Yeast Genetics

A Manual of Methods

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With 10 Figures

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Introduction

The manual consists of two main sections. The first includes the essential, sometimes laborious, procedures for handling yeasts, for inducing mating and isolation of hybrids, for inducing sporulation and isolation of single-spore clones, with some details of tetrad analysis, and including techniques and ancillary equipment for use of the micromanipulator. There are also procedures for induction of mutants by physical and chemical agents, and for isolation of particular types of mutants, such as to temperature sensitivity, for increased frequency of mutations, for mutations in the mitochondrial genome, both to the petite colonie form and to resistance to antibiotics, for mutations in that part of the yeast genome controlling the glycolytic cycle, and numerous others. Mapping of mutations is discussed briefly, though this aspect of yeast genetics is probably one which should not be undertaken until the investigator has gained a certain amount of experience in the field. However, as is pointed out in the pertinent part of the manual, the task of mapping has been tremendously simplified by the availability from the Yeast Genetics Stock Center at the University of California at Berkeley of a set of auxotrophic strains designed to permit mapping of most unknown genes with a minimum number of crosses and tetrad analyses. The first section concludes with the description of methods for hybridization of yeasts by protoplast fusion, which has been described as the poor man's system for genetic engineering. Be that as it may, the procedure has a great many applications in the construction of improved industrial yeast strains, both by fusion of strains of the same species, usually but not necessarily *Saccharomyces cerevisiae*, and for introduction of desirable genes from other species from different genera, by gene transfer associated with cytoduction and cybrid formation.

The second, and very important section, deals with the more up-market methods used in yeast genetics and genetic engineering by recombinant DNA techniques. In particular, Dr. Bruce emphasizes the use of pulsed-field gel electrophoresis, including OFAGE, the grand old procedure of them all, FIGE, and CHEF, the most sophisticated of the three procedures, which achieves sharper separations of a range of sizes of high-molecular-weight

DNA molecules by contour clamping of individual electrodes in arrays. Of the three systems, FIGE is probably the simplest for the beginner to use, and it also gives excellent separations of large molecules of DNA, the size range separated being variable by simple changes in the pulse frequency and timing. Also included are techniques for isolation and analysis of high molecular weight DNA and RNA and their characterization.

Recombinant DNA techniques, which permit isolation of individual genes and their re-insertion into the yeast genome at different sites, fusion of genes with promoters, leader sequences, fusion with genes from other organisms (lacZ gene, for example), any many other such operations, have greatly enhanced the range of problems which can be solved by the yeast geneticist. While it is not possible to discuss all of the possible uses of gene cloning and related recombinant DNA techniques, we have described some of the more important of the basic methods, which will enable the yeast geneticist to go on to the solution of many problems, which could not even be formulated, let alone attempted, before the advent of these methods.

The manual assumes a basic knowledge of microbiological and biochemical techniques, but given this, our hope is that it will enable both the serious student and the experienced investigator to gain a wider knowledge of the current methods in use in this field, and to accelerate the pace of their investigations.

Part I. "Classical" Yeast Genetics

This stage began, essentially, with the discovery of haploid and diploid phases in the life cycle of *Saccharomyces cerevisiae* by Winge (1935), and of Mendelian segregation in *Saccharomycodes ludwigii* (Winge and Lautsten 1939) and the isolation of heterozygous strains in which haploid clones, obtained by dissection of yeast asci and separation of the individual spores, did not self-diploidize and could be used to re-form the diploid state by mating of vegetative cells of opposite mating types, or by pairing of spores or of spores and vegetative haploid cells by micromanipulation. Auxotrophic mutants of the haploid clones can be obtained by mutagenesis with X-rays, UV-irradiation, chemical mutagens or by other methods. Complementing mutations in clones of opposite mating types can be used to obtain prototrophic diploids, and these can be sporulated and haploid clones can be isolated and used to demonstrate recombination of characters during meiosis, linkage of characters, and the construction of maps of the yeast chromosomes. These techniques have been most widely used in the study of the genetics of *Saccharomyces cerevisiae*, but have also been extremely useful in the study of the genetics of other yeast species, including *Schizosaccharomyces pombe*, *Yarrowia lipolytica* (a species of importance for its ability to metabolize alkanes and lipids, and to produce extracellular proteases), *Kluyveromyces lactis*, *Pichia pinus*, *Hansenula polymorpha* and other methylotrophic yeasts, and various other species. Some years ago, mating types and a sexual cycle were observed in the basidiomycetous species, *Rhodosporidium toruloides* (imperfect stage *Rhodotorula* sp.) and *Leucosporidium scottii*, and in a group of ascomycetous yeasts forming needle-shaped spores, of the genus *Metschnikowia*.

It might be thought that the discovery of restriction enzymes, cloning of genes, and transformation of yeasts with various plasmid-like vectors, would have by now superseded the use of the methods of classical genetics in the production of new yeast strains and in characterization of known strains, but in fact, the one group of techniques usually complements the other, one taking up where the other leaves off, so that a knowledge of the methods of "classical" yeast genetics as well as of those based on recombinant DNA

techniques is highly desirable, especially for the yeast geneticist who desires to construct improved strains of industrial yeasts.

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1. Mating

Mating of haploid yeast strains can be done in a number of ways, either by mixing the yeast strains on a rich medium and isolating the resulting zygotes by micromanipulation, or by some variant of the drop-overlay method. The latter is frequently used for mating auxotrophic strains having complementary requirements. For prototrophic parental strains, of course, hybrids can only be isolated by micromanipulation of the zygotes from the mating mixture.

a) Drop-Overlay Method

Required

1. 24-h cultures of auxotrophic haploid strains (opposite matings types, having complementary nutritional requirements), to be mated.
2. Plates of minimal medium containing a carbon source (yeast-nitrogen base-glucose (2%), for instance).
3. Sterile water blanks, 9.0 and 9.9 ml, sterile pipettes, Pasteur pipettes and other glassware.

Procedure

1. Dilute the culture approximately 1:10 with sterile water and wash once or twice with sterile water and make back to original volume.
2. With a Pasteur pipette, put 2 drops of the suspension of the first strain on a plate of minimal medium (as above), in well-separated locations. Let dry.
3. Put a drop of the second suspension on top of one of the dried drops of the first, and another drop in a new location. Let dry. There should be one drop containing cells of both strains, and two other drops of one of each of the other strains, as controls (Fig. 1).

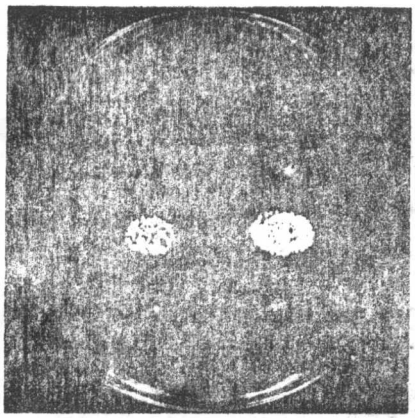

Fig. 1. Mating of haploid strains. Drop overlay pattern

4. Incubate 3–4 days. Microcolonies should occur in profusion if the strains are active maters, in the drop containing both strains. Occasional microcolonies may arise in the controls if only a single auxotrophic marker is present, brought about by reversion of the marker. These are not normally observed where two or more markers are present.
5. Restreak the prototrophic diploids on minimal medium (Fig. 2). Test for sporulation. Haploid revertants do not sporulate.

b) Mating of Prototrophic Haploid Strains

This can be done in the same way as for auxotrophic haploids, though isolation of the resulting diploids is more laborious, as the complementation method on minimal medium cannot be used. Two methods at least are possible:

1. Isolation of diploids by streaking out the mating mixture and random isolation of colonies, which are then tested for the ability to sporulate. This method can be varied by selecting and isolating a number of large cells by micromanipulation, followed by testing the strains for sporulation as before. This method has the advantage of ensuring that the strains arise from single cells. If homothallic strains are to be mated, spore-spore or spore-cell pairing (using haploid mating strains for the vegetative cells) by micromanipulation can be used. The difficulty in this case is to determine whether the resulting colonies are actually a cross, unless some marker is introduced in the haploid strain, which can be detected later in the hybrid. Markers such as are used in the "rare-mating" method are probably the most satisfactory.

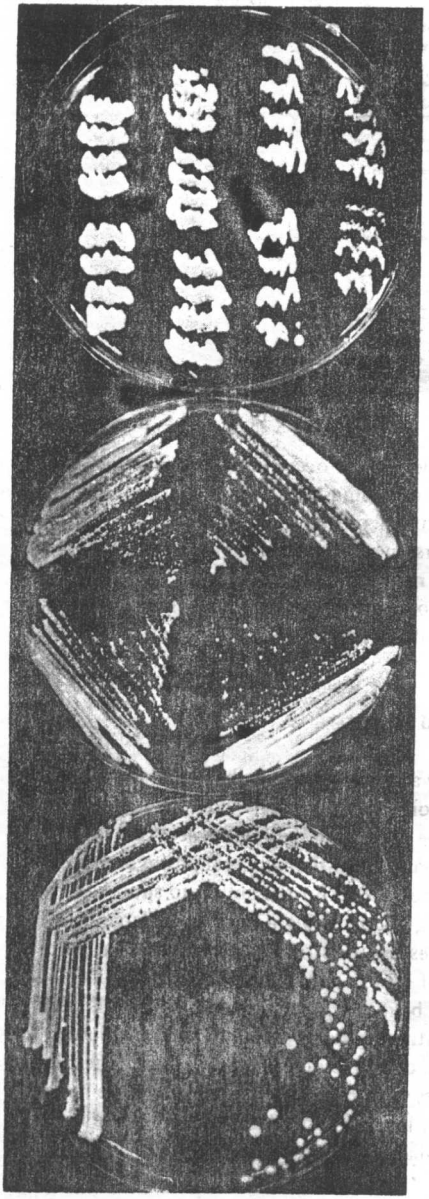

Fig. 2. Patterns for restreaking yeast colonies

2. Possibly the quickest method is to isolate zygotes from the mating mixture. These appear, usually from 2-6 h after mixing of the strains on a complete medium, though in some cases at least, zygotes can be observed in the mating mixture for 24-48 h after mating begins. Individual zygotes can be isolated from the mixture with a micromanipulator, though zygotes are not as readily picked up as tetrads or individual spores (see Section "Micromanipulation").

3. "Rare-mating" techniques. This method is based on the observation by Gunge and Nakatomi (1971) that mating-type switching occurs at low frequencies in *Saccharomyces cerevisiae*, in diploid cells or cells of higher ploidy, and leading to the appearance of mating cells, which will mate and form hybrids if cells of another mating type are present. The method was adapted by us for use in obtaining hybrids from industrial yeast strains, which often sporulate poorly or not at all, and which generally form nonviable spores if any are produced (Spencer and Spencer 1977). The diploid (or polyploid) strain is converted to the petite form by any one of numerous methods, including treatment with acriflavin or ethidium bromide, by starvation, or simply spreading an appropriate dilution of the yeast culture on a plate of normal medium, incubating for a few days, and selecting small colonies, which are then tested for inability to grow on non-fermentable substrates (glycerol or lactate). The petite strain is then mated with an auxotrophic mating strain which may be haploid or diploid, of either mating type, as in most cases there will be switching to either mating type in the petite. Hybrids are isolated on selective medium, as follows:

Required

1. Petite mutants of the industrial strain (see petite mutants).
2. Mating strain of laboratory yeast, carrying any auxotrophic markers desired.
3. Culture media: 1. Yeast extract-glucose broth.
   2. Yeast-nitrogen base (YNB)-glycerol agar, containing 3% ethanol to inhibit sporulation in the hybrids.
4. Sterile pipettes, centrifuge tubes, and possibly sterile membrane (Millipore) filters; sterile McCartney bottles, if this variant of the method of mating is to be used.

Procedure

1. Grow cultures, 24-48 h in YEP-glucose broth. Usually enough culture can be grown in 5 ml of medium, aerated on a roller drum or rotary shaker held in an inclined rack.
2. Either: mix cultures in YEP broth and incubate in still culture in shallow layers to allow aeration, for 5-6 days,

or, mix cultures and filter on a membrane filter, to pack the cells tightly. Make sure the cells do not dry out.
Or: mix the cultures and centrifuge to pack the cells tightly.

3. After allowing sufficient time for mating to take place (a few hours for the two latter variants), recover and wash cells twice in sterile water or buffer.
4. Spread a heavy suspension of the cells on the YNB-glycerol-ethanol agar, and incubate until colonies appear. The auxotrophic laboratory strain will not grow on minimal medium, and the petite of the industrial strain will not grow on a non-fermentable carbon source.
5. Restreak the colonies of respiratory-competent prototrophs which appear, on YNB-glycerol-ethanol agar. Isolate and store.

Note

Homothallic strains may be held in the haploid state by dissecting the asci directly on to a slab of acetate agar (McClary's medium), and using the small colonies arising from the spores in mass mating with known laboratory haploids, or in pairing by micromanipulation with haploid cells or spores of other strains. If the strains are prototrophic, then the usual system of isolating zygotes directly by micromanipulation must be used. If some kind of marker is naturally present, or can be introduced into the strains involved, identification of the hybrids is simplified (see Palleroni 1961).

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2. Sporulation

Diploid hybrids (or hybrids of higher ploidy) having been obtained, sporulation can then be induced in a number of ways, mostly involving growth of the culture on a medium containing potassium acetate, glycerol or other non-fermentable substrate. Sporulation may also be induced in raffinose-acetate solutions. The presence of K ions improves sporulation. For most yeast strains, McClary's medium (Fowell 1969) gives adequate sporulation. Growth on a relatively rich pre-sporulation medium such as yeast extract-peptone glucose medium is desirable. Sporulation may be induced in liquid or on solid medium, or in protoplasts as well as in intact cells. Induction of sporulation in protoplasts has the advantage that the unsporulated protoplasts may

be burst by addition of water to the osmotically stabilized sporulation medium which must be used in this method, and the spores can either be isolated by micromanipulation of the naked tetrads or harvested, free of unsporulated vegetative cells, and subjected to random spore analysis.

Required

1. Presporulation medium. Yeast extract-peptone-glucose medium, liquid or solidified with agar.
2. McClary's sporulation medium:

Potassium acetate	1 g
Yeast extract	0.25 g
Glucose	0.1 g
Agar (if desired)	1.5 g
Water	100 ml

If this medium is used in liquid form, it must be aerated adequately for good sporulation.

Procedure

1. Inoculate the yeast strains on to YEP-glucose medium and incubate for 48–72 h at 25–30°. If sporulation in liquid medium is required, inoculate 4 ml of the above medium in a 16-mm culture tube and incubate with shaking for the same time and temperature.
2. Inoculate a plate or tube of McClary's medium from the presporulation medium (for solid medium, streak on the medium, for liquid medium, inoculate from the broth culture at a ratio of 1:10. Two ml of sporulation medium in a 16 mm culture tube, with 0.2 ml of presporulation culture, for instance).
3. For sporulation on solid medium, incubate at 20–30° for up to 7–10 days. Cultures which sporulate readily will generally show the first asci in 48 to 72 h. Some cultures sporulate better at lower temperatures, in which case, it may be necessary to incubate for a week or more.
4. Cultures sporulated in liquid cultures should be incubated on a shaker operated at a high enough speed to give good aeration. Sporulation usually occurs in 3–4 days. Confirm sporulation, in both methods, by microscopic observation.
5. Centrifuge cultures sporulated in liquid medium, and wash cells and asci once or twice in sterile water, and store in the refrigerator. If the tubes are sealed with parafilm to prevent drying, the spores will remain viable, normally, for several weeks.
6. Cultures sporulated on solid medium may be stored successfully for a week or two at refrigerator temperature, if kept from drying.

Notes

1. Better sporulation and spore viability can be obtained in some strains by incubating at 18-20° instead of at 25-30°.
2. Some strains may be sporulated adequately in raffinose-acetate medium (2% + 1%), or on media containing glycerol as sole carbon source.
3. Strains of *Saccharomyces diastaticus*, and some of our intergeneric fusion hybrids, having *S. diastaticus* as a parent, will often sporulate well on yeast extract-soluble starch agar.
   Sometimes yeasts sporulated on media other than those containing acetate form an ascus wall which is very difficult to dissolve enzymatically.
4. *Schizosaccharomyces pombe* will often sporulate adequately on malt extract agar. In this species, the ascus wall dissolves without the use of enzymatic treatment, which makes the isolation of the ascospores less complicated.
5. The above procedures using acetate medium for sporulation, apply normally to *Saccharomyces cerevisiae* strains. Other yeast species (*Yarrowia lipolytica, Saccharomycopsis fibuligera, Saccharomycopsis capsularis, Hansenula* and *Pichia* species, *Debaryomyces* species, and various others) can be sporulated readily on malt extract agar, Gorodkowa agar and other specialized media (Lodder 1970, 1984).

a) Spore Isolation

Random Spore Isolation. In these techniques, some way of eliminating the unsporulated vegetative cells is essential. This can be done either by killing the vegetative cells, or by separating them physically from the spores in some way.

i) Heat Killing of Vegetative Cells

This method is based on the slightly greater heat resistance of spores as compared to vegetative cells. However, different strains differ in the resistance of the vegetative cells and spores to heat, so that the actual times and temperatures used should be determined for each strain used. The temperature will normally be between 54 and 60 °C.

Required

1. Yeast cells, sporulated as above.
2. Sterile water and culture tubes.
3. Water bath, set at the predetermined temperature.

Procedure

Suspend the sporulated yeast culture in sterile water, and hold in the water bath at the desired temperature (example, 59°), as determined previously, for 10–15 min. Plate the treated spore suspension on malt agar or YEP-glucose agar.

Notes

1. Industrial yeast strains, if polyploid or aneuploid, may form spores of varying ploidy, so that diploid strains as well as haploids, which generally form smaller colonies, may be isolated. The isolates should be tested for the ability to sporulate as well as for mating type.
2. Industrial yeast strains are frequently homothallic, so that single spore clones which were originally haploid may well be diploid when isolated.
3. Strains forming few viables spores will often form asci with no more than one viable spore each, which facilitates the isolation of single-spore clones by this and the following method. It is necessary to verify the purity of the clones if possible.

ii) Ether Killing of Vegetative Cells (Dawes and Hardie 1974)

This method is based on the differential sensitivity of the vegetative cells and spores to exposure to diethyl ether.

Required

1. Sporulated yeast culture.
2. Sterile water.
3. Diethyl ether.
4. McCartney bottles or roller tubes.

Procedure

1. Make a suspension of the yeast culture in water.
2. Place 5 ml of suspension in roller tubes or McCartney bottles, cool to 4 °C, add an equal volume of ether, close tube tightly and place on roller apparatus in cold room, or otherwise agitate for 10 min.
3. Separate ether and water layers, and remove ether from water layers under vacuum.
4. Plate out suspension on malt agar or YEP glucose agar.

Notes as for heat killing also apply to ether killing.

REFERENCE

Dawes IW, Hardie ID (1974) Selective killing of vegetative cells in sporulated yeast cultures by exposure to diethyl ether. Molec Gen Genet 131:281–289

iii) Sporulation of Protoplasts and Bursting of Vegetative Cells

Strains which form protoplasts readily can be grown on presporulation medium, converted to protoplasts (see section on protoplast formation) and the protoplast suspension sporulated, after which the protoplasts can be burst, giving a relatively pure spore suspension.

Required

1. Yeast culture, 16–24-h-old (exponential growth phase), in YEP-glucose medium.
2. Pretreatment and protoplasting solutions, as in Section 8, (Protoplast Fusion), 0.6 M KCl solution (all solutions sterile).
3. Snail enzyme, Zymolyase, Novozyme or other enzyme suitable for digesting yeast cell walls.
4. Osmotically stabilized sporulation medium.
5. Sterile water, culture tubes, pipettes, etc.
6. Ultrasonicating apparatus or Potter-Elvehjem homogenizer with Teflon pestle (sterile).

Procedure

1. Grow yeasts to exponential phase and convert to protoplasts (Section 8, Protoplast Fusion).
2. Transfer protoplasts to osmotically stabilized sporulation medium (liquid) and incubate (shallow layers for better aeration) until spore formation has taken place.
3. Recover sporulated cells by centrifugation if necessary and add sterile water to burst unsporulated vegetative cells.
4. Lightly sonicate the suspension, or give 1 or 2 strokes in the Potter-Elvehjem homogenizer, to separate the spores.
5. Plate on malt agar or YEP-glucose agar. Various substances (15% gelatin solution, etc.), may be added to aid in keeping the spores separated during plating.