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Short Communication

High-Density Cultivation of the Marine Ciliate *Uronema marinum* (Ciliophora, Oligohymenophorea) in Axenic Medium

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Abstract. *Uronema marinum* is a cosmopolitan marine ciliate. It is a facultative parasite and the main causative agent of outbreaks of scuticociliatosis in aquaculture fish. This study reports a method for the axenic cultivation of *U. marinum* in high densities in an artificial medium comprising proteose peptone, glucose and yeast extract powder as its basic components. The absence of bacteria in the cultures was confirmed by fluorescence microscopy of DAPI-stained samples and the failure to recover bacterial SSU-rDNA using standard PCR methods. Using this axenic medium, a maximum cell density of 420,000 ciliate cells/ml was achieved, which is significantly higher than in cultures using living bacteria as food or in other axenic media reported previously. This method for high-density axenic cultivation of *U. marinum* should facilitate future research on this economically important facultative fish parasite.

Key words: Axenic cultivation, ciliates, fish parasite, scuticociliatosis, *Uronema marinum.*

INTRODUCTION

Axenic cultures of ciliates have proved to be extremely valuable in a wide range of research fields including growth, nutrition, respiration, genetics, facultative parasitism, and molecular biology. The lack of efficient, cost-effective methods for axenic cultivation is a major constraint for research on most ciliates (Soldo and Merlin 1972). Nevertheless comparatively few ciliate species, mostly from fresh-waters, have been cultured axenically. The peniculine species *Paramecium caudatum* was the first ciliate to be successfully cultivated axenically and was grown in a medium containing dead yeast cells, liver extract and kidney tissue (Glaser *et al.* 1933). Pure cultures of strains belonging to the "*Colpidium-Glaucoma-Leucophrys-Tetrahymena* group" were subsequently established (Corliss 1952). In each case the main nutritional sources were proteose peptone and yeast extract. In such cultures, the cell densities of *Tetrahymena* and *Paramecium* can reach 75 cells/µl and 38 cells/µl, respectively (Martin *et al.* 1976, Thiele *et al.* 1980). The axenic cultivation of marine ciliates is far less common and is largely restricted to philasterid scuticociliates such as *Uronema nigricans, Parauronema virginiatum* and *Miamiensis avidus*. In these cultures, maximum populations are usually obtained after 4–5 days and range from several hundred to 3 or 4×10^3 cells/µl (Soldo *et al.* 1972, Lee *et al.* 2003).

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Scuticociliates are generally small in size and characterized by the presence of a scutica, which is a transient kinetosomal structure located near and slightly to the right of the posterior end of the paroral in both the proter and opisthe during late stomatogenesis (Foissner and Wilbert 1981, Wang *et al.* 2009). Many scuticociliates are pathogenic and commonly found in the haemolymph or tissues of infected aquacultured fish and certain invertebrates. They display high potential for systemic invasion and destruction of host tissues, resulting in a parasitological condition called "scuticociliatosis" (Jee *et al.* 2001, Azad *et al.* 2007, Jin *et al.* 2009). *Uronema marinum* Dujardin, 1841 is a cosmopolitan, facultatively parasitic scuticociliate and the main causative agent of outbreaks of scuticociliatosis in aquaculture fish (Jee *et al.* 2001). When free-living, *U. marinum* can be found in a wide range of marine habitats, but occurs most commonly in the sediment of eutrophic waters (Gast 1985, Song *et al.* 2009). Alternatively it can occur as an endoparasite of fishes and has often been associated with mortalities in cultured fish such as flounder, sea bass and turbot resulting significant economic loss to the aquaculture industry worldwide (Jee *et al.* 2001).

When free-living under natural conditions, *Uronema marinum* feeds on bacteria, typically those associated with decomposing seaweed. It can readily adapt to cultivation in the laboratory with bacteria as a food source (Parker 1979, Kim *et al.* 2004). This method, however, limits the utility of the culture for certain investigations because it is expensive to remove the bacteria and ciliate cell densities are relatively low. In order to obtain high-density pure cultures of *U. marinum*, axenic cultivation is necessary. *U. marinum* has previously been successfully cultured under axenic conditions using a chemically defined medium or commercial cell culture medium, such as MEM (Eagle's minimum essential medium) and Leibovitz L-15 medium. However, the components of such media are rather complex and high in price (Hanna and Lilly 1974, Kwon *et al.* 2003, Lee *et al.* 2003, Anderson *et al.* 2009).

A population of *Uronema marinum* was isolated from the Yellow Sea coastal waters at Qingdao, China, giving the opportunity to investigate different methods of its cultivation in the laboratory. The main aim of the present study was to establish a method for cultivating *U. marinum* whereby it could be grown axenically in high densities in a low-cost, chemically defined medium.

MATERIALS AND METHODS

Organisms

Uronema marinum was collected using the Polyurethane Foam Unit (PFU) method from coastal waters in a harbor of Qingdao (36°05′N, 120°33′E), China in 2011. The PFU were used as artificial substrates to collect ciliates and were retrieved after being immersed in seawater for about two weeks. Samples were squeezed out from the PFU and transferred into Petri dishes with seawater from the sampling site. Isolated specimens were maintained in the laboratory in Petri dishes in filtered, autoclaved seawater, at a temperature of ca. 14°C, with rice grains added to promote the growth of bacteria as food for the ciliate. *Uronema marinum* was identified by Dr. Xuming Pan, Ocean University of China, on the basis of morphological characters observed both *in vivo* and after protargol staining (Fig. 1A–D) according to (Pan *et al.* 2010).

Composition and preparation of the culturing medium

The composition of the growth medium (hereinafter referred to as "PGY medium") is: proteose peptone, 1.5 g; glucose, 0.5 g; yeast extract powder, 0.5 g; seawater filtered through a 0.22 μm filter membrane, 100 ml. The medium was adjusted to pH 8.0 with saturated sodium hydroxide, and autoclaved at 120°C for 20 min. The sterilized PGY medium is orange in colour. All reagents used in the PGY medium were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

Determination of optimal concentration of antibiotics

In order to determine the optimal concentration of antibiotics required to achieve bacterial sterility, 5 ml PGY medium was added to each well of 15 ml six-well plates. Each well was inoculated with *U. marinum* by adding a 50 μl aliquot of bacterized filtered seawater containing about 2000 ciliate cells. A combination of penicillin-streptomycin (penicillin 6 mg/ml and streptomycin 10 mg/ml, Sangon Biotech Co., Ltd., Shanghai, China, cat. no. BS7011) was added to each of 5 wells to give the following final concentrations of penicillin and streptomycin respectively (mg/ml): (0.015, 0.025), (0.030, 0.050), (0.045, 0.075), (0.060, 0.100), and (0.075, 0.125). The 6th well (control) received no antibiotics. Two replicates of this antibiotic experiment were conducted. The concentration of antibiotics was considered to be effective if the medium was free of bacteria and remained limpid for at least two days after inoculation. The minimum concentration of penicillin-streptomycin to maintain axenic conditions in the culture medium was found to be penicillin 0.06 mg/ml and streptomycin 0.1 mg/ml.

Axenic cultivation

For convenience of observation, a 260 ml cell culture flask (Nunc, Denmark, cat. no. 156800) was used to cultivate *U. marinum* axenically. In order to guarantee the axenic condition of medium, inoculation was performed using aseptic inoculation technique. A 500 μl aliquot of penicillin-streptomycin solution was added to 50 ml PGY medium, giving the final concentration of penicillin 0.06 mg/ml and streptomycin 0.1 mg/ml. A 50 μl aliquot of bac-

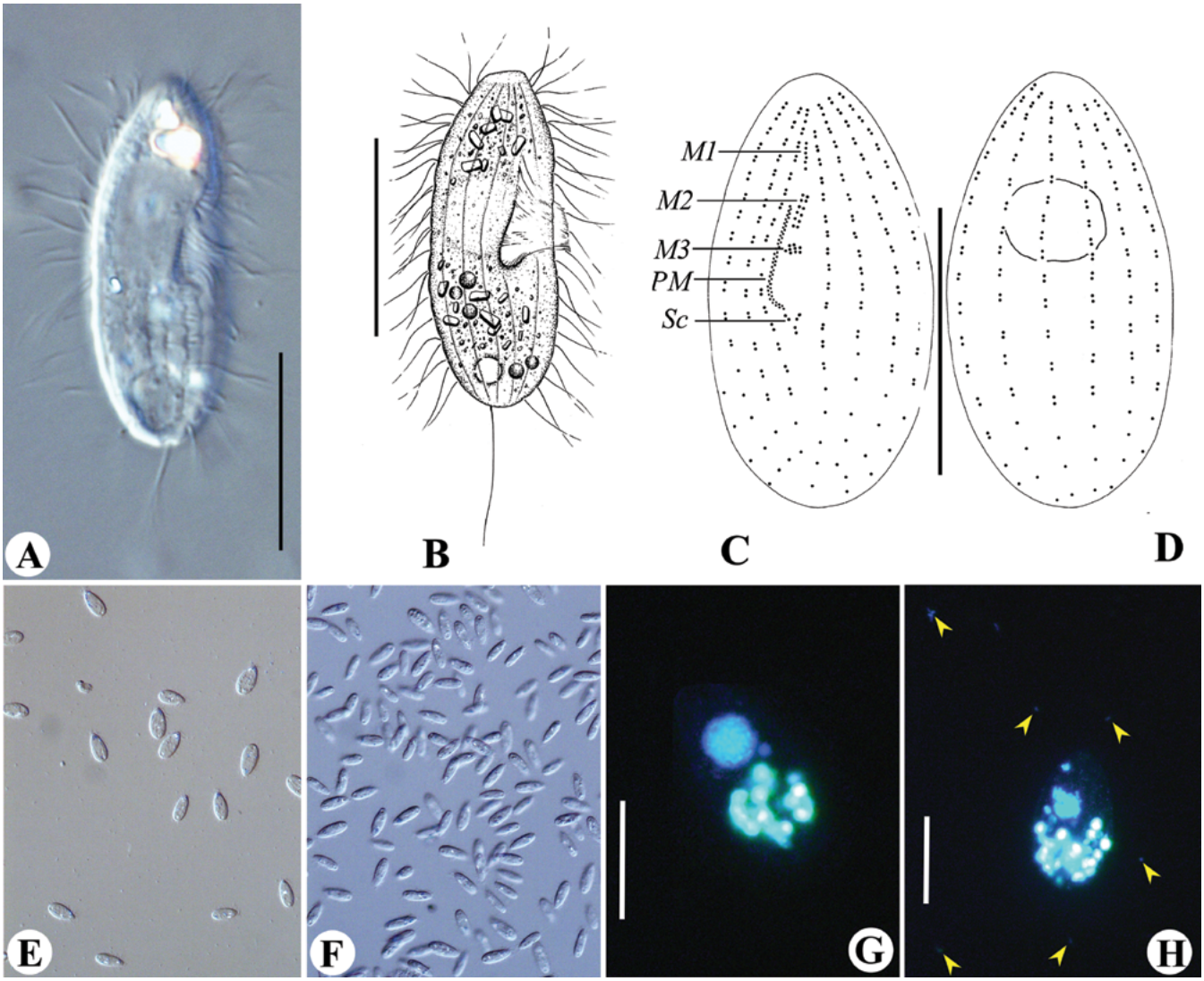

Fig. 1. *Uronema marinum* from life (A, B, E, F), after protargol (C, D) and DAPI-staining (G, H). **A, B** – lateral-ventral view of typical cell (B, from Pan *et al.* 2010); **C, D** – ventral and dorsal view of the same specimen (from Pan *et al.* 2010); **E** – 72 hours after inoculating into PGY medium; **F** – 168 hours after inoculating into PGY medium; **G** – *Uronema marinum* in axenic culture, demonstrating the absence of bacteria; **H** – *Uronema marinum* in bacterized filtered seawater cultivating system, arrowheads indicate bacteria that are active in the real-time viewing conditions; M1–3 – membranelles 1–3, PM – paroral membrane, Sc – scutica. Scale bars: 20 μm.

terized filtered seawater containing about 250 *U. marinum* individuals was inoculated into the medium. The flask was incubated at 25°C in the dark. The culture was observed every 24 hours with stereomicroscope.

Detection of the presence of bacteria

Both fluorescence microscopy and PCR were used to check for the presence of bacteria in the axenic cultures with three replicates when the axenic cultivating system was established. The bacterized filtered seawater culture system served as the positive control.

For fluorescence microscopy observations, DAPI stain was added to a sample of culture to give a final concentration of 10 μg/ml of DAPI (4'-6-diamidine-2-phenylindole, Sangon Biotech Co., Ltd., Shanghai, China, cat. no. D6584). Staining was carried out at room temperature for 30 min according to the manufacturer's protocol. Organisms were observed with a fluorescence microscope (DMR Leica Microsystems, Mannheim, Germany). Fluorochromes were visualized with an excitation wavelength of 350 nm.

For the PCR method, the bacterial small subunit ribosomal RNA gene (SSU-rDNA) was used as the target to detect the pres-

ence of bacteria. PCR amplifications were performed using Ex Taq Polymerase (Takara Biotechnology Co., Ltd, Dalian, China, cat. no. RR902Q). The primers and PCR conditions are as described by (Osborne *et al.* 2005). The PCR products were screened on 1% agarose gel.

Growth curves of *U. marinum* cultivated in axenic PGY medium and bacterized filtered seawater

In order to compare the efficacy of the two methods of cultivating *U. marinum*, second round of parallel cultures were set up in 260 ml cell culture flasks, i.e. one containing 50 ml PGY medium and the other 50 ml bacterized filtered seawater. Two replicates were set for each cultivating system. Each flask was inoculated with 8000 cells of *U. marinum*. The density of cells was measured using improved Neubauer hemocytometer every 12 hours after inoculation for a period of 8 days. On each occasion two samples per flask were counted, and the mean of the four samples was calculated.

RESULTS AND DISCUSSION

Establishment of axenic cultivating system in PGY medium

Observation by light microscopy of living ciliate cells immediately after inoculation into PGY medium revealed *U. marinum* to behave normally and did not display any visible morphological abnormalities when compared to cells grown in bacterized filtered seawater (Fig. 1E, F). Subsequent observations performed every 24 hours over the following eight days likewise revealed no observable abnormalities in form or behaviour. Proliferation of ciliates grown in the PGY medium was detected after 72 hours (Fig. 1E), and the density of cells kept increasing thereafter. After seven days the ciliate density was 375 cells/µl (Fig. 1F).

After the establishment of axenic cultivating system, both culture systems were checked for the presence of bacteria using fluorescence microscopy and PCR with three replicates. Bacteria were not detected in the PGY cultivation system whereas large numbers of bacteria were found in the bacterized filtered seawater cultivation system that served as the positive control (Figs 1G, H, 2). These results indicate that the PGY culture was axenic.

The axenic strain of *U. marinum* (strain no. QDSW2011) is maintained in the laboratory at the Ocean University of China, Qingdao, at ca. 14°C. Cells are transferred to fresh media every month. After one year in axenic culture, the growth status of strain QDSW2011 remains stable. A subculture of this strain has been deposited in the Culture Centre of Algae and Protozoa, SAMS, Dunstaffnage, UK (http://www.ccap.ac.uk/).

Growth curves of *U. marinum* cultivated in PGY medium and in bacterized filtered seawater

In order to compare the cell densities of *U. marinum* grown axenically in PGY medium vs in bacterized filtered seawater, its growth curve in each cultivation system was drawn (Fig. 3). In bacterized filtered seawater, *U. marinum* reached maximum density (18 cells/µl) after 36 hours and remained at about this density throughout the rest of the experiment. By contrast, *U. marinum* in PGY medium grew more slowly in the first 48 hours but more rapidly thereafter reaching a density of 419 cells/µl by the end of the experiment, which is about 40 times of the density in bacterized filtered seawater (11 cells/µl) after the same period of cultivation.

The low density of ciliate cells in bacterized filtered seawater was probably due to competition for food between *U. marinum* and the bacteria. It has long been known that the growth rate of bacterivorous ciliates is dependent on both bacterial and ciliate concentrations (Cutler and Crump 1924): if the ratio of bacteria:ciliates is either too low or too high, ciliate growth will be restricted. By contrast, food is not limiting in our axenic cultivation system so the ciliate can grow to a much

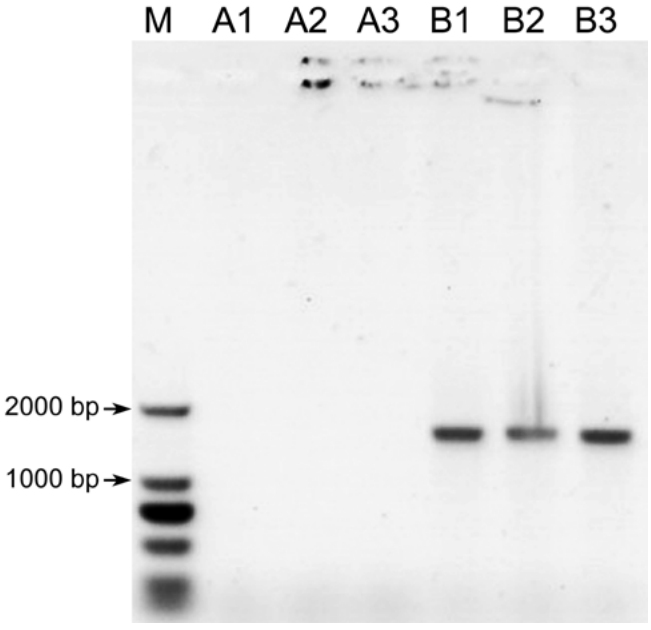

Fig. 2. PCR amplification of the bacterial SSU-rDNA on 1% agarose gel. A1, A2, and A3 are parallel samples extracted from the axenic culture in PGY medium. B1, B2, and B3 are parallel samples extracted from the culture in bacterized filtered seawater. M – DNA ladder.

higher density. It is noteworthy that there are also differences in the cell densities of *U. marinum* that have been achieved among different axenic media. Previous investigations have reported maximum cell densities ranging from 100 to 350 cells/µl (Hanna *et al.* 1974, Kwon *et al.* 2003, Lee *et al.* 2003, Anderson *et al.* 2009), whereas in the present study a cell density of 419 cells/µl was achieved. Possible explanations include the use of different strains of *U. marinum* in each study or differences in the nutrients present in the culture media.

When *U. marinum* was first inoculated into PGY medium there was a lag phase of about 3 days when there was no or very limited cell proliferation. This suggests that the ciliate might have been adapting to its new growth medium as it switched from a particulate, bacterial food source to a dissolved organic one. Such a mechanism of adaptation is likely to have evolved in *U. marinum* due to its lifestyle, e.g. it can be either a free-living phagotroph or an endoparasitic osmotroph. Our attempts to axenically cultivate the entirely free-living phagotrophic ciliates *Euplotes vannus* and *Uroleptopsis citrina* have so far failed, suggesting that these taxa probably lack the osmotrophic feeding mechanism present in the facultatively endoparasitic *U. marinum*.

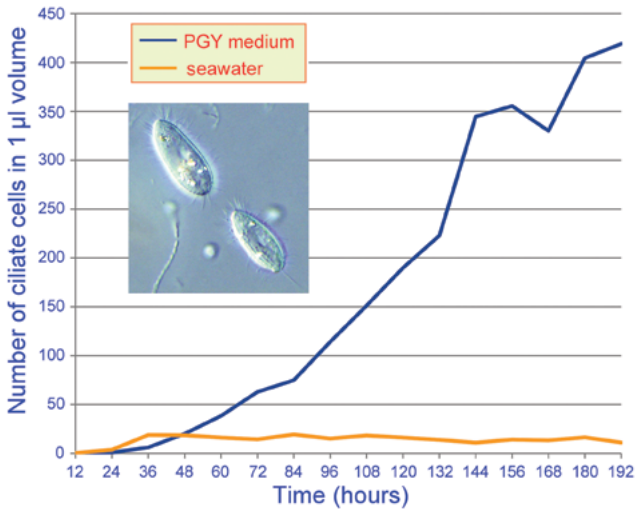

Fig. 3. Growth chart of *U. marinum* in PGY medium and bacterized filtered seawater. The density of ciliate cells was measured every 12 hours after inoculation using a hemocytometer. The final data points are 419 cells/µl in PGY medium and 11 cells/µl in bacterized filtered seawater.

Axenic cultivation of *U. marinum* was firstly achieved in a medium containing yeast supernatant, dextrose, proteose peptone and homogenate of the sea lettuce *Ulva* (Hanna *et al.* 1970). *Uronema marinum* has also been cultured under axenic conditions in chemically defined media made from commercial products (Hanna *et al.* 1974, Kwon *et al.* 2003, Lee *et al.* 2003, Anderson *et al.* 2009). The PGY medium in our experiment is a modified form of the medium used for cultivating the freshwater hymenostome ciliate *Tetrahymena pyriformis* (Curds *et al.* 1968). In our case, we substituted filtered seawater for freshwater and adjusted the pH to 8.0. Compared with the more complex media used previously for the axenic cultivation of *U. marinum* (Hanna *et al.* 1974, Kwon *et al.* 2003, Lee *et al.* 2003, Anderson *et al.* 2009), the PGY medium is easier to prepare, less expensive, and more effective in terms of the cell densities achieved.

In summary, an axenic strain of *U. marinum* (QDSW2011) was successfully cultivated in a simple artificial medium and grew to very high densities. With this axenic strain, large numbers of *U. marinum* cells can be harvested in a short time for use in a wide range of research fields such as genomics, infection experiments, disease pathology and the testing of chemical and physical therapies for disease treatment and parasite control.

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