial deoxyribonucleic acid of 6 μm

(The original description of the method used in this experiment)

EXPERIMENT H

KILLER YEASTS

Certain strains of yeast secrete a toxin which is lethal for other strains. These strains are named ***killer*** strains and are themselves immune against the toxin they produce . The killer phenotype is determined by the presence of a particular double stranded RNA (ds-RNA) plasmid encapsulated in virus-like particles. Therefore the killer trait is inherited in a non-Mendelian way. Two types of ds-RNA have been decribed: L (larger) and M. L ds RNA is present in both killer and non-killer yeasts. It codes for the major protein of the particles in which L and M forms are encapsulated. But it is M dsRNA that codes for the killer toxin and is therefore responsible for the killer phenotype. Maintenance of this ds RNA requires a series of genes (*mak* genes, of which more than 26 have been reported) and its expression is dependent on other genes (KEX and REX) . The killer trait is easily eliminated by treatment of the yeast with cycloheximide or exposition to high temperatures.

Three different killer traits have been described: K_1,K_2 and K_3. K_1 trait is mainly found among laboratory strains while K_2 and K_3 traits are found among wine yeasts, brewery and in general wild type yeasts. Strains K_1 and K_2 are sensitive to each other's toxin while K_2 and K_3 are cross immune . Killer strains are indicated as KIL- K (with the corresponding number 1,2 or 3) and the lack of killer trait is noted KIL-K_0. Resistance is indicated as R.

Killer activity is easily visualized on plates by the inhibition of growth of sensitive strains exerted by killer strains. Usually 0.07% methylene blue (a vital stain) is added to the plate. A lawn of a sensitive strain is inoculated on a plate and strains to be tested are spotted on top. Killer strains produce a halo of inhibition of growth bounded by a blue ring of dead cells.

In this experiment (KIL-K_1) and KIL-K_2) strains will be tested. Elimination of the killer phenotype by incubation at high temperature will be also assayed.

The following strains will be supplied:

Saccharomyces cerevisiae: 17/17 : MAT α *his* (KIL-Ko) (K^-, R^-)

Saccharomyces cerevisiae 10701C : MAT a *thr4* (KIL-K_1) (K^+, R^+)

Saccahromyces cerevisiae 9744C : MAT α *leu 2* (KIL-K_1) (K^+, R^+) This strain is easily cured at 37 ºC.

Saccharomyces cerevisiae SMR 4: Wine yeast, (KIL-K_2) (K^+,R^+).

Day 1

Streak strains SMR 4,10701C and 9744C on YPD plates to get isolated colonies. Make two plates for 9744C . Incubate one of the plates of 9744C at 37°C and the others at 30°C

Day 2

Streak 17/17 and 107o1 C on YPD. Incubate at 30ºC.

Day 3

Make lawns of 17/17 and 10701C on plates with "killer medium". Prepare four plates of 17/17 and one of 10701C. To prepare lawns suspend in 1 ml water a portion of biomass and plate 0.2 ml of this suspension (a barely turbid suspension contains *ca.* 10^6 cells /ml).

With sterile toothpicks transfer single colonies from SMR4, 10701C, 9774C incubated at 30ºC and 9744C incubated at 37ºC to the 17/17 lawn. In the same way transfer SMR4 to the 10701C lawn.

Day 4

Score the results.

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Wickner R.B., Killer systems in S.cerevisiae. In "The molecular biology of the yeast Saccharomyces" Life cycle and Inheritance, pp 415-444 (1981) Stathern J.N. et al eds. Cold Spring Harbor Laboratory

EXPERIMENT I

MAPPING WITH BENOMYL

Benomyl (methyl benzimidazole -2- carbamate)is a fungicide which inhibits b-tubulin polymerization in fungi and yeast. Very likely due to this action it induces chromosome losses if applied during vegetative growth of Saccharomyces cerevisiae. This is the basis for a method of parasexual mapping in yeast that will be shown in this experiment. Since chromosome loss takes place without concomitant intrachromosomal recombination the method can be used to adscribe genes to particular chromosomes.

A diploid strain D 649 will be supplied with the following characteristics in different chromosomes:

Chromosome I : *+/ade 1*

ChromosomeII : *+/lys 2*

Chromosome III MAT a MAL 2 + + +/MAT α *mal2 his 4 leu2 thr 4*

Chromosome IV : *+/trp 1*

Chromosome XV : *+/ade 2*

Day 1

Refresh the strain on YPD. Incubate at 30ºC.

Day 2

Resuspend a loop of refreshed biomass in ca. 5 ml of sterile water so as to obtain a suspension of milky appearance (ca. 10^8 cells/ml). Make dilutions 10^{-3},10^{-5},10^{-6} and 10^{-7} in sterile water.

Plate 0.1-0.2 ml of each of the 10^{-3},10^{-4},10^{-5} and 10^{-6} dilutions on five plates of YPD+benomyl.

Plate 0.1-0.2 ml of each of the 10^{-5},10^{-6} and 10^{-7} dilutions on three plates of YPD. Incubate at 30ºC

Day 3

Count colonies and calculate the rate of survival. Take about 200 colonies from the plates of YPD+ benomyl and make a pattern on YPD plates.

Day 4

Replicaplate the patterns to SD medium. Keep the YPD plate in the refrigerator.

Day 5

Observe the SD replica and identify auxotrophic clones. Calculate the frequency of auxotrophies. Make a pattern on YPD of these colonies using the corresponding spots of the original YPD plate that was kept in the refrigerator on Day 4. Incubate at 30 ºC.

Day 6

Replicaplate the YPD pattern of auxotrophs to media with the adequate requirements to characterize the particular auxotrophies. Incubate at 30 ºC.

Day 7

Evaluate the results.

REFERENCES

Wood J.S. Genetic effects of methyl benzimidazole - 2 - carbamate on Saccharomyces cerevisiae Mol.Cell.Biol., 2, 1064-1079 (1982).

EXPERIMENT J

SEPARATION OF CHROMOSOMAL DNAs OF S.cerevisiae BY PULSED FIELD ELECTROPHORESIS

Chromosomal DNAs are extracted from cells that have been previously included in agarose. This allows to maintain them intact. Separation of chromosomal DNAs is achieved by an electric field whose orientation is changed periodically (pulsed field).

Chromosome preparation.

(Composition of solutions and reagents is given at the end of the experimental protocol.)

-10 ml of YPD in a 100 ml erlenmeyer are inoculated with 0.1 - 0.2 ml of a preculture in the same medium with a cell density of ca. 2.106 cells/ml and shaken at 30 C during 24h. In this time the culture reaches early stationary phase where synthesis of DNA is stopped. At this moment the number of cells are counted in a cell counter or haematocytometer.
- Weight a sterile centrifuge tube and centrifuge the culture at 1500 xg during five minutes at room temperature .
- Discard the supernatant and wash the pellet with 5 ml of 50 mM EDTA pH 8 . Centrifuge as above.
- Decant carefully without leaving liquid in the tube. Weight now the tube with the cells. Determine by subtraction the weight of cells. Assume that the weight in grams is equivalent to the volume in milliliters. Calculate how much volume of liquid is needed to suspend these cells so that the final suspension has a cell density of $3x10^9$ cells / ml for haploid strains or $1.5x10^9$ cells / ml in case of diploid strains. Add to the cells the calculated volume of the CPES buffer to which Zymolyase 20000 has been added just prior to use. Resuspend the cells using a mixer and

maintain this suspension at 28 C. This suspension should be mixed as soon as possible with the agarose solution described next .

- Prepare a solution of 1% agarose in buffer CPE. It is important to avoid a prolonged heating when melting the agarose. Maintain the solution at 55 C.
- Equal volumes of the suspension of cells and the agarose solution are mixed carefully with a mixer assuring a perfect homogeneity. This is an important step of the preparation.
- The mixture is poured in a plexiglas frame that allows to form agarose blocks of 2 x 2 cm in surface with a thickness of 1 mm. The filled frame is placed on crushed ice to speed up the gelification of the agarose
- After ca 15 minutes the agarose blocks are removed from the frame and each is cut in four pieces to facilitate subsequent manipulations.
-The four pieces are placed in a sterile test tube , covered with CPE and maintained 4 hours at 28 C without shaking. During this time the lytic enzyme digests away the cell walls.
- After this time discard the buffer and add 5 ml of TESP to which 1 mg / ml proteinase K has been added just before use. Keep the tubes overnight at 50 C.
- Decant the solution . The agarose blocks are now ready for use. If not used immediately they may be conserved in the refrigerator up to four months covered with 0.5M EDTA pH 8.
- Before electrophoresis the blocks are washed with 5 ml of TE buffer at 50 C without shaking for 30 minutes. This treatment is repeated three times. These washings are followed by six washings with the same buffer but at room temperature .

Preparation of the gel and electrophoresis

In this demonstration we will use the equipment Geneline (Beckman) designed for transverse alternating field electrophoresis (TAFE) . The gel is positioned vertically within the electrophoresis chamber between two sets of linear electrodes : one in front and one behind the gel (Figure 1) . When the electric field pulses between the two sets of electrodes the DNA

molecules move down in zigzag through the thickness of the gel not across its surface .
- Prepare a solution of 550 mg of agarose in 55 ml of TAFE heating as before. Cool at 55 C and pour the solution in the gel casting mould . Place the comb adequately.
-Keep some agarose (1 or 2 ml) at 55 C for the next operations. Allow the gel to settle at room temperature (ca. 20 minutes).
- Remove the glass cover and the comb from the casting mould.
- Take the agarose blocks previously prepared with the included digested yeast. Cut each piece so as to fit exactly in the slots of the gel. Be sure that these pieces are well adjusted to the lower part of the slot.
- Add some drops of melted agarose to stabilize the piece. Put the whole in a freezer during 3 to 5 minutes.
- The electrophoresis chamber is filled with 3.5 l of freshly prepared TAFE buffer (Do not reutilize !). Connect the refrigeration and the pump for circulation of the buffer.The temperature in the electrophoresis chamber is maintained between 12 C to 14 C during the run.
- Switch on current. Standard conditions for karyotyping S.cerevisiae : Constant current 150 mAmp . A first run of 18 hours with switching intervals of 1 minute is followed by a second run of 6 hours with switching intervals of 30 seconds.

Staining and observation of the gel.

At the end of the two runs the gel is taken out of the elctrophoresis chamber and put in TAFE with ethidium bromide (final concentration of 0.2 mg /ml). (This compound is TOXIC !) . Allow the gel to remain in this solution for 30 minutes or one hour with moderate shaking. Visualize the gel under UV light at 312 nm (Wear protective eye goggles!). To improve contrast the gel may be destained by putting it during 30 minutes in distilled water. A picture of the gel may be done with a Polaroid camera (Film 3000 ISO, exposure time 1/4 sec. , diafragm 11 for a CUS type camera) or with a reflex camera (Film 100 ISO , exposure time 10 to 20 sec. , diafragm 11 for a 24 x 36 equipment)

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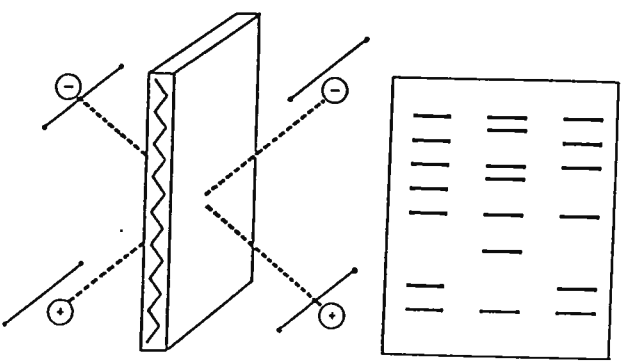


Figure 1: Transverse Alternating Field Electrophoresis. Pulses at 115° cause DNA to move in a zigzag pattern through the thickness of the gel, not across its face. The resulting lanes are straight.

Composition of media and buffers used in the practical

Separation of chromosomal DNAs of *S. cerevisiae* by pulsed field gel electrophoresis.

YPD.

Yeast extract	10 g/l
Peptone	20 g/l
Glucose (Dextrose)	20 g/l.

CPES

Citric acid pH 6	40 mM
Sodium phosphate pH 6	120 mM
EDTA-Na_2	20 mM
D-sorbitol	1.2 M
Dithiothreitol	5 mM

Just prior to its use 0.2 mg/ ml of Zymolyase 20000 are added (Laboratoires Miles, Tour Bayer, 13 Rue Jean Jaurés 92807 Poteaux Cedex, France). Alternatively you may use Novozyme 234 at 7 mg/ml. (Novo Industrie Enzymes S.A., 26 rue Fortuny 75017 Paris, France).

CPE

Same as above but without sorbitol and dithiothreitol.

TESP

TRIS /HCl pH 8	10 mM
EDTA-Na_2 pH 8	450 mM
SDS	10 g/ l

Just prior to use 1 mg / ml of proteinase K is added. (Appligene, Parc d´innovation. BP 72., 67402 Illkirch Cedex.

France) . ***Remark*** : This solution does not remain clear during storage. Before use it is clarified by submerging the container in a water bath at 50 C.

TAFE buffer

The quantities given are for a 20 X concentrated solution .

TRIS (Trizma base)	24.2 g
EDTA (free acid)	2.9 g
Acetic acid	5 ml
Water up to	1 liter.

No adjustement of pH is done

TE buffer

TRIS	10 mM
EDTA-Na_2	1mM

Adjust to pH 7.5

All the solutions are prepared with ultrapure water . They are not sterilized but they are conserved in sterile containers

EXPERIMENT K

ISOLATION OF TOTAL YEAST DNA

(Composition of reagents is given at the end of the protocol)

All along this assignement USE STERILE MATERIAL AND MAINTAIN STERILE CONDITIONS.

Day 1
Inoculate one colony of the yeast strain CJM 006 into 10 ml YPD. Shake at 30ºC until the culture enters stationary phase.

Day 3
With 0.1 ml of the above culture inoculate 200 ml of YPD in a 1 liter flask. Incubate with shaking at 30ºC.

Day 4
After ca.17 hours growth (stationary phase) harvest the yeast by centrifugation.
Resuspend the yeast cells in 12.5 ml of a solution containing 0.9M sorbitol,0.1M EDTA pH 8, 0.1% mercaptoethanol and transfer the suspension to a polycarbonate tube.
Add to the suspension 2.5 ml of a Zymolyase solution (2mg/ml of Zymolyase 5000) . Incubate for one hour with gentle shaking at 37ºC.
Centrifuge at about 500 x g (position 3 of the Wifug centrifuge) for 10 min at room temperature. Be careful when eliminating supernatant, protoplasts do not adhere tightly to the tube.
Resuspend the sediment by pipetting into 25 ml of 50 mM Tris pH 8, 20 mM EDTA.
Add 2.5 ml 10% sodium dodecylsulfate . Incubate at 65ºC for 30 min.
Add 7.5 ml potassium acetate and leave in ice for 60 min.

Centrifuge in the cold at 10,000xg for 10 min (ca. 10,000 rpm in a SS-34 Sorval rotor).

Filter the supernatant through a cheesecloth and distribute it into two Falcon tubes, add to each 2 volumes of absolute ethanol and leave the mixture for at least 15 min at room temperature.

Centrifuge at about 3,000xg for 15 min at room temperature. Discard the supernatant and drain the precipitate thoroughly.

Resuspend the sediment of both tubes into 5ml of TE buffer using a sterile glass rod.

Keep the solution at 4ºC.

Day 5

To the solution, add 75 µl of ribonuclease (1mg/ml) and incubate 30 min at 37ºC. Transfer to a 25ml erlenmeyer flask.

Add 1 volume isopropanol to precipitate the DNA. It will precipitate as a loose "cocoon".

Decant the supernatant carefully. Wash repeatedly the DNA with 5 ml 70% ethanol until the washings remain clear (3-4 fold). Wash once with absolute ethanol. Let DNA adhere to the vessel and drain over filter paper.

Resuspend the DNA in 1 ml TE and precipitate it again with 1 ml isopropanol. Decant the liquid and wash the DNA with 2ml 70% ethanol as above. Drain thoroughly.

Transfer the DNA to an Eppendorf tube and dissolve it in 0.5 ml TE. Take 10 ml to 0.5 ml water to measure the optical density at 260 and 280 nm. Pure DNA gives a quotient 260/280 = 1.8. A preparation of pure DNA gives an O.D.260nm of 1.0 for a concentration of 50 micrograms/ml.

Store at -20ºC.

Composition of the solutions used in the Experiment K

Isolation of total yeast DNA

Zymolyase solution
Zymolyase 5000 from Seikagaku Kogyo Co Ltd. is dissolved in 0.9 M sorbitol, 0.1 M EDTA pH 8, 0,1% β mercaptoethanol. The solution is prepared inmediately before use and sterilized by filtration

10 % Sodium dodecylsulfate
This solution does not need to be sterilized. Weight the necessary amount of SDS in a sterile vessel and use sterile water to dissolve it; warming at 65ºC may be necessary to ensure complete dissolution.

Ribonuclease solution
Dissolve pancreatic ribonuclease A (Sigma R5000) in TE at 1 mg/ml. Heat for 30 minutes at 80 ºC. Aliquot it and store frozen at - 20 ºC.

Potassium acetate.
To 60 ml of 5M potassium acetate add 11.5 ml of glacial acetic acid and 28.5 ml of H20. The resulting solution is 3M with respect to potassium and 5M with respect to acetate. It has a pH of about 5.5.

Ethanol and isopropanol are not sterilized. They are taken directly from the bottle and measured with sterile material.

EXPERIMENT L

PREPARATION OF A YEAST LIBRARY IN A HYBRID E.COLI-YEAST PLASMID.

In this practical we will construct a yeast library in the E.coli - yeast shuttle vector YEp352 (Hill et al, 1986).

Fragments of yeast DNA of a certain length will be generated by partial digestion of total yeast DNA and isolated after electrophoresis. These fragments will be inserted into YEp352 at a unique Bam HI site. To enhance efficiency of insertion the digested vector will be dephosphorylated before ligation.

Use in all steps sterile material

Day 1

Digestion and dephosphorylation of plasmid DNA

Digestion

Pipette into a sterile Eppendorf tube:

2.5 µl buffer Bx10 (Boehringer restriction buffer)

5 µl YEp352 (1mg/ml)

16.5 µl H20

1µl BamH1 (10.000 u/ml)

Put 10 seconds in the microfuge to collect all reagents at the bottom of the tube.

Incubate for 2 h at 37ºC

To check the digestion mix in an Eppendorf tube 2 µl of the digested plasmid with 8 µl of water and 2 µl of stop solution. Place in a well of a 0.8% agarose gel. Run electrophoresis with molecular weight markers for 1h at 4 V/cm. Observe the gel under UV light. A single band of 5.1 kb should be seen.

Add to the digestion mixture 150 µl TE buffer and extract proteins once with 175 µl phenol once with 175 µl phenol-

chloroform and once with 175 µl chloroform-isoamyl alcohol. **DNA remains always in the upper,aqueous phase .**

Add to the plasmid solution 0.1 volume of 3M sodium acetate (pH 5.2) and 2 .2 volumes of absolute ethanol.

Let stand at -20ºC for at least 30min. Centrifuge for 15 min in a microfuge in the cold and discard the supernatant. Cover the tube with a Parafilm and punch in it some holes with a fine needle. Dry briefly (ca. 10 min) in a dessicator under vacuum. Dissolve the sediment in 20 µl TE.

Dephosphorylation

In the same tube where you have your digested plasmid pipette the following.

20 µl H20

5 µl buffer CIPx10

5 µl calf intestine alkaline phosphatase 40 units/ml (CIP)

Incubate 30 min at 37ºC.

To inactivate the phosphatase add:

6 µl 50 mM EDTA pH 8

3 µl 10% SDS

Heat at 75 ºC for 10 min.

To deproteinize extract once with phenol and once with phenol- chloroform.

To precipitate the DNA add 0.1 volume of 3M sodium acetate (pH 7) and 2 .2volumes of ethanol.

Let stand at -20ºC for at least 30 min.

Partial digestion of yeast DNA and isolation of 5-10 kb fragments.

Choosing conditions for digestion

Before performing the actual preparation a previous assay of the yeast DNA digestion by the Mbo I enzyme in the actual local conditions is recommended. (To save time this will done by the instructors.). This is done as follows :In sterile Eppendorf tubes the following incubation mixtures are prepared

10 µl total yeast DNA (1mg/ml)

5 µl buffer Mx10 (Boehringer restriction buffer)
34 µl H_2O
1 µl (or 2) of Mbo I (0.4 u/ µl)

Incubate at 37 ºC. At different times (2,4,and 8 min) 10 µl aliquots of the digestion mixture are removed and added to Eppendorf tubes containing 5 µl of stop solution. The samples are run with molecular weight references on a 0.8% agarose gel. The amount of Mbo I and the digestion time which give a digest with a maximum of DNA fragments between 5 and 10 kb is choosen.

Actual digestion

To a sterile Eppendorf tube add
40 µl total yeast DNA (1mg/ml)
20 µl buffer Mx10
Mbo I (0.4 u/ µl) as deduced from the preliminary experiment
Water up to 200 µl.

Incubate at 37ºC for the time deduced from the preliminary experiment.

Precipitate the DNA with 400 µl absolute ethanol. Let stand at -20ºC for at least 30 min.

Isolation of DNA fragments of 5 to 10 kilobases

Centrifuge the digested DNA for 15 min in the cold. Resuspend sediment in 40 µl TE buffer and add 10 µl stop solution. Load into a gel of 0.8% of low gelling agarose and run the electrophoresis with molecular weight reference markers . Take a picture of the gel under UV light. With the help of the markers determine the position of the fragments between 5 and 10 kb. Cut the corresponding strip of agarose with a spatula or scalpel. DO NOT cut on the UV transparent plate!

Transfer the agarose to a sterile tube and melt it at 65-70ºC. Add about 1 volume of TE buffer and incubate 5 min at 65ºC.

Cool the solution at room temperature. Extract the agarose twice with phenol (some agarose remains at the interphase in the first extraction), once with phenol-chloroform and once with chloroform.

To the aqueous phase add 0.2 volumes of 10 M ammonium acetate and 2.4 volumes of absolute ethanol. Store at least 30 min at -20ºC.

Day 2

Ligation of the yeast DNA to the plasmid DNA

The yeast DNA and the digested plasmid DNA you prepared on Day 1 are centrifuged separately for 15 min in a microfuge in the cold. They are washed once with 0.6 ml of 70% ethanol, centrifuged and dried in a dessicator under vacuum for 5-10 min. Each DNA is suspended in 20 µl distilled water.

Pipette into a sterile Eppendorf tube
9.5 µl digested plasmid
9.5 µl digested yeast DNA
2.5 µl ligation buffer x10
2.5 µl 10 mM ATP
1 µl of T4 DNA ligase (1 Weiss unit/µl)
Incubate for at least 4h at 16ºC. Store at 4ºC.

Day 3

Transformation of E.coli

Place 4 Eppendorf tubes in ice and pipette 0.1ml of the competent E.coli TG1 cells in each of them (Be careful not to take competent cells out of the ice bath)

The first tube will be used as a blank, the second as a control to which 1 µl of plasmid YEp352 (1µg/ ml) will be added, to the third and fourth tubes add 2 µl of the ligation mixture stored at 4ºC. Mix gently.

Incubate for 15 min at 0ºC, then 2 min at 42 ºC and again 2 min at 0 ºC. Add 0.4 ml LB transfer to 10 ml plastic tubes and shake for 60 min.at 37 ºC From these cultures plate on LBamp plates 0.1ml of the first three tubes, 50 µl of a 1/10 dilution of tubes 2 and 3, the whole content of tube 4 (centrifuge it first and resuspend the pellet in 0.1 ml LB).Incubate the plates at 37 ºC.

Day 4

Examine the LB amp plates and estimate the number of clones per plate. The method used for the preparation of the ligation mixture should produce a majority of clones containing an insert in the vector. To check this a number of transformants will be tested in a plate of LBamp containing an inductor of the β-galactosidase gene (IPTG) and a substrate of the corresponding enzyme (X-gal). The cells which contain a plasmid without insert should express the β-galactosidase gene and give a blue color, the cells which contain a plasmid with an insert should remain white.

With a toothpick transfer a total of about 20 transformants from any of the plates containing bacteria transformed with the ligation mixture to a plate of LBamp with IPTG and X-Gal. As a control transfer to the same plate about 10 transformants from the plate containing bacteria transformed with the plasmid YEp352 Incubate at 37ºC.

(Another way to test the efficacy of insertion could be to analyze minipreps from a number of transformants)

Day 5

Observe the plates with IPTG and X-gal and count the white clones . Calculate the proportion of the clones containing insert.

To obtain a library which covers 10 times the yeast genome 200,000 independent clones should be used. (In the course each group will try to obtain about 30,000 clones and the transformants of all groups will be pooled).

From the amount of transformed bacteria obtained on Day 4 and the proportion of transformants containing an insertion calculate the number of tubes of competent E.coli cells necessary to obtain 30,000 transformants containing an insertion.

Defreeze this number of tubes by leaving them 15 min in ice. To each tube add 2 µl of your remaining ligation mixture.

Incubate for 15 min at 0 ºC, then 2 min at 42 ºC and again 2 min at 0º C. Add 0.4 ml of LB and incubate with shaking for 1 hour at 37 ºC.

The tubes are centrifuged, the pellets recovered in 0.1 ml of LB and each is spread on a LB amp plate. Incubate the plates at 37 ºC.

Day 6

Stocking the pool

The clones on ampicillin plates inoculated on Day 5 are resuspended with a spreader in liquid LB amp with 25 % glycerol. Use 6 ml to resuspend all your colonies. The colonies of all groups will be pooled resulting in about 50 ml of suspension containing the 200,000 clones. The usual way to store the library is to distribute 1 ml samples of the above suspension in small sterile tubes and keep them at -70 ºC or in liquid nitrogen for further experiments.

If you wish to take home the constructed library:

Inoculate 3 well dried plates of LB amp with 0.3 ml each of the above suspension. When grown,store the plates in the refrigerator

Preparing DNA from the pool

To prepare DNA from this library . proceed as follows:

Suspend with a spreader the E.coli from the 3 plates into 5 ml of LB amp. Use 4 ml of this suspension to inoculate 40 plates of LB amp (0.1 ml per plate). To the other milliliter of suspension add glycerol to obtain a final concentration of 25 %. The suspension may be stored at -70 ºC as reserve library.

After one day of incubation at 37 ºC the cells are washed out of the plates and plasmid DNA is prepared according to standard techniques.

REFERENCES

Hill J.E.,Myers A.M., Koerner T.J. & Tzagoloff A. Yeast 2, 163-167 (1986)

Reagents used in Experiment L

Preparation of a yeast library in a hybrid E. coli- yeast plasmid

10x CIP dephosphorylation buffer
10 mM $ZnCl_2$
10 mM $MgCl_2$
100 mM Tris/HCl pH 6.3

10X Ligation buffer
0.5 M Tris/HCl pH 7.6
100 mM Mg Cl_2
100 mM dithioerythritol
500 µg/ml bovine serum albumin (Fraction V,Sigma)

IPTG (isopropyl β -D-thio galactopyranoside)
0.1 M solution in water

X-Gal (5 bromo 4 chloro 3 indolyl β -D- galactopyranoside)
2% solution in dimethylformamide

IPTG-X-Gal plates.
Spread on a plate 10 µl IPTG and 50 µl of the above X-gal solution.

Competent E.coli cells
Refresh the bacteria in LB broth overnight. Inoculate the actively growing cells in fresh LB medium to give an $O.D._{660}$= 0.05. Incubate at 37ºC with vigorous shaking until an O.D. of 0.25-0.3 has been reached (about 1.5-2 hours).

Transfer 40 ml of the culture to a cold Falcon tube. From now on, ***all the operations should be performed in the cold using cold solutions.***

Centrifuge 5 min at 5000 rpm in a tabletop centrifuge. Resuspend the cells in 25 ml 0.1 M $CaCl_2$ using a cold sterile Pasteur pipette.

Place the suspension in an ice bath for 20 min and centrifuge 5 min at 5000 rpm.

Resuspend the cells in 2 ml of 0.1 M $CaCl_2$, 14% glycerol. Store the cells at 0ºC for several hours. (The best results are obtained with 12-24h storage. After this time the suspension can be divided into 0.2 ml aliquots and stored at -70 ºC for months).

EXPERIMENT M

TRANSFORMATION OF YEAST

The goal of this practical is to illustrate different approaches to the problem of transforming yeast cells. We will consider two cases: selection of transformants complementing an auxotrophic requirement (complementation of the *leu2* mutation) and selection of transformants complementing a deficiency of a regulatory protein (complementation of a *cat1* mutant. The CAT 1 gene is a gene implicated in catabolite repression, mutants *cat 1* do not grow on non-fermentable carbon sources). Two methods of transformation will be used. One of them involves the use of protoplasts while the other utilizes whole cells treated with a cation to make them permeable to DNA. When looking for complementation of the *cat1* mutation a two-step selection procedure will be used.

(In this protocol several non-standard abbreviations to designate solutions will appear. The composition of these solutions is given at the end of the practical)

Day 1

Transformation of the cells

All subsequent operations should be performed in sterile conditions. IDENTIFY CAREFULLY YOUR TUBES. Write your group number on adhesive tape and fix it to the tubes. Write it also on your plates.

Groups 1,2,3,5,6 and 7 will use whole cells

Groups 1,2,3,5,and 6 will select transformants complementing *leu2*.

Group 7 will select transformants complementing *cat 1* in a two-step procedure.

Each group will receive 80 ml of an exponentially growing yeast culture in YPD (ca. 3mg wet weight/ml). **Groups 1,2** and 3 will use strain TS5 (*a ,ura3 ,leu2*). **Groups 5,6** and **7** will use strain CJM096α *cat1 ,ura3 ,leu2*).

Centrifuge the culture in two plastic tubes (Falcon) for 5 minutes at 4000 rpm at room temperature.

Wash the pellet of each tube as follows:

once with 25 ml water

once with 25 ml TELI

After this, put 5 ml TELI in one of the tubes and resuspend the pellet. Transfer the suspension to the other tube and resuspend the sediment. Centrifuge as before and resuspend the sediment in 0.5 ml TELI

Incubate 1 hour at 30°C with gentle shaking .

After this time transfer 0.1 ml aliquots to five Eppendorf tubes Add 20 µl carrier DNA (10 mg/ml) to each tube. To tubes 1 to 4 add 2 µl DNA solution (3 mg/ml) prepared from a yeast library in the centromeric plasmid YCp 50. Tube 5 is a control and does not receive DNA.

Incubate for 30 minutes at 30°C without shaking.

Mix with a vortex and add 0.7 ml TELIPEG to each tube. This solution is highly viscous, make sure that the pipette has delivered all its contents. Mix well by inverting the Eppendorf tube several times.

Incubate for one hour at 30°C.

Incubate 10 minutes at 42°C.

Centrifuge in an Eppendorf centrifuge 2 minutes at maximal speed.

Eliminate supernatant with a pipette.

Resuspend carefully in 0.2 ml water using a sterile glass rod.

Groups 1,2,3,5 and 6

Take two aliquots of 100 µl from tubes 1 to 3 and one of 100 µl from tube 4 and plate them on seven plates of YNB glucose. Label plates 1 to 7.

Make a 1/10 dilution from tube 4 and plate 50 µl of this dilution and 50 µl of the original suspension on plates of YNB leu glucose. This is a control of the efficacy of transformation. Label plates 1L and 2L.

Plate the whole content of tube 5 on a plate of YNB leu glucose. Label it C. This is a control of reversion of the ura3 mutation. Incubate all plates at 30ºC

Group 7

Take two aliquots of 100 µl from tubes 1 to 3 and one of 100 µl from tube 4 and plate them on seven plates of YNB leu glucose. Label plates 1G to 7G.

Make a 1/10 dilution from tube 4 and plate 50 µl of this dilution and 50 µl of the original suspension on plates of YNB leu glucose. This is a control of the efficacy of transformation. Label plates 1L and 2 L.

Plate the whole content of tube 5 on a plate of YNB leu glucose. Label it C. This is a control of reversion of the ura3 mutation.

Incubate all plates at 30º C.

Groups 4 and 8 will use protoplasts.

Each group will receive 50 ml of an exponentially growing yeast culture on YPD (*ca* . 1mg wet weight /ml).

Group 4 will use strain TS5 (*a,ura3,leu2*) and select transformants complementing *leu2* .

Group 8 will use strain CJM096(*αcat1,ura3 ,leu2*) and select transformants complementing *cat 1* in a two-step procedure.

Centrifuge the culture in a Falcon tube 5 min at 4000rpm at room temperature.

Resuspend the sediment in 5 ml Tris-sorbitol. Make 10^{-2} and 10^{-5} dilutions from a 10 µl sample of the suspension. As a control

for contamination plate 0.1 ml of the 10^{-2} dilution on 2 plates of YNB-glucose. As a control of the initial cells plate 0.1 ml aliquots of the 10^{-5} dilution on 2 plates of YPD. Transfer the suspension to a 25 ml erlenmeyer flask and add 60 µl of lyticase (2.5mg/ml).

Shake at 30ºC gently .In the conditions used in about 30 min , 80 to 90% of the cells will be converted into protoplasts. PROTOPLASTS ARE FRAGILE CREATURES. Perform all subsequent operations very gently.

Centrifuge the protoplasts in a Falcon tube 5 min at 2500 rpm in a desk centrifuge at room temperature. Wash the protoplasts carefully twice with 5 ml 1M sorbitol. Do not shake vigorously to suspend the protoplasts, do it by gently mixing with the liquid.

Wash the protoplasts once with 5 ml SORTRIS, centrifuge as above and resuspend them in 0.4ml of the same solution.

Take 4 sterile plastic tubes. Number them 1 to 4. Into each of them pipette 100 µl of the protoplast suspension. To tubes 1 to 3 add 10 µl of a DNA solution (3mg/ml) prepared from a yeast library in the centromeric plasmid YCp50 . Tube 4 is a control and no DNA is added to it. Mix gently and let stand for 10 min at room temperature.

Add to all tubes 1 ml PEGTRIS . Mix and let stand for 10 min at room temperature.

Centrifuge 5 min at 2500 rpm. Resuspend in 150 µl SOS. Incubate 20 min at 30ºC with gentle shaking as before.

Cells can now be plated as described below. However if necessary they can remain at 4ºC up to a few days before proceeding further.

Group 4

Take 2 aliquots of 75µl from tubes 1 and 2 and a 100 µl aliquot from tube 3 and add them to tubes containing 20 ml regeneration agar RG (YNB sorbitol glucose). This agar is maintained in a water bath at 48 ºC . Mix gently by inverting the tube twice. Pour inmediately on empty plates labelled 1 to 5.

Using 1M sorbitol make 10^{-2} and 10^{-4} dilutions from a 10 µl aliquot from tube 3.

Take 5 µl from tube 3 and 50 µl from the 10^{-2} dilution and add them to the tubes containing 20 ml of regeneration agar RLG (YNB sorbitol leu glucose). Treat as before and label the plates G5 and G50. These are controls of the efficiency of transformation.

Add 10 µl and 50 µl of the 10^{-4} dilution from tube 3 to tubes containing 20 ml of regeneration agar RLGU (YNB sorbitol leu ura glucose). Mix and plate as before and label plates U10 and U50. These are controls of the regeneration of protoplasts.

Take from tube 4 one aliquot of 0.1ml and add it to a tube of RLG. Treat as before and label plate C. This is a control of reversion of the ura3 mutation.

After the plates have gellified, incubate them at 30ºC. Make a 10^{-2} dilution of tube 3 with water. Plate 0.1 ml on a YPD plate. This is a control for intact cells in the protoplast preparation.

Group 8

Take 2 aliquots of 75 µl from tubes 1 and 2 and a 0.1 ml aliquot from tube 3 and add them to tubes containing 20 ml regeneration agar RLG (YNB sorbitol leu glucose). This agar is maintained in a water bath at 48 ºC . Mix gently by inverting the tube twice. Pour inmediately on empty sterile plates labelled 1 to 5.

Using 1M sorbitol make 10^{-2} and 10^{-4} dilutions from a 10 µl aliquot from tube 3.

Take 5 µl from tube 3 and 50 µl from the 10^{-2} dilution and add them to the tubes containing 20 ml of regeneration agar RLG (YNB sorbitol leu glucose). Mix and plate as before and label the plates G5 and G50. These are controls of the efficiency of transformation.

Add 10 µl and 50 µl of the 10^{-4} dilution from tube 3 to tubes containing 20 ml of regeneration agar RLGU (YNB sorbitol leu ura glucose). Treat as before and label plates U10 and U50. These are controls of the regeneration of protoplasts.

Take from tube 4 one aliquot of 0.1 ml and add it to a tube of RLG. Treat as before and label plate C. . That is a control of reversion of the ura3 mutation.

After the plates have gellified, incubate them at 30ºC. Make a 10^{-2} dilution of tube 3 with water. Plate 0.1 ml on a YPD plate. This is a control for intact cells in the protoplast preparation.

Day 5

Recovery of transformants

All groups: score the plates.

Groups 1,2,3,5 and 6

Inspect plate C. No colonies should appear.

Inspect plates 1L and 2L . Count the colonies to have an estimate of the efficiency of transformation.

Look for colonies on plates 1 to 7. With a sterile toothpick take these colonies and streak them on plates of YNB glucose forming a patch of ca. 1 x 1cm . This will give a "standard" quantity of yeast for succesive operations. Incubate at 30ºC.

Group 4

Inspect plate C. No colonies should appear.

Inspect plates G5 and G50. Count the colonies to have an estimate of the efficiency of transformation.

Count colonies on U10 and U50 plates to calculate the efficiency of regeneration of protoplasts.

Look for colonies on plates 1 to 5 . With a sterile toothpick take some colonies and streak them on plates of YNB glucose forming a patch of ca. 1 x 1cm . This will give a "standard" quantity of yeast for succesive operations.Incubate at 30ºC.

Group 7

Inspect plate C. No colonies should appear.

Inspect plates 1 L and 2 L. Count the colonies to have an estimate of the efficiency of transformation.

Take plates 1 to 7 . The colonies growing there are cells that have been transformed and have been selected on the basis that they no longer require uracil. Proceed with these plates as follows:

Wash each plate with 1 ml sterile water. Mix the yeast suspensions thus obtained and adjust to 20 ml.

Plate three aliquots of 0.1 ml of this suspension on plates of YNB leu pyruvate and three others on YNB plates. Incubate at 30ºC

Group 8

Inspect plate C. No colonies should appear.

Inspect plates G5 and G50. Count the colonies to have an estimate of the efficiency of transformation.

Count colonies on U10 and U50 plates to calculate the efficiency of regeneration of protoplasts.

Take plates 1 to 5. The colonies growing there are cells that have been transformed and have been selected on the basis that they no longer require uracil. Proceed with these plates as follows:

Attach the handle of a sterile sieve to a firm basis and place the sieve on a 250 ml plastic beaker (see figure at the end of protocol). Take a spatula, dip it in alcohol and flame it. Cut the agar of the plates in 4 to 6 portions. Remove the aluminum cover of the beaker and of the sieve. Transfer the pieces of one plate to the sieve and grind the agar through the sieve with a sterile pestle. Do the same with the contents of two other plates. Add some sterile water after each plate has been ground. When the three plates have been processed add more water (total volume including the intermediary washes shall not exceed 100 ml) to elute the yeast cells. To eliminate most of the agar filter through a double layer of sterile gauze placed on a sterile funnel. Collect the liquid in a 500 ml erlenmeyer flask. Proceed in the same way for the other two plates.

Centrifuge the yeast suspension in Falcon tubes 5 min at 5000 rpm and resuspend the sediment in 5 ml sterile water.

Plate three aliquots of 0.1 ml of this suspension on plates of YNB leu pyruvate and three others on YNB plates. Incubate at 30ºC

Day 7 to 11

Groups 7 and 8 observe their YNB glucose and YNB leu pyruvate plates.

Day 8

Preliminary characterization of transformants

All groups will utilize the LEU2 URA3 transformants obtained by groups 1 to 6.

Using a sterile toothpick transfer a whole patch of the cells streaked on day 5 to an erlenmeyer flask with 10 ml of YNB glucose. Incubate with shaking at 30ºC.

Day 9

Minipreparations of yeast DNA

Collect the cells of transformed yeast inoculated on day 8 by centrifugation.

Resuspend the cells in 1ml TE. Centrifuge for 5 min in a microfuge. Wash the pellet once with 1 ml TE and resuspend the cells in 0.25 ml extraction buffer (50 mM Tris/HCl; 50 mM EDTA; 0.2M NaCl; 0.2% Triton X-100 ; pH 8).

Add sterile glass beads (0.5 mm diameter) until 2mm below the surface of the suspension and mix energetically in a vortex for 90 seconds. Centrifuge 5 min in a microfuge and collect the supernatant.

Add 0.2 ml TE and extract proteins 3 times with an equal volume of phenol-chloroform and twice with chloroform.

Precipitate the DNA with two volumes absolute ethanol in the presence of 0.3 M sodium acetate.

Let at least 30 min at -20ºC. Centrifuge for 15 min in a microfuge, wash the sediment with 70% ethanol ,dry under vacuum and resuspend in 20 µl TE.

Transformation of E.coli

Put in an ice bath four Eppendorf tubes . Defreeze in the same bath the competent E.coli TG1 cells. Pipette 0.1 ml of the cells in each of the tubes. To tube 1 add 1 µl of YEp 352 (1µg/ml). To tubes 2 and 3 add 5 and 15 µl respectively of the DNA isolated from the yeast. Tube 4 is a control and receives no DNA.

Incubate 15 minutes in the ice bath, then 2 minutes at 42 ºC and again 2 minutes at 0ºC. Add 0.4 ml LB and incubate with shaking at 37ºC for one hour. Plate 0.1 ml of each culture in LBamp plates. Incubate overnight at 37ºC

Day 10

Characterization of the E.coli transformants

Evaluate the results of the E.coli transformation.

Inoculate 2 transformants in 2 ml LBamp. Incubate with shaking at 37 ºC. (For practical reasons, only two transformants will be analyzed. In normal laboratory practice it is advisable to examine in parallel a dozen of transformants).

Day 11

Minipreparations of E.coli plasmid DNA

Centrifuge in an Eppendorf tube1.5 ml of each of the cultures inoculated yesterday. Keep the rest of the culture. Decant the supernatant and drain carefully with a pipette.

Resuspend with 0.1 ml lysis 1 solution. Leave in ice and shake occasionally.

Add 0.2 ml of lysis 2 solution. Mix several times by vigorous inversion of the tube. Leave 5 minutes in ice.

Add 0.15 ml of 3M potassium acetate pH 5.5 . Mix by inversion. Leave 30 minutes in ice.

Centrifuge 15 minutes in the cold.Take supernatant .

Add 0.5 ml of phenol-chloroform, mix and centrifuge for 1 minute. Take upper phase.

Add 2 volumes absolute ethanol and leave 30 minutes at -20ºC. Centrifuge 15 minutes in the cold. Decant immediately and dry 5 minutes under vacuum. Dissolve in 20 µl of distilled ultra pure water.

Day 14

Digest the plasmid DNA from the transformants isolated on Day 11 with restriction endonuclease Hind III as follows:

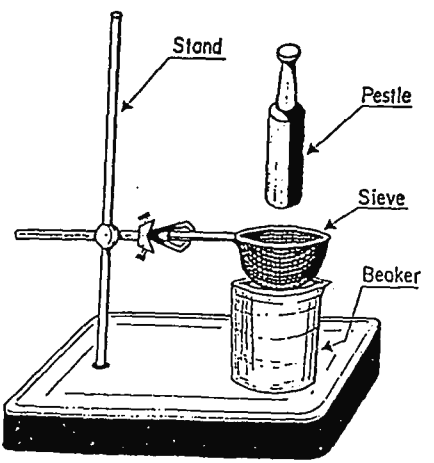
H_2O	8.5 µl
Plasmid DNA	3 µl
Buffer B x 10	1.5 µl
HindIII(10 u/µl)	1 µl

Incubate 90 minutes at 37ºC.

Add 1 µl RNase (1mg/ml). Incubate 30 minutes at the same temperature.

Add to each tube 3 µl stop solution. Give a pulse in the centrifuge and load all the sample in a 0.8% agarose gel with molecular weight markers. Run electrophoresis at 4 V/cm for 1 to 2 hours. Photograph the gel under UV light. Analyze results.

Figure 1.-Recovery of yeast cells entrapped in agar.



Composition of solutions used in Experiment M
Transformation of yeast

Carrier DNA .-Sonicated and boiled salmon sperm DNA (10 mg/ml)

Electrophoresis buffer (TAE).- A 50 x concentrated stock solution contains per liter 242 g Tris base, 57.1ml glacial acetic acid and 100 ml 0.5 M EDTA pH 8.

Lysis 1 solution.- 50 mM glucose, 25 mM Tris.HCl,10 mM EDTA, pH 8.

Lysis 2 solution.-0.2M NaOH (freshly diluted from a 10M stock),1 % SDS.

Lyticase.-From Sigma, L8137, 2.5mg/ml in **Tris-sorbitol**.

PEGTRIS.- 20% polyethylenglycol 4000,10 mM Tris.HCl pH8, 10 mM$CaCl_2$.

Potassium acetate.- Mix 60 ml 5M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml H_20. The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

Regeneration agar.- 2% agar with 1M sorbitol.

RNase .- Dissolve pancreatic ribonuclease (Sigma R-5000) in 1 ml TE (final concentration 1 mg/ml) and heat at 80ºC for 30 minutes . Store frozen at - 20º C.

SORTRIS .- 1M sorbitol,10 mM Tris.HCl pH 8, 10 mM $CaCl_2$.

SOS .- 10 ml 2 M sorbitol, 3.35 ml 2% yeast extract, 3.35 ml 2 % peptone, 0.13 ml 1M $CaCl_2$, 27 µl 1% of the requirement to be selected (e.g. ura), 3.17 ml water.

Stop solution .- 0.125 M EDTA, 30% glycerol, 0.25 % bromophenol blue.

TE .- 10 mM Tris, 1 mM EDTA. Adjust pH to 8 with HCl.

TELI .- 50 mM $LiS0_4$ in **TE.**

TELIPEG .- 40 % poliethylenglycol 4000 in **TELI**.

Tris-sorbitol.- 20 mM Tris.HCl pH 8, O.9 M sorbitol.

EXPERIMENT N

Use of the β galactosidase and endoglucanase A genes as reporters in studies of gene expression and protein secretion

Studies on gene expression are greatly facilitated if the promoters of the corresponding genes can be fused to a structural gene that codes for an easily detectable protein . Measurements of the activity coded by this gene (***reporter gene***) can be directly related to the function of the promoter under consideration. The same applies for the study of the signals for protein secretion.

The ability of transformed S. cerevisiae to synthesize active β - galactosidase has been extensively utilized to identify and delineate regulatory sequences in yeast genes. In this assignement we will use this reporter gene and also introduce a new one : the gene coding for endoglucanase A from Chlostridium thermocellum . This gene codes for a single polypeptide chain of 55000 Da molecular weight. There exist sensitive and rapid assays for measuring endoglucanase activity in extracts, gels and agar plates (Beguin, 1983) The excreted activity can be measured on plates after wiping out the colonies thus providing a method to distinguish between intracellular and excreted activity.

Use of β galactosidase as reporter gene

We will use a fusion that places synthesis of β-galactosidase under the control of the promoter of fructose-1,6-bisphosphatase (plasmid pJM 8) With this construction we will follow the activity of the promoter in yeasts growing in different media and in yeast carrying different alleles of the gene CAT1. CAT 1 is a gene implicated in catabolite repression. Mutants cat 1 do not derepress a series of enzymes (Gancedo and Gancedo 1986) . Yeast cells carrying either the wild type allele CAT 1 (strain CJM 095) or the mutant cat 1 (Strain CJM 096) transformed with pJM 8 will be supplied

Day 1

A suspension of ca. $2x10^3$ cells/ml of CJM 095 and CJM 096 transformed with pJM 8 will be supplied. Spread 0.1 ml of these suspensions on plates of YNB glucose 8% + X-gal and YNB pyruvate1%+ glucose 0.4 %+ X-gal. (In the plates for CJM 096 leucine is incorporated)

Days 4-8

Observe the plates for the appearance of blue color in the colonies and around them

Use of endoglucanase A as reporter gene

We will use this reporter gene to study the expression of an enzyme subjected to catabolite repression, fructose-1,6-bisphosphatase and to study secretion of invertase.

Plasmid pAS 5 contains the promoter of fructose 1-6 bisphosphatase fused with the endoglucanase gene (Silva et al. unpublished results). A culture of strain CJM 088 carrying this plasmid will be supplied.

Plasmid pBS 7 carries the promoter and secretion signals of the invertase gene fused to the endoglucanase gene (Benítez et al. 1989). Yeast strain SEY 2202 transformed with this plasmid will also be supplied.

Day 1.

Spot in a row 5 µl from each of the yeast suspensions on plates of rich medium containing 2 % glycerol as carbon source . Spot an identical second row . As a control spot in each row 5 µl of the yeasts strains without plasmids. Be careful not to scratch the agar surface. Incubate at 30º C.

Day 2.

From the same suspensions spot as above but on plates of rich medium containing 5 % glucose as carbon source . Be careful not to scratch the agar surface. Incubate at 30 º C.

Day 3

In one of the rows of each plate wipp off the colonies with a cotton swab. Cover both plates with an overlay of agar containing carboxymethyl

cellùlose at 60 C. Incubate the plates 2 h at 60º C. Flood the plates with a Congo red solution and let them stand at room temperature during 30 minutes. Decant the Congo red solution and destain several times with a solution of 1 M NaCl until halos are visible. Observe the halos around the colonies and the halos in the wipped off spots. (Viable colonies may be recovered if the incubation with the carboxymethylcellulose is performed at 37º C). An alternative procedure is the use of ostazin brilliant red- hydroxyethyl cellulose (available from Sigma Chemical Co.) incorporated at 0.2 % in the plates. This procedure gives good results in the case of secreted proteins while the halo is not clearly visible with intracellular proteins.

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Gancedo J.,M & Gancedo C., FEMS Microbiol. Rev., 32,179-187 (1986)

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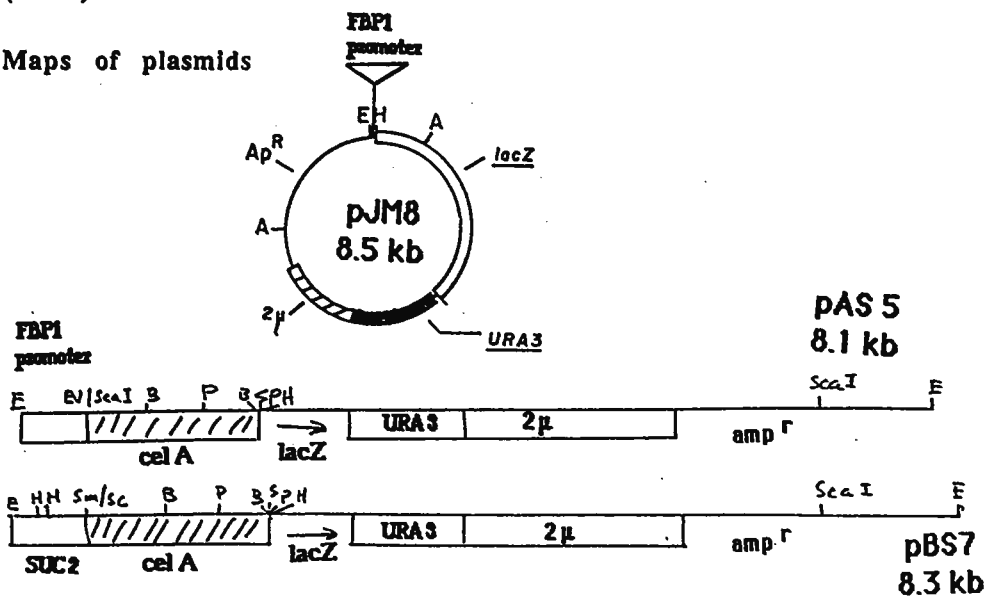
Maps of plasmids



(Plasmids pAS5 and pBS 7 were kindly given by Drs. A.Silva and J. Benítez, CENIC, Havanna, Cuba)

Composition of the reagents used in the practical
Use of the β galactosidase and endoglucanase A genes as reporters in studies of gene expression and protein secretion

X-gal (5 bromo -4 -chloro- 3 indolyl β -D- galacto pyranoside)
2% solution in dimethyl formamide

X-gal plates
Spread 50 µ l of X- gal on the adequate plate

Carboxymethyl cellulose overlay.
0.7 % agar
0.5% carboxymethly cellulose (sodium salt, medium viscosity) in 50 mM K2HPO4, 12 mM citric acid pH 6.3

Congo red stain.
1 % Congo Red in water

Destain solution
1 M NaCl.

Relevant phenotype of yeast strains used:
CJM 095, ura 3, CAT 1.
CJM 096 , ura 3,leu 2, cat 1. These strains were derived from strain JS87.11-8C kindly provided by Prof. Dr. K.D. Entian. Institut für Mikrobiologie der Johan-Wolfgang Goethe Universität. Frankfurt (FRG).
SEY 2022
CJM 088 a ura 3 can 1 FBP 1.

EXPERIMENT P

GENE DISRUPTION AND REPLACEMENT

In this practical we will show the use of the "integrative disruption/ replacement approach" (Shortle et al. PNAS 81,48889,1984) to replace the constitutive promoter of the ATPase gene by a galactose dependent promoter.

The strategy for replacing the promoter of the ATPase gene is shown in figure 1 . Plasmid pRS-61 has been constructed by ligation of the following 3 fragments: a 0.75 kb EcoR1-Xho1 fragment which contains the promoter of the galactokinase gene (R-X bar), a 1.65 kb Xho1-BamH1 fragment (X-B bar) containing a portion of the ATPase gene which does not include the promoter and a 5.1 kb EcoR1-BamH1 fragment containing the yeast URA3 gene, the bacterial origin of replication and the amp^r gene (see Cid et al. Curr. Genet. 12,105 (1987) for further details). Since this plasmid lacks sequences for autonomous replication in yeast, transformation of yeast cells to uracil prototrophy must occur by integration into the yeast genome resulting from homologous recombination. In order to target the recombination to the ATPase sequences the plasmid can be digested with Xba1 which cuts within the ATPase sequence. Integration at the ATPase locus results in a constitutive promoter in front of a truncated coding region and the galactose-dependent promoter in front of a complete coding region as shown in the lower part of the figure 1.

Due to lack of time the transformation will be performed by the instructors. They will transform strain W303-1A (a ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3) with plasmid pRS-61 digested with Xbal , using the lithium procedure described in the practical **Transformation of Yeast (Experiment M)**

Day 1

A plate of YNB ade,trp,leu,his, galactose inoculated with the transformation mixture will be supplied. Incubate at 30 ºC.

Day 4

Replicate transformants onto an YNB glucose plate (to check for contaminants), an YNB ade,trp,leu,his glucose plate and an YNB ade,trp,leu,his, galactose plate.

Day 6

Collect results. Discussion.

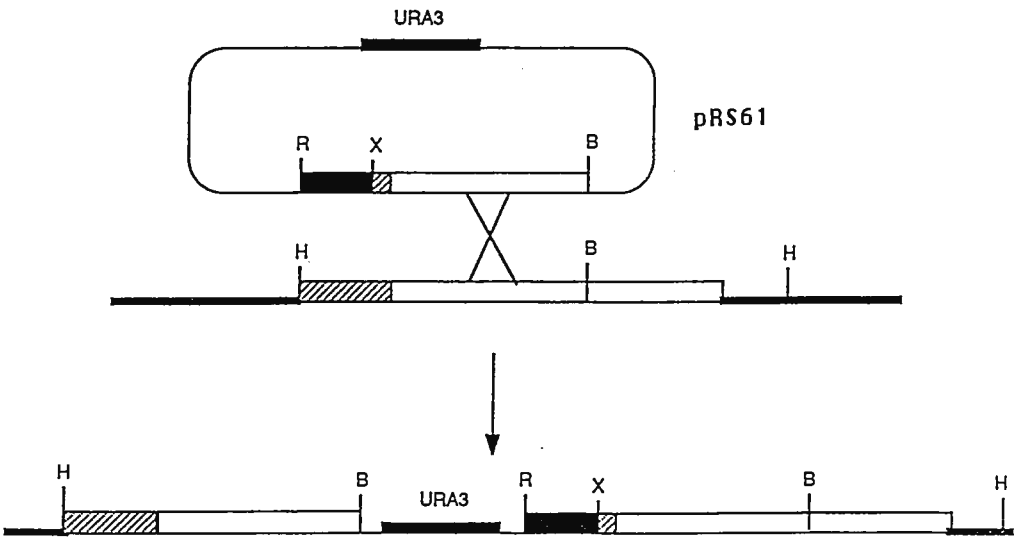


Figure 1.- Integration of pRS 61 into the yeast genome. R=EcoRI, X=XbaI, B=BamHI, H=HindIII

CULTURE MEDIA USED ALONG THE EXPERIMENTS

YPD Complete medium for routine work.

Yeast extract	1%	w/v
Peptone	2%	"
Glucose	2%	"
Agar	2%	"
Distilled water		

YPG
Complete medium with glycerol as carbon source. Used to differentiate "*petite* " strains, unable to grow on it.

Yeast extract	1%	w/v
Peptone	2%	"
Glycerol	3%	v/v
Agar	2%	w/v
Distilled water		

GNA. Presporulation medium

Yeast extract	1%	w/v
Peptone	0.5%	"
Glucose	10%	"
Agar	2%	"
Distilled water		

SPO. Sporulation medium

Potassium acetate	1%	w/v
Yeast extract	0.1%	"
Glucose	0.05%	"
Agar	2%	"
Distilled water		

SD Synthetic minimal medium

Yeast Nitrogen Base w/o aminoacids
and ammonium sulphate.....0.17 % w/v
Ammonium sulphate.....0.5 %
Glucose.....2 %
Agar.....2 %
Distilled water

YNB

As SD but may contain carbon sources different from glucose.

Requirements and inhibitors.
Prepared as stock solutions and added to SD at the following final concentrations

	stock solut. (g/l)	Final concent.(mg/l)
adenine sulphate	1.2	20
uracil	1.2	20
L-arginine-HCl	2.4	20
L-tryptophan	2.4	20
L-histidine-HCl	2.4	20
L-methionine	2.4	20
L-tyrosine	0.9	30
L-isoleucine	3.6	30
L-leucine	3.6	30
L-lysine-HCl	3.6	30
L-phenylalanine	3.0	50
L-aspartic acid	4.0	100
L-glutamic acid	6.0	100
L-valine	18.0	150
L-threonine	24.0	200
L-serine	45.0	375
Canavanine sulphate	6.0	60
Cycloheximide		5

LB

For one liter: 10 g bacto tryptone, 5 g yeast extract, 10 g sodium chloride. Adjust to pH 7.5. For plates add agar to a final concentration of 2 %.

LB amp

Add to sterile LB 50 µg/ ml ampicillin., Stock solution sterilized by filtration (25 mg/ml in water).

THE LECTURERS

Isabel L. CALDERON did her studies at the University of Seville (Spain) where she got a PhD for her work on nitroso guanidine mutagenesis in yeast. With a post-doctoral Fellowship of the Juan March Foundation she worked with R.K. Mortimer at the University of California at Berkeley on DNA recombination and repair. She is presently Assistant Professor of Genetics at the University of Seville. Her interests are the regulation of threonine biosynthesis in both laboratory and industrial strains of S. cerevisiae. She has participated in another FEBS Advanced Course on Biochemistry and Genetics of Yeast .

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Marco A. DELGADO got his Ph D. degree for his work on aminoacid overproduction in S. cerevisiae. After this he moved to the fermentation industry and later on to La Cruz del Campo S.A. in Seville where he is now Head of the Research Department . His current research is concerned with brewing technology , improvement of brewing yeasts and other basic aspects of the brewing process. He has participated in several FEBS Advanced Courses on Biochemistry and Genetics of Yeast .

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Horst FELDMANN graduated in Organic Chemistry at the University of Cologne. He worked in the Institute of Genetics in Cologne from 1962-1967. Since 1967 he is Professor of Biochemistry at the Medical Faculty of the University of Munich. He is presently the Chairman of the FEBS Advanced Courses Committee. His present research interests are the analysis and sequencing of the yeast genome, the study of retro elements and tRNA genes and the regulation of gene expression in yeast.

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Supervivencia de patógenos bacterianos y virales de peces en sistemas de cultivo.

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Caracterización molecular de las estructuras de colágeno.

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Clonación de genes de «Saccharomyces cerevisiae» implicados en la reparación y la recombinación.

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Mecanismo de expresión de la PBP-3 de «E. coli»: Obtención de una cepa hiperproductora de la proteína.

240 **Genetic Strategies in Development.**
Symposium in honour of Antonio García Bellido. Lectures by S. Ochoa, S. Brenner, G. S. Stent, E. B. Lewis, D. S. Hogness, E. H. Davidson, J. B. Gurdon and F. Jacob.

244 **Course on Genome Evolution.**
Organized by E. Viñuelas. Lectures by R. F. Doolittle, A. M. Weiner/N. Maizels, G. A. Dover, J. A. Lake, J. E. Walker, J. J. Beintema, A. J. Gibbs, W. M. Fitch, P. Palese, G. Bernardi and J. M. Lowenstein.

246 **Workshop on Tolerance: Mechanisms and implications.**
Organized by P. Marrack and C. Martínez-A. Lectures by H. von Boehmer, J. W. Kappler, C. Martínez-A., H. Waldmann, N. Le Douarin, J. Sprent, P. Matzinger, R. H. Schwartz, M. Weigert, A. Coutinho, C. C. Goodnow, A. L. DeFranco and P. Marrack.

247 **Workshop on Pathogenesis-related Proteins in Plants.**
Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
Course on DNA - Protein Interaction.

249 **Workshop on Molecular Diagnosis of Cancer.**
Organized by M. Perucho and P. García Barreno. Lectures by F. McCormick, A. Pellicer, J. L. Bos, M. Perucho, R. A. Weinberg, E. Harlow, E. R. Fearon, M. Schwab, F. W. Alt, R. Dalla Favera, P. E. Reddy, E. M. de Villiers, D. Slamon, I. B. Roninson, J. Groffen and M. Barbacid.

251 **Lecture Course on Approaches to Plant Development.**
Organized by P. Puigdoménech and T. Nelson. Lectures by I. Sussex, R. S. Poethig, M. Delseny, M. Freeling, S. C. de Vries, J. H. Rothman, J. Modolell, F. Salamini, M. A. Estelle, J. M. Martínez Zapater, A. Spena, P. J. J. Hooykaas, T. Nelson, P. Puigdoménech and M. Pagès.

252 **Curso Experimental de Electroforesis Bidimensional de Alta Resolución.**
Organizado por Juan F. Santarén. Seminarios por Julio E. Celis, James I. Garrels, Joël Vandekerckhove, Juan F. Santarén y Rosa Assiego.

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening,

J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

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