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Differences in physiological properties and sexual substances between heterothallic strains of *Closterium*

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Sexual substances involved in interaction between sexually opposite strains of *Closterium peracerosum-strigosum-littorale*, KAS-4-29 (mating type minus) and KAS-4-30 (mating type plus) were investigated with a "agar-agarose culture system". In this system, sexual substances interact through agar and agarose layers without cell-cell contact. When cultured in a mating medium with this system, mating type plus cells were sexually activated and disrupted by male-specific sexual substance, a protoplast-releasing substance. The activation was induced by mating type minus cells which were cultured together with mating type plus cells. The substance secreted from mating type plus cells may have a role in induction of the male-specific substance.

In the "agar-agarose culture system", the addition of minute amounts of growth medium on the surface of agar-disk including mating type minus cells markedly stimulated release and disruption of protoplasts of mating type plus cells. Cell division was limited within 3 times during mating culture in liquid and agar media.

A male-specific sexual hormone, a "protoplast-releasing substance" (PRS) was found in *Closterium peracerosum-strigosum-littorale* (KATO *et al.* 1981). PRS is formed in mating type minus cells (strain KAS-4-29) and induces the formation of the mating-specific cell-wall lytic enzyme in mating type plus cells (strain KAS-4-30). By the action of the enzyme, gametic protoplasts are released and fused in distended conjugation papilla in paired cells in liquid mating medium, and protoplasts are released and disrupted in unpaired mating type plus cells in liquid and an agar-agarose culture system which prevents cell-cell contact. In this culture system, mating type plus cells were inoculated in agar-plate and mating type minus cells in agar-disk, and they were separated by an agarose layer acting as a barrier to prevent contact between the cells moving out from the agar layers. Since there are

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Abbreviation: PRS, protoplast-releasing substance.

different events during the mating process, different sexual substances may be formed and function in the different events. With the agar-agarose system, it is possible to detect PRS and the other sexual substances involved in the early phases of the mating process, and to know the interaction between sexually opposite cells. Two sexual substances from mating type minus and plus cells have been found : one is a stimulator of cell multiplication and the other is its inhibitor (OBOKATA and SASAKI 1983). Cell division in the mating culture is very important for sexual reproduction in the *Closterium* used here (KATO *et al.* 1981, 1983).

In this paper, we describe evidence for the formation of a female-specific substance involved in induction of PRS in mating type minus cells and intracellular and external conditions for sexual reproductions of 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 in liquid medium for vegetative growth and sexual reproduction were the same as described previously (KATO *et al.* 1981, 1983). An "agar-agarose barrier culture system" was designed for estimation of sexual substances (Fig. 1) : mt^+-cells in agar plate (0.6%, 1 mm thickness) and mt^--cells in agar disk (1%, 7 mm diameter, 3 mm height) were inoculated at a cell density of about 10^4/m*l* in a Petri dish (45 mm diameter). Agarose gel layer (1.2%, 0.8 mm thickness) was used as a barrier and placed under the mt^--disk and on the mt^+-plate. Using this system, contact between the cells that moved out from mt^+-plate and mt^--disk was prevented, while sexual substances secreted from the cells can contact with cells through the agarose layer. The standard light-dark condition was set as 16 hr light (25°C, 10,000 to 11,000 lux) and 8 hr dark (20°C). Total cells and sexually activated cells (zygotes and disrupted mt^+-cells in liquid medium, or disrupted mt^+-cells in agar-plate) were measured after various periods of culturing. In some cases, mt^--disk was removed from the system and mt^+-cells in agar plate were further cultured for 1 to 3 days. By the procedure, male-substance secreted from mt^--cell and diffused into agar and agarose layers may be detected. To investigate effects of female-product on mt^--cells, mt^--disk preincubated with or without mt^+-plate was recombined with fresh mt^+-disk and recultured (Fig. 2). By the recombination with fresh mt^+-plate, female-substance which induces the ability to form PRS in mt^--cells may be detected.

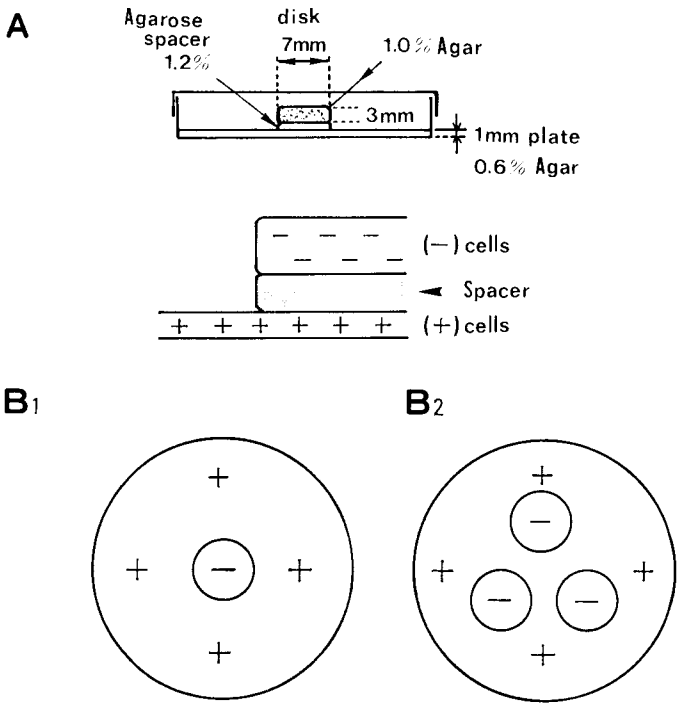

Fig. 1. The "agar-agarose culture system" for sexual activation of mt$^+$-cells by sexual substance. Mt$^+$-cells were dispersed in the agar-plate and mt$^-$-cells in the agar disk. The mt$^+$-plate and mt$^-$-disk were placed under and on the agarose gel which functions as a barrier ("spacer") to prevent contact between cells moving out from the agar plate and disk. A, "agar-agarose system". B, combinations: B1, "one disk on one plate" B2, "three disks on one plate".

In the "agar-agarose system", sexually activated mt$^+$-cells in the agar plate appeared in the area under the mt$^-$-disk. Here, about 900 cells are present and sexual activity is shown as numbers of sexually activated cells /100 or /900 cells. The values are the means of three measurements: three systems in three Petri dishes (Fig. 1, B1) or three systems in one dish (Fig. 1, B 2). In a case, cells were cultured in 0.6% agar-mating medium.

Results

Growth rates of mt$^+$- and mt$^-$-cells in liquid medium

Under the standard light-dark conditions, cell division occurred in the dark period during vegetative growth and in the light period during the

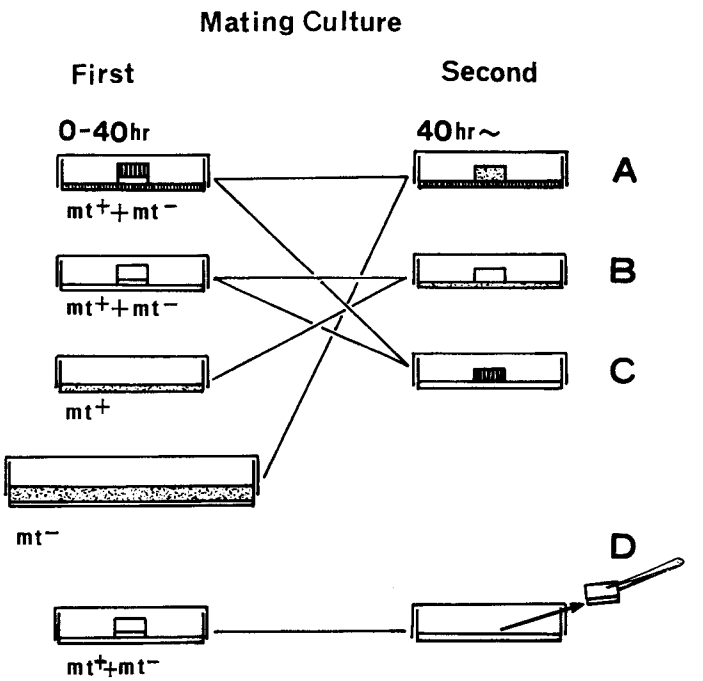

Fig. 2. Experimental procedure for the effects of mt$^+$-cells on PRS formation in the "agar-agarose culture system". First culture: preculture for 40 hr in combination of mt$^-$-disk and mt$^+$-plate (combined preculture) and mt$^-$-disk or mt$^+$-plate alone (independent preculture). Second culture, further culture in different combinations: A, mt$^-$-disk from "independent preculture" and mt$^+$-plate from "combined preculture"; B, mt$^-$-disk from "combined preculture" and mt$^+$-plate from "independent preculture"; C, mt$^-$-disk and mt$^+$-plate from "combined preculture"; D, mt$^+$-plate separated from mt$^-$-disk.

mating process. From the cell number (and optical density at 660 nm), the growth rate was a little higher in mt$^+$-cells than in mt$^-$-cells, as was the ratio of cells/OD_{660} (Fig. 3).

Effects of age of inoculum cells on sexual activation

In liquid mating medium, sexually activated cells, many zygotes and a few protoplast-released and disrupted mt$^+$-cells appeared in the second (about 20%), third (about 60%), and sometimes fourth (only few) light periods. Cells harvested from 12, 16, 19, 24, and 28 or 29 day of vegetative cultures were inoculated into fresh liquid mating medium and the rates of cell division and sexual activation were measured at 48, 72, and 96 hr of mating culture (Table 1).

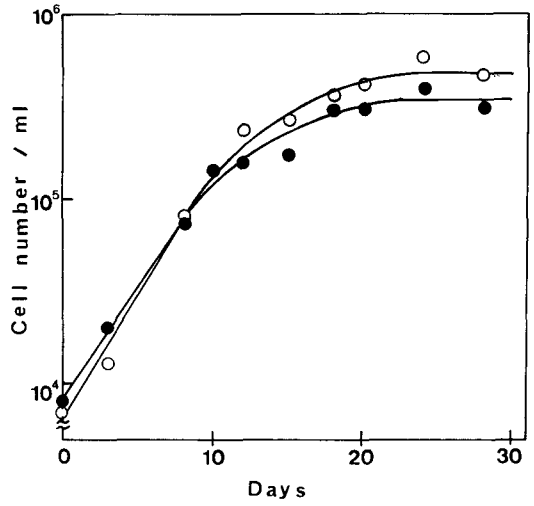

Fig. 3. Growth curves of mt$^+$- and mt$^-$-cells in liquid growth medium under the standard light-dark condition. About 1.2×10^3 cells/m*l* were inoculated into 50 m*l* medium in a 100 m*l*-Erlenmeyer flask. ○, mt$^+$-cells; ●, mt$^-$-cells.

Table 1. Effects of age of inoculum cells on sexual cell division and sexual activation in liquid and agar mating media

Mating medium	Inoculum cells		Cell division (times)			Sexual activity (%)		
			Mating culture (hr)			Mating culture (hr)		
	Age (days)	No./m*l* ($\times10^{-3}$)	48	72	96	48	72	96
Liquid	12	1	—	—	—	53	80	70
		2	3.0	3.0	3.7	65	80	87
		4	2.8	2.4	2.4	70	80	83
	16	1	—	—	—	65	78	85
		2	2.0	1.7	2.5	75	80	80
		4	2.2	2.0	2.5	70	82	86
	19	1	—	—	—	8	38	58
		2	2.3	2.6	2.0	67	70	90
		4	2.6	2.5	2.4	67	75	73
	24	1	—	—	—	5	10	20
		2	1.5	1.8	1.4	7	20	23
		4	1.1	1.1	1.1	0.	0.	15
	28	1	—	—	—	4	23	35[a]
		2	1.3	1.5	1.3	0.	8	17
		4	—	—	—	0.	4	6
Agar	13	0.9	2.4	2.3	2.5	5	7	5
		1.4	2.1	2.3	1.7	8	7	7
		2.8	1.6	1.6	1.7	5	7	7

[a] Sexual activity was measured with inoculum cells from 29 day-growth culture.
Sexual activity, % of zygotes and sexually disrupted mt$^+$-cells in total cells in a Petri dish filled 1 mm depth with the liquid or agar mating medium.

Using 2000 and 4000 cells/ml as inoculum cells from the 16 day culture in growth medium, sexually activated cells appeared with the highest frequency : the rate was about 70% at 48 hr, 80% at 72 hr, and 90% at 96 hr. Similar experiments were made with inoculum cells from 19, 24, and 28 or 29 day-growth cultures : using 19 day-cultured inoculum cells with 2000 and 4000/ml, the rate of sexually activated cells at 48 and 72 hr of mating culture were about 70% and at 96 hr 80% : using 1000/ml, the rates were very low at 48 (the lowest), 72 and 96 hr of culturing. Cell multiplication times were as follows : with 2000 cells/ml, cell divition times were about 3 in 12 day-inoculum cells, 2 in 16 and 19 day-inoculum cells, 1.5 in 24 day-inoculum cells, and 1.2 in 28 day-inoculum cells ; using 4000 cells/ml, cell division times were about 2.8 in 12 day-inoculum cells, 2 in 16 day-cells, 1.5 in 19 day-cells, and 1.1 to 1.2 with 24 day-cells (Table 1). A stationary phase was observed from 16 to 19 days. In liquid mating medium, mt^+- and mt^--cells divided once independently with the same rate, and the sexual activity was

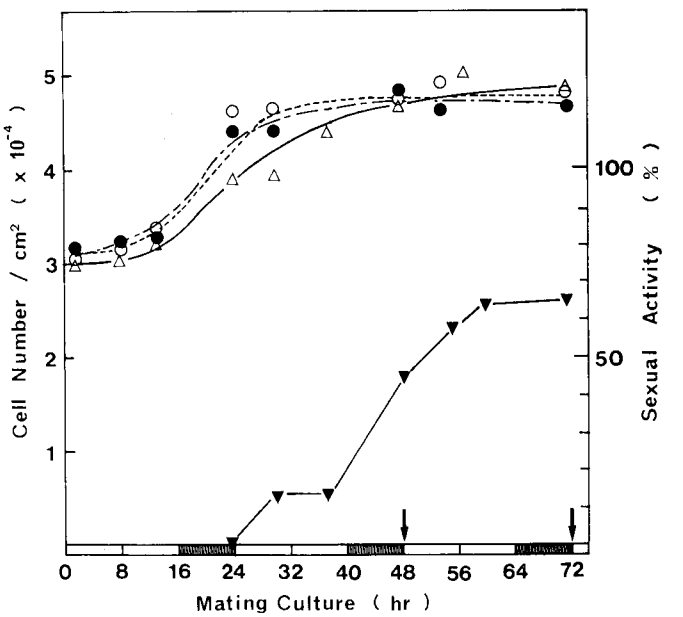

Fig. 4. Cell division and sexual activity of mt^+-cells, mt^--cells, or a mixture of both in liquid mating culture under the standard light-dark condition. Cell number/cm² of surface at bottom of Petri dish was measured, since cells were present on the surface. Cell number: ●, mt^+-cells ; ○, mt^-cells ; △, mixture. Sexual activity : ▼, mixture. At arrows, equal numbers of mt^+-and mt^--cells precultured independently were mixed (see Fig. 5). □, light period ; ▥, dark period.

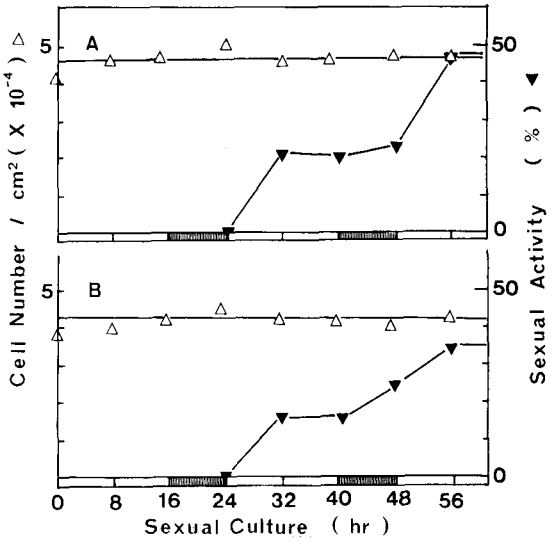

Fig. 5. Sexual activity of the cells precultured independently. A, mixing after 48 hr preculturing; B, mixing after 72 hr preculturing. Cells in Fig. 5 were used. △, cell number; ▼, sexual activity. □, light period; ▨, dark period.

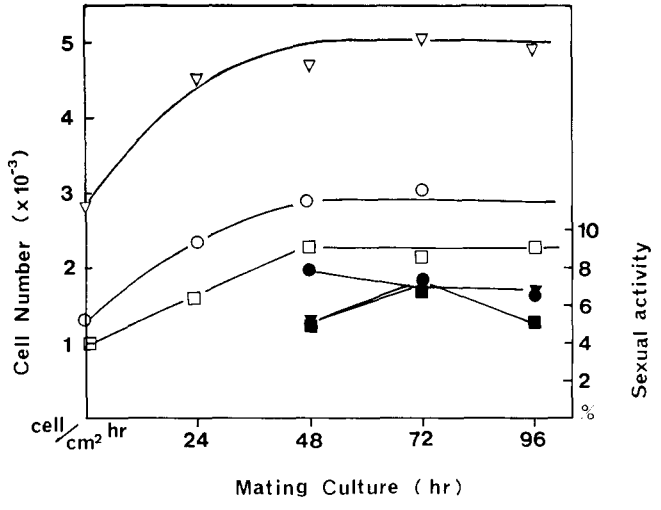

Fig. 6. Cell division and sexual activity in agar mating medium. Mixture of equal numbers of mt^+- and mt^--cells from 13 day-growth culture was inoculated in 0.6% agar medium, with different cell numbers/m*l*. Cell number: □, with about 900/m*l*; ○, with 1400/m*l*; ▽, with 2800/m*l*. Sexual activity: ■, with 900/m*l*; ●, with 1400/m*l*; ▼, with 2800/m*l*.

decreased by preculture in the absence of partner cells (Figs. 4 and 5).

Cell division occurred within 48 hr of culturing in 0.6% agar mating medium and sexually activated cells appeared after 48 hr of culturing (Fig. 6). Cell multiplication times were about 2.5 with about 900 cells/m*l*, 2 with 1400 cells/m*l*, and 1.5 with 2800 cells/m*l* (Table 1). In this case, 16 day-inoculum cells were used.

Sexual activation in "agar-agarose barrier culture system"

Mt^+-cells in agar plate (mt^+-plate) and mt^--cells in agar disk (mt^--disk) were inoculated, and they were placed under and on an agarose gel used as a barrier layer (Fig. 1). In this system, mt^+- and mt^--cells can not contact, while cell and sexual substance secreted from sexually opposite cells contact each other. Sexually activated mt^+-cells appeared in two steps under the standard light-dark condition : first, in the third light period and, second, in the fourth light period. They appeared from about 2 hr of light periods but not after 8 hr until the next light period (Fig. 7).

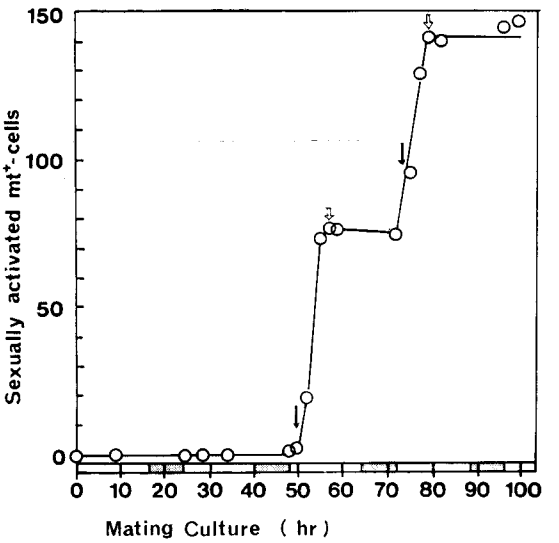

Fig. 7. Sexual activation of mt^+-cells in the "agar-agarose culture system" under the standard light-dark condition. ↓, times of appearance of sexually activated mt^+-cells; ⇩, times of decrease in sexual activation of mt^+-cells.

Effects of substances secreted from mt^+- and mt^--cells on sexual activation in "agar-agarose system"

Mt^+-plate and mt^--disk were precultured for 40 hr in "agar-agarose

culture system" in various combinations (first culture), and then the combinations were exchanged to investigate sexually activated mt$^+$-cells (second culture) (Fig. 2). Sexually activated cells appeared rapidly only in a combination of mt$^-$-disk and mt$^+$-plate which were precultured (in first culture) with their partner cells in agar layers (Fig. 8, curve C). They appeared from 8 to 20 hr of the second culture in this combination, but not in the other combinations within 48 hr of the culture.

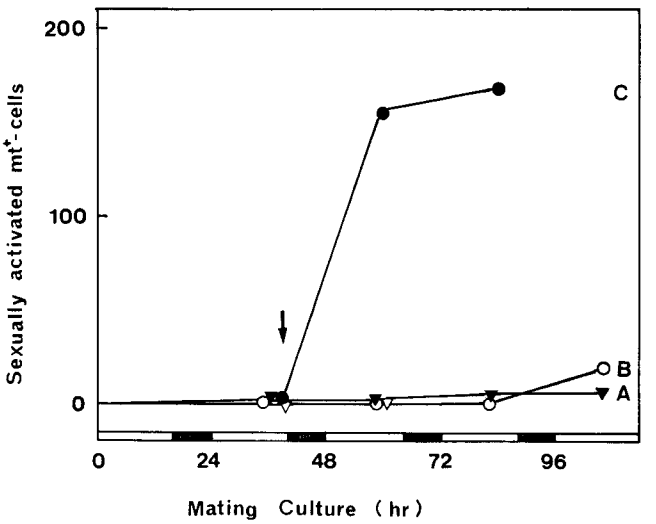

Fig. 8. Effects of mt$^+$-cells on PRS formation in mt$^-$-cells in the "agar-agarose culture system". Effects of sexual substance of mt$^+$-cells was estimated as described in Fig. 2. Number of sexually activated mt$^+$-cells in the area under the mt$^-$-disk was measured. Combinations in second culture: A, mt$^-$-disk from "independent preculture" and mt$^+$-plate from "combined preculture"; B, mt$^-$-disk from "combined preculture" and mt$^+$-plate from "independent preculture"; C, mt$^-$-disk and mt$^+$-plate from "combined preculture"; D (▽), mt$^+$-plate separated from mt$^-$-disk. □, light period; ▥, dark period.

Effects of nitrogen sources on sexual activation in "agar-agarose system"

Adding growth medium in minute amounts (about 0.1 m*l* on surface of mt$^-$-disk) in "agar-agarose system", mt$^+$-cells in agar-plate under the mt$^-$-disk were sexually activated with high frequency (Fig. 9). Adding of a mixture of Na_2CO_3 and $NaNO_3$, sexually activated cells increased markedly. Effects of Na_2CO_3, $NaNO_3$, and glucose were not clarified in this experiment. In this system omitted the agarose layer, mt$^+$-cells were activated at higher rate than in the system with the layer.

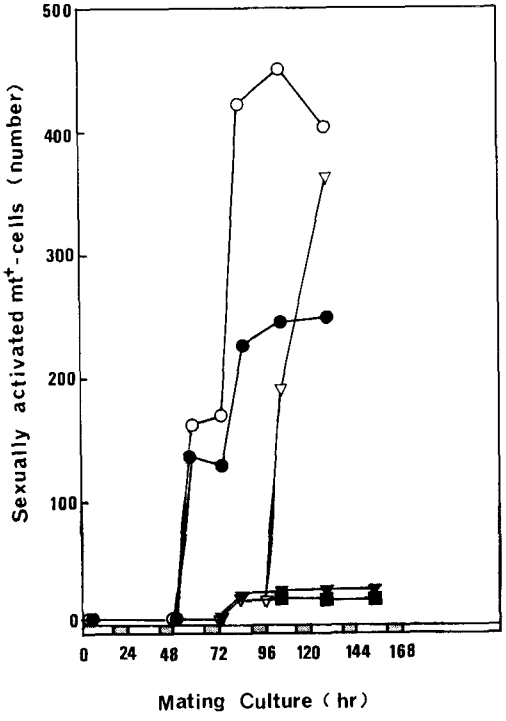

Fig. 9. Effects of growth medium on sexual activation of mt$^+$-cells in "agar-agarose culture system" under the standard light-dark condition. Combination of mt$^+$-plate and mt$^-$-disk from the beginning of culturing: ●, without growth medium; ○, with growth medium. Combination after 40 hr of culturing: ■, without growth medium; ▽, with growth medium; ▼ with mating medium. □, light period; ▨, dark period.

Effects of growth medium on cell division of mt$^+$-cells cultured in mating medium

Mt$^+$-cells divided once in liquid mating medium within 24–32 hr of culturing (Fig. 4). To investigate whether sexually divided cells have an ability to grow vegetatively, growth medium was added after 48 hr of mating culture of mt$^+$-cells in liquid medium and recultured (Fig. 10). Adding growth medium, mt$^+$-cells divided again in the dark period as shown in vegetatively growing cells.

Discussion

The formation and function of sexual substances during the mating process were determined by use of an "agar-agarose barrier culture system"

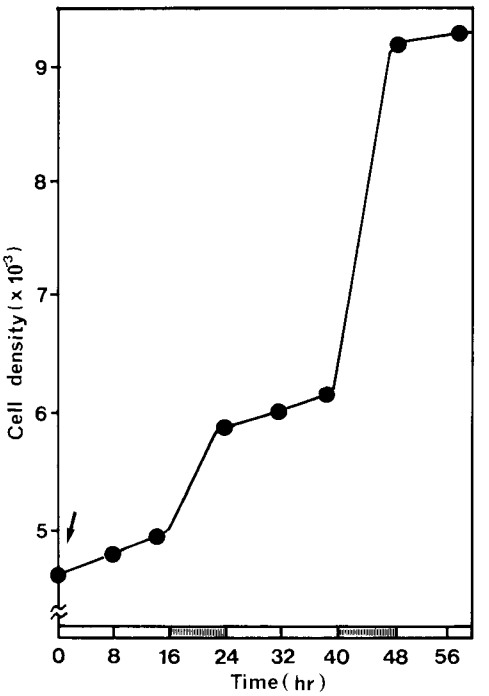

Fig. 10. Effects of growth medium on cell multiplication of mt$^+$-cells divided in mating medium. Mt$^+$-cells were cultured for 48 hr in liquid medium. At arrow growth medium was added. ▭, light period; ▥, dark period.

(Fig. 1). In this system, mt$^+$-cells were incubated in agar-plate and mt$^-$-cells in agar-disk and contacted via an agarose layer used as a barrier gel to prevent contact of mt$^+$-cells with mt$^-$-cells. Using this system, sexual substances secreted from the cells can contact with cells in the agar through the agarose layer. After precultivation of mt$^+$-plate and mt$^-$-disk for 40 hr with (combined preculture) or without (independent preculture) their partner cells in agar layers, they were cultured further in different combinations to investigate sexually activated mt$^+$-cells (Fig. 8). When mt$^+$-plate and mt$^-$-disk from "combined preculture" were recombined, sexually activated mt$^+$-cells appeared within 16 to 18 hr of further culturing (Fig. 8, combination C), while the cells did not appear in the combination of mt$^-$-disk and mt$^+$-plate from "independent preculture" or mt$^-$-disk from "independent preculture" and mt$^+$-plate from "combined preculture" (Fig. 8, combinations A and B). These results indicate that a sexual substance is secreted from the mt$^+$-cells and acts to induce PRS formation in the mt$^-$-cells (IPRS), and a

male-substance is secreted and acts to induce IPRS in the mt^+-cells (IIPRS). The formations seem to be induced by interaction between sexually opposite cells within 40 hr. The chemical properites and action mechanism of the substances were not clarified in this study. Since the early phase of the sexual reproduction process depends on light, the formation of the substances may depend on light.

In paired cells in liquid mating medium, the formation of conjugation-papilla and the fusion of gametic protoplasts in distended papilla are induced by the action of the mating-specific cell-wall lytic enzyme (KATO and SASAKI 1983); In unpaired cells, conjugation papilla-like budding took place at a site in semicells and their protoplasts were released and disrupted by the action of the enzyme, as they can not fuse with their partner cells. The timing of the enzyme formation and action seems to be limited to a short interval from 2 to 8 hr of the light period, since sexually activated mt^+-cells appeared from 2 to 8 hr of the light periods in culture with "agar-agarose system" (Fig. 7). This suggests that substances involved in formation of the lytic enzyme and the enzyme itself are very labile.

Cell division finished within 24 hr (most cells) to 32 hr in liquid mating medium (Fig. 3) and within 48 (most cells) to 56 hr of culturing in the "agar-agarose system" (Fig. 7) or in 0.6% agar medium (Fig. 6). Under the mating conditions, cell division was limited: about 3 times with inoculum cells from 12 day-growth culture: 2 times with 16 day-cells; 1.5 times with 20 day-cells; and 1.2 times with 29 day-cells (inoculum cells, 2000/m*l*) (Table 1). These data indicate that limitation of cell division and growth may be important to transform vegetative cells to gametes and to the regular process of mating: by the limitation of further cell multiplication and growth, nutrients turn on for mating-specific protein and nucleic acid synthesis under nitrogen-limited conditions. Nitrogen-deficient conditions may be necessary for the formation and/or function of proteolytic enzymes: endogenous protease has a role in the mating process by providing substrates for synthesis of new protein and nucleic acid which are used for zygote formation and maturation (JONES *et al.* 1968, STARR *et al.* 1980, TAKAGI *et al.* in preparation). The sexual activity of mt^+-cells was enhanced by the addition of minute amounts of growth medium in the "agar-agarose system" (Fig. 9). Nitrogen source deficiency is required for initiation of sexual processes in the *Closterium* used here. These data suggest that the synthesis of PRS itself and/or mating-specific cell-wall lytic enzyme is increased by increasing nitrogen supply, while the induction is inhibited.

The mt^--cells are slightly larger than the mt^+-cells (WATANABE and ICHIMURA 1978). Although the speed of cell division is almost the same for

the cells, the final cell number is somewhat higher for mt^+-cells than for mt^--cells (Fig. 3). Protoplast formation with external cellulase is more difficult in mt^--cells than in mt^+-cells (KATO *et al.* 1983). Reactivity to the mating-specific cell-wall lytic enzyme, which is formed in mt^+-cells by the action of PRS, was different: in liquid mating medium, first the mt^+- and second mt^--cells formed conjugation-papilla at the side of adhesion, and released and fused protoplasts under the action of the cell-wall lytic enzyme; in the "agar-agarose system", mt^+-cells were disrupted by the enzyme, while mt^--cells were not (Fig. 8). These results suggest that the chemical composition of the mt^--cell wall is partly different from that of the mt^+-cells and the mt^--cell wall is not lysed unless contacting with mt^+-cells binding cell-wall lytic enzyme around their surface. By the adhesion, mt^--cells are in contact with many enzyme molecules and the cell-wall is lysed for formations of conjugation-papilla and protoplasts in liquid mating medium. In the "agar-agarose system, the mt^--cell wall is not lysed, since the amounts of female-specific cell-wall lytic enzyme diffused from the mt^+-cells is too little different from the amounts binding on the mt^+-cells.

The formation of the cell-wall lytic enzyme seems to be repressed and the enzyme is inactivated after fusion of gametic protoplasts in paired cells in liquid mating medium, but not in unpaired mt^+-cells in liquid medium and in "agar-agarose system" preventing adhesion of sexually opposite cells. The differences in physiological and chemical properties between mt^+- and mt^--cells may have a role in cell-cell recognition, cell-cell adhesion, and cell-cell interaction for the sexual reproduction in *Closterium*.

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