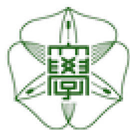

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Synchronous sexual cell division in L_{200}-cells of *Closterium acerosum*

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Synchronous sexual cell division and conjugation were achieved in L_{200}-cells on an agar plate containing nitrogen-deficient mating medium in a 16 hr light (3,000 lux)—8 hr dark regime at 20°C. L_{200}-cells were prepared by the following procedure: the cells were collected in an exponential phase of vegetative growth, washed with the mating medium, and recultivated in a nitrogen-limited (tenfold diluted) growth medium under illumination at 200 lux in the 16 hr light—8 hr dark regime at 20°C for 2 weeks. These treated cells were designated as "L_{200}-cells". The L_{200}-cells divided once in the dark period between 3 and 4 days of the mating culture and about 95% of the divided cells conjugated from 4 to 7 days of the culture.

It is very difficult to distinguish sexual cell division from vegetative cell division in some unicellular algae. It has been reported that in *Chlamydomonas* sexual differentiation is induced when vegetative cells are incubated in a nitrogen-free mating medium (KATES and JONES, 1964; SAGER and GRANICK, 1954) and that the number of daughter cells is different between the vegetative and sexual cell divisions as well as enzymatic activities (KATES and JONES, 1964) and degradation rate of ribosomes (SIERSMA and CHIANG, 1971). These reports suggest that a specific cell division, designated as "sexual cell division", is strictly required for sexual differentiation in *Chlamydomonas* and some algae belonging to Zygnematales. ICHIMURA (1971) has proposed that the sexual cell division is one of the processes of sexual differentiation in *Closterium strigosum*. In a previous paper (UENO and SASAKI, 1978), we reported that the sexual cell division was strictly needed for induction of the mating process in a homothallic strain of *Closterium acerosum* as well as in a heterothallic strain of *Closterium strigosum*. For studies of the biochemical properties of induction and procession of the mating process of *Closterium*, synchronization of cell division and zygote formation must be achieved.

In this paper, conditions for synchronous sexual cell division and zygote formation and also effects of nitrogen compounds on the mating process in *Closterium acerosum* are reported.

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Materials and Methods

Closterium acerosum, a homothallic alga, was purely cultivated in a standard light regime, 16 hr light—8 hr dark, at 20°C. Other culture conditions were the same as in a previous paper (UENO and SASAKI, 1978).

Synchronous sexual cell division and zygote formation were achieved with L_{200}-cells. L_{200}-cells were prepared by the following procedure: cells grown in the growth medium at 2,000 lux were collected in the exponential phase, and cultivated at 200 lux in a nitrogen-limited (tenfold diluted) growth medium for 2 weeks at 20°C. These treated cells were designated as "L_{200}-cells". The cells were washed with mating medium, inoculated onto an agar plate containing the mating medium, and cultivated under 3,000 lux at 20°C for conjugation. The conjugation rate (% of zygote formation) of L_{200}-cells was compared with that of the cells in the exponential and stationary phases of vegetative growth.

The number of cell divisions was obtained by periodical recording the increase in the number of cells in the mating culture. The conjugation rate (%) was obtained by counting zygotes per total cells.

Growth rate of the cells in 450 m*l* of the culture medium was measured spectrophotometrically at 550 nm with a Hitachi spectrophotometer Type 139. At 0.3 of OD_{550}, cell number was about 2,000 cells/m*l* (equivalent to about 0.18 mg dry weight).

Results and Discussion

Growth curve under standard culture conditions: As shown in Fig. 1, maximum growth was achieved on the 23 rd day of the culture, and the zygote was not formed for 23 days. The zygote was gradually formed at slight rate after 23 days under illumination at light intensity higher than 3,000 lux but not under illumination at lower than 2,000 lux.

Conjugation ability of vegetative cells in different growth phases: The cells were collected in the different growth phases indicated in Fig. 1, in initial, middle, and late exponential phases (A, B, and C), and late stationary phase (D). Identical amounts of cells in the different phases were washed separately with the mating medium and inoculated onto each the agar-mating medium. Total cell number and zygotes were periodically counted during 8 days of the mating culture. As shown in Fig. 2, the older cells in the later phase were more rapidly conjugated than the younger cells in the earlier phase, although maximum conjugation rate was the same; about 90 to 95%. These results suggest that older cells have great ability to initiate sexual cell division and

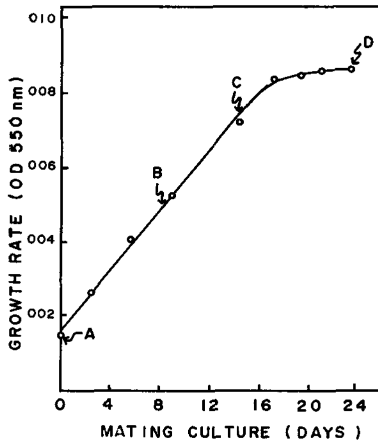

Fig. 1. Growth curve of L_{200}-cells in a standard growth medium. L_{200}-cells were cultured in a standard growth culture medium at a light intensity of 2,000 lux in a 16 hr light—8 hr dark regime at 20°C under aeration. Growth phases are indicated as A, B, C, and D. A, initial exponential phase; B, middle exponential phase; C, late exponential phase; D, late stationary phase. Cells in each of A, B, C, and D phases were used in the experiments in Fig. 2.

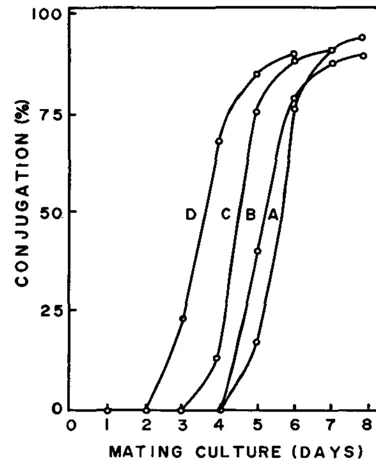

Fig. 2. Zygote formation from the cells harvested in different growth phases in the mating culture. Growth phases of inoculum cells were: A, initial exponential phase; B, middle exponetial phase; C, late exponential phase; D, late stationary phase.

that younger cells have a certain inhibitor of induction of the sexual process.

Effects of nitrogen source on mating of exponential phase cells: Cells in the exponential phase of vegetative growth were collected, washed with the mating medium, and inoculated onto the agar-mating medium with or without a KNO_3 (0.1 g/l) and $(NH_4)_2HPO_4$ (0.01 g/l) mixture. As shown in Fig. 3, in the absence of the nitrogen source, cell number gradually increased for 6 days to a maximum level and zygote formation rapidly increased from 5 days to 8 days to a maximum, whereas in the presence of the nitrogen source, the former linearly increased for 10 days and the latter gradually increased at a low rate from 6 days. These results suggest that the amounts of nitrogen compound utilized for cell division and cell growth are larger in the exponential phase cells than in L_{200}-cells (the former twice and the latter once divide), and that nitrogen-rich conditions delay and inhibit sexual differentiation.

Conjugation ability of L_{200}-cells and effects of nitrogen source on mating: L_{200}-cells were inoculated onto the agar-mating medium with or without a KNO_3

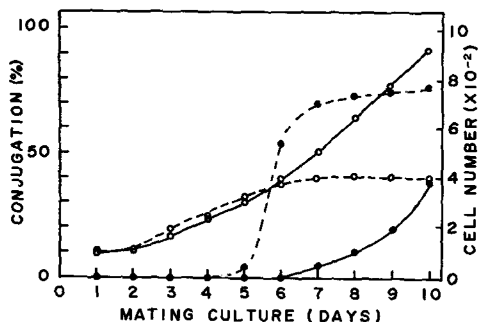

Fig. 3. Effects of nitrogen source on the relationship between cell division and conjugation of exponential phase cells in the mating culture. Cells in the exponential phase were cultured on the agar plate containing mating medium under conditions of nitrogen source existence (——) and deficiency (---). Nitrogen source was a KNO_3-$(NH_4)_2HPO_4$ mixture at the same concentration as in Pringshein's culture medium. ○, cell number; ●, conjugation rate (% of zygote).

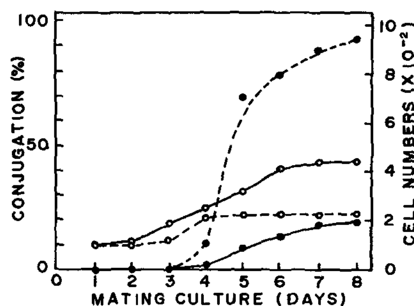

Fig. 4. Effects of nitrogen source on the relationship between cell division and conjugation of L_{200}-cells in the mating culture. L_{200}-cells were cultured on the agar plate containing mating medium under conditions of nitrogen source existence (——) and deficiency (---). Nitrogen source was a KNO_3-$(NH_4)_2HPO_4$ mixture at the same concentration as in Pringsheim's culture medium. ○, cell number; ●, conjugation rate (% of zygote).

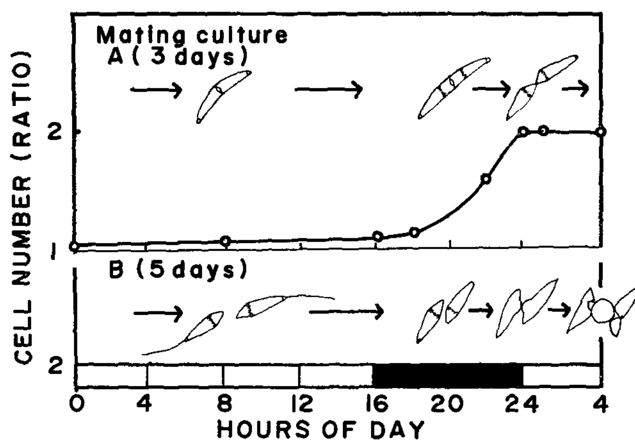

Fig. 5. Relationship between light-dark cycle and sexual cell division or conjugation in the cource of mating culture. L_{200}-cells were cultured on the agar plate under the same conditions as described in "Materials and Methods". Cell number was counted and morphological changes were observed in the courses of sexual cell division and conjugation. A: sexual cell division (in the dark period between 3 and 4 days of the mating culture). B: zygote formation (pairing occurred in the dark period and conjugating in the light period after 5 days of the mating culture).

and $(NH_4)_2HPO_4$ (0.1 g and 0.01 g/*l*) mixture. As shown in Fig. 4, in the absence of the nitrogen source, cell number synchronously increased 2 times, between 3 and 4 days of the culture, and zygote formation rapidly increased from 4 to 8 days, whereas in the presence of the nitrogen source, the former gradually increased 4 times for 6 days and the latter gradually increased from 5 to 7 days and ceased at a low level. These results suggest thta in the nitrogen-free mating medium L_{200}-cells divide once between 3 and 4 days of the culture and the zygote is formed at a great rate, and that nitrogen compounds inhibit synchronous sexual cell division and conjugation.

In Fig. 5, morphological changes during synchronous sexual cell division and zygote formation were illustrated. After the cell division in the dark period between 3 and 4 days, slightly elongated cells were paired in the dark period, and conjugated in the light period of the next day.

Effects of different kinds of nitrogen compounds on cell multiplication and zygote formation: Different kinds of nitrogen compounds were added in the mating medium, and L_{200}-cells were inoculated. As summarized in Table 1, 10^{-3} M KNO_3, 10^{-3} M $NaNO_3$, 10^{-4} M NH_4NO_3, and 10^{-4} M urea enhanced cell multiplication, delayed initiation of mating, and yielded many zygotes, while 10^{-3} M NH_4NO_3 and 10^{-4} M and 10^{-3} M NH_4Cl strongly inhibited cell multiplication and completely inhibited zygote formation. $NaNO_2$ and urea at 10^{-3} M partially inhibited the zygote formation. These results suggest that starvation of nitrogen source, at the limited level lower than 10^{-4}M, except NH_4Cl, may be strict conditions for induction of sexual differen-

TABLE 1 Effects of nitrogen sources on zygote formation and cell multiplication

Nitrogen source	Conc. (M)	Zygote (%)	Number of cell divisions
KNO_3	10^{-3}	77.5	5.7
$NaNO_3$	10^{-3}	67.6	6.2
$NaNO_2$	10^{-3}	44.6	3.9
NH_4Cl	10^{-3}	0	1.6
"	10^{-4}	0	1.0
NH_4NO_3	10^{-3}	0	1.5
"	10^{-4}	86.5	5.0
Urea	10^{-3}	43.3	—
"	10^{-4}	70.0	4.2
None		78.8	2.0

Cell number increased in mating culture for 8 days is indicatad as number of cell divisions and zygote number as % of total cells.

tiation, and that metabolic pathways for vegetative growth turn off and those for sexual reproduction turn on under the nitrogen-limited conditions.

In several bacteria and fungi, nitrogen defficiency induced the sporulation process (REINESS *et al.*, 1975; RHAESE and GROSCURTH, 1976; LÉ JOHN *et al.*, 1975; MCNAUGHTON *et al.*, 1975; TRAVERS, 1976): in spore-forming microorganisms, nitrogen-deficiency induced accumulation of uncharged tRNA and repressed RNA and protein synthesis; under nitrogen-deficient conditions large amounts of nucleoside polyphosphates, such as ppGpp, pppGppp, or ppApp, accumulated and repressed synthesis of rRNA and vegetative growth-specific mRNA and enhanced synthesis of sporulation-specific RNA. These results reported suggest that in *Closterium acerusum* sexual differentiation-specific substance is synthesized under nitrogen-deficient conditions, and a part of the substance is related to ppGpp or ppApp which is produced from GTP or ATP. In addition to nitrogen-deficiency, light is needed for induction and procession of sexual differentiation (UENO and SASAKI, 1978). Now it is probable that a part of the role of light for induction of sexual differentiation may be related to the synthesis of mating-specific substance from a photosynthetic product such as ATP; ppApp from ATP and ppGpp or pppGppp from GTP which may be induced from ATP. Induction of sexual cell division by nitrogen deficiency under light illumination is a very interesting problem from the viewpoint of environmental control of transition from vegetative growth to sexual reproduction in this alga. Relationships between a regulatory substance synthesized in the nitrogen-deficient mating medium, such as ppGpp, pppGppp, or ppApp, and induction of sexual differentiation should be investigated in detail using homothallic and heterothallic strains of *Closterium* under synchronous growth and mating conditions.

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