Original

Bacteria-free culture of a colorless euglenoid, *Peranema trichophorum*, and establishment of a method for flagellar isolation

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SUMMARY

We have recently shown that the anterior flagellum and its mastigonemes play an important role in gliding movement in the colorless euglenoid flagellate, *Peranema trichophorum*. To facilitate further biochemical analysis of the molecular mechanism of flagellar surface motility, we have developed an improved monoxenic sterile culture method and a method for flagellar isolation. With initial cell densities of *Peranema* and co-existing food flagellates, *Chlorogonium elongatum*, at 800 and 30,000 cells/ml, respectively, *Peranema* reached its maximum cell density of about 10,000 cells/ml in one week. A cold-shock treatment allowed efficient isolation of flagella from the cell body, and light and electron microscope observations confirmed that the isolated flagella retained their intact structure with abundant mastigonemes. SDS-PAGE analysis showed that the preparation contained a predominant protein band of 100 kDa, which was not stained by silver and may therefore be highly glycosylated.

INTRODUCTION

Gliding motility in *Peranema trichophorum* has been poorly studied and its mechanism continues to be elusive (Chang, 1966; Chen, 1950; Saito et al., 2003). *P. trichophorum*, like many other related euglenoid flagellates, exhibits cell body deforma-

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tion (Triemer, 1997). It has long been controversial whether locomotion of *P. trichophorum* along a solid substratum is attributable to the motility of the cell body or to the anterior flagellum (Chen, 1950). We have recently demonstrated that interaction between the surface of the anterior flagellum and the substratum is crucial for gliding motility in *P. trichophorum* and that flagellar length is proportionally related to the rate of whole cell gliding (Saito et al., 2003). As in other gliding protists, such as *Chlamydomonas*, flagellar surface motility can be visualized by translocation of polystyrene microspheres. Microspheres were observed to move in close association with the extensive coat of mastigonemes on the anterior flagellum, indicating that the mastigonemes are involved in the force-generating mechanism responsible for cell gliding (Saito et al., 2003). To further understand the role of the mastigonemes in the mechanism of gliding motility in *P. trichophorum*, biochemical and molecular biological approaches are necessary, and these require the rapid production of large numbers of cells. Recently, we described a monoxenic culture method for *P. trichophorum,* with *Chlorogonium elongatum* as a single food source (Saito et al., 2003). Although this method gave a constant yield of *P. trichophorum*, further improvement is needed to prepare sufficient cells in a short time and to completely eliminate co-existing food flagellates at the time of cell harvest. Accordingly, the present study attempted to improve the culture method and to establish a procedure for isolating flagella from the cell body.

MATERIALS AND METHODS

Peranema trichophorum was purchased from Carolina Biological Supply (USA). A conventional subculturing was carried out according to the method used in previous studies (Saito et al., 2003), but with slight modifications. One hundred milliliters of a culture medium consisting of 0.24 mM $Ca(NO_3)_2$, 0.14 mM KNO_3, 58 μM $MgSO_4$, 0.1mM KH_2PO_4, 1.47 mM sodium acetate, 0.02% polypeptone (Wako, Tokyo), 0.04% tryptone (Difco, Detroit), 0.04% yeast extract (Difco, Detroit), and 13.6 μM $CaCl_2$ was put into a 200-ml Erlenmeyer flask (IWAKI No.4442FK-200, Iwaki Glass, Co. Ltd., Tokyo). After being sterilized by autoclaving (115°C for 20 min), the medium was inoculated with *P. trichophorum* (about 4–5 cells/ml) and food *Chlorogonium elongatum* (about 140 cells/ml), and kept still in an incubator (MIR-253, Sanyo Electric Co. Ltd., Osaka) at 23 ± 1°C in a 12:12 LD cycle with white fluorescent lamp illumination, unless otherwise stated.

To examine the effect of shaking on the growth rate of *P. trichophorum*, 200-ml culture flasks containing 100 ml of culture medium with *P. trichophorum* (about 2,500 cells/ml) and food *C. elongatum* (about 500,000 cells/ml) were placed on a shaker and rotated at a speed of 87 rpm at 20°C under constant illumination. Cell density was monitored for more than 2 weeks.

The effect of the depth of the culture medium was tested as illustrated schematically in Fig. 2. Different amounts of culture medium in the same flask size (flasks A, B and C) and the same amount of culture medium in different flask sizes (flasks C and D) were compared.

For isolation of flagella from *P. trichophorum*, culture flasks at 1 week after subculturing were shaken well to detach cells from the bottom, and the suspended cells were collected by centrifugation (400 × g, 5 min). After being washed with a solution consisting of 0.24 mM $Ca(NO_3)_2$, 0.14 mM KNO_3, 58 μM $MgSO_4$, 0.1 mM KH_2PO_4 and 10 mM Hepes–KOH (pH 7.0), the cells were condensed into 1 ml and put into a 1.5-ml plastic test tube. In the "slow" cold treatment, the suspension of *P. trichophorum* was poured into a 1.5 ml plastic test tube that had been pre-cooled in ice-

cold water. In the "quick" treatment, a condensed suspension of *P. trichophorum* (about 0.1 ml) was quickly mixed with 1 ml of pre-cooled medium in a plastic test tube which was then dipped in ice-cold water for further cooling. The cells were cold-shocked using a "test-tube rack for rapid cooling" (IR-1, Towa Lab. Co. Ltd., Tokyo). The tubes were placed in the rack, and the gap between the tubes and the inner surface of the rack was filled with 70% ethanol for better heat conduction. Then the rack was dipped into iced water, and kept there for 1 min. After cooling, the rack was returned to room temperature for 5 min. To increase flagellar detachment, this step was repeated 3–5 times. The tubes were removed from the rack, and centrifuged for 5 min at 400 × g using a swing rotor to sediment cell bodies. Finally, the supernatant was centrifuged at 10,000 × g for 30 min to precipitate isolated flagella.

Living cells were observed under a differential interference microscope (BH-2 and BX-50, Olympus, Japan) or an inverted microscope (Axiovert-25, Carl Zeiss, Germany). Images were taken with a CCD video camera (KY-F30, Victor, Japan) or a digital camera (DP-11, Olympus, Japan), and were transferred to a personal computer. For ultrastructural observations, isolated flagella were negatively stained with 3% aqueous uranyl acetate before being observed with a transmission electron microscope (H-7100, HITACHI, Japan).

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue (CBB) or with a Silver Staining Kit "Daiichi" (Daiichi Chemical, Japan).

RESULTS

As aeration continuously supplies oxygen into the culture medium, shaking culture vessels at about 100 rpm has been used to facilitate growth of other euglenoid flagellates. However, as shown in Fig. 1, growth of *P. trichophorum* was completely suppressed by shaking. The inhibitory effect of fluid mixing on cell growth was observed at shaking speeds greater than 30 rpm.

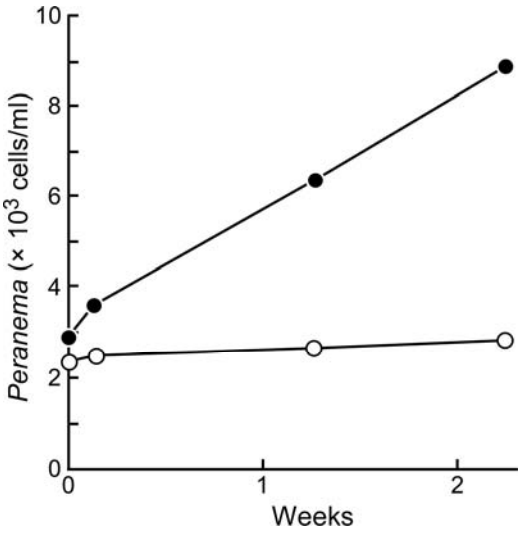
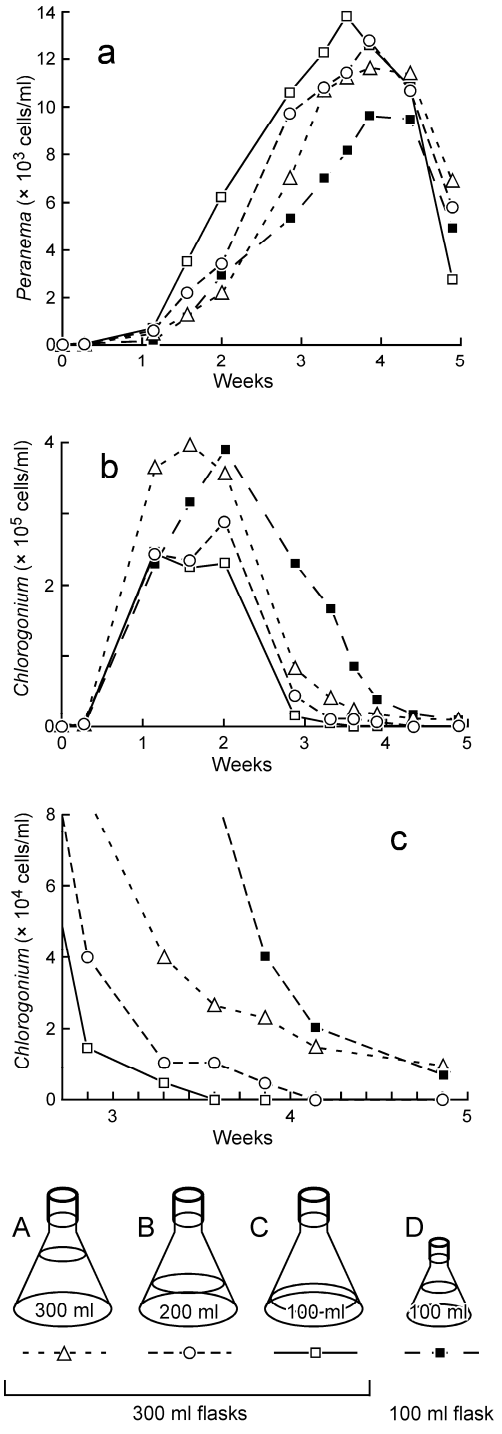


Fig. 1. Effect of shaking culture flasks on growth of *Peranema trichophorum*. Compared with the control culture method (no shaking, closed circles), cell growth was suppressed when the culture flask was shaken at a speed of 87 rpm (open circles).

P. trichophorum usually inhabits the bottom surface of the culture vessel. We therefore examined whether depth of the culture medium affects the growth rate of *P. trichophorum*. Among the four different conditions shown in Fig. 2, similar maximum cell densities were achieved in all flasks, despite flasks B and C showing faster cell growth at the initial growing stage. However, as shown in Fig. 2c, rate of diminution of the food flagellates appeared to be dependent on the depth of the culture medium. With 100 ml of culture medium in flask C, for example, food *C. elongatum* disappeared at 3.5 weeks after inoculation, while food flagellates persisted longer in the 300-ml culture medium (flask A). These results show that 1) the initial cell growth is dependent on the



depth of the culture medium, although the maximum cell density is influenced by neither the flask size nor the depth of the culture medium, and 2) the time required to eliminate co-existing food flagellates is dependent on the depth of the culture medium in the flask.

For cytochemical and physiological research, good quality samples are required, so it is necessary to eliminate food *C. elongatum* cells as soon as possible after the growth of *P. trichophorum* reaches a plateau. Figure 3 shows that the growth characteristics of *P. trichophorum* were dependent on the cell density of *C. elongatum* at the time of subculturing. When the initial ratio of cell density of *C. elongatum* to that of *P. trichophorum* was high (for example, filled triangles in Fig. 3), *C. elongatum* showed faster growth and greatest maximum cell density. In such conditions, *P. trichophorum* showed a slower initial increase in cell number, and took about two weeks to reach a plateau. When the initial cell density of *C. elongatum* was as low as 15,000–30,000 cells/ml, *P. trichophorum* reached a similar plateau level but in a shorter time (about 1 week after inoculation), and the density of food flagellates remained low.

Based on these results, optimal conditions for the monoxenic culture of *P. trichophorum* was determined to be: 100 ml of culture medium in a 300-ml flask, and initial densities of *P. trichophorum* and *C. elongatum* of 800 cells/ml and 30,000 cells/ml, respectively. In these conditions, cell density of *P. trichophorum* reaches a maximum in about 1 week, and almost all the food flagellates disappear within a few days, as shown in Fig. 4b. Compared with the culture method used previously

Fig. 2. Growth curves for *Peranema trichophorum* (a) and food *Chlorogonium elongatum* (b and c) in different sizes of culture flask and with different amounts of culture medium. The last part of Fig. 2b is expanded in Fig. 2c. Symbols indicate different culture conditions as schematically represented at the bottom. Although no significant differences in the growth of *P. trichophorum* were observed, the disappearance of *C. elongatum* seems to be related to the size of flask and the amount of culture medium.

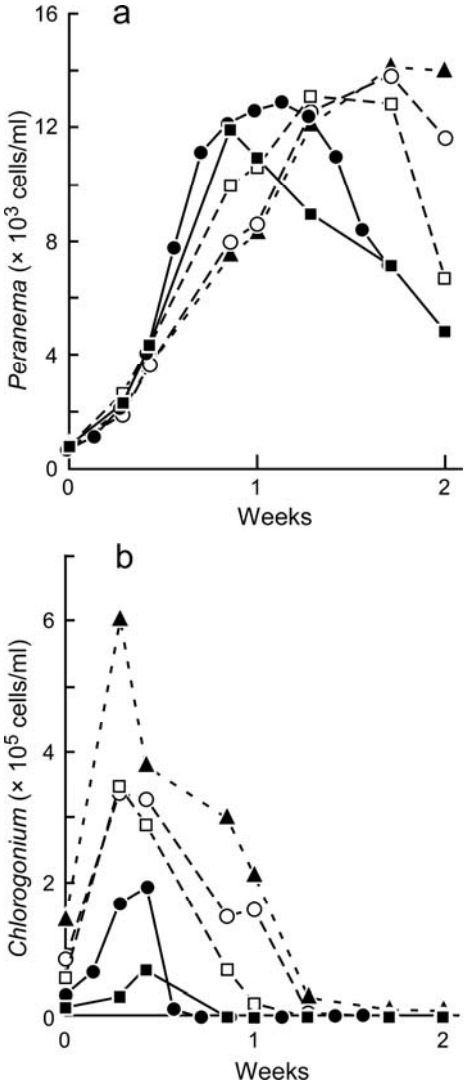


Fig. 3. Growth curves for *Peranema trichophorum* (a) and food *Chlorogonium elongatum* (b). Initial densities of *C. elongatum* were 15,000 (closed squares), 30,000 (closed circles), 60,000 (open squares), 90,000 (open circles), and 150,000 (closed triangles) cells/ml, while the initial density of *P. trichophorum* was constant at 800 cells/ml in all flasks. When initial densities of *C. elongatum* were 15,000 or 30,000 cells/ml, the number of *P. trichophorum* reached a plateau at about 1 week without contamination with *C. elongatum*.

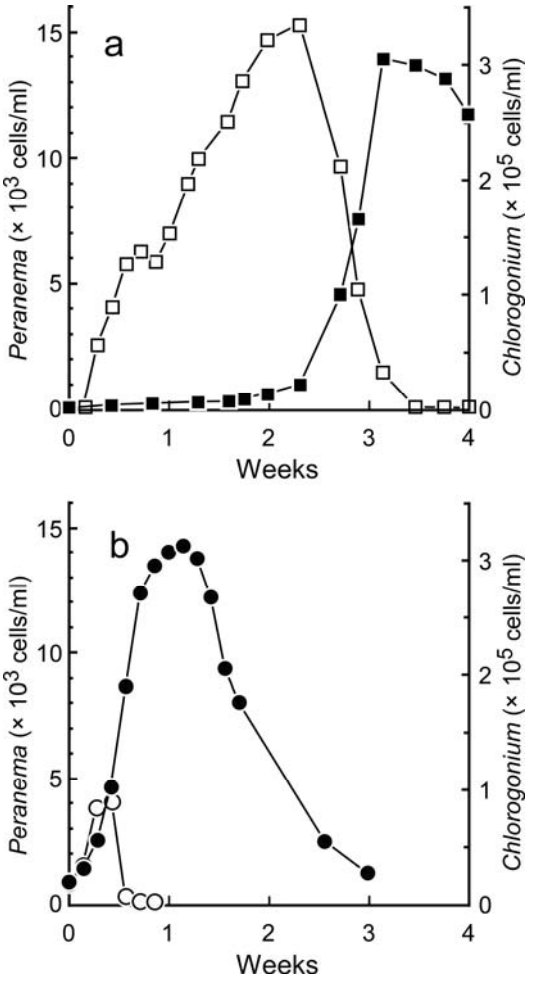


Fig. 4. Comparison of the newly-established culture method with the method used in previous studies. a: Growth curves of *Peranema trichophorum* (filled squares) and *Chlorogonium elongatum* (open squares) in the conventional culture conditions. The size of the flask was 200 ml with 100 ml of culture medium. The initial cell densities of *P. trichophorum* and *C. elongatum* were 4–5 cells/ml and 140 cells/ml, respectively. b: Growth curves of *P. trichophorum* (filled circles) and *C. elongatum* (open circles) in the newly-established culture conditions. The size of the flask was 300 ml with 100 ml of culture medium. The initial cell densities of *P. trichophorum* and *C. elongatum* were 800 cells/ml and 30,000 cells/ml, respectively.

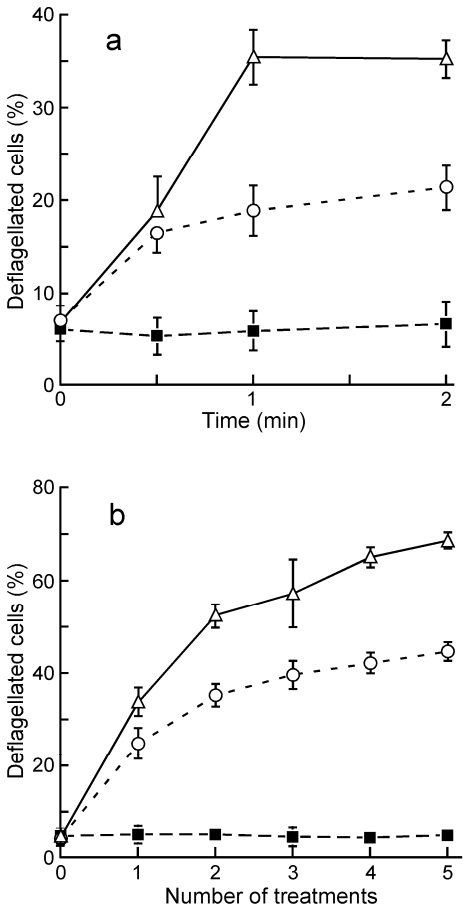


Fig. 5. The effect of cold-shock treatment on the rate of deflagellation. a: Time course of deflagellation in different cold-shock protocols. The percentage of cells deflagellated by slow cold-shock treatment (open triangles) was almost double that for quick cold-shock treatment (open circles), and reached a plateau at 1 min. b: The effect of repetition of cold-shock treatment on deflagellation. In both types of cold treatment, the percentage of cells without flagella was increased by repetition of the treatment. In both panels, the rate of deflagellation of non-treated cells is shown as the "control" (closed squares). Error bars show ± SD.

(Fig. 4a), the new method yielded almost as many cells in a shorter period, with no contaminating *C. elongatum.*

To establish an efficient method of flagellar isolation, we compared two different cold treatments, namely "slow" and "quick" methods. As shown in Fig. 5a, the "slow" method of cold-shock yielded more isolated flagella than the "quick" method. Repetition of the cold treatment yielded further flagella (Fig. 5b). Cells that had been cold-treated for 1 minute by either method were returned to room temperature for 5 minutes. This was repeated several times, and the percentage of cells with detached flagella determined. After five repetitions of the "slow" treatment, about two-thirds of the total flagella were detached, while only about 40% of the total flagella were detached by the "quick" treatment after five repetitions. The flagella retained their intact structure even after repetitions of cold treatment (Fig. 6b and c).

When *P. trichophorum* cells cultured for 1 week were observed with an inverted microscope, large amounts of waste from the cells were seen at the bottom of the flask (small black particles in Fig. 6a). After this material was removed by gentle centrifugation at 200 × g for 2 min, the cells were subjected to a cold-shock treatment as described above to detach flagella from the cell bodies (Fig. 6b). Isolated flagella appeared like twisted ribbons because of the paraxial rod that is characteristic of the flagellum in euglenoid flagellates (Fig. 6c). Negatively-stained isolated flagella were examined by transmission electron microscopy. The mastigonemes were detached from the isolated flagella by gentle vortexing and were observed as thick ribbons (arrows in Fig. 6d), the "short mastigoneme ribbons" that surround the flagellar shafts. As shown in the enlarged micrograph, individual mastigonemal filaments arise from the surface of the ribbons (Fig. 6e).

Isolated flagella were collected and sampled for SDS-PAGE analysis (Fig. 7). The CBB stained gel

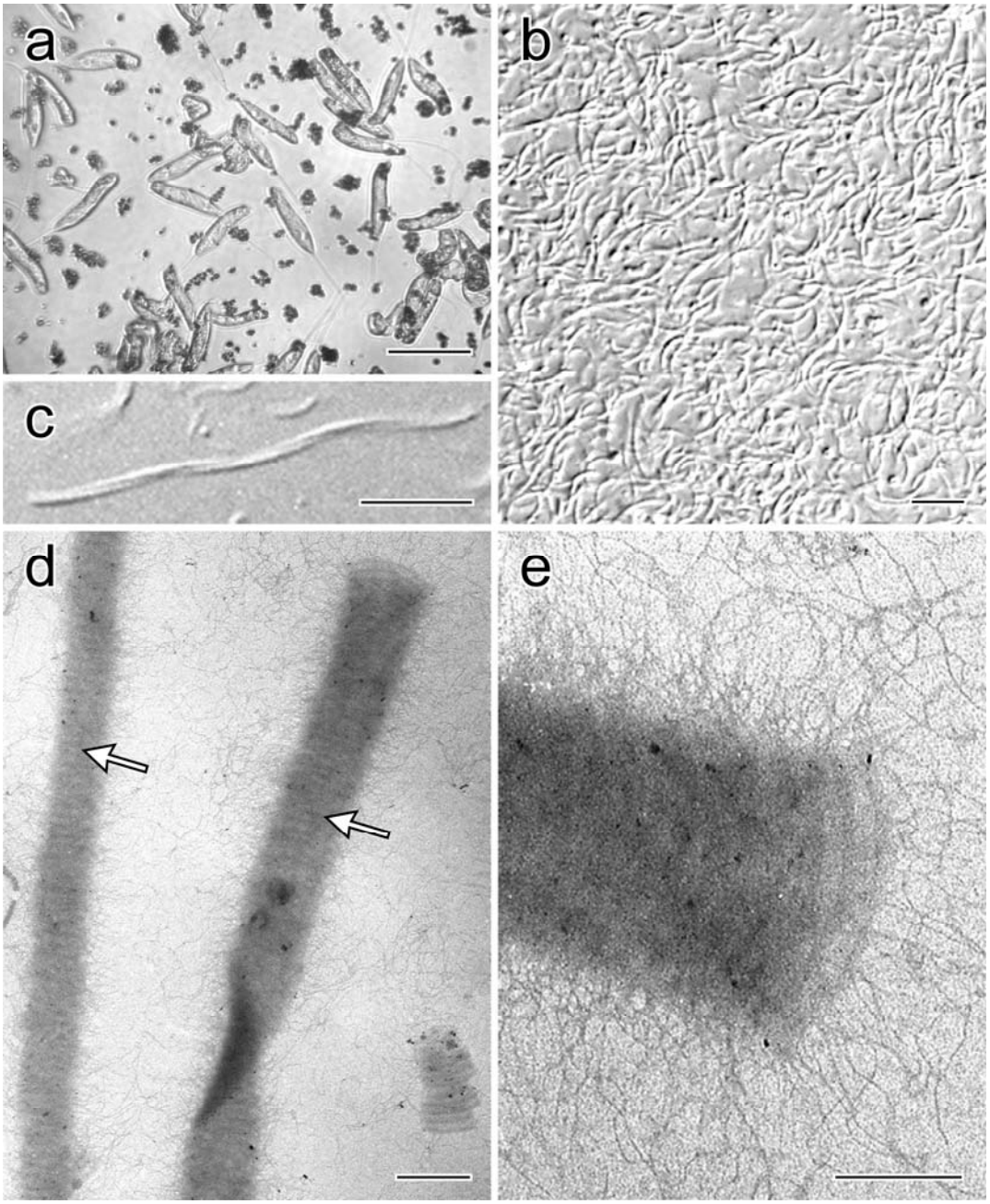


Fig. 6. Light and electron micrographs showing living *Peranema trichophorum* cells and isolated flagella. a: Gliding *P. trichophorum* on the bottom of a culture flask was easily observed with an inverted microscope. b and c: Low and high magnification light micrographs of flagella isolated by cold-shock treatment. Flagella retained their intact structure after isolation, and showed a characteristic twisted ribbon-like appearance. d and e: Transmission electron micrographs of isolated mastigonemes stained with uranyl acetate. Mastigoneme ribbons (arrows in d) were composed of many mastigonemes. A large number of mastigonemes terminate at the surface of the ribbon. Bars are 50 μm (a), 10 μm (b and c), 1 μm (d) and 500 nm (e).

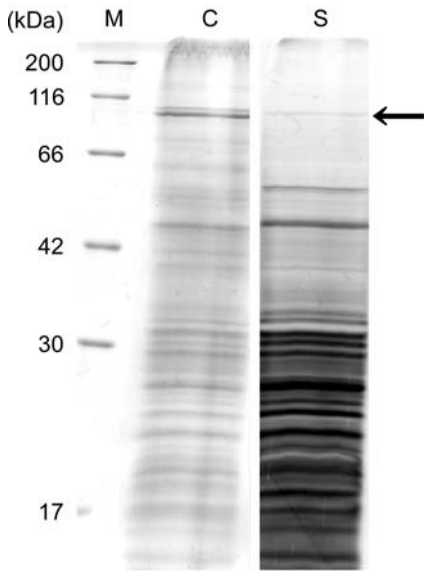


Fig. 7. SDS-PAGE analysis of isolated flagella of *P. trichophorum*. The gel was stained with CBB (lane C) and with silver (lane S). Molecular weights of marker proteins (lane M) are indicated at the left side of the figure. The arrow shows the protein band that is most heavily stained by CBB, but which is not stained by silver, indicating that this major component of flagella is highly glycosylated.

shows that isolated flagella contain a large number of constituent proteins and that the apparent molecular weight of the predominant protein is about 100 kDa (arrow). This protein was strongly stained by CBB but not by silver stain.

DISCUSSION

Recently, we showed that mastigonemes radiating from the surface of the anterior flagellum play an important role in the gliding motility of *Peranema trichophorum* (Saito et al., 2003), possibly providing the force for flagellar surface motility. To further characterize the function of the mastigoneme, biochemical and molecular biological approaches will be required. We have previously succeeded in producing bacteria-free cultures of *P. trichophorum* with a single species of food flagellate, *Chlorogonium elongatum*. However, this monoxenic culture method 1) took a long time (usually more than 3 weeks till the cell number reached a plateau), and 2) food flagellates always remained with *P. trichophorum* when the cells were harvested. In this study, we have tried to improve the culture of *P. trichophorum* and to establish a method for isolating flagella and mastigonemes.

We first tested the effect of shaking the culture flask on the growth rate of *P. trichophorum*. In many protozoan species, including *Euglena*, a genus closely related to *Peranema*, better cultivation has been achieved by shaking the culture flasks, which increases oxygen supply to the culture medium. However, contrary to our expectation, shaking of the culture flask was actually deleterious to growth of *P. trichophorum*, as shown in Fig. 1. *P. trichophorum* is a heterotrophic flagellate that glides on the bottom of the culture vessel and usually captures prey organisms using the anterior flagellum. It is probable that shaking of the culture flask disrupts cell gliding, so that cells cannot capture food organisms efficiently and cell growth is consequently suppressed. The fact that a broader-based culture flask was more favorable for the growth of *P. trichophorum* also indicates that gliding on the substratum is essential for *P. trichophorum* to capture food organisms.

In this study, it was shown that various culture conditions, such as the size of culture flask, the depth of the culture medium and the ratio of prey to predators influence the growth rate of *P. trichophorum*. The culture method we developed yields more cells in a shorter time than the previous procedure, and also eliminates contamination with the food flagellates.

Bouck and co-workers reported that, in *Euglena gracilis*, cold-shock treatment could induce flagellar detachment without any structural damage (Bouck et al., 1978). In other flagellates as well, cold-shock has been used as an effective method

that causes minimal damage to the flagellar structure (Ngô et al., 1995). We confirmed that cold treatment was also effective for isolating flagella in *P. trichophorum*. Furthermore, we compared slow and quick cold-shock treatments for flagellar isolation. As shown in Fig. 5, the slow treatment yielded more isolated flagella than the quick one, and repetition of the cold treatment increased the yield without damage to the flagellar structure. Using this slow cold treatment procedure, biochemical and physiological approaches may be possible in the future to identify molecular components that are essential for gliding motility in *P. trichophorum*.

SDS-PAGE analysis showed that the isolated flagella still contain a large number of proteins and that the molecular weight of the predominant protein is 100 kDa. This protein may be highly glycosylated as judged by its appearance in the silver stained gel (Fig. 7). Electron microscopy, in Fig. 6, showed that mastigonemes were abundant around the flagellum, and were bundled into mastigoneme ribbons (Hilenski et al., 1985). The 100-kDa protein is a candidate for the main component of the mastigoneme. In *E. gracilis*, a 200-kDa glycoprotein has been identified as the major mastigonemal component (Rogalski et al., 1982). The 100-kDa protein of *P. trichophorum* could be a counterpart of the major mastigoneme glycoprotein of *E. gracilis*. Purification and characterization of the 100-kDa protein are expected to lead to better understanding of the role of mastigonemes in gliding motility in *P. trichophorum*.

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REFERENCES

Bouck, G. B., Rogalski, A. and Valaitis, A. (1978) Surface organization and composition of *Euglena*. II. Flagellar Mastigonemes. J. Cell Biol. 77, 805-826.

Chang, S. L. (1966) Observations on the organelles, movement, and feeding of *Peranema trichophorum*. Trans. Am. Microsc. Soc. 85, 29-45.

Chen, Y. T. (1950) Investigations of biology of *Peranema trichophorum*. Q. J. Microscop. Soc. 91, 279-308.

Hilenski, L. L. and Walne, P. L. (1985) Ultrastructure of the flagella of colorless phagotroph *Peranema trichophorum*. I. Flagellar mastigonemes. J. Phycol. 21, 114-125.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Ngô, H. M. and Bouck, G. B. (1995) Isolation of *Euglena* flagella. Methods Cell Biol. 47, 25-29.

Rogalski, A. A. and Bouck, G. B. (1982) Flagellar surface antigens in *Euglena*: Immunological evidence for an external glycoprotein pool and its transfer to the regenerating flagellum. J. Cell Biol. 93, 758-766.

Saito, A., Suetomo, Y., Arikawa, M., Monguchi, T., Omura, G., Khan, S. M. M. K., Kakuta, S., and Suzaki, T. (2003) Gliding movement in *Peranema trichophorum* is powered by rapid flagellar surface motility. Cell Motil. Cytoskel. 55, 244-253.

Triemer, R. E. (1997) Feeding in *Peranema trichophorum* revisited. J. Phycol. 33, 649-654.