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Growth-Inhibitory Activities in *Tetrahymena* Culture Medium of the Stationary Phase

KAZU-MICHI SUZUKI, NAOHISA NISHIHARA, TADAO TAKAHASHI, TOSHIKAZU KOSAKA AND HIROSHI HOSOYA

Department of Biological Science, Faculty of Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739, Japan

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ABSTRACT

Culture medium in the stationary phase of *Tetrahymena pyriformis* (strain W) had activities to reduce the growth rate of the ciliate markedly, while the culture medium in the logarithmic phase had no growth-inhibitory activities. These activities were heat stable and reduced by dialysis against a fresh culture medium. When the pH of the culture medium in the stationary phase (pH 7.4) was changed to that of fresh culture medium (pH 6.5), the growth rate of the ciliate was recovered. Further, when the pH of the fresh culture medium (pH 6.5) was changed to that of the culture medium in the stationary phase (pH 7.4), the growth rate of the ciliate was reduced. These results suggest that the pH in the culture medium has very important roles for cell growth.

INTRODUCTION

Tetrahymena cells inoculated in a fresh culture medium at a low cell density grow logarithmically for 1-2 days and then stop their growing. To elucidate mechanisms of the growth regulation of *Tetrahymena*, effects of a variety of environmental factors such as nutrition (Cameron 1973; Prescott 1957; Hofmann and Cleffmann 1981), metabolic products (Hofmann and Cleffmann 1981; Larsen et al. 1988; Gillies and Deamer 1980) and cell to cell collisions (Saitoh and Asai 1982) have been reported. Furthermore, recent studies have suggested the presence of compounds that *Tetrahymena* cells produce and release to promote the cell growth of *Tetrahymena* cells (Christensen and Rasmussen 1992) and

Paramecium cells (Tokusumi et al. 1996), and to arrest the cell cycle of *Tetrahymena* cells (Murata-Hori and Fujishima, 1996). However, the mechanisms in regulating the growth of *Tetrahymena* by such compounds are presently unknown.

During the growth of *Tetrahymena*, alterations in the culture medium occur. For example, it is well known that the culture medium in the stationary phase has an increased pH (Larsen et al. 1988). Thus, we examined the effects of pH on the growth of *Tetrahymena* as a responsible factor for cessation of cell growth. Here, we found that the culture medium in the stationary phase had activities to reduce the growth rate of *Tetrahymena* markedly. These growth-inhibitory activities were due to the increased pH of the culture medium in the stationary phase. This report suggests that the pH in the culture medium is very important for cell growth.

MATERIALS AND METHODS

Cell Culture

The ciliate *Tetrahymena pyriformis* strain W was cultured in a PY medium consisting of 1% (w/v) proteose peptone (Difco Labs., Michigan, USA) and 0.5% (w/v) yeast extract (Oriental Yeast Co. Ltd., Tokyo, Japan) (Hosoya et al. 1995). Logarithmically growing cells were inoculated at an initial density of 25 cells per ml and kept at 26°C without shaking. Conditioned medium in the stationary phase (4 days after start of cell culture) was prepared as described previously (Suzuki et al. 1997).

Assay for Growth-Inhibitory Activities in the Culture Medium of the Stationary Phase

Tetrahymena cells were inoculated at an initial density of 25 cells per ml and incubated at 26°C in 10-ml Erlenmyer flasks containing 2 ml of a fresh PY medium, which is diluted with the conditioned medium in various ratios. Cell density was measured once a day as described previously (Suzuki et al. 1997).

Characterization of the Growth-Inhibitory Activities in the Culture Medium of Stationary Phase

To determine whether the growth-inhibitory activities in the culture medium of stationary phase were inactivated by heating, the conditioned medium in the stationary phase was boiled for 10 min at 98°C. To examine the effect of dialysis, another conditioned medium was dialyzed against the fresh culture medium overnight at 4°C. After sterilization by filtration through a MILLEX-GS filter (0.22 μm; Millipore Co. Ltd., USA), these two media were assayed for their

growth-inhibitory activities as described above.

Effects of pH on the Cell Growth

To examine the effects of pH on the cell growth, pH of the conditioned medium or the fresh culture medium was adjusted with 50 mM 2-(*N*-morphorino) ethane sulfonic acid (MES) or *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (HEPES), respectively. After sterilization by filtration through a MILLEX-GS filter, these media were assayed for their growth-inhibitory activities as described above.

RESULTS AND DISCUSSION

Growth-Inhibitory Activities in the Culture Medium of the Stationary Phase

The effects of the conditioned medium in the stationary phase on the growth of *Tetrahymena* were examined. Cells were cultured in the fresh culture medium which was diluted with the conditioned medium in various ratios. When the ratio of the conditioned medium was raised from 75 to 100%, the growth rate was markedly reduced in a dose-dependent manner (Fig. 1A). The conditioned medium in the logarithmic phase did not have these growth-inhibitory activities (data not shown).

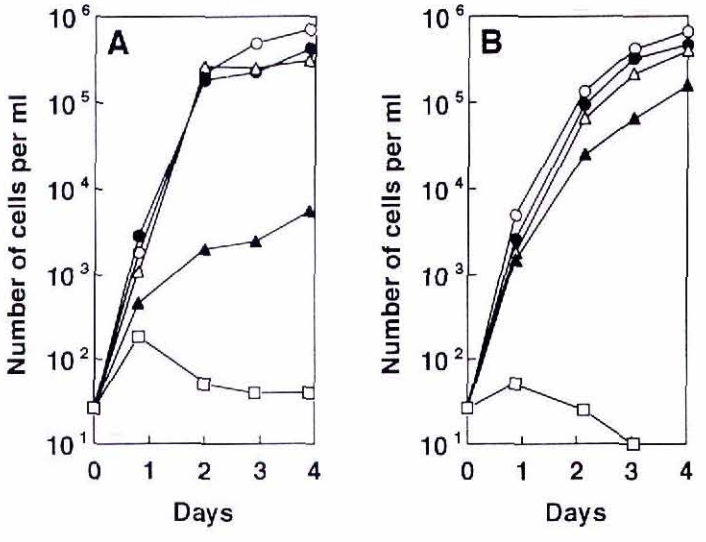

Fig. 1. Growth-inhibitory activities in the culture medium of the stationary phase. Cells were cultured in the fresh culture medium, which was diluted with the conditioned medium in the stationary phase (A) or distilled water (B) in various ratios. The ratios of the conditioned medium or distilled water were 0% (○), 25% (●), 50% (△), 75% (▲) and 100% (□), respectively. Cell density was measured daily and the mean of three experiments was shown.

It is reported that the amount of nutrients decreases in the culture medium of stationary phase (Larsen et al. 1988). So it might be speculated that the cell growth is inhibited due to depletion of nutrients in the conditioned medium of the stationary phase. To ascertain this speculation, cells were cultured in the fresh culture medium which was diluted with distilled water in various ratios instead of the conditioned medium. When the ratio of distilled water was raised up to 75%, the growth rate was not so markedly decreased, although cells could not grow in the 100% distilled water (Fig. 1B). These results suggest that the growth-inhibitory activities in the culture medium of the stationary phase are not due to the depletion of nutrients in the conditioned medium.

The growth-inhibitory activities were not lost by heat treatment (data not shown), but were reduced by dialysis (Fig. 2), suggesting that the growth-inhibitory activities could be resistant to heating and were not composed of macromolecules.

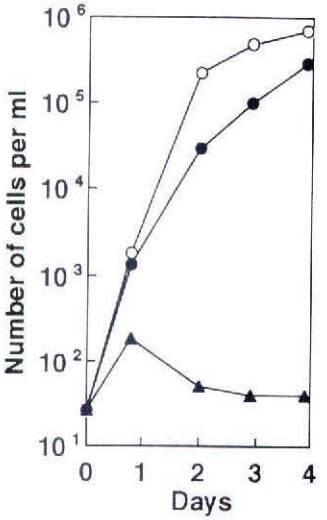

Fig. 2. Effects of dialysis on the growth-inhibitory activity in the culture medium of the stationary phase. Conditioned medium was dialyzed against fresh culture medium and incubated with cells (●). As controls, cells were cultured in the fresh culture medium (○) or conditioned medium (▲). Cell density was measured daily and the mean of three experiments was shown.

Effects of pH on the Cell Growth

As the pH of the culture medium increases during the cell growth in *Tetrahymena* culture (Fig. 3), the effects of pH on the cell growth were examined. Several studies have excluded the increased pH of the culture medium as a responsible factor for cessation of cell growth in the stationary phase (Hofmann and Cleffmann 1981; Larsen et al. 1988). Larsen et al. (1988) have adjusted initial pH of the culture medium using NaOH, and then tested the effects of an increased pH on the cell growth. Because the culture medium was not bufferized, however, it is not uncertain whether the initial pH was kept constant during cell growth. Then, we adjusted the pH of the culture medium using MES-NaOH or HEPES-

NaOH to minimize the variation of pH during the cell growth. When pH of the fresh culture medium (pH 6.5) was changed to that of the culture medium in the stationary phase (pH 7.4) using HEPES-NaOH, the growth rate of *Tetrahymena* was markedly reduced (Fig. 4A). Furthermore, pH of the conditioned medium in the stationary phase (pH 7.4) was changed to that of the fresh culture medium (pH 6.5) using MES-NaOH. As shown in Fig. 4B, the growth-inhibitory activities in the conditioned medium were reduced. These results suggest that most, but not all, of the growth-inhibitory activities are due to the increased pH of the culture medium in the stationary phase.

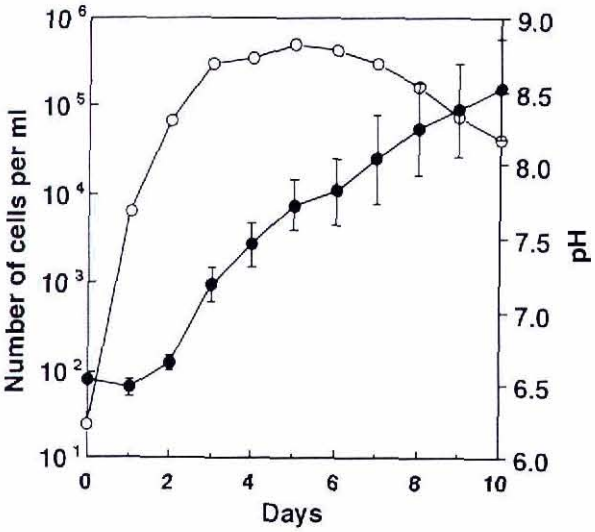

Fig. 3. Increase of pH in the culture medium during the cell growth. Cells were cultured in fresh culture medium and cell density (○) and pH of the culture medium (●) were measured daily. The mean of three experiments was shown, respectively. Vertical bars indicate the standard deviation of the mean.

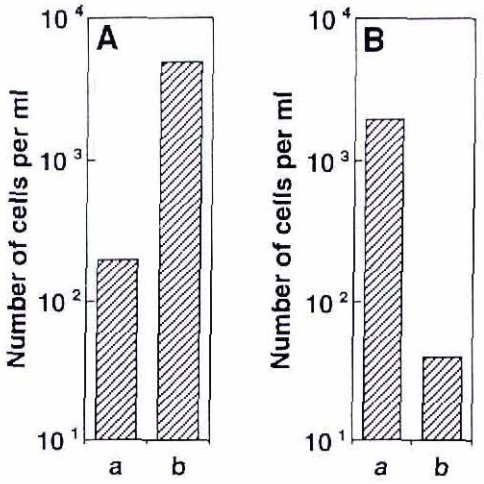

Fig. 4. Effects of pH on the cell growth. (A) pH of the fresh culture medium (pH 6.5) was changed to 7.4 (pH of the culture medium in the stationary phase) using HEPES-NaOH (a). As a control, pH of the fresh culture medium was adjusted to 6.5 using MES-NaOH (b). (B) pH of the conditioned medium in the stationary phase (pH 7.4) was changed to 6.5 (pH of the fresh culture medium) using MES-NaOH (a). As a control, pH of the conditioned medium was adjusted to 7.4 using HEPES-NaOH (b). Cells were cultured in these media for 96 h at 26 ℃ and then the cell density was measured. The mean of three experiments was shown, respectively.

Although pH of the culture medium is important for the cell growth, the growth-inhibitory activities were not lost completely by dialysis (Fig. 2) or pH adjustment (Fig. 4B). A key consideration in the interpretation of these findings is that the observed inhibitory activities in the conditioned medium of the stationary phase are composed of component other than pH itself. Previously, we have found that the protease activity in the culture medium of *Tetrahymena* increased markedly during the cell growth (Suzuki et al. 1997). The protease activity was partially purified, identified as a member of cysteine proteases, and designated as tetrain, a *Tetrahymena* cysteine protease (Suzuki et al. 1997). The enzyme tetrain, which has high protease activity at neutral to alkaline pH values, may have functional roles in the culture medium of the stationary phase in a pH dependent manner.

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