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Synchronous Mating in Yeast

(zygote isolation/cell fusion/mating efficiency)

ELISSA P. SENA, DAVID N. RADIN, SEYMOUR FOGEL

Genetics Department, University of California, Berkeley, Calif. 94720

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ABSTRACT **Homogeneous *a* and *α* unbudded yeast cells in logarithmic phase, grown in supplemented minimal medium and isolated by zonal gradient centrifugation, are used for mating. When these cells are resuspended in aerated defined medium, highly synchronous mating rapidly occurs. Within 20 min of incubation at 30° early sexual pairing is evident; extensive agglutination is observed by 60 min, and cell fusion and bud initiation in zygotes occurs after 60–140 min. Sorbitol gradient fractionation of mating mixtures taken at various times during incubation allows the isolation of zygotes or unmated cells. Zygote preparations 90–95% purified are obtained in quantities suitable for genetic and biochemical analysis. The mating procedure is predictable and reproducible.**

Mating by cellular fusion is essential for segregational and recombinational analysis of nuclear or cytoplasmic inheritance in *Saccharomyces cerevisiae*. However, a detailed description of the mating reaction at the molecular level has not been established. In part, this reflects the inadequacy of current experimental methods for studying mating in yeast. In heterothallic yeasts mating occurs when cells with complementary mating types are mixed under appropriate conditions. The conjugation reaction (1, 2) then progresses as a sequence of ordered events that can be subdivided on a gross visual and cytological basis into three principal phases: (*a*) mating initiation, which involves conjugant pairing and sexual agglutination, (*b*) cell fusion (plasmogamy), and (*c*) nuclear fusion (karyogamy). The completed reaction sequence yields a morphologically distinct zygote that can, depending on nutritional conditions, either proliferate mitotically or enter into meiotic development and ascospore formation.

A prerequisite for studies aimed at understanding mating and sexuality is the ability to obtain sizable, homogeneous cell populations that undergo synchronous mating under defined conditions. We report here our initial studies on mating and a protocol for producing highly efficient synchronously mating populations of yeast in liquid minimal medium. From these populations, purified zygote suspensions are readily isolated in quantities suitable for extensive genetic and biochemical analyses.

MATERIALS AND METHODS

Strains. Two heterothallic haploid strains of *S. cerevisiae* containing complementary leucine and tryptophan markers were isolated as single spore clones: 5032A *a leu*2-27 *try*1-1 and 5032B *α leu*1-12 *trp*4.

Medium. A defined liquid minimal medium (YNB) containing 1.45 g of yeast nitrogen base (Difco), supplemented with 3 g of $(NH_4)_2SO_4$, 20 g of glucose, 40 mg of L-leucine, and 30 mg of L-tryptophan per liter was used for all culture growth and mating.

Growth. Both *a* and *α* cells were inoculated into separate 2-liter flasks each containing 1 liter of YNB medium at a density of about 1.2×10^4 cells per ml and grown overnight at 30° on a rotary shaker at 250 rpm.

Cell Isolation. Mid- to late-log phase *a* and *α* cultures with 4 to 8×10^6 cells per ml were mixed in varying proportions. The mixtures were collected by centrifugation and resuspended in 20–25 ml of YNB. They were then treated with a Heat Systems Sonifier at 50 W for 5 sec to break up cell clumps and release mature buds. Cells were harvested as rapidly as possible to prevent premature agglutination. The sonicated *a* and *α* cell mixture was immediately layered onto a sterile 8–35% linear sorbitol gradient in an MSE zonal rotor (1300 ml) and centrifuged at 1200 rpm for 10–12 min. A detailed description of zonal rotor methodology for yeast cell separations has been published (3). Fractions of 15 ml were collected from the gradient by use of a displacement pump and an automatic fraction collector. The unbudded cell fractions were identified by microscopic examination and pooled for subsequent use in the mating procedure. This selected premating *a* and *α* cell population contained about 95% single, unbudded cells and represented 8–12% of the initial mixed cell population or about 30% of the unbudded cells introduced into the rotor. The initial sample contained about 10^{10} cells, but this may be increased 3–4 times without loss of resolution. Also, the method can be scaled down, and unbudded cells may be isolated in smaller quantities in 20- to 50- ml sorbitol gradients (4).

Mating Procedure. The single cell fraction obtained from the zonal rotor was centrifuged to remove the sorbitol, resuspended in 100 ml of YNB to a density of 6 to 10×10^6 cells per ml in a 500-ml Erlenmyer flask, and shaken at 30°. The beginning of incubation is defined as zero time for the mating reaction. A cell sample was immediately withdrawn from the mating mixture to determine the ratio of *a* and *α* unbudded cells obtained from the rotor. Samples taken throughout the first 180 min of the mating reaction were fixed immediately with an equal volume of 3.7% formaldehyde in 0.05 M phosphate buffer (pH 7.0).

Zygote Isolation. After 120 and 150 min of incubation, 30–40 ml of the cell suspension was harvested by centrifugation, resuspended in 1 ml of water, sonicated for 5 sec, and then layered onto a 25-ml linear 10–30% sorbitol gradient. The gradient was centrifuged at 1000 rpm in a Sorvall HB-4

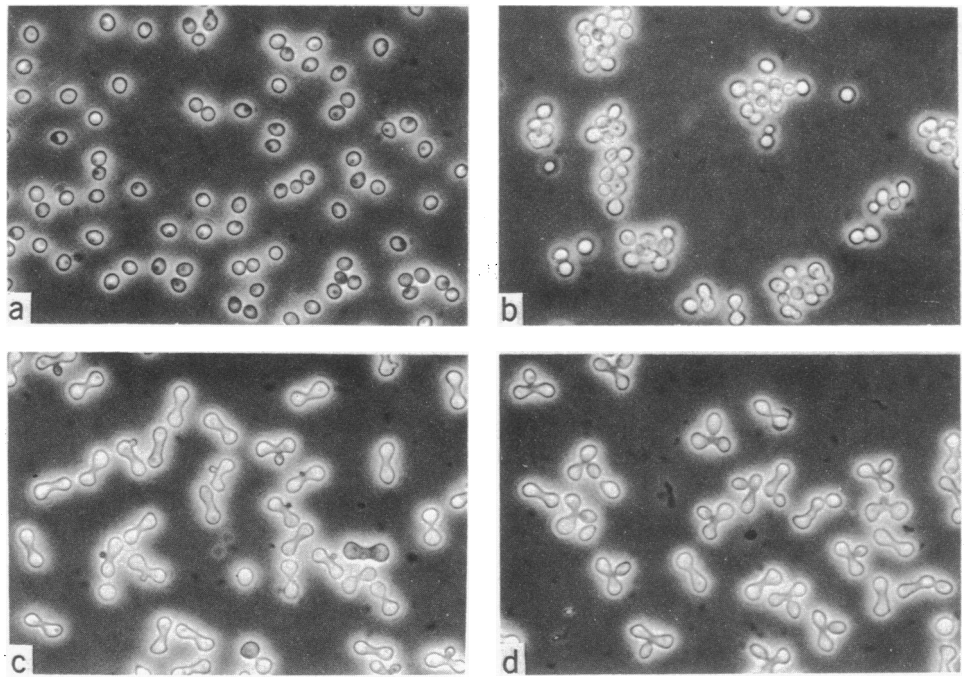

FIG. 1. Sequence of events during synchronous mating. (*a*) Cells after 20 min. (*b*) Cells after 60 min. (*c*) Purified zygotes after 120 min. (*d*) Purified zygotes after 150 min. Micrographs were taken with phase optics at a magnification of 552.

rotor for 4.5 min. Overloading the gradient leads to rapid agglutination, which in turn increases streaming and pellet formation. Twenty-five 1-ml fractions were collected from top to bottom with a 1-ml syringe. The optical density of the fractions at 600 nm was determined on a Gilford spectrophotometer.

Calculations: Percent Mating. The formaldehyde-fixed samples were sonicated to disrupt agglutinated clumps and counted for budded cells, unbudded cells, and mating pairs by use of a hemacytometer. Cells were scored as zygotes when they appeared fused. The time-course of the mating reaction was calculated by defining unmated budded and unbudded single cells as one unit and budded and unbudded zygotes as two units. When percentages are given to assess zygote purification each zygote is counted as one unit. At least 100 cells were scored for each determination.

Mating-Type Testing. The ratio of *a* to α cells obtained from the zonal rotor was determined by dilution plating and conventional mating-type test procedures (5).

RESULTS

Time course of mating

A homogeneous unbudded cell fraction from a mixture of two exponentially growing haploid cultures was isolated by the sorbitol zonal gradient technique as described in *Methods*. When the *a* and α cell mixture was removed from sorbitol by low-speed centrifugation, resuspended, and incubated in YNB medium, the initial events of the mating reaction rapidly ensued. Within 20 min early sexual pairing was evident (Fig. 1*a*). After 60 min, marked cell agglutination was clearly evident macroscopically as a particulate suspension, and microscopically as clumps of cells (Fig. 1*b*). Both cell pairs and agglutinated clumps could be disrupted by sonication. However, such treatment did not disrupt cell pairs that had initiated zygotic fusion. Sonication affected neither cell viability nor mating efficiency, and pairing and agglutination would reoccur rapidly. Extensive mating occurred within the agglutinated clumps between 60 and 140 min, as illustrated in Fig. 2 (total zygotes), while budded zygotes appeared about 30 min after the onset of plasmogamy.

The mating reaction is essentially complete after 150 min when 66% of the cells fuse. Cell number per ml remained constant during the initial 170 min, and subsequently unmated cells displayed a round of budding. Since the zygotic bud matures about 120 min after emergence, the small decrease in the percent budded zygotes observed at 210 min probably reflects the separation of the first diploid cell from the parental zygote.

To assess the effect of prior mixing on the time-course of mating, unbudded log-phase *a* or α cells were isolated on separate 25-ml sorbitol gradients and inoculated either separately or as a mixture into fresh YNB medium. The mating time-course of the newly mixed unbudded cells was identical to that of the cells mixed before centrifugation on the zonal rotor. Thus, prior mixing, which facilitates our cell-selection procedure, does not appear to have a major effect on the premating activities. However, mitotic budding in the separately maintained *a* and α cultures occurred with half the lag time observed for unmated cells in the mating mixture. Therefore, mixing induces a delay of mitotic division among the unmated residual cells regardless of the isolation procedure.

Zygote isolation and purification

Highly purified zygote preparations were obtained by fractionation of the mating mixture into two bands on a 10–30% sorbitol gradient (Fig. 3). Depending upon the age of the sample, the upper band (*A*) contained only unmated cells while the lower band (*B*) contained 40–95% zygotes along with unmated cells. Zygote purification was about doubled after fractionation on the sorbitol gradient. Accordingly, an

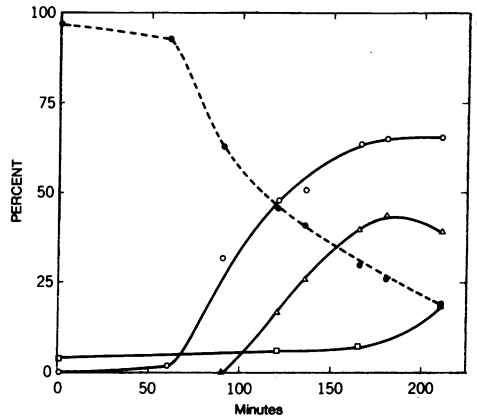

FIG. 2. Time course of the mating reaction. A mixture of *a* and *α* unbudded cells isolated from exponentially growing cultures by zonal gradient centrifugation was incubated at 30° in liquid YNB medium. Procedures for sampling and counting of budded and unbudded cells and zygotes are described in *Methods*. Averaged results from several experiments are shown. ●– – –●, unbudded cells; O——O, total zygotes; Δ——Δ, budded zygotes; □— —□, budded cells.

initial population containing 20% zygotes would contain 40–50% zygotes in band *B* (Fig. 3). If the pooled fractions from this band were concentrated and repartitioned on a similar sorbitol gradient, zygote preparations of 90–95% purity could be obtained. However, if the zygotes amounted to 50–60% in the initial mating mixture, a single separation would yield an 80–95% zygote preparation in the lower band. When heterogeneous zygote mixtures were fractionated, a partial separation between budded and unbudded zygotes could be detected. The upper portion of band *B* contained mostly unbudded zygotes, while large budded zygotes predominated in the lower fractions.

A purified zygote preparation (95%) isolated from a 120-min synchronously mating culture containing 65% unbudded zygotes and 30% zygotes with small buds is illustrated in Fig. 1*c*. The zygote preparation (90%) illustrated in Fig. 1*d* was recovered from a 150-min incubation mixture in which 70% of the zygotes have large buds. Typically, zygotes develop a first bud at the cell-fusion juncture, though occasionally it is generated elsewhere.

Properties of isolated zygotes

The viability and sporulative capacity of purified zygotes was excellent. Purified zygote preparations sporulated readily, efficiently, and at normal rates upon transfer to 1% potassium acetate. Four-spored zygotic asci were analyzed for marker transmission and segregation by microdissection and testing of ascosporal clones for nutritional requirements and mating type. In all, five heterozygous markers exhibited conventional segregational and recombinational behavior among 25 tetrads analyzed.

Mating optimization

In addition to maximal sexual agglutination, several considerations were essential to produce efficient, synchronous mating. These include growth phase, cell concentration, and physical homogeneity of the haploid cells in the mating mixture.

Haploid strains 5032A and 5032B approach the end of log-

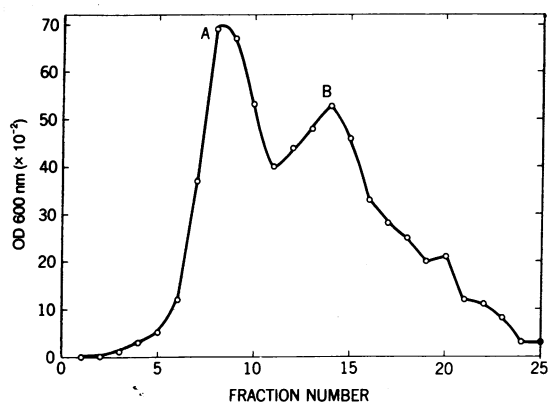

FIG. 3. Purification of zygotes on a sorbitol gradient. Cells from a synchronously mating culture containing 25% zygotes were fractionated and analyzed as described in *Methods*. The top of the gradient is on the left. (*A*) Unbudded cells; (*B*) zygotes.

phase growth in YNB medium at a density of 2×10^7 cells per ml. When unfractionated late-log phase or early stationary phase cultures were mixed, the observed zygote frequency was reduced 10 times compared to mixtures made with younger mid-log phase cultures harvested at 5×10^6 cells per ml. The former mixtures display an inhibition of vegetative budding. Also, they contain distinctive elongated, pear-shaped cells. The morphological alteration noted is indistinguishable from the effect previously described by Duntze and coworkers (6) and attributed by them to the action of an extracellular factor produced by *α* strains on cells of the opposite mating type. The coincident inhibition of mating and change of cell shape were also observed in mixtures of fractionated cells taken from late-log-phase cultures. Identical effects could be produced in mixtures of fractionated cells from mid-log phase cultures by (*a*) increasing the cell density in the mating mixture considerably above 5×10^6 cells per ml, (*b*) introducing a large

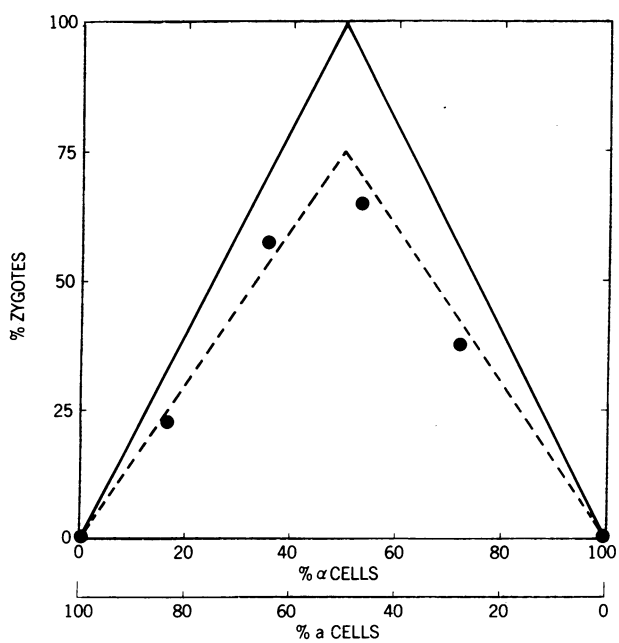

FIG. 4. Mating efficiency. Theoretical (——) and corrected theoretical (– – –) mating efficiencies compared with observed (●) results at various *a* and *α* cell ratios. The method for determination of the corrections is given in *Results*.

excess of α cells into the mixture, or (*c*) allowing the liquid cultures to stand without shaking.

To test the notion that the observed effects were attributable to the diffusible peptide factor secreted by α cells, exogenous α-factor prepared by the procedure of Duntze *et al.* (6) was added into an otherwise normal mating mixture at time zero. These cultures produced a reaction identical to that described above. Taken collectively, the observations define more precisely the optimal conditions for the synchronous mating procedure outlined in *Methods*.

The degree of synchrony in the procedure described above is defined by the rate of the mating reaction as well as by the initiation and development of the first zygotic bud. Cell fusion is restricted to a specific time period after mating initiation and rapidly reaches a maximal level. Zygotic buds appear about 30 min after cell fusion. Taken collectively, these observations indicate that zygotes present at 150 min were initiated synchronously and exhibit approximately the same level of bud development. Although mating synchrony strongly depends upon the selection of homogeneous unbudded cell fractions from the zonal rotor, total mating was not obtained. Rather, the maximal observed frequency of mating did not exceed 66%.

In unfractionated mating cultures, the initial burst of mating involved only unbudded cells and occurred with rates similar to those of fractionated cultures. Because the zygotes amounted to only 20–25% of the total cells present (a fraction equivalent to about half the unbudded cells in the mixture), only a limited fraction of the unbudded cells taken from exponentially growing haploid cultures undergo mating. In cultures partitioned through the zonal rotor gradients, we found the highest mating efficiency in the fractions containing smaller unbudded cells. As a general rule, the more heterogeneous selections of unbudded cells resulted in higher frequencies of mitotic budding rather than mating. Thus, to optimize mating, only the smaller 30% of the unbudded cells from logarithmically growing cultures were used. About 10% of these unbudded cells initiated vegetative buds instead of mating. This bud initiation adds to the 5% of budding cells initially present in the cell mixture and accounts for the increase in budded cells during incubation of the mating cells. This observation was corroborated by isolation of the unmated cells from a 120-min mating mixture in a 25-ml sorbitol gradient. When these cells were reinoculated into fresh YNB medium, the equivalent of 10% of the cells from the original incubation mixture initiated bud production within 60 min. Reincubation of these previously unmated cells also produced 10% additional matings. That these apparently competent cells failed to mate initially probably reflects the steric arrangement of cells within the agglutinated clumps. Thus, one might expect to find that a small number of competent cells rigidly held in an agglutinated clump would have contact only with incompetent partners of the same or opposite mating type and/or with cells that had already initiated mating with other partners.

Fig. 4 shows the total mating obtained from incubated mating mixtures of selected unbudded cultures with various ratios of *a* and α cells. The *solid line* indicates the theoretical mating frequency at any given ratio if all cells are competent to mate. The *broken line* shows the predicted mating frequency in cultures where 25% of the cells present are unable to mate; i.e., 5% of the cells are initially budded, 10% proceed to initiate buds rather than mate, and 10% fail to mate due to steric factors. Since the observations agree closely with the corrected expectations, the system exhibits an optimal mating capacity.

A strong agglutination reaction was a necessary but insufficient prerequisite for initiation of mass matings in the vigorously agitated liquid system. The haploid strains used were selected for nonclumpiness, and they agglutinated only after cells of opposite mating types were added. Sexual agglutination does not occur immediately, but attains completion only after a 1-hr incubation period. Recently two varieties of *a* cultures were distinguished (7); those that agglutinate immediately upon admixture with α cells and those that require a period of mutual incubation before the agglutination reaction is observed. Since mixing of *a* and α cells before incubation does not result in immediate agglutination, our strains are probably inducible. Furthermore, mixed cells will not agglutinate unless the aerated medium contains all nutrients essential for growth; e.g., glucose, amino acids, or nitrogen. Rather wide variations in cell density and growth stage of the haploid cultures did not affect the efficiency of sexual agglutination. Maximal agglutination was observed when the *a* to α cell ratio was unity, and diminished agglutination occurred when cells of either mating type were present in excess. The addition of diploid cells to mixtures of *a* and α cells also decreased the observed agglutination. Finally, agglutination is not restricted to cells competent to mate, since budded cells from an unfractionated mixture are not excluded from agglutinated clumps.

DISCUSSION

The mating efficiency in yeast is dependent upon the age and growth conditions of cultures before mating as well as on the cell-mixing technique. In the typical procedure for hybridizing yeast, separate *a* and α cultures are grown to early stationary phase and mixed on solid complex media containing yeast extract and peptone. This procedure produces average zygote frequencies between 10 and 15% after 3–5 hr, a yield sufficient for routine genetic analyses. When stationary phase *a* and α cultures are starved for 48 hr on buffered solid media, the resulting unbudded cells mate at a frequency of >95% when paired by direct micromanipulation (8). Jacob (9) attempted to increase the zygotic yield in mass cultures by a synchronization technique using stationary-phase cells grown and mated in complex liquid medium. Although apparently high mating efficiency was reported, the absence of specific details concerning zygote enumeration does not permit a comparison with our protocol.

The mating procedure reported here produces synchronous mating with high efficiency. Under defined conditions, zygote yields are predictable and reproducible. The advantages of our procedure are: (*a*) A single chemically defined liquid minimal medium is used both for cell growth and mating. (*b*) Large populations of log-phase cells at a defined stage are used. (*c*) A short time span is required for completion of the mating reaction. (*d*) The isolation of purified preparations of unmated cells and zygotes in high yield at well-defined stages of the mating process facilitates critical and perhaps novel analysis. Resuspension of zygotes in fresh or altered medium permits further growth studies or the synchronous induction of meiotic development and sporulation without intervening mitotic cell divisions. In addition, the procedure allows for a

more precise definition of the early events associated with cell pairing and sexual agglutination.

The results suggest that unbudded haploid yeast cells mate during a stage of the cell-division cycle before bud initiation. Previously reported genetic, biochemical, and cytological studies (6, 10–12), and especially MacKay's analysis of mating sterile and α-factor-deficient mutants (13), have implicated the diffusible factor produced by α strains to be involved in phasing a cells for mating. Indeed, our observation that mitotic budding of all unmated cells is delayed in a mating mixture beyond the normal lag time for separately maintained a and α cultures, supports a postulated reciprocal phasing mechanism (10–12) in which α cells are staged by an a factor. However, the actual role of these factors in the mating process is not clear. Our observations indicate a negative involvement of α factor in mating, since we found a virtually complete inhibition of mating concomitant with the morphological effects reported earlier. If the diffusible mating substances are the major factors affecting mating inhibition, then the observed mating inhibition may be related to a high concentration of factors in the incubation medium, or to the high rates of their production. Conceivably, the optimal factor concentration affecting mating is well below that which produces morphological changes. In conclusion, it is not known whether the presence of factors is essential for mating. It is possible that they merely enhance mating efficiency in a population of mixed cells.

The controlled synchronous mating procedure described here has allowed us to initiate investigations concerning sexuality in a simple eukaryote. Conditional mutants, variously blocked at as yet undefined execution points of functions essential to the mating process have been isolated by us and they will allow for parallel molecular, cytological, and genetic studies. Furthermore, the ability to induce meiotic development and sporulation in highly synchronous, purified zygote populations, with or without intervening diploid vegetative growth cycles, provides unique opportunities for developmental analysis of the entire sexual cycle in yeast. Particularly, a study of the time course of macromolecular synthesis will allow us to correlate specific molecular events with observations on both nuclear and cytoplasmic inheritance, especially on the segregation of mitochondrial genes. In addition, dissolution of the cell wall and cell fusion may allow RNA, DNA, or other macromolecules and organelles not commonly taken up by yeast to be incorporated into the zygote (14). Hence, these could be studied with respect to their interactions within the yeast cell. Also, an understanding of the mating reaction at the molecular level could illuminate the basis for mating incompatibility between certain yeasts. The production of viable matings between previously nonhybridizable species would provide favorable material for analyzing the evolution of species differences.

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