Journ. Fac. Sci., Hokkaido Univ. Ser. V (Botany), 13 (3, 4): 261-266 (1984).

The effects of several metabolic inhibitors on sexual reproduction in heterothallic strains of *Closterium*

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Chymostatin, 50 μg/m*l*, phenylmethylsulfonyl fluoride, 10^{-3} M, cycloheximide, 1 μg/m*l*, actinomycin D, 10^{-5} M, and cordycepin, 3.6×10^{-4} M, produced maked inhibition of the mating process in heterothallic strains of *Closterium peracerosum-strigosum-littorale*, KAS-4-29 (mating type minus) and KAS-4-30 (mating type plus). When added at the beginning of the mating culture in liquid medium, each of the drugs inhibited the conjugation completely and addition after 48 hr of culturing inhibited about 20% of the conjugation. Phenylmethylsulfonyl fluoride, 1 mM, added after 24 hr of culturing stimulated sexually induced disruption of mating type plus cells.

Heterothallic strains of *Closterium peracerosum-strigosum-littorale*,KAS-4-29 (mating type minus) and KAS-4-30 (mating type plus), were conjugated synchronously in a liquid mating medium in a 16 hr light and 8 hr dark cycle: sexual cell division occurred within 24 hr of culturing, about 20% achieved first conjugation in the second light period, from 26 to 32 hr, and about 60% reached the second conjugation in the third light period, from 50 to 56 hr (KATO *et al.* 1981, 1983). During the period, about 80% of the cells conjugated and a few mating type plus cells disrupted. Since this was induced by interaction between the sexually opposite cells, the sum of the zygotes and the disrupted cells was considered the "sexually activated cells". In a previous paper (KATO *et al.* 1981), we reported a male-specific substance which induces the release of protoplasts in paired cells and in unpaired mating type plus cells. This protoplast-releasing substance (PRS) is assumed to produce the cell wall lytic enzyme in mating type plus cells which has a role in the formation of conjugation papilla and the release and fusion of gametic protoplasts in paired cells and in the release and disruption of protoplasts of unpaired mating type plus cells.

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

We are attempting to clarify the mechanisms of cell-cell recognition and interaction in the sexual reproduction process of *Closterium*.

In this paper, we describe the effects of metabolic inhibitors on sexual cell division and conjugation in heterothallic strains of *Closterium*.

Materials and Methods

Algal materials and culture conditions

Closterium peracerosum-strigosum-littorale, KAS-4-29 (mating type minus, mt^-) and KAS-4-30 (mating type plus, mt^+) were used. Culture conditions for vegetative growth and sexual reproduction in liquid media were as descrived in a previous paper (KATO *et al.* 1981). Light conditions for mating was a cycle of 16 hr light (25°C, 10,000 to 11,000 lux) and 8 hr dark (20°C).

Effects of inhibitors on mating

Chymostatin, 1 to 50 μg/m*l*, and phenylmethylsulfonyl fluoride (PMSF), 10^{-4} to 10^{-3} M, were used as protease inhibitors, since the sexual processes may depend on protease-products originated from endogenous protein in inoculum cells in nitrogen-deficient mating media (JONES *et al.* 1968, STARR *et al.* 1980). Inhibitors of RNA synthesis, actinomycin D, 10^{-5} to 10^{-3} M, and cordycepin, 3.6×10^{-5} to 5.6×10^{-3} M, and inhibitor of cytoplasmic protein synthesis, cycloheximide, 1 to 100 μg/m*l*, were added to the mating culture at the beginning or after various culturing times. Total cells and sexually activated cells were measured at various culturing times, and % of inhibition was calculated.

Chemicals

Actinomycin D, chymostatin, cordycepin, cycloheximide, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co.

Results and Discussion

Effects of inhibitors added at the beginning of culturing

After addition of the inhibitors, cell numbers were counted at 24 hr of culturing (when cell division was finished) and sexually activated cells, zygotes and protoplast-disrupted mt^+-cells were measured at 72 hr (when conjugation was finished). Chymostatin at a concentration of 50 μg/m*l* inhibited conjugation strongly and cell division weakly, but not at concentrations below 10 μg/m*l* (Table 1). PMSF at concentrations above 10^{-3} M inhibited conjugation completely and cell division partially (Table 1). These results indicate

that intracellular protease is involved in cell division for formation of gametangial cells, sexual activation of gametes, and zygote formation in nitrogen-deficient mating medium.

Actinomycin *D* at 10^{-5} M and cordycepin at 3.6×10^{-4} M inhibited zygote formation completely and cell division partially (Table 1). These results suggest that mating-specific RNA synthesis is involved in whole processes in

Table 1. Effects of several metabolic inhibitors on the mating process of heterothallic *Closterium*

Inhibitor Added	Conc.	Cell multiplication		Sexual activity	
		Times	Inhibition[a] (%)	Frequency[b]	Inhibition[a] (%)
1. Chymostatin	0	2.11		79	
	1 μg/ml	2.28	0	81	0
	10 μg/ml	2.16	0	79	0
	50 μg/ml	1.80	28	26	67
2. PMSF	0	2.31		78	
	1×10^{-4} M	2.01	23	63	20
	5×10^{-4} M	1.89	32	45	42
	1×10^{-3} M	1.77	44	0	100
	5×10^{-3} M	1.24	82	0	100
3. Actinomycin D	0	2.04		72	
	1×10^{-5} M	1.97	7	0	100
	1×10^{-4} M	1.72	31	0	100
	1×10^{-3} M	1.08	93	0	100
4. Cordycepin	0	2.11		75	
	3.6×10^{-5} M	1.86	23	64	15
	3.6×10^{-4} M	1.96	17	0	100
	5.6×10^{-3} M	1.88	20	0	100
5. Cycloheximide	0	1.99		72	
	1 μg/ml	1.95	4	4	95-100
	10 μg/ml	1.34	65	0	100
	100 μg/ml	1.24	76	0	100

Inhibitors were added at beginning of the mating culture (0 hr), total cell number was counted at 24 hr and the sexually activated cell number (zygotes and sexually disrupted mt^+-cells) were counted at 72 hr of culturing. Inoculum cells were about 8×10^4/ml in Experiments 1, 2, and 5, about 9×10^4/ml in Exp. 4, and about 10^5/ml in Exp. 3. [a] Control, without inhibitors. [b] Sexually activated cell number (number of zygotes $\times2$ plus sexually disrupted mt^+-cells), % of total cell number.

sexual reproduction at different levels in different periods.

Cycloheximide at 10 μg/ml inhibited zygote formation completely and about 40% of cell divisions (Table 1), suggesting a requirement for RNA and protein synthesis in mating which is depending on the nuclear genes.

All of the drugs used here inhibited zygote formation more strongly than cell division.

Effects of inhibitors added at various culturing times

All inhibitors added at 0 and 12 hr of culturing stopped conjugation completely, except that chymostatin added at 12 hr and 24 hr inhibited about 70% of conjugations; about 60% at 36 hr; 15 to 32% at 48 hr; 4 to 12% at 60 hr; and the drugs added at 72 hr caused no inhibition (Table 2). At 12 hr of culturing, about a half of inoculum cells divided and PRS was synthesized; at 24 hr, cell division was finished and PRS was forming, but conjugation did not appear until 26 to 32 hr; at 36 hr, first conjugation was finished and the PRS for second conjugation was forming; at 48 hr, PRS was forming but conjugation did not occur until 50 to 56 hr; at 60 hr, second conjugation was finished and there was almost no formation of PRS for the third conjugation (sometimes detectable); at 72 hr, conjugation was finished and occasionally a few zygotes (third conjugation) were formed until 80 hr. These data suggest that the drugs inhibited a number of steps of the sexual reproduction: sexual cell division, activation of gametangial cells to active gametes, PRS formation, protoplast-release and fusion, etc. Inhibitor additions at beginning and at 12 hr of culturing may inhibit the whole sexual process; additions after 24 hr inhibited the induction of pro-

Table 2. Effects of metabolic inhibitors added at various times of the mating culture on the sexual activity of heterothallic *Closterium*

Inhibitor Added	Conc.	Inhibition (%) of sexual activity[a]						
		Addition at mating times (hr)						
		0	12	24	36	48	60	72
Chymostatin	50 μg/ml	68	73	73	63	38	10	0
PMSF	1×10^{-3} M	100	100	88	60	15	4	0
Actinomycin D	1×10^{-5} M	100	98	86	65	20	8	0
Cordycepin	3.6×10^{-4} M	100	100	93	80	32	10	0
Cycloheximide	1 μg/ml	100	100	85	80	20	12	0

Each of inhibitors was added at various times of the mating process, and sexually activated cells (zygotes and disrupted mt^+-cells) were counted at 72 hr of culturing.
[a] Control, without inhibitors.

toplast formation by PRS and conjugation; additions at 36 hr inhibited the PRS formation for the induction of the second conjugation; additions at 48 hr, the induction of protoplast release by PRS; additions at 60 hr inhibited the PRS formation for induction of the third conjugation (below 10%, when it appeared).

Effects of PMSF on release of gametic protoplasts

When PMSF at 1 mM was added at 24 hr of culturing, further cell division stopped completely, while conjugation continued with a very slow rate (Fig. 1). In liquid medium, sexually activated cells consisted of many zygotes and a few protoplast-released and disrupted mt^+-cells (KATO *et al.*

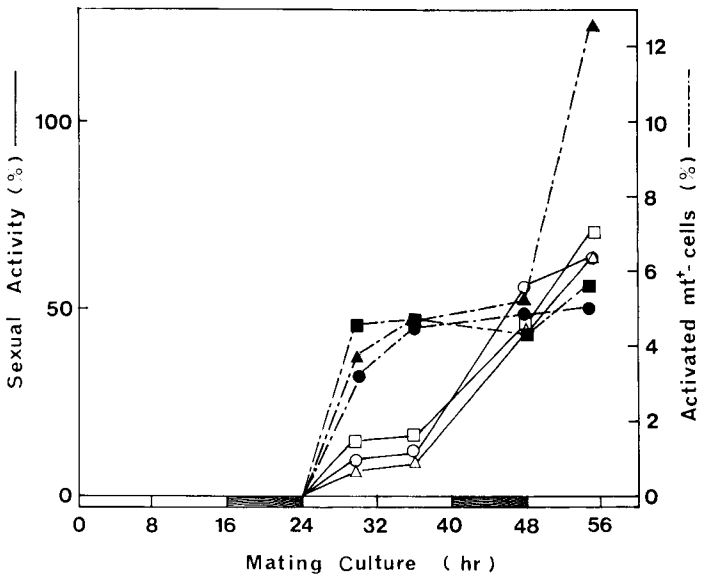

Fig. 1. Effects of PMSF on conjugation and release and disruption of gametic protoplasts during mating culture. PMSF was added at 7.4×10^{-4} M from the beginning and at 10^{-3} M after 24 hr of culturing. Numbers of sexually activated cells (zygotes and sexually induced disrupted mt^+-cells) were measured at various times of culturing, and sexual activity was expressed as the percent of sexually activated cells in the total cells.

——, sexual activity (%); — — —, activated mt^+-cells (% of the sexually disrupted mt^+-cells in the total cells sexually activated). □■, without PMSF; ○●, addition of 7.4×10^{-4} M PMSF at the beginning of culturing; △▲, addition of 10^{-3} M PMSF after 24 hr of culturing. □, light period; ■, dark period.

1981, 1983). The ratio of disrupted mt^+-cells to the total number of sexually activated cells increased by incubation with PMSF : One mM PMSF added at 24 hr of culturing increased numbers of disrupted mt^+-cells. Release and disruption of protoplasts in unparied mt^+-cells was induced by the action of PRS together with conjugation papilla formation and release and fusion of protoplasts in paired cells. These data suggest that PMSF enhances cell wall lysis, in other words, it stimulates the formation of mating-specific cell wall lytic enzyme in mt^+-cells, and inhibits the action of endogenous protease. PRS seemed exactly labile : conjugation stopped after 8 hr in the second and third light periods and was inhibited by prolonged light (KATO *et al.* 1983). The lability of PRS may be due to degradation by intracellular protease. Since PMSF inhibits protease action, inactivation of PRS by intracellular protease may be protected with the drug, therefore the function of PRS is more active in the presence of the drug than in the absence.

Isolation and characterization of PRS and the cell wall lytic enzyme which has a role in mating process, and studies on the control mechanism of genes involved in sexual reproduction are in progress.

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