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Effect of Environmental Factors on Growth Characteristics of the Ciliate Protozoan *Aspidisca costata*

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Abstract Ciliate protozoan (Hymenostomatida) *Aspidisca costata* is one of the typical protozoa found frequently in activated sludge and bio-film from waste-water treatment processes. Effects of environmental factors on the growth characteristics of *A. costata* were investigated in this study. Using a bacterial strain of *Acinetobacter calcoaceticus* as a food source for *Aspidisca costata*, the effects of culture temperature, potential of hydrogen (pH), phosphate buffer solution (PBS) concentration, shake stress and food concentration on the monoxenic growth rate of *A. costata* were investigated. The optimum temperature for *A. costata* was 30℃. Activation energy estimated from the Arrhenius plot was determined as 76.8 $kJ \cdot mol^{-1}$. None of the PBS concentrations, pH conditions, bacterial concentrations were serious for the growth of *A. costata* within our observation ranges. The endurance of *A. costata* against shake stress was higher than those of Peritrichida, Rotatoria and Oligochaeta. *A. costata* was able to grow by feeding on 5 bacterial strains of the 11 strains investigated in this paper. The maximam specific growth rate (μ max) was 2.5 day^{-1}, and the Ks value estimated from Monod kinetics was 18 $mg \cdot l^{-1}$.

Key words: Protozoa, *Aspidisca costata*, *Aspidisca cicada*, growth characteristics, environmental factor.

Biological waste-water treatment processes generally utilize a complex ecosystem composed of bacteria, fungi, protozoa and small metazoa (Hynes, 1960; Bick, 1972; Curds *et al.*, 1975). In these processes, bacteria can degrade polluted organic materials to carbon dioxide and other inorganic small molecules (Sudo and Aiba, 1984). Protozoa and small metazoa graze the bacteria and, in turn, become prey for higher-order species in the food web such as fly larvae (Sudo and Aiba, 1984). Protozoa have been found in various types of biological waste-water treatment processes that were operated successfully (Ward, 1978; Brenda, 1984). There have been studies on about the role of protozoa in the waste-water treatment processes (Taylor and Berger, 1976; Güde, 1979; Curds, 1982; Liang *et al.*, 1982; Sinclair and Alexander, 1984). However, these experimental studies have been restricted to few species so far, and it remains unclear how they actually contribute to the waste-water treatment processes.

We focused on ciliate protozoan (Spirotrichea) *Aspidisca costata* Dujardin, 1842 (*Syn. Aspidisca cicada* Claparede & Lachmann, 1859). This species is common in various kinds of biological waste-water treatment facilities (Martin *et al.*, 1996; Lee *et al.*, 2004; Salvadó *et al.*, 2004). Furthermore, this genus has been taken up through physiological approaches (Banchetti *et al.*, 2003; Song, 2003), though the effects of environmental factors remain unsolved. In the present paper, we atteempt to clarify the effects of environmental factors, such as water temperature, pH, qualities and quantities of bacteria as food source and so forth, on the growth characteristics of ciliate protozoan *A. costata*.

Materials and Methods

1. Protozoa strain

Aspidisca costata (Dujardin, 1842) isolated from biofilm samples in a waste-water treatment plant, was used throughout this study. We picked it up by using a capillary pipette from biofilm samples, and rinsed it in phosphate buffer solution (PBS) repeatedly until other potozoa species are absent. Then the monoxenic *A. costata* strain was replanted with known bacterial strains in PBS where any bacteria cannot grow (Inamori *et al.*, 1990).

A. costata belongs to the phylum Ciliophora, class Spirotrichea, subclass Hypotrichia, order Euplotida, suborder Euplotina, family Aspidiscidae of Protozoa (Kahl, 1931). *Aspidisca cicada* is a synonym of *A. costata*. A body size of A. costata is about 20-25 μm in length and 20-25 μm in width. *A. costata* is colorless and resembles a bisected orange, with the cur flat surface being the ventral surface and the almost hemispheric scalloped (ridged) convex outline, the dorsal site, as described in Figure 1. *A. costata* preys bacteria and small fungi. *A. costata* propagates asexually by means of transverse division.

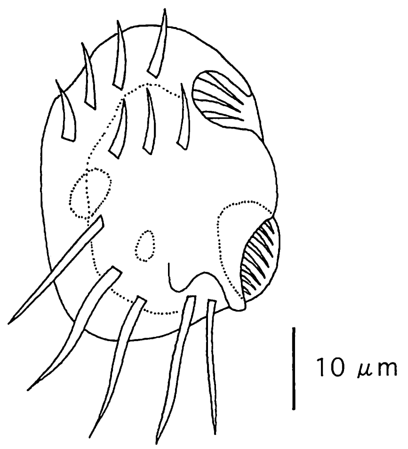

Fig. 1. Shape of Protozoa"*Aspidisca costata*".

2. Bacterial strains

The bacterial strains used as food source were obtained from the Institute of Molecular and Cellular Bioscience (IAM), University of Tokyo, Japan, and also from the Institute of Fermentation (IFO), Osaka, Japan. These strains of bacteria were isolated from biofilm and activated sludge samples from a wastewater treatment plant. The characteristics of these bacterial strains are shown in Table 1.

3. Experimental conditions

From preliminary growth experiments with propagate, we chose 1/750 M for PBS, 20℃ for temperature, and potential of hydrogen (pH) 6.5, as well as dark and static conditions. We fed *A. costata* with a complex of known-bacteria mixed resting cells for food, as the basic condition to estimate various kinds of environmental conditions : (1) water temperatures 5, 10, 15, 20, 25, 30, 35 and 40℃; (2) pH ranged form 4.2 to 9.7; (3) buffer concentration ranged from 0 to 40 mM; (4) bacterial species as food composed of 11 bacterial strains shown in Table 1; (5) bacterial food concentrations (initial bacterial concentrations) ranging from 5 to 5,000 mg$\cdot$l^{-1}; (6) shake stress ranging from 0 (static) to 0.9 G by a rotary and reciprocate shaker. The growth characteristics of *A. costata* were tested in 5.6 cm diameter petri dishes with initial 100 individuals triplicated by batch cultures.

4. Growth measurements

The growth of *A. costata* was monitored during the 6-day incubation period, and the specific growth rates in the logarithmic phase of growth were calculated using the following equation :

Table 1. Bacterial strains used as food for *Aspidisca costata*.

Strain	Strain Lot.*	Gram stain	Cell	Oxygen Demand	Characeristics
Pseudomonas putida	IAM1002	Negative	Rod	Aerobe	Glugonate oxidation
Bacillus cereus	IAM1029	Positive	Rod	Aerobe	Endospore, *Bacillus cereus* toxin
Bacillus subtilis	IAM1069	Positive	Rod	Aerobe	Endospore
Escherichia coli	IAM1239	Negative	Rod	Facultative anaerobe	No endospore
Acinetobacter calcoasceticus	IAM1517	Negative	Rod	Aerobe	No movement, Catalase positive, Oxidase negative
Micrococcus luteus	IAM1313	Positive	Coccus	Aerobe	Catalase positive, Salt tolerant, Dry tolerant
Klebsiera pneumoniae	IAM1102	Negative	Rod	Facultative anaerobe	No movement, No endospore
Achromobacer cycloclastus	IAM1013	Negative	Rod	Aerobe	Nitrogen fixing abilitiy
Flavobacterium luteus	IAM1667	Negative	Rod	Aerobe	Catalase positive, Oxidase positive
Flavobacterium suaveolens	IFO3752	Negative	Rod	Aerobe	Catalase positive, Oxidase positive
Streptococcus acidominius	—	Positive	Coccus	Facultative anaerobe	Catalase negative, Oxidase negative

* IAM : Institite of Molecular and Cellular Bioscience (Univ. of Tokyo).
IFO : Institute for Fermentation, Osaka. — : isoleted from biofilm.

$\mu = 2.303 \log (N - N_0) / (t - t_0)$

where μ = the specific growth rate (day^{-1}), N = the population number at time t (individuals ml^{-1}), and N_0 = the population number at time t_0 (individuals ml^{-1}).

Results and Discussion

1. Water temperature

Water temperature is an important environmental factor for the growth of protozoa (Sudo and Aiba, 1984). Figure 2 shows the effect of temperature on the growth of A. costata ranging from 5 to 40 ℃ using the Arrhenius plot (Hall, 1953). *A. costata* was acclimatized for each water temperature at every series of cultures. Figure 2 suggests that the optimum water temperature condition was 30℃, and the specific growth rate of *A. costata* at 30℃ was 2.6 $days^{-1}$. Activation energy (Sudo and Aiba, 1984) calculated from the linear relationship within the limits illustrated in Figure 2 is 52.4 $kJ \cdot mol^{-1}$. There are some alternateve approaches to determine activation energy. Although there is no space to describe every relevant thing about activation energy, we show a few typical examples on the species that are common in waste-water treatment processes. Activation energies of Protozoan ciliata *Vorticella microstoma* (Peritrichida) and *V. convallaria* were reported as 76. 8 $kJ \cdot mol^{-1}$ and 67.1 $kJ \cdot mol^{-1}$, respectively (Hayashi *et al.*, 1999). Similarly, the activation energy of Protozoan ciliata *Colpidium campylum* was reported as 76.8 $kJ \cdot mol^{-1}$ (Hayashi *et al.*, 2003). These data with a partial linear relationship to temperature in Fig. 2 suggest that the sensitivity of *A. costata* against water temperature is lower than those of Protozoan ciliata *Vorticella microstoma, V. convalla* (Peritrichida) and *Colpidium campylum* (Hymenostomatida). This means that *A. costata* can grow in waste-water treatment processes regardless of water temperature condition.

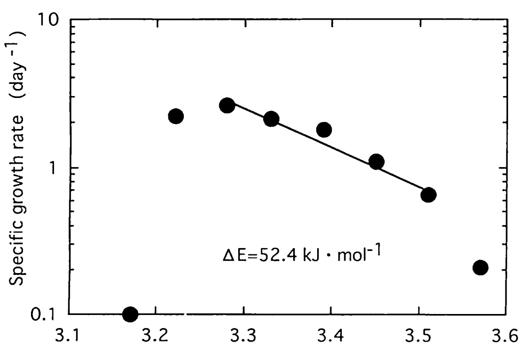

Fig. 2. Effect of temperature on the growth of *Aspidisca costata*

2. pH

Conditions of pH are also known as an important environmental factor for the growth characteristics of protozoa (Sudo and Aiba, 1984). Figure 3 shows the effect of pH on growth of *A. costata* with the initial pH ranging from 4.2 to 9.7. *A. costata* could grow in every initial pH condition within our observation range, where as the growth of protozoan ciliata *Vorticella microstoma* was restricted to the pH ranges under 5.2 and over 8.2 (Hayashi *et al.*, 1998). Concerning Oligochaeta, the growths of *Aeolosoma hemprichi, Pristina longiseta* and *Nais variabilis* were restricted to the range under pH 4.7 conditions (Kuniyasu *et al.*, 1997). On the other hand, the growth of Rotatoria *Lecane luna* requires over pH 9.2 conditions (Hayashi *et al.*, 1998). These results suggest that the pH condition is not an important factor for the growth of *A. costata*. *A. costata* can appear in waste-water treatment processes regardless of pH conditions.

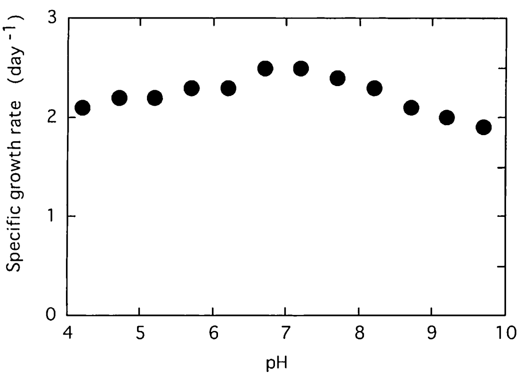

Fig. 3. Effect of initial pH condistion on the growth of *Aspidisca costata*.

3. Buffer concentration

Figure 4 shows the effect of buffer concentration on growth of *A. costata* ranging from 0 to 40 mM under pH 6.5 conditions. *A. costata* could grow in every condition of buffer concentration within our observation range. The growth of protozoan ciliata *Vorticella microstoma* was restricted to the ranges under 1.3×10^{-3}M and over 4×10^{-3}M (Hayashi *et al.*, 1999). Concerning Oligochaeta, the growths *of Aeolosoma hemprichi, Pristina longiseta* and *Nais variabilis* require over 1.3×10^{-2}M conditions (*A. hemprichi*) or over 4×10^{-3}M (*P. longiseta* and *N. variabilis*) (Kuniyasu *et al.*, 1997). On the other hand, Rotatoria *Philodina erythrophthalma, Rotaria rotatoria* and *Lecane luna* could grow in every condition as buffer concentration ranging

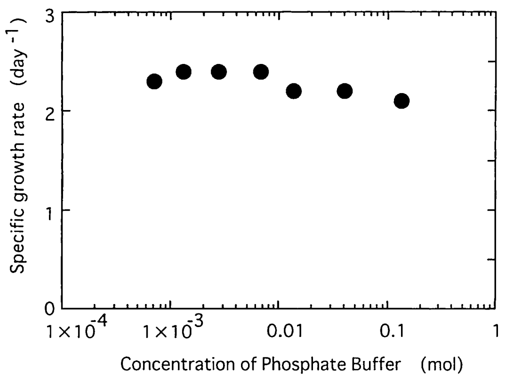

Fig. 4. Effect of phosphate buffer consentration on the growth of *Aspidisca costata*.

from 0 to 40 mM under pH 6.5 conditions (Hayashi *et al.*, 1998). These results suggest that the buffer concentration is not important for the growth of *A. costata*. Basically *A. costata* appears under high-loading conditions at waste-water treatment facilities (Curds *et al.*, 1975; Curds, 1982). These facts support our result showing high tolerance of *A. costata* against wide buffer concentration ranges.

4. Bacterial species as food

Aspidisca costata was conducted using various strains of bacteria as food source to test the prey specificity. *A. costata* could grow by feeding on the following five bacterial species : *Escherichia coli, Pseudomonas putida, Acinetobacter calcoasceticus, Klebsiera pneumoniae* and *Achromobacer cycloclastus*. The specific growth rate of *A. costata* with these bacterial strain was in the range from 0.3 to 2.5 day^{-1}. In case of the culture with *Acinetobacter calcoasceticus* and *Achromobacer cycloclastus*, *A. costata* recorded maximum specific growth rate as 2.5 day^{-1}. Other six bacterial strains of *Streptococcus acidominius, Micrococcus luteus, Bacillus cereus, Bacillus subtilis, Flabobacterium suaveolens* and *Flavobacterium luteus* could not become the food source for *A. costata*. These results indicate that *A. costata* has a specific preference on food. In case of other researches on Protozoa (Hayashi *et al.*, 2003), Oligochaeta (Kuniyasu *et al.*, 1997) or Rotatoria (Hayashi *et al.*, 1998), there has been the same food preference. We suggest some factors on bacteria such as bacterial size, bacterial shape, flocculate characteristics, and gram positive or negative, as a clue to elucidate the food preferences of Protozoa (Table 1).

A characteristic common to the two bacterial strains such as *Acinetobacter calcoasceticus* and *Achromobacer cycloclastus*, which become to good food for *A. costata*, is that they do not move themselves. We tried to give other bacterial strains under restricted movement conditions using ultrasonic pre-treatments. However, these experiments could not clarify the influence of bacterial movement on the predation of *A. costata*. Ultimately we could not clarify the preferences in bacterial food, requiring further researches on food preferences of many other protozoa and small metazoa as basic information. Especially the metabolic materials from bacterial strains need to be focused in next steps. This will allow to clarify why some bacteria do not meet *A. costata*'s taste.

5. Bacterial food concentration

Figure 5 shows the effect of bacterial food concentration on growth of *A. costata*. Initial bacterial concentrations ranged from 5 to 5,000 mg･l^{-1}. There are hyperbolas relations between the bacterial concentrations and the specific growth rate of *A. costata*. However, an increase in the bacterial concentration beyond a critical level resulted in a decrease in the specific growth rate, unlike the prediction by conventional Monod kinetics. We tried to apply the Monod kinetics to these hyperbolas relationships. Under the Monod-type saturation model, the maximum specific growth rate (μmax) and the saturation constant (Ks) were estimated from Lineweaver-Burk plots (Hayashi *et al.*, 1998) as follows :

μmax = 2.5 day^{-1}, Ks = 18 mg･l^{-1}

In the cases of Protozoan *Vorticella microstoma* and *V. convallaria*, their μmax and Ks values were reported as 2.3 day^{-1}, 2.9 day^{-1} (μmax) and 33 mg･l^{-1}, 38 mg･l^{-1} (Ks) respectively (Hayashi *et al.*, 1999). Similarly the μmax and Ks values of Protozoan *Colpidium campylum* have been reported as 2.6 day^{-1} and 20 mg･l^{-1}, respectively (Hayashi *et al.*, 2003). The Ks value of *A. costata* is clearly lower than those of *Vorticella microstoma* and *V. convallaria*, and slightly

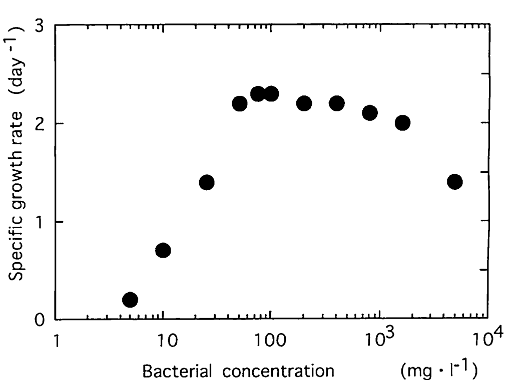

Fig. 5. Effect of bacterial food consentrations on the growth of *Aspidisca costata*.

higher than *C. campylum* in Ks values. This means that the affinity of *A. costata* for bacteria is lower than these other protozoans, which can be explained by grazing impacts. Namely *A. costata* graze while moving itself, whereas *Vorticella microstoma* and *V. convallaria* draw food without moving itself using their cilia around the corona. In the case of Rotatoria *Rotaria rotatoria* and *Lecane luna*, their μ max and Ks values have been reported as 0.50 day^{-1}, 0.19 day^{-1} (μ max) and 27 $mg \cdot l^{-1}$, 23 $mg \cdot l^{-1}$ (Ks), respectively (Hayashi *et al.*, 1998). These results support the above discussion, because *Lecane luna* grazes while swimming, similarly to *A. costata*. On the other hand, other Rotatoria taxa have two corona for moving and feeding. This is a reason why the Rotatoria achieves high Ks values. Protozoan *Colpidium campylum* recorded a low Ks value as 18 $mg \cdot l^{-1}$ because of the same reason. Therefore, the bacterial food concentration is an important factor for the growth of *A. costata*.

6. Shake stress

Both rotary and reciprocate shake intensities are converted to the maximum acceleration. Figure 6 shows the relationship between the shake stress as the maximum acceleration and the specific growth rate of *A. costata*. The maximum acceleration exceeding 0.25 G clearly influenced the specific growth rate of *A. costata*. In the case of *Colpidium campylum*, the specific growth rate were not influenced throughout the range from 0 to 0.94 G. On the other hand, *Vorticella microstoma* and *V. convallaria*, show clear decreases in their specific growth rates 0.3 G (Hayashi *et al.*, 1999). Furthermore, growth activities were perfectly blocked in the range over 0.6 G (*V. convallaria*) and 0.94 G (*V. microstoma*) shake stress conditions (Hayashi *et al.*, 1999). Similarly, the growth activities of Oligochaeta *Pristina longiseta* and *Nais variabilis* were perfectly blocked under 0.94 G shake stress conditions (Kuniyasu *et al.*, 1997). These results suggest that *A. costata* is sensitive to shake stresses as an environmental factor. Practically, *A. costata* is common in biofilm or activated sludge processes despite the presence of shake stress. These practical habitats is constructed with many colonized bacterial flocs and other protozoan communities. Meanwhile we tested the effect of shake stress in batch monoxenic culture conditions. This is a reason why *A. costata* can appear in full-scale waste-water treatment processes regardless of the shake stress conditions.

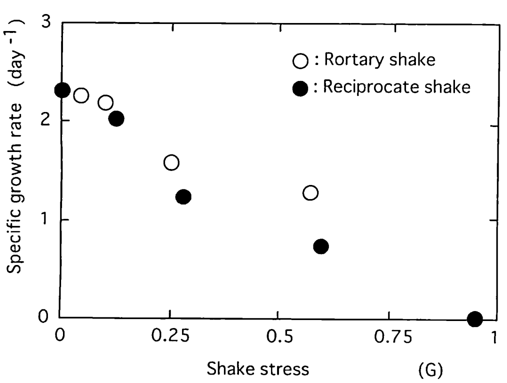

Fig. 6. Effect of shake stress on the growth of *Aspidisca costata*.

Conclusion

The effects of environmental factors on growth characteristics of ciliate protozoan *Aspidisca costata* were investigated. Results are summarized as follows; (1) *A. costata* is able to grow in a wide waste-water temperature range from 5 to 40℃. (2) The buffer concentration and pH conditions are not an important factors for the growth of *A. costata*. (3) *A. costata* could grow by feeding on 5 bacterial species, such as *Escherichia coli*, *Pseudomonas putida*, *Acinetobacter calcoasceticus*, *Klebsiera pneumoniae* and *Achromobacer cycloclastus*, from the 11 bacterial strains tested in this paper. Especially *Acinetobacter calcoasceticus* and *Achromobacer cycloclastus* are suitable food source for *A. costata*. (4) The variety and quantity of bacteria as food are important for the growth characteristics of *A. costata*. (5) The maximam specific growth rate (μ max) is 2.5 day^{-1}, whereas the Ks value estimated from the Monod kinetics is 18 $mg \cdot l^{-1}$. (6) The growth of *A. costata* is severely affected by shake stress at over 0.25 G in monoxenic culture conditions. (7) *A. costata* can survive in wide environmental condition ranges as described above. This is one of reasons why *A. costata* is common in many waste-water treatment processes.

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原生動物 *Aspidisca costata* の増殖に及ぼす環境因子の影響

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原生動物繊毛虫類に属する *Aspidisca costata* は，排水の生物処理施設において活性汚泥および生物膜中にしばしば大量に出現する微小動物として知られる.しかしながら，その増殖に及ぼす環境因子の影響に関しては明らかにされていない点が多い.そこで本研究では，その生理・生態学的諸特性にかかわる基礎的知見を得ることを目的として実験的検討を行なった.得られた知見は次のようにまとめられる. (1) 水温の至適条件は，30℃であり，夏期の高水温時に増殖活性が高まるが，冬期の5℃程度の低水温時にも増殖の十分可能である.アレニウスプロットから算出した活性化エネルギーは，52.4 kJ·mol^{-1} である. (2) 塩濃度は，リン酸緩衝液濃度で 0～40 mM の範囲において増殖に大きな影響を及ぼさない. (3) pH は，4.2 から 9.7 の範囲において増殖に大きな影響を及ぼさない. (4) 食物源としての細菌濃度の影響を，Monod 式の適用できる範囲で計算すると，最大比増殖速度は 2.5 day^{-1}，Ks 値は 18 mg・l^{-1}. (5) 攪拌強度への耐性は，原生動物縁毛類，袋形動物輪虫類，環形動物貧毛類に比較して低く，0.25G 以上の攪拌強度において増殖に影響が認められ，0.94G では増殖が完全に阻害される. (6) 食物源としての細菌の種類は，供試細菌 11 種の内，*Escherichia coli*，*Acinetobacter calcoasceticus*，*Klebsiera pneumoniae*，*Achromobacer cycloclastus*，*Pseudomonas putida* の 5 種の単独細菌を用いた培養で増殖可能であり，*Bacillus cereus*, *Bacillus subtilis*, *Streptococcus acidominius*，*Micrococcus luteus*，*Flavobacterium luteus*, *Flavobacterium suaveolens* の 6 種の単独細菌では増殖できない.