Conjugation in Chemostat Cultures of *Schizosaccharomyces pombe*

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In chemostat cultures, *Schizosaccharomyces pombe* (NCYC 132 and derivative strains) formed conjugants in glucose-limited but not in ammonia-limited synthetic medium. Conjugation occurred at a low growth rate (doubling time, t_d approx. 11 h), low aeration rate (0.1 fermenter vol. air min⁻¹) and 32 °C, but not at an increased growth rate (t_d approx. 4.5 h) or at lower temperatures (20–25 °C). Conjugation of *S. pombe* 968^{h90} in chemostat culture was greater under glucose-limitation than ammonia-limitation. Batch cultures of *S. pombe* 968^{h90} in synthetic medium produced conjugants when the glucose supply was limited, but when glucose was in excess the amount of nitrogen influenced the degree of conjugation. Conjugation of strain 968^{h90} was inhibited in batch cultures in synthetic medium under conditions such that a large quantity of glucose was metabolized, and in cultures grown in synthetic medium containing added ethanol (0.5%). Inhibition of conjugation also occurred in batch cultures of *S. pombe* NCYC 132 and 968^{h90} in malt extract broth containing added ethanol.

INTRODUCTION

Cultivation of *Schizosaccharomyces pombe* in chemostat culture in this laboratory was undertaken primarily to allow a study of cell division under controlled environmental conditions. It became evident, however, that other facets of growth of the fission yeast could be studied by this technique when conjugants and asci were observed in chemostat cultures growing in synthetic medium under carbon (glucose)-limitation. This observation was of considerable interest in view of reports in the literature concerning the effects of carbon and nitrogen on conjugation and sporulation of *S. pombe*. Fowell (1969) showed that conjugation in batch cultures of *S. pombe* in a yeast extract medium reaches a maximum with an initial glucose concentration of 1% but is completely inhibited with an initial concentration of 5%. *Schizosaccharomyces pombe* NCYC 132 does not conjugate when grown in yeast nitrogen base containing 1% maltose (Calleja & Johnson, 1971). It has also been shown that the addition of glucose to malt extract broth results in a severe inhibition of the process (Calleja, 1974; Calleja *et al.*, 1980). Such results strongly suggest that carbon-limitation in batch cultures results in conjugation and that excess carbon is inhibitory. On the other hand, Egel (1971) found that *S. pombe* 968^{h90} conjugates and sporulates in a synthetic medium when the nitrogen source (aspartate) is completely consumed, a result which was interpreted as suggesting that nitrogen-limitation is required for conjugation. This conclusion was supported by results obtained with *S. japonicum* (Tsuboi *et al.*, 1978).

Thus, conjugation in different strains and in different species of *Schizosaccharomyces* apparently occurs under different conditions with respect to nutrient limitation. Our observation that *S. pombe* NCYC 132 conjugated and sporulated under defined conditions in chemostat cultures suggested further experiments designed to explore the conditions for conjugation of *S. pombe* in both chemostat and batch cultures. The results obtained are presented below.

METHODS

The following strains of *Schizosaccharomyces pombe* NCYC 132 were used: S2-2, a twice recloned strain; 360-2, a high frequency conjugating strain; ade-2, an adenine-requiring strain; and Yt, a colonial morphology mutant.

Other strains of *S. pombe* were: 968^{h90} (for chemostat culture; a strain isolated from an iodine-positive colony); 975h⁺; 972h⁻; and leu3-241, a leucine-requiring strain of 972h⁻. Strains were maintained by periodic transfer in 2% (w/v) malt extract broth (Oxoid) incubated at 32 °C.

Chemostat cultures were grown in glucose-limited (2.0 mg glucose ml⁻¹, 5.0 mg NH₄Cl ml⁻¹) or ammonia-limited (20 mg glucose ml⁻¹, 0.1 mg NH₄Cl ml⁻¹) EMM2 (Mitchison, 1970). Low levels of glucose and ammonia in the media were chosen so that they were undetectable in supernatants of nutrient-limited chemostat cultures. In the experiments reported, chemostats were sampled periodically, and the culture supernatant was tested for glucose or ammonia, or the culture was tested for stimulation of growth as a batch culture after addition of the nutrient in question (Johnson *et al.*, 1978). The growth rate was controlled by the rate of addition of nutrient-limited medium, i.e. $D = F/V$, in which D is the dilution rate (h⁻¹), F is the flow rate (ml h⁻¹), and V is the culture volume (ml). (The time required for the concentration of organisms to double, $t_d = \log_2/D$.) At low values of F , the rate at which medium was delivered to chemostats tended to vary slightly during the course of extended experiments and, as a consequence, calculated dilution rates and mass doubling times varied. The variation was deemed to be acceptably low, however, since comparisons were made between the effects of relatively low growth rates (approx. 10 h) and those of moderately rapid growth rates (approx. 5 h). Therefore, the values of D at low growth rates are given as the range that applied to the data.

Chemostats were custom-made (Pegasus Industrial Specialists, Agincourt, Ontario, Canada) and consisted of jacketed fermenter jars with an overflow side-arm located so as to maintain a volume of 500 ml, as described previously (Johnson *et al.*, 1978). Agitation and aeration of cultures was accomplished by use of a Vibro Mixer (Chemopac, Hoboken, N.J., U.S.A.) with a hollow stainless-steel shaft through which sterile air was passed (Martin, 1980). The temperature of cultures was controlled by the circulation of water through the fermenter jackets. Cultures were maintained at designated pH values by means of a pH control system (pH 40, New Brunswick Scientific Co.) through the addition of sterile 1 M-KOH. Batch cultures in 125 ml Erlenmeyer flasks were incubated at 32 °C either unshaken or shaken at 150 rev. min⁻¹ (Psychrotherm, New Brunswick Scientific Co.).

Growth of cultures was measured as the turbidity at 660 nm using a Coleman Jr spectrophotometer. Using phase contrast microscopy, a total of 1000 cells, scored as conjugants, asci or vegetative cells, were counted in pronase-treated samples, either of cells suspended in buffer at pH 7.0 or of cultures adjusted to pH 7.0. The percentage of conjugants plus asci was scored as:

$$\frac{[\text{Conjugate pairs} + (\text{asci} \times 2)]}{[\text{Conjugate pairs} + (\text{asci} \times 2)] + \text{vegetative cells}} \times 100$$

A Petroff-Hauser chamber was used for total cell counts.

Glucose was measured by the Nelson-Somogyi method (Neish, 1952) and ammonia by the Berthelot method (Weatherburn, 1967).

RESULTS

In preliminary experiments, glucose-limited chemostat cultures of *S. pombe* 360-2 grown at low dilution rates were found to contain conjugants and asci. For example, 24 h after the start of dilution ($D = 0.06$ h⁻¹, $t_d = 11.5$ h) a glucose-limited culture at pH 5.4–5.6 contained about 6% conjugants plus asci, a level that was maintained for 192 h of continuous growth. Under similar conditions, ammonia-limited chemostat cultures did not contain conjugants or asci. When the medium supply to a glucose-limited chemostat culture of *S. pombe* 360-2 was changed to one with ammonia as the limiting nutrient, the percentage of conjugants plus asci dropped to zero after 52 h and remained at zero for a further 72 h of ammonia-limited growth (Fig. 1*a*). On changing the medium supply back to one with glucose as the limiting nutrient, the percentage of conjugants plus asci rose as the culture again became glucose-limited (Fig. 1*a*). These results with chemostat cultures agree with the conclusions from studies using batch cultures (Calleja, 1974; Fowell, 1969), namely, that conjugation in *S. pombe* occurs under conditions of limited glucose supply.

The frequency of conjugation in chemostat cultures of *S. pombe* 360-2 could be controlled by changing the imposed growth rate (Fig. 1*b*). Conjugation which had occurred at a low growth rate (approx. 10%, at $t_d = 11$ h) disappeared when the growth rate was increased ($D = 0.15$ h⁻¹, $t_d = 4.6$ h). Nevertheless, when the dilution rate was returned to the lower value, conjugation again occurred (Fig. 1*b*). [It should be noted that conjugants were not seen in several other

Fig. 1. Conjugation in chemostat cultures of *S. pombe* in EMM (see Methods) with growth limited by glucose (2.0 mg glucose ml⁻¹) or ammonia (0.1 mg NH₄Cl ml⁻¹). Unless otherwise stated, conditions were: carbon-limitation, 32 °C, pH 5.4–5.6, $D = 0.06$ – 0.07 h⁻¹, 0.1 fermenter vol. air min⁻¹.

(a) Effect of nutrient limitation (carbon-limitation, 64 h; nitrogen-limitation, 124 h; carbon-limitation, 93 h) on strain 360-2. (b) Effect of growth rate ($D = 0.06$ h⁻¹, 48 h; $D = 0.15$ h⁻¹, 24 h; $D = 0.06$ h⁻¹, 47 h) on strain 360-2. (c) Effect of temperature (32 °C, 14 d; 26 °C, 5 d; 20 °C, 4 d; 32 °C, 3 d; 20 °C, 5 d) and of aeration rate (A→B, 12→14 d, 0.8 vol. air min⁻¹) on strain S2-2.

chemostat cultures growing at high D (0.15–0.25 h⁻¹) for periods of 3–6 d.] These results are consistent with the observation that conjugation of strain 360-2 in batch cultures occurs after the end of the exponential phase, i.e. when the growth rate has slowed down (Calleja & Johnson, 1971).

Schizosaccharomyces pombe also formed conjugants in a chemostat culture limited by maltose rather than glucose. After 5 d at $D = 0.07$ h⁻¹, conjugants plus asci accounted for about 3.0% of the total cell population whereas 40 h after a change from $D = 0.07$ h⁻¹ to $D = 0.17$ h⁻¹, conjugants plus asci accounted for only about 0.4% of the cell population (data not shown).

Glycerol was another carbon source examined. A batch culture of *S. pombe* 360-2 in a chemostat vessel contained 1.4% conjugants after growth for 40 h at 32 °C in EMM with 0.2% (v/v) glycerol. Dilution was begun at $D = 0.03$ h⁻¹ ($t_d = 23$ h) and after 24, 48 and 120 h the percentages of conjugants were 4.9, 9.5 and 0.5%, respectively. Although this culture did not reach a steady state, the results showed that conjugation occurred with glycerol as the carbon source. In other experiments, batch cultures of *S. pombe* 360-2 grown in shaken flasks at 32 °C in EMM with 0.2% glycerol contained up to 10% conjugants and asci. Also, conjugants were

produced by *S. pombe* strains S2-2, *ade-2* and Yt (but not by strains 972^{h-}, 975^{h+} or leu 3-241) when they were grown in shaken flasks at 32 °C in EMM with 4% glycerol. (With xylulose as the carbon source, *S. pombe* NCYC 132 grows poorly but produces conjugants; H. Schneider, personal communication.)

Attempts to demonstrate conjugation of *S. pombe* 360-2 in batch cultures in EMM with glucose have not been successful. Growth in shaken flasks in EMM with a low initial concentration of glucose (0.05 mg ml⁻¹) and a low nitrogen concentration (0.05 mg NH₄Cl ml⁻¹), with or without the periodic (daily) addition of glucose (0.05 mg ml⁻¹), did not result in conjugation. Thus, in experiments to date, with glucose as the carbon source in EMM, *S. pombe* 360-2 produced conjugants only in carbon-limited chemostat cultures.

Examination of chemostat cultures of *S. pombe* S2-2 showed that the temperature and the rate of aeration during growth affect conjugation (Fig. 1c). At 32 °C and a low rate of aeration (0.1 fermenter vol. air min⁻¹) conjugation occurred, but when the rate of aeration was increased (from 0.1 to 0.8 vol. air min⁻¹) the percentage of conjugants decreased rapidly (A, Fig. 1c). No change occurred in this percentage when the aeration rate was returned to 0.1 vol. air min⁻¹ and the temperature was simultaneously reduced from 32 to 26 °C (B, Fig. 1c). Lowering the temperature further to 20 °C had no effect. However, with the culture at low aeration rate, raising the temperature back to 32 °C resulted in a rapid increase in the percentage of conjugants. This percentage dropped rapidly again when the temperature was lowered to 20 °C. Thus, at a fixed growth rate, conjugation at 32 °C appeared to be inhibited at a high aeration rate, while at a low aeration rate, conjugation was inhibited at low temperature (20–26 °C). Since oxygen solubility increases at low temperatures, these two results may be related.

The results for chemostat cultures of *S. pombe* 360-2 in defined medium were in good agreement with those obtained with this strain in batch cultures in non-defined medium, particularly with respect to the effect of carbon availability (Calleja, 1974). On the other hand, results with *S. pombe* 968^{h90} in batch culture in defined medium (Egel, 1971) indicated that nitrogen-limitation rather than carbon-limitation was the important factor in conjugation. Therefore, to compare results with those for *S. pombe* 360-2, the effects of carbon- and nitrogen-limitation on conjugation and sporulation of *S. pombe* strain 968^{h90} were studied in chemostat cultures.

The organism was grown as a batch culture in a chemostat vessel for 23 h in EMM (20 mg glucose ml⁻¹) and then as a glucose-limited chemostat culture (EMM, 2 mg glucose ml⁻¹) at $D = 0.05 \text{ h}^{-1}$ for 72 h. This culture had a conjugation percentage of 50%. It was divided into two 250 ml portions which were each made up to 500 ml in chemostat vessels, one with glucose-limited EMM, the other with ammonia-limited EMM. Dilution with the respective media was then recommenced at $D = 0.05\text{--}0.06 \text{ h}^{-1}$. The results (Fig. 2) show that although conjugation in *S. pombe* 968^{h90} was reduced markedly under both limiting conditions, glucose-limitation was more favourable for conjugation than was nitrogen-limitation.

These results agree with those obtained in chemostat cultures of *S. pombe* 360-2 but not with those for batch cultures of strain 968^{h90} (Egel, 1971). To obtain more information, we examined batch cultures of *S. pombe* 968^{h90} in defined medium containing various concentrations of glucose and ammonia, and various other nitrogen sources (Table 1). The results can be summarized as follows: (a) at a low level of glucose (0.1%), increasing the level of ammonia so that it was in excess did not affect conjugation; (b) at intermediate levels of glucose (0.5, 1.0%), increasing the level of ammonia so that it was in excess at 0.5% but not at 1% glucose resulted in inhibition of conjugation at both glucose levels; (c) at a low level of nitrogen, markedly increasing the glucose level from 1.0 to 2.5% resulted in inhibition of conjugation; (d) in the presence of excess nitrogen, inhibition of conjugation depended upon the level of glucose supplied; (e) inorganic and organic nitrogen behaved similarly with respect to conjugation; and (f) the final pH attained by the culture did not greatly influence the degree of conjugation.

The results, in addition to indicating that conjugation was inhibited by an excess of glucose when the level of nitrogen was low and growth-limiting, suggest that at high, non-growth-limiting levels of nitrogen, excess glucose is metabolized to a product or products which may be involved in the control of conjugation in *S. pombe* 968^{h90}. Two possible metabolic products, acetate and ethanol, were considered. The organism was grown with shaking in EMM (50 mg

Fig. 2. Conjugation in chemostat cultures of *S. pombe* 968^{h90} in EMM with growth limited by glucose or ammonia (see Fig. 1). Conditions were: 32 °C, pH 5.4–5.6, $D = 0.05\text{--}0.06\text{ h}^{-1}$, 0.2 fermenter vol. air min^{-1} . The organism was grown as a batch culture in EMM for 32 h, then as a glucose-limited chemostat culture for 72 h at $D = 0.05\text{--}0.06\text{ h}^{-1}$. At this time (time 0) the culture was divided into two 250 ml portions which were each made up to 500 ml in separate chemostat vessels, one with glucose-limited EMM (○) and the other with ammonia-limited EMM (●).

Table 1. Effect of glucose and nitrogen sources on conjugation of *S. pombe* 968^{h90}

Cultures in EMM (see Methods), with the carbon and nitrogen sources indicated, were shaken at 32 °C for 65 h [NH_4Cl as nitrogen source], 89 h [$(\text{NH}_4)_2\text{CO}_3$] or 24 h [aspartate].

Glucose		Final pH	Nitrogen source		Conjugants + asci (%)
Initial concn (mg ml ⁻¹)	Residual concn (mg ml ⁻¹)		Initial concn (μg ml ⁻¹)	Residual concn (μg ml ⁻¹)	
<i>NH₄Cl</i>					
1.0	0.05	—	100	50	47
10.0	0.6	—	100	0	45
25.0	5.5	—	100	0	14
1.0	0.06	5.3	50	0	55
1.0	0.06	5.3	100	44	52
5.0	0.25	5.2	100	0	59
5.0	0.25	5.0	200	11	1.1
10.0	1.2	3.9	100	0	49
10.0	0.64	3.6	200	0	2.9
<i>(NH₄)₂CO₃</i>					
5.0	0.3	5.5	100	0	60
5.0	0.3	6.0	200	3.6	0.7
<i>Aspartate</i>					
5.0*	(0)†	5.0	200	—	44 (39)‡
10.0	(+2)	4.7	200	—	16 (37)
20.0	(+5)	4.7	200	—	11 (13)
1.0	(0)	—	2500§	—	33
5.0	(0)	—	2500	—	10

* At 5.0 $\text{mg glucose ml}^{-1}$, asparagine (100 $\mu\text{g ml}^{-1}$), glutamate (200 $\mu\text{g ml}^{-1}$), and glutamine (100 $\mu\text{g ml}^{-1}$) gave the same result as aspartate.

† Qualitative estimation of residual glucose.

‡ Subcultures, incubated for 90 h at 32 °C.

§ Glutamate (2500 $\mu\text{g ml}^{-1}$) gave the same result.

glucose ml^{-1} , 10 $\text{mg NH}_4\text{Cl ml}^{-1}$) to which either sodium acetate (1, 5 or 10 mg ml^{-1}) or ethanol (0.25, 0.5, 2.5 or 5.0 mg ml^{-1}) was added. All cultures grew to about the same extent (72 h, $A_{660} = 0.42\text{--}0.52$) but conjugation was affected only in the culture with ethanol added at 5.0 mg ml^{-1} ; this culture contained 23% conjugants plus asci whereas the others all contained between

Fig. 3. Effect of ethanol on growth and conjugation in batch cultures of *S. pombe* 968^{h90}. Cultures in EMM (see Methods) with 5 mg glucose ml⁻¹ (○) and EMM with 5 mg glucose ml⁻¹ and 10 mg ethanol ml⁻¹ (●) were incubated at 32 °C with shaking at 150 rev. min⁻¹.

50 and 60% conjugants plus asci. That ethanol interfered with conjugation without affecting growth is clear from the data obtained from batch cultures grown with and without added ethanol (Fig. 3). In other experiments, addition of ethanol (20 mg ml⁻¹) to malt extract broth prior to inoculation resulted in a reduction in the percentage of conjugants in cultures of *S. pombe* strain 968^{h90} from 38 to 7.5%, and in strain 360-2 from 11 to 3.75%.

DISCUSSION

The occurrence and control of conjugation in species of *Schizosaccharomyces* has been attributed to either the exhaustion or absence of a nitrogen source for *S. pombe* 968^{h90} (Egel, 1971) and *S. japonicum* (Tsuboi *et al.*, 1978), or the supply of a limited quantity of glucose for other strains of *S. pombe* (Fowell, 1969; Calleja *et al.*, 1980). The latter authors found that exogenous glucose inhibited conjugation in strain 360-2, a result which appeared contradictory to those of Egel (1971).

The results presented in this study clearly indicate that nitrogen-limitation is not required for conjugation in batch or continuous cultures of *S. pombe*. Limiting the availability of the carbon source, on the other hand, allowed conjugation to occur, a finding that does not agree with the statement of Crandall *et al.* (1977), namely, that starvation for sugar does not induce conjugation. In batch cultures, when glucose is essentially used up conjugation may or may not occur, depending upon the amount of nitrogen present. Excess glucose in batch cultures of strain 360-2 causes inhibition of conjugation (Calleja *et al.*, 1980) and it is now clear that this is also true for strain 968^{h90}. It is also true that inhibition of conjugation occurs in media containing high concentrations of glucose together with sufficient nitrogen source to attain almost complete utilization of the glucose. The effect under these conditions could be caused by the additional ethanol produced, since it is now evident that ethanol is inhibitory to conjugation. Thus, repression of conjugation appears to be related to glucose metabolism as suggested by Crandall *et al.* (1977).

The chemostat results with *S. pombe* S2-2 indicate that under glucose-limiting growth conditions, conjugation is favoured by a slow growth rate, a low aeration rate, and a growth temperature of 32 °C. An increase in the growth rate or aeration rate or a decrease in the temperature resulted in an inhibition of conjugation. It is possible that at the increased dilution rate required

to increase the growth rate, sufficient increase in the glucose concentration in the chemostat culture occurred to account for inhibition of conjugation. Similarly, the inhibition of conjugation occurring at the low temperature could have been a result of an increase in the amount of oxygen dissolved in the medium at this temperature. However, these possibilities have not been investigated.

Chemostat results show that conjugation occurs when *S. pombe* is forced to grow at a slow rate under glucose-limiting conditions. The results indicate that the sequence of rapid growth (e.g. exponential phase) followed by slow growth (e.g. stationary phase) is not necessary for the initiation of flocculation and conjugation. Conditions favouring conjugation are achieved in chemostat cultures by imposing a slow growth rate under glucose-limiting conditions and in batch cultures by supplying glycerol as the carbon source. It seems clear that under slow growth conditions some cells continue to multiply vegetatively whereas others become committed, conjugate and eventually sporulate. The nature of controls modulating the switch from mitotic cell cycles to meiotic cell cycles remains unclear.

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