Sexual Reproduction and Cyst Formation in the Freshwater Dinoflagellate *Peridinium cunningtonii*

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In order to make clear the mechanism of the bloom of *Peridinium cunningtonii*, which periodically occur in summer in Nagase Reservoir, Kochi Prefecture, the conditions regulating encystment of this species were examined by use of clonal population and a synthetic medium (MW1). The maximum cell concentration was 1×10^6 vegetative cells $\cdot \text{ml}^{-1}$ in the chemically defined medium (MW1) for about 2 weeks at 20°C. Sexual reproduction was induced in the dinoflagellate *Peridinium cunningtonii* when late logarithmic phase cells in MW1 medium were inoculated into nitrogen and phosphorus deficient medium.

Sexual reproduction was isogamous and homothallic. Small, naked vegetative cells produced by division of thecate cells acted as gametes. The planozygote retained two red bodies and remained motile for 8-10 days. About 10 days following plasmogamy the zygote became non-motile, the protoplast expanded, a large red body appeared and the wall thickened. The theca of planozygote split at the girdle region and the hypnozygote (cyst) was produced.

The cysts were produced under 10,000 lx illumination at 15°C in N- and P-free medium at the maximum frequency of 70%. The frequencies of encystment of transferring the cell into N-free medium, P-free medium, and fresh medium containing N and P were about 63, 41, and 0%, respectively. Optimum temperature for encystment was 15 to 20°C and optimum light intensity was 5,000 to 10,000 lx. The dormant cysts were resistant to low temperature. It is strongly suggested that the cysts of *P. cunningtonii* develop into the seed population for blooms of this dinoflagellate for the next year.

In recent years it has been often reported that some microalgae belonging to *Peridinium* (Dinophyceae) cause remarkable blooms (so-called freshwater red tide) in reservoirs and man-made lakes in Japan.¹⁾ We found that large blooms of *Peridinium cunningtonii* occurred in every summer in Nagase Reservoir in Kochi Prefecture. When temperature of water is above 17°C in late spring to early summer, the succession of dominant species from *P. penardii* to *P. cunningtonii* occurs. In Nagase Reservoir, a little bloom of *P. cunningtonii* initiated in early June, 1981, a maximum density of 40,000 cells $\cdot \text{ml}^{-1}$ was observed at the end of August, and the bloom disappeared in late September. During late fall, winter, and spring it is possible that this alga keeps cyst stage (dormant cell), and is associated with bottom sediments in the reservoir.

It has been believed that the excystment of overwintering cysts of *Gonyaulax tamarensis* pro-

vides the seed population for toxic blooms of this dinoflagellate.^{2,3)} ANDERSON and MOREL⁴⁾, and ANDERSON⁵⁾ found the cysts from sediments samples and demonstrated the seeding of a spring and a fall blooms of the toxic dinoflagellate *G. tamarensis* by the temperature-induced germination of benthic cysts. The sexual reproduction and cysts (hypnozygotes) of *G. tamarensis*⁶⁾ and *G. monilata*⁷⁾ were observed in nitrogen-deficient medium, although the incidence of sexual fusion was very low.

The sexual reproduction and hypnozygote formation have been shown for several freshwater dinoflagellates, of which sexuality was induced by manipulation of nutrients, light intensity, photoperiod, or temperature in laboratory cultures.⁸⁻¹³⁾ In *Peridinium cinctum*, for example, nitrogen depletion induced gamete formation. The small, naked, isogamous gametes fused and formed planozygotes which remained active for 2 weeks before

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they were transformed into thick-walled hypnozygotes. Unfortunately, these studies are not quantitative, because of application from the taxonomical point of view. WATANABE *et al.**¹ has quantitatively determined encystment for a marine culture of *Scripsiella trochoidea*, which caused a large scale of red tides at the Osaka Bay. They have shown that this alga produces zygotes (cysts) at a high rate when inoculated into either nitrogen- or phosphate-depleted medium. To the present the encystment has not been established for cultures of freshwater dinoflagellates.

The first objective of this paper is to report the previously unknown sexual life history of *P. cunningtonii*, which periodically blooms in summer in Nagase Reservoir, Kochi Prefecture. The second objective of this paper is to make clear the conditions regulating encystment of this species by use of clonal population and synthetic medium.

Materials and Methods

Organism

Peridinium cunningtonii was isolated from Nagase Reservoir, Kochi Prefecture. Clonal culture was utilized in all experiments.

Culture

All cultures of *P. cunningtonii* were grown in 50- and 100-ml capacity Erlenmeyer flasks. Cells were grown at 20°C on a 14: 10 h light-dark cycle under illumination of 15,000 lx provided by banks of 30-w cool white fluorescent lamps. No bubbling or shaking of culture was employed.

P. cunningtonii was grown in MW1 medium which is the modified medium of WATANABE's medium (W1).^{*2} The composition of MW1 medium was shown in Table 1.

Cell numbers were determined with a hemocytometer after fixation with formalin.

Encystment Experiment

When cell density of the cultures reached 1×10^5 cells $\cdot$ ml⁻¹ for 2 weeks, the cells were transferred at a final concentration of ca. 400 cells $\cdot$ ml⁻¹ to fresh MW1 medium without nitrogen and/or phosphorus. Unless otherwise noted the standard conditions for sexual reproduction and encystment were: i) N- and P-free MW1 medium; ii) 15°C incubation temperature; and, iii) illumina-

Table 1. Composition of MW1 medium for *Peridinium cunningtonii*

Ingredient	Concentration	(l ⁻¹)
Urea	8.5	mg
NaNO ₃	1.7	mg
NH ₄ Cl	0.425	mg
Ca(NO ₃) ₂ · 4H ₂ O	100	mg
CaCO ₃	10	mg
CaCl ₂ · 2H ₂ O	14	mg
KNO ₃	10	mg
KHCO ₃	9	mg
β-Glycerophosphoric acid disodium salt	20	mg
MgSO ₄ · 7H ₂ O	15	mg
PIV metal ^a	0.5	ml
Vitamin B ₁₂	0.2	μg
Thiamine hydrochloride	20	μg
Biotin	0.2	μg
Glycylglycine	100	mg

pH 7.2

^a PIV metal: 19.6 mg FeCl₃ · 6H₂O, 3.6 mg MnCl₂ · 4H₂O, 1.05 mg ZnCl₂, 0.4 mg CoCl₂ · 6H₂O, 0.25 mg Na₂MoO₄ · 2H₂O, 100 mg Na₂EDTA in 100 ml.

tion of 10,000 lx on 14: 10 LD cycle. Conditions for nutrient, light and temperature during sexual reproduction were modified according to experimental design. At short intervals the number of planozygotes which contain two red bodies and hypnozygotes (cysts) was counted. A frequency of cyst formation (%) were calculated using the following formula*¹:

Frequency of cyst formation (%)

$$= \frac{2 \times \left[\frac{\text{number of}}{\text{cysts} \cdot \text{ml}^{-1}} \right]}{\left[\frac{\text{number of vegetative}}{\text{cells} \cdot \text{ml}^{-1}} \right] + 2 \times \left[\frac{\text{number of}}{\text{cysts} \cdot \text{ml}^{-1}} \right]} \times 100$$

Observation of Life Cycle

The sexual life cycle was followed by isolating single pairs of fusing gametes in 0.2 ml of media poured into each hole in Microtest Plates (Nunc). These zygotes formed were checked daily by light microscope.

Scanning Electron Microscopy

Cells which were collected on nylon filters (NHD 5, 5 μ mesh), were fixed in 2% glutaraldehyde-0.2 M sodium phosphate buffer (pH 7.2) for 30 min and washed well with distilled water. These samples on filters were dehydrated in ethanol and isoamyl acetate and dried using a critical point

*¹ M. M. WATANABE, M. WATANABE, and Y. FUKUYO: Res. Rep. Natl. Inst. Environ. Std., No. 30, 27-42 (1982).

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Fig. 1. Growth curve of *P. cunningtonii* in MW1 medium.

A, Culture was incubated at 20°C on a 14: 10 h light-dark cycle under illumination of 15,000 lx. B, Temperature effect. C, pH effect (○, MW1 with Piperazine-N,N'-bis(2-ethanesulfonic Acid) in place of glycylglycine; ●, MW1 medium) D, Light intensity effect.

drier. The filters were stuck onto specimen stubs and coated with gold using a JFC-1100, before examination with a scanning electron microscope (JSM-P 15).

Results

Vegetative Growth

A typical growth curve of *P. cunningtonii* in MW1 medium under 15,000 lx at 20°C was shown in Fig. 1A. The cell of exponential phase divided into two daughter cells. The maximum cell concentration was 1×10^5 vegetative cells $\cdot$ ml $^{-1}$ for about 2 weeks. The optimum temperature for the maximum growth rate and cell yields was 20°C (Fig. 1B). The optimum pH for growth was approximately 7.5 (Fig. 1C). The growth rate and cell yields were maximal at 5,000 lx and

higher intensity, and the growth reductions were not observed at 15,000 lx.

Morphological Observation in Encystment

Vegetative cells (Fig. 2) of *Peridinium cunningtonii* measured 25–30 μ m in length and 22–26 μ m in width, and were ovoidal in ventral view. Vegetative cells had an evident eyespot on the lower ventral side, and had 2–4 antapical spines. Numerous yellow-brown or yellow-green chromatophores were equally distributed. The thecal debris were usually observed in cultured material. Scanning electron microscopy revealed fine structural plates of this vegetative cell (Figs. 3 and 4).

Sexual reproduction was induced when late logarithmic phase cells were inoculated into N- and P-free MW1 medium. Gametes formed within 2 days on a 14: 10 LD cycle. Cells divid-

Figs. 2-7. Light and scanning electron micrographs of fusing process in *P. cunningtonii*. Scale bars = 10 μ m.

Fig. 2. Vegetative cell.

Fig. 3. Vegetative cell (ventral view).

Fig. 4. Vegetative cell (dorsal view).

Fig. 5. Gamete.

Fig. 6, 7. Fusing gametes.

Figs. 8-12. Light and scanning electron micrographs of encystment in *P. cunningtonii*. Scale bars = 10 μ m.

Fig. 8. Planozygote.

Figs. 9, 10. Release of cyst.

Fig. 11. Cyst (hypnozygote).

Fig. 12. Cysts (hypnozygotes).

ed longitudinally into two gametes within the parental theca. Gametes ranged from 20 to 25 μm in length and were smaller than asexual cells which ranged from 25 to 30 μm . Gametes were naked or possessed only thin thecae (Fig. 5). Gametes were lighter in colour than vegetative cells.

Gametes were isogamous with fusion (Figs. 6 and 7). Fusing gametes were large in number at 3–7 h during the dark cycle. We could not find the initial point of contact between the fusing gametes. It seems, however, that fusion was proceeded gradually from the upper to the lower regions of the cells. Sexual fusion required about 60 min from gametic contact to completion. Sexual reproduction was thus isogamous and homothallic.

These planozygotes had two longitudinal flagella, developed thecae and retained two red bodies (Fig. 8). Eight to ten days following cellular fusion the planozygotes, which slowly enlarged to 30–35 μm , became darkened, and remained motile, lost motility and became lighter in colour. After the protoplasts were expanded and the thecae split at the girdle region, cysts (hypnozygotes) were released (Figs. 9 and 10). The cyst of *P. cunningtonii* was round and the surface of cyst was relatively smooth (Fig. 11). The cyst contained yellow-brown or yellow-green chromatophores and retained a large red body (Fig. 12). Cysts always precipitated to the bottom of flasks or test tubes. By excreting mucilaginous material the cysts were easily aggregated or stuck the thecae of vegetative cells around each cyst. A detailed study on excystment of this alga will be published in the next paper.

Effects of Environmental Conditions on Encystment.

The effect of nutrient deficiency on the encystment of *P. cunningtonii* was investigated. Results obtained are shown in Fig. 13. After 20 days following inoculating of the vegetative cells into N- and P-free medium cysts were produced at the high frequency of 71% under 10,000 lx illumination at 15°C. In N-free medium cysts were produced at the frequency of 63%, and in P-free medium, at the frequency of 41%. After further 20 days' incubation without being transferred cysts were formed at the frequency of 7%. No cyst was produced when vegetative cells were inoculated to MW1 medium and normal vegetative growth occurred.

The encystment in N- and P-free MW1 medium

Fig. 13. Effect of nutrient deficiency on the encystment in *P. cunningtonii*. The cells were transferred to the following media and were incubated at 15°C and 10,000 lx.

○, nitrogen and phosphorus deficient medium; ●, nitrogen deficient; △, phosphorus deficient; ■, control without transfer; ▲, complete medium (MW1).

Fig. 14. Effect of temperature on the encystment of *P. cunningtonii*. All cultures were incubated in nitrogen- and phosphorus-deficient medium under illumination of 10,000 lx. ●, 20°C; ○, 15°C; □, 10°C; ■, 5°C.

is illustrated in Fig. 14, as a function of incubation temperature, at 5, 10, 15, and 20°C. The optimum temperature for encystment was 15°C, and the frequency was 71%. The frequencies of encystment at 20 and 10°C were 68 and 13%, respectively. No encystment was observed at 5°C within 20 days' incubation. Most cells remained at vegetative cells.

The dependence on light intensity of encystment

Fig. 15. Effect of light intensity on the encystment of *P. cunningtonii*. All cultures were incubated in nitrogen- and phosphorus- deficient medium at 15°C. ○, 10,000 lx; △, 5,000 lx; ●, 2,000 lx; □, 1,000 lx; ■, dark.

in N- and P-free MW1 medium at 15°C was studied and the results is shown in Fig. 15. The cyst production was followed for 20 days. At 10,000 lx (14:10 LD cycle) cysts were produced at the frequency of 60% for 8 days' incubation and 72% for 20 days'. The optimum light intensity for encystment was 10,000 lx. The frequencies of encystment at 5,000, 2,000, and 1,000 lx were 69, 60, and 9%, respectively. The cyst was not produced in continuous darkness.

Discussion

The present culture study has established sexual cyst formation of *P. cunningtonii*. The sexual life cycle is similar to the cycle of *P. cinctum*,⁹⁾ *P. willei*,¹⁰⁾ and *P. gatunense*¹¹⁾ in the following: i) sexuality is induced in nitrogen deficient medium; ii) two gametes are formed within the parental theca; iii) gametes are similar in shape to vegetative cells; iv) sexual reproduction is isogamous and homothallic; and v) gametes fuse during dark period of LD cycle. The differences include; i) the planozygotes of *P. cunningtonii* have two large red bodies as compared with those of other species; ii) the mature cysts of *P. cunningtonii* are released by splitting at the girdle region of the theca of hypnozygote. Sexual reproduction of *P. limbatum*, which is induced in nitrogen deficient medium, is also homothallic and similar to that reported for other *Peridinium* species.¹³⁾ On the other hand, sexual reproduction of *P. volzii* is iso-

gamous and heterothallic with two mating types.¹²⁾

Observation by scanning electron microscopy showed that planozygote of *P. volzii* conspicuously enlarges with unequal enlargement of intercalary bands between plates, and then theca distorts.¹²⁾ In the present study intercalary bands of *P. cunningtonii* planozygote did not remarkably enlarge.

P. cinctum formed round, dark thin-walled asexual cysts when being inoculated in N-free medium,⁹⁾ but in *P. cunningtonii* asexual cysts are not observed.

Sexual cysts of *P. cunningtonii* are produced in N- and P-free medium under 10,000 lx illumination at 15°C at the maximum frequency of 71%. In N-free medium cysts are produced at the frequency of 63%, and in P-free medium cysts are produced at the frequency of 41%. Cysts of *P. cunningtonii* are produced in stationary culture (35 days) at the frequency of 7%. These encystment frequency is high in comparison with that of *Dinobryon cylindricum* (0.01–29%)¹⁴⁾ and *Uroglena americana* (0.5%) (H. NAKAHARA, personal communication). In other five species of freshwater *Peridinium*,^{9–13)} sexual encystment was observed in only N-deficient medium and the other conditions regulating encystment were not studied. The other dinoflagellates, *G. tamarensis*⁶⁾ and *G. monilata*⁷⁾ produced hypnozygotes in nitrogen-deficient medium, and the cyst of the latter was also observed in late stationary cultures. These nutrient stress may stimulate a sexual response or induce production of some mating substances, although no information is available as to these speculations.

Encystment of *P. cunningtonii* is maximal at light intensity of 5,000 to 10,000 lx, and at temperature of 15 to 20°C. The cyst is not produced in continuous darkness as well as in other dinoflagellates.^{6–13)} These physical conditions are similar to the condition for growth in *P. cunningtonii*. According to VON STOSCH, gametes of *Gymnodinium pseudopalustre* were formed when a culture actively growing at 21°C was subjected to a short-day treatment (10 h light) at 15°C and gametes of *Woloszynskia apiculata* were formed when vegetative cells were inoculated in moderate N and P deficient medium and put into a lower light intensity (from 14,000 to 2,000 lx).⁸⁾ If we consider these environmental factors influencing encystment of *P. cunningtonii* in relation to those of other dinoflagellates, it is assumed that while light irradiation and temperature only support photosynthesis of vegetative cells, gametes and plano-

zygotes, the nutrient stress induces gametes formation and causes sexual reproduction of *P. cunningtonii*.

In an ecological point of view encystment and excystment of the alga will play an important role in the red tide outbreak in Nagase reservoir. During the bloom of *Peridinium cunningtonii* the concentrations of dissolved NO_3^- and PO_4^{2-} in the reservoir decreased to $0.01\text{--}0.02\text{ mgN}\cdot\text{l}^{-1}$ and to less than $0.002\text{ mg P}\cdot\text{l}^{-1}$, respectively.* The decreasing means that the environmental conditions favoring encystment would be provided for this alga as if this alga were incubated in N and P deficient medium. Therefore, the cysts formed must sink to sediment in the reservoir and revive as a seed population after overwintering.

Further studies on the environmental conditions regulating the encystment and excystment of *P. cunningtonii* are now being carried out.

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References

- 1) T. ITOH: *Bull. Plankton Soc. Japan*, **26**, 113–116 (1979).
- 2) A. PRAKASH: *J. Fish. Res. Board Can.*, **24**, 1589–1606 (1967).
- 3) K. A. STEIDINGER: in "Toxic Dinoflagellate Blooms" (ed. by V. R. LOICICERO), Proc. 1st. Int. Conf. Mass. Sci. Technol. Found., 1975, pp. 153–162.
- 4) D. M. ANDERSON and F. M. M. MOREL: *Estuar. Coast. Mar. Sci.*, **8**, 279–293 (1979).
- 5) D. M. ANDERSON: *J. Phycol.*, **16**, 166–172 (1980).
- 6) D. H. TURPIN, P. E. R. DOBELL, and F. J. R. TAYLOR: *J. Phycol.*, **14**, 235–238 (1978).
- 7) L. M. WALKER and K. A. STEIDINGER: *J. Phycol.*, **15**, 312–315 (1979).
- 8) H. A. VON STOSCH: *Br. Phycol. J.*, **8**, 105–134 (1973).
- 9) L. A. PFIESTER: *J. Phycol.*, **11**, 259–265 (1975).
- 10) L. A. PFIESTER: *J. Phycol.*, **12**, 234–238 (1976).
- 11) L. A. PFIESTER: *J. Phycol.*, **13**, 92–95 (1977).
- 12) L. A. PFIESTER and J. J. SKVARLA: *Phycologia*, **18**, 13–18 (1979).
- 13) L. A. PFIESTER and J. J. SKVARLA: *Amer. J. Bot.*, **67**, 955–958 (1980).
- 14) C. D. SANDGREN: *J. Phycol.*, **17**, 199–210 (1981).

* Y. HATA: Res. Data Natl. Inst. Environ. Std. No. 24, 15–28 (1983).