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a and α Supernatant Pretreatment of *Saccharomyces cerevisiae* Cells Affects Both the Kinetics and Efficiency of Mating

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The effects of culture supernatant treatment on subsequent matings between pretreated **a** and α *Saccharomyces cerevisiae* cells were studied. For each experiment, pairs of **a** and α [*rho*$^+$] or [*rho*$^-$ *rho*^{0}] cells in the logarithmic growth phase in defined minimal medium were pretreated for a total of 15 min (by exchanging their cell-free supernatants or by mixing samples of **a** and α cell cultures) and then mated in defined minimal (YNB) or enriched (YEP) liquid medium. All pretreated cells, regardless of treatment procedure, initiated cell fusion 15 to 35 min faster than did their nontreated counterparts. In all cases, pretreated cells mated 8 to 20% more efficiently than did nonpretreated ones. Regardless of the strains, the hierarchy of mating efficiency was always treated YEP > untreated YEP > treated YNB > untreated YNB. The cell fusion kinetics in α [*rho*$^+$] × **a** [*rho*$^-$] crosses were most affected by pretreatment (Δ30 to 35 min), whereas [*rho*$^+$] × [*rho*$^+$] crosses were least affected (Δ15 min). These results are discussed in relation to the functions known for **a** and α pheromones. The successful pretreatment regimes were used to design new rapid and efficient techniques for mating YNB-grown log-phase cells in either YNB or YEP liquid media. These techniques can be used for small- or large-scale mating, and because of their inherent media flexibility, they have many potential applications to future studies on mating-specific or intrazygotic phenomena.

The mating reaction between haploid *Saccharomyces cerevisiae* cells of opposite mating type progresses through three principal phases. The earliest is characterized by G_1 cell cycle arrest and sexual agglutination, or adhesion, of **a** and α cells. This is followed by cell fusion (plasmogamy or zygote formation) and finally by haploid nuclear fusion (karyogamy) to form a diploid nucleus. The first mating stage is mediated by **a** and α mating pheromones produced constitutively during cell growth. These pheromones specifically affect cells of the opposite mating type (see reference 16 for a review). Cells exposed to the proper pheromone become transiently arrested in the G_1 phase of the cell cycle as unbudded cells (5, 10). Concomitantly with these effects, inducible **a** and α cells become agglutination competent (see reference 30 for a review).

Although most laboratory strains can produce and respond to mating pheromone, the mere mixing of **a** and α cells does not ensure that efficient mating will result. If high mating efficiency is required for studying morphological, physiological, biochemical, or genetic events occurring during mating or zygote maturation or both, specific mating protocols must be followed. This requirement has led to the development of several efficient mating techniques (3, 4, 9, 13, 18, 22, 23), including the new methods described in this paper. These techniques differ in methodological variables such as (i) the cell growth stage used for mating, (ii) the pregrowth and mating media employed, (iii) the cell concentrations used, (iv) the cell manipulations required during mating, and (v) the conditions required for maintaining cell contact before cell fusion. For example, my own experiments on the molecular basis of mitochondrial inheritance during mating and zygote maturation required the use of a defined medium for pregrowth and mating. The existing techniques (22, 23) proved variable and often inefficient for matings between the respiratory-sufficient (grande [*rho*$^+$]) and respiratory-deficient (petite [*rho*$^-$]) strains used. This led me to search for alternative rapid and efficient mating techniques.

During technique development, I discovered that a brief pretreatment of **a** and α cells with parental culture supernatants could shorten the time required for cell fusion and improve mating efficiency. These effects are documented here. The mating protocols described are adaptable to either small- or large-scale zygote production. In

the simplest procedure, log-phase cells grown in defined medium are mixed and pretreated for 15 min before being suspended in either defined or complex medium for mating. If required, the zygotes formed can be separated from mating cell mixtures and used for further study in any medium. The methods will probably have wide applicability because their inherent media flexibility make them potentially useful for many studies on mating-specific events, mitotic and meiotic development of zygotes, and interactions between parental components brought together after cell fusion.

MATERIALS AND METHODS

Strains. The heterothallic haploid yeast strains used for these experiments are listed in Table 1. These respiratory-sufficient ([*rho*$^+$]) and respiratory-deficient ([*rho*$^-$ *rho*^{0}]) strains were used to develop and test the mating methods. Strains α-4, 7b, S1 and α-4[S1] (21), F13 (15), and *kar1-1* (7) have been described elsewhere.

Media. All premating cultures were grown in a defined liquid minimal medium (YNB) and mated in either liquid YNB or enriched medium (YEP) containing 2% dextrose (wt/vol) at pH 4.5 (21). Premating ([*rho*$^+$] and [*rho*$^-$]) YNB medium contained 1% dextrose (wt/vol). Standard dropout media were used for scoring parental nuclear and mitochondrial genotypes and diploid yields from matings (21). Medium for scoring inositol requirement contained 16.7 g of Vitamin Free Yeast Base (Difco Laboratories), 20 g of agar (Difco), 1 ml of vitamin mixture (0.2 mg of biotin, 40 mg of pantothenate, 40 mg of pyridoxine per 100 ml) per liter, and amino acid additions (21) as needed.

Treatment and mating procedures. Overnight YNB cultures of **a** and α parental strains, aerated at 200 rpm in a New Brunswick G25 shaker at 30°C, were used for mating in their mid to late logarithmic phase of growth when cell concentrations were 3×10^6 to 6×10^6 cells per ml or approximately 10 Klett units when read in a Klett-Summerson colorimeter fitted with a no. 66 red filter. Before mating, a sample of each culture was sonicated for 3 to 5 s with a Heat Systems microprobe set at a 50-W output, and the sonicated samples were counted with a hemacytometer. For mating purposes, the cell count per milliliter was calculated from the unbudded cell number plus 1.5 times the budded cell number. This correction factor helped maintain an approximate one-to-one ratio between parental cells when the parental cultures contained very different percentages of unbudded and budded cells, due to their very different growth rates. Individual matings were routinely done in a final volume of 50 ml of mating medium. To simplify cell manipulations, cell pretreatment, centrifugation, and suspension in mating medium were all done in the same sterile 50-ml round-bottom polycarbonate centrifuge tube (Nalge/Sybron Corp.).

To begin the matings, samples of parental cell cultures, each containing a total of 1.25×10^8 cells according to the previous calculations, were transferred into centrifuge tubes. **a** and α samples to be untreated or to be pretreated separately were placed in different tubes and spun at top speed for 4 to 5 min in an International table top clinical centrifuge (model CL). If pretreated, the culture medium was decanted, and the **a** or α cells were suspended in 15 ml of cell-free supernatant obtained from the other parental culture. Untreated cell samples were resuspended in their own supernatants. Samples pretreated as **a**, α cell mixtures were added to the same centrifuge tube and incubated in the resulting supernatant mixture. After a 10-min incubation period at room temperature, all cell samples were centrifuged for 4 to 5 min, and the supernatants were decanted. Cells were then resuspended in about 5 ml of mating medium (YEP or YNB, preheated to 30°C) and sonicated for 3 to 5 s. Then more of the same mating medium was added to each sample. **a**, α cell mixtures were resuspended in a final volume of 50 ml and added to a 500-ml Erlenmeyer flask. The separate **a** and α cell samples were each suspended in a final volume of 25 ml of medium and then poured into the same 500-ml Erlenmeyer flask. The time at which

TABLE 1. Strains used for study

Strain	Origin	Nuclear markers	Mitochondrial respiratory sufficiency
α-4	Laboratory stock 5032B	α *trp4-48 leu1-12*	[*rho*$^+$]
7b	Laboratory stock	**a** *trp1-1*	[*rho*$^+$]
S1	P. Slonimski, IL8-8C/E41/S1	α *his1 trp1*	[*rho*$^-$]
α-4[S1]	New laboratory strain with the α-4 nuclear genome and the S1 mitochondrial genome[a]	α *trp4-48 leu1-12*	[*rho*$^-$]
kar1-1	G. Fink, 6430-20A	**a** *his3-5 ino1 ino4 kar1-1*	[*rho*$^+$]
7b [*rho*^{0}]	Isolate of 7b after treatment with ethidium bromide	**a** *trp1-1*	[*rho*^{0}]
F13	P. Slonimski, IL8-8C/F13	α *his1 trp1*	[*rho*$^-$]
α-4[F13]	New laboratory strain containing the α-4 nuclear genome and the F13 mitochondrial genome[a]	α *trp4-48 leu1-12*	[*rho*$^-$]

[a] These strains were produced via cytoduction (E. Sena, Curr. Genet., in press).

samples were added to the flasks was considered zero time for all mating protocols. All matings were initially aerated at 100 to 120 rpm in a New Brunswick G25 shaker at 30°C. After about 1.5 h of incubation, mating mixtures were centrifuged as before to pellet the cells. Pelleting ensures cell contact and allows a quick check for a and α sexual agglutination capability. An agglutination-competent mixture produces a flattened cell pellet. When the tube is gently shaken, the pellet flakes into large cell clumps which are dispersed in clear medium. A cell mixture which is poorly agglutinated produces a more typical soft and pasty yeast cell pellet. After gentle shaking of the tube, only a few large cell clumps are visible, and the medium appears opaque due to the resuspension of non-agglutinated cells. Poorly agglutinated mating mixes have always produced poor zygote yields with these mating methods.

After the agglutination check, each suspension of agglutinated cells was poured back into its flask and reincubated at 30°C at 120 to 200 rpm until mating was completed or until the cell mixtures were harvested for zygote isolation. All 50-ml matings were done in duplicate.

Zygote isolation. Samples for zygote isolation were harvested as soon as zygote production was maximal for a particular mating protocol. Zygote purification was most efficient if done before the unbudded, unmated cells in the mating culture reinitiated mitotic budding. Both small- and large-scale zygote isolation have been described (22, 23). Large-scale zygote isolation for these experiments was done in an IEC Z-15 zonal rotor.

Mating efficiency. To assess the efficacy of cell pretreatment and the efficiency of each mating, samples were taken during mating, sonicated briefly, and either counted immediately or fixed in 3.7% formaldehyde and then counted to determine their cell and zygote composition (22). Mating progress was followed by calculating the percentage of unbudded cells, zygotes, and mated cells. The percentage of unbudded cells was derived only from unmated cell counts. The percentage of zygotes was calculated by counting all cells and zygotes (either budded or unbudded) as one unit. The percentage of mated cells was calculated by counting unbudded and budded cells as one unit and unbudded and budded zygotes as two units. For example, a mating sample with 90 unbudded cells, 10 budded cells, and 50 zygotes would have 90% unbudded cells, 33% zygotes, and 50% mated cells.

Genetic analysis. The composition of mixtures of parental haploids and unmated haploids and zygotes was ascertained after dilution plating by conventional genetic testing procedures.

RESULTS

Established methods for mating cells in YNB minimal medium (22, 23) produced both inefficient and inconsistent zygote yields (0 to 15%) when the [*rho*$^-$] strains listed in Table 1 were crossed with [*rho*$^+$] strain 7b. These unexpected results led me to develop the YNB and YEP mating techniques described here. In the process, I discovered that treating premating cells with cell-free supernatants from cell cultures of opposite mating type could have a significant effect on the mating reaction. Various pretreatment schemes were tried. The results of two of these are given below.

Effect of pretreatment on mating. (i) Separate a and α treatment method. Supernatant treatment of a and α cells before mixing had two basic effects on the mating reaction (Fig. 1). Treated a and α cells initiated cell fusion earlier than did nontreated ones. The time required to produce one-half maximum mating cell yields for treated and untreated cells was shortened by about 35 min in both YNB and YEP media. In addition, mating efficiency was also improved by pretreatment. Treated YEP matings were the most efficient and produced a maximum of 48% mated cells at 3.5 h compared with a 4-h maximum of 40% for untreated cells in the same medium. Matings between treated cells in YNB produced a maximum of 24% mated cells after 4.5 h. The least efficient mating protocol (untreated cells mated in YNB) showed 18% mated cells at 5.5 h.

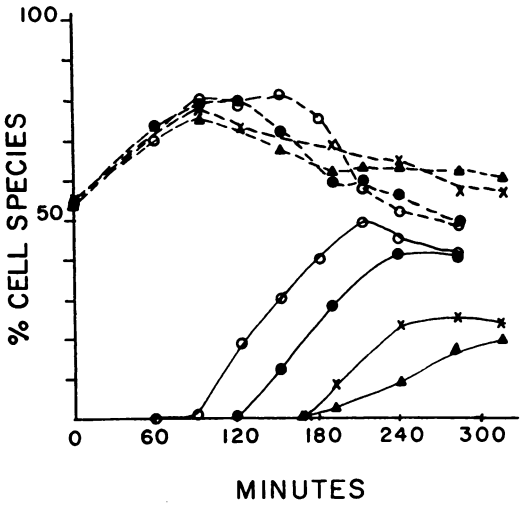

FIG. 1. Effect of separate pretreatment on the time course of [*rho*$^+$] × [*rho*$^-$] mating. Log-phase a 7b and α-4[S1] cell samples, containing equivalent cell numbers, were either individually treated for 10 min with cell-free α or a supernatants, respectively, or incubated for an equivalent nonpretreatment period in their own supernatants as described in the text. After incubation and a 4- to 5-min sample centrifugation, a and α cell pellets were each suspended in 25 ml of either YNB or YEP mating medium. (Total pretreatment time equals 15 min: 10 min for incubation and 5 min for centrifugation and supernatant removal.) Similarly treated a and α cells in identical media were then mixed to begin the mating. The percentages of mated (———) and unbudded cells (– – –) during mating are shown. All points were averaged from duplicate matings run in parallel. (○) Treated YEP, (●) untreated YEP, (×) treated YNB, and (▲) untreated YNB matings.

Since separately pretreated **a** and α cells do not have an opportunity to come into physical contact until after treatment, when the cells are mixed, the altered mating kinetics must result from cell exposure or "conditioning" (8) by the supernatants used for treatment.

Pretreatment does not seem to significantly affect the kinetics of cell arrest in an unbudded G_1 state. The gradual increase in the percentage of unbudded cells from 53% followed similar kinetics for all matings. YEP mating mixtures accumulated slightly more unbudded cells than YNB matings did (80 versus 74%). After 5 h, only 45% of unmated YEP cells remained unbudded compared with about 60% of YNB unmated cells. Treatment appeared to slow down the bud reinitiation of YEP unmated cells relative to the other matings.

(ii) Mixed a and α treatment method. Once the initial effects of pretreatment on the 7b and α-4[S1] mating were established (Fig. 1), a simplification of the treatment method was tried. Samples of cells containing the correct number of cells for a 50-ml mating were mixed immediately after removal from overnight cultures. This eliminated the first centrifugation and supernatant suspension steps of the separate treatment method. Therefore, **a** and α cell mixtures were treated in an essentially one-to-one mixture of **a** and α cell culture supernatants. This method was tried first on a YEP mating between 7b and α-4[S1] (Fig. 2). The percentage of both mating cells and unbudded cells followed the kinetics observed in the comparable matings in Fig. 1. Again, pretreated cells initiated cell fusion about 30 min earlier than untreated ones.

This simple pretreatment method was then tested on YEP and YNB matings between two separate pairs of [*rho*$^+$] strains (Fig. 3 and Table 2) and a [*rho*^{0}] and a [*rho*$^-$] (Table 2). When 7b and α-4 [*rho*$^+$] cells were mated after mixed pretreatment, the temporal hierarchy of mating in the four experiments was identical to that shown in Fig. 1. Treated cells mated in YEP produced a maximum of 48% mated cells within 3 h, and untreated YEP mated cells produced a maximum of 40% mated cells by 3.5 h. Treatment, as before, improved the YEP mating efficiency and accelerated the time course of cell fusion by about 15 min.

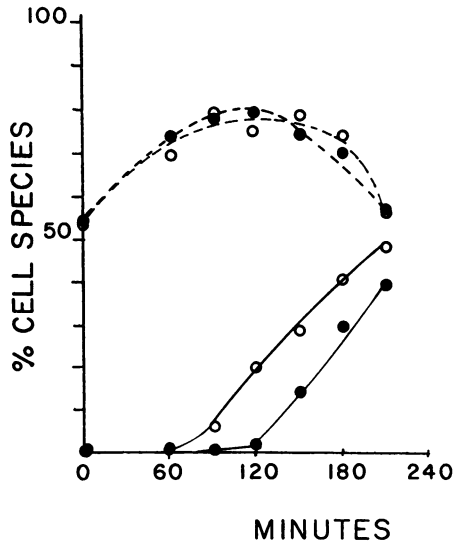

FIG. 2. Effect of parental cell culture mixing on the time course of [*rho*$^+$] × [*rho*$^-$] mating. Log-phase **a** 7b and α-4[S1] cell samples, containing equivalent cell numbers, were either mixed and incubated in their supernatant mixture or incubated separately, without mixing, for 10 min as described in the text. Incubation was followed by 4- to 5-min of centrifugation, after which the cell pellets of untreated **a** and α cells were suspended in 25 ml of YEP mating medium and then mixed together. The pretreated mixed cells were suspended directly into 50 ml of YEP. The percentages of mated (———) and unbudded cells (------) during mating are shown. All points were averaged from duplicate matings run in parallel. (○) Treated and (●) untreated matings.

This 15-min displacement is not simply a result of bringing **a** and α cells together 15 min earlier than the untreated ones, for separately pretreated α-4 and 7b cells also showed this 15-min difference (data not shown). Treatment had

TABLE 2. Effect of parental cell culture mixing on the kinetics of mating[a]

Strains mated	Premix treatment	Mating medium	Maximum % mated cells	Time of half-maximum mating (min)
kar1-1 × α-4	Yes	YEP	39	150
	No	YEP	32	165
	Yes	YNB	17	205
	No	YNB	11	225
7b × α-4[S1]	Yes	YEP	45	140
	No	YEP	36	160
	Yes	YNB	19	220
	No	YNB	12	240

[a] Cell cultures were grown, treated, mated, and analyzed as described in the legend to Fig. 3.

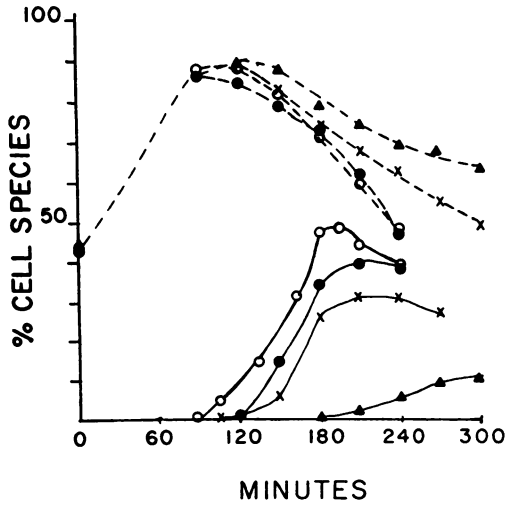

FIG. 3. Effect of parental cell culture mixing on the kinetics of [*rho*$^+$] × [*rho*$^+$] mating. Log-phase YNB-grown 7b [*rho*$^+$] and α-4 [*rho*$^+$] parental cells, either mixed and pretreated or untreated as described in the legend to Fig. 2, were mated in 50 ml of mating medium. All data points were averged from parallel duplicate experiments. The percentages of mated (———) and unbudded cells (------) during mating are shown. (○) Treated YEP, (●) untreated YEP, (×) treated YNB, and (▲) untreated YNB matings.

a great effect on YNB mating. Treated cells produced a maximum of 32% mated cells after 3.5 h, whereas untreated cells mated slowly, and only 10% were mated by 5 h. All mating mixtures contained about 90% unbudded cells by 1.5 h, almost 10% higher than in the other matings (Fig. 1 and 2). Again, as in Fig. 1, unmated cells in YEP reinitiated budding more rapidly than did those in YNB.

The results of the final two matings to test the effect of the premix treatment on mating in YEP and YNB media are summarized in Table 2. Again, for both crosses, pretreatment affected both mating kinetics and mating efficiency. Since every mating pair tested responded to pretreatment, regardless of whether the parents were [*rho*$^+$], [*rho*$^-$], or [*rho*^{0}], I conclude that the time course of mating can be accelerated and the efficiency of mating can be improved by a brief 15-min parental cell pretreatment with log-phase parental culture supernatants. Mating medium also affected zygote yields and mating kinetics. YEP matings were always more efficient and more rapid than YNB ones, a probable result of differences in cell metabolism due to growth in enriched versus minimal medium.

Resultant mating methods. The mixed pretreatment method was adopted because of its simplicity, efficiency, and reproducibility. Samples of a and α log-phase YNB-grown cells, each with 1.25×10^8 cells, are mixed and incubated at room temperature for 10 min. Then the cells are pelleted for 4 to 5 min, and the old medium is replaced with 50 ml of either YEP or YNB mating medium. A brief sonication is often used after the addition of 5 ml of mating medium to ensure the separation of mothers from their mature daughters. This step is optional if the strains do not contain groups of three or more cells. Matings are aerated at 100 to 120 rpm. When the fraction of unbudded cells is maximal at about 1.5 h, the cells are briefly pelleted by centrifugation, gently resuspended in their supernatants, and incubated at 120 to 200 rpm until mating completion. Although the procedure routinely incorporates this cell pelleting step, strains exhibiting excellent sexual agglutination show clumps of cells dispersed in clear medium even without this step, and pelleting does not improve their mating efficiency. If large agglutinated cell clumps are not apparent at 1.5 h, this pelleting step improves mating efficiency.

Yeast strains that grow as sonication-resistant clumps of cells because mothers do not drop mature daughter buds show poor mating with both the YEP and YNB protocols. Two such strains, S1 and F13, when sonicated and then crossed to 7b, produced only 10 to 20% mated cells by either mating method. However, unbudded S1 cells crossed with log-phase 7b cells mated as efficiently as the α-4[S1] × 7b crosses (Fig. 1 and 2). Therefore, clumpiness must interfere with the formation of 1:1 a-to-α cell contacts via agglutination, thereby decreasing mating efficiency.

Matings can be scaled down if fewer zygotes are required for a particular experiment. For large-scale matings, we simply scale up the procedure, running 10 to 24 500-ml flasks simultaneously. This method of scale-up has been reproducible and is as efficient as the small-scale matings. When 24 flasks are processed, there is about a 15-min time differential between cell resuspension in the initial flasks and the final samples. Processing parental cell mixtures in larger volumes and in larger flasks has not given either as high or as reproducible zygote yields as running separate flasks.

Zygote isolation. If a mating sample for a specific experiment must contain more than the 30 to 48% of mated cells normally produced, the zygotes can be separated from unmated cells on sorbitol gradients (23). Zygote-containing fractions are then pooled to produce samples with higher percentages of mating cells. In this manner, a 50-ml mating mixture with 47% mated cells (from the treated YEP mating in Fig. 2) was fractionated to produce a sample with 73% mated cells after just one small sorbitol gradient (Fig. 4). Large zygote preparations can be ob-

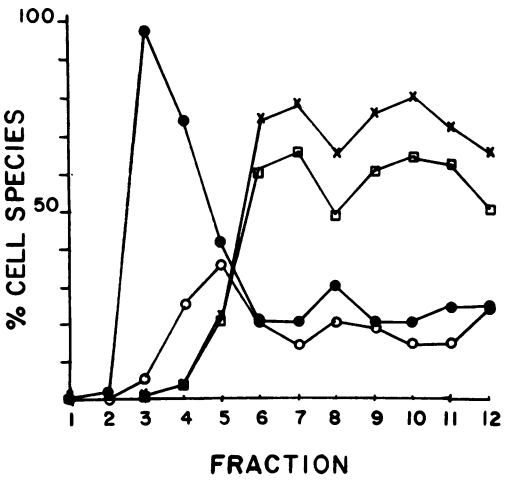

FIG. 4. Small-scale gradient fractionation of a treated YEP mating culture. After 3.5 h, the cells and zygotes from the treated YEP mating mixture (Fig. 2) were fractionated on a linear 50-ml 8 to 35% sorbitol gradient in an HB-4 Sorvall rotor for 4 min at 1000 rpm. Fractions containing about 4 ml were collected from the top to the bottom of the tube with a pipette. The relative percentages of unbudded (●), budded (○), and mated (×) cells and zygotes (□) per fraction are shown. The last fraction (12) also includes the cells and zygotes from the tube bottom. The decrease in percentages of mated cells and zygotes in fraction 9 was caused by a partial separation of budded zygotes from unbudded ones.

tained from large-scale matings. Several large YNB matings of 7b and α-4[F13] with the premix treatment technique yielded mating preparations containing 86 to 91% mated cells after zonal rotor fractionation. The viability of zygotes from all matings and gradients was greater than 95% as determined by methylene blue staining and standard viability plating assays.

DISCUSSION

The exposure of unbudded log-phase cells to supernatants from cultures of the opposite mating type has been reported to improve mating efficiency (23; D. N. Radin, Ph.D. thesis, University of California, Berkeley, 1976). Lee and co-workers (14) were unable to detect differences between YEP matings of log-phase cells pretreated for 2 h with cell-free culture supernatants and untreated ones. In light of the data presented here, their "untreated" a and α cultures, which had been mixed before centrifugation and old medium removal, were probably pretreated enough so that no differences were apparent.

Although no direct proof that improved mating kinetics and efficiency result from purified pheromone treatment is presented here, it is reasonable to assume that pretreatment is effective because the culture supernatants contain a and α pheromones (2, 11, 20, 26). Other supernatant components, however, cannot be ruled out. Recent studies on agglutination induction have shown that sexual agglutination can be induced in responsive cells after a brief 5- to 30-min treatment with the appropriate purified pheromone or cell-free culture supernatant (8, 30). In addition, cells are sensitive to agglutination induction over a wide range of pheromone concentrations (8) and are capable of responding to hormone throughout the cell cycle (29, 30).

After complete agglutination induction (1, 8, 24, 29, 30), mating efficiency in a one-to-one a-to-α cell mixture should be limited by the number of arrested, unbudded, G_1 cells available for mating (5, 10, 12, 17, 19, 28). Since agglutination-competent cells retain their competence even after initially escaping from G_1 arrest (unpublished data), the effectiveness of pretreatment for matings which agglutinate well must be related to the efficiency of cell cycle G_1 arrest.

In contrast to agglutination induction, only cells before the cell cycle "start" can arrest rapidly after pretreatment. Cells beyond "start" must complete their cell cycles before G_1 arrest (5, 12, 28). The pheromone concentration seems to control both the number of arrested cells and their mean G_1 arrest recovery times (6, 16, 25). Concentrations effective at prolonging cell escape times beyond those required for cell fusion (Fig. 1) would be expected to increase mating efficiency. Mating would cease as cells within agglutinated cell clumps escaped from cell cycle arrest. There would be a limit to pheromone-induced mating improvement since high pheromone concentrations would produce cell shape changes and decrease mating efficiency (23, 27). The pheromone levels in the matings studied here were sufficient to yield 80 to 90% unbudded cells after 1.5 h of mating and were below the levels required to produce cell shape changes.

As discussed above, all matings between pretreated cells showed both an earlier cell fusion pattern and an increased zygote yield over those of nontreated matings. Since all matings except one (*kar1-1* × α-4 [Table 2]) were done with an isonuclear series of a or α parents with different combinations of mitochondrial genomes, the time variations observed could not result from nuclear genetic differences between strains. The smallest effect (15 min) occurred when both parents were [*rho*$^+$] (Fig. 3), the next smallest (20 min) occurred between [*rho*$^-$] and [*rho*^{0}] (Table 2), and the greatest effect (30 to 35 min) occurred in matings of [*rho*$^+$] and [*rho*$^-$] (Fig. 1 and 2). In fact, if differences between the mean generation times of parental pairs are ordered from least to greatest, the hierarchy is identical

to that observed during mating. This order suggests that pretreatment may lead to increased mating efficiency because it overcomes some metabolic constraint (perhaps related to pheromone production or response or both) which normally controls the temporal pattern of cell fusion events in untreated cell mixtures. Further studies are required to ascertain the molecular basis of the pretreatment effects seen here.

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