Effects of Protozoa and Glass Beads on Bacterial Biomass and Metabolism in a Food Chain System of Glucose-*Pseudomonas* sp. strain DP-4-*Tetrahymena thermophila*

MITSUNORI TARAO^{1*}, YOSHIRO YAMASHITA¹, AKIRA SATO¹ and MASAYUKI SETO¹

¹ Laboratory of Environmental Microbiology, Department of Environmental and Natural Resource Science, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183–8509, Japan

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The effect of the protozoa *Tetrahymena thermophila* or glass beads on the biomass of the bacterium *Pseudomonas* sp. strain DP-4 and metabolism of glucose in a steady state was studied in a food chain system of glucose-*Pseudomonas* sp. strain DP-4-*T. thermophila*. Glucose was added to 1.5 ml cultures in 50 ml vials at a rate of 50 $\mu\text{g C/vial/day}$. In the 1.5 ml cultures without glass beads, the bacterial biomass was ca. 450 and 26 $\mu\text{g C/vial}$ when the protozoa was absent and present, respectively. The bacterial biomass with glass beads was ca. 650 and 34 $\mu\text{g C/vial}$ when the protozoa was absent and present, respectively. Protozoan biomass was 7 and 20 $\mu\text{g C/vial}$ without or with glass beads, respectively. Irrespective of presence of the protozoa or glass beads, the rate of consumption of glucose per vial (50 $\mu\text{g C/vial/day}$) was considered to be almost the same as the rate of CO_2 evolution per vial (45–47 $\mu\text{g C/vial/day}$). The diameter of the glass beads (50, 70 or 100 μm) had no effect on the bacterial biomass in the absence of the protozoa. While, in the presence of the protozoa, the bacterial biomass was greatest when the diameter of glass beads was just 50 μm . These results suggested that the rate of glucose consumption per unit bacterial biomass of 0.11 $\mu\text{g glucose-C}/\mu\text{g biomass-C/day}$ was increased to 1.9 $\mu\text{g glucose-C}/\mu\text{g biomass-C/day}$ by protozoan predation, while the value was decreased to 0.077 $\mu\text{g glucose-C}/\mu\text{g biomass-C/day}$ by glass beads. The significance of protozoan predation and particles for bacterial biomass and metabolic activity was discussed.

Key words: carbon balance, biomass, respiration rate, food chain, particulate matrix

Bacteria comprise 18–50% of the total microbial biomass in arable soil¹⁶, and are considered to play a significant role in the carbon cycle in soil through the detritus food chain. However the mechanisms that regulate bacterial biomass, substrate consumption, and CO_2 evolution in soil, are poorly understood.

A number of studies reported that, in an aquatic environment or liquid medium, protozoan predation decreased bacterial biomass^{4,19,21,23} or enhanced the rate of bacterial turnover^{1,11,22}. While, in soils, studies of the effect of protozoan predation on bacterial biomass, substrate consumption, and CO_2 evolution are limited¹⁰. Particles, a distinctive fea-

ture of soil environment, are another factor influencing bacterial metabolic activity^{2,3,6,9,14}, but their effect on bacterial biomass, substrate consumption, and CO_2 evolution remains unknown. There is evidence to suggest that particles affect the protozoan predation of bacteria by providing small porous habitats that protect bacteria^{5,24} or due to the development of biofilms on their surface⁴. However, the combined effect of protozoan predation and particles on bacterial biomass, substrate consumption, and CO_2 evolution is limited.

In order to elucidate the limiting factor for bacterial biomass, substrate consumption, or CO_2 evolution in soil, we focused on the effect of protozoan predation, particles, and a combination of the two. We compared the microbial biomass, the rate of substrate consumption, and the rate of CO_2 evolution in a food chain system consisting of a bacterial

* Corresponding author; E-mail: tarao@cc.tuat.ac.jp, Tel: +81-42-367-5852, Fax: +81-42-367-5731

isolate and a protozoan isolate in culture without or with glass beads where glucose was supplied continuously.

Materials and Methods

Bacterial and protozoan strains

Pseudomonas sp. strain DP-4²⁰) was used as a model bacterium. It was precultured in an inorganic salt solution containing 1.6 mg C/ml of glucose for four days with shaking at 25°C in the dark. *Tetrahymena thermophila* inbred strain B provided by Dr. T. Sugai of Ibaraki University was used as a model protozoa. It was precultured in an autoclaved bacterial suspension (150 µg C/ml of biomass of strain DP-4) for four days with gentle shaking at 25°C in the dark.

Culture system

Pure cultures of the bacterium and mixed cultures of the bacterium with the protozoa were grown in the presence and absence of glass beads. Thus, four culture systems were used. One milliliter of the bacterial suspension and 0.25 ml of autoclaved inorganic salt medium containing 100 µg C of glucose were added to a sterilized 50 ml screw-cap vial. For the cultures with glass beads, a vial containing 5 g of glass beads (diameter, 50, 70, or 100 µm) was used. Unless otherwise indicated, 70 µm glass beads were used. Then, 0.25 ml of the protozoan suspension was added when the bacterium was to be cultured in the presence of protozoa. When the bacterium was cultured in the absence of protozoa, 0.25 ml of an autoclaved protozoan suspension was added. All the cultures were incubated at 25°C in the dark in a desiccator in which the humidity was kept at 20%. An amount of inorganic salt solution containing 100 µg C of glucose equal to that lost by evaporation during the incubation was added every two days. Therefore, glucose was estimated to be added at 50 µg C/vial/day. Unless otherwise indicated, the size of the bacterium and protozoa was 470 and 10 µg biomass-C/vial, respectively.

Composition of inorganic salt solution

The inorganic salt solution contained 6.9 mM Na₂HPO₄, 2.97 mM KH₂PO₄, 3.2 mM NH₄NO₃, 33 µM MgSO₄, 33 µM CaCl₂, 9.9 µM Na₂SiO₃, 3.3 µM MnCl₂, 3.3 µM H₃BO₃, 3.3 µM Na₂MoO₄, 0.66 µM FeCl₃, 16.5 nM CuSO₄, 16.5 nM ZnSO₄, and 16.5 nM CoSO₄ in deionized water. The pH was adjusted to 7.0.

Measurement of microbial biomass carbon

The bacterial biomass in terms of carbon amount was calculated from the difference between the total amount of or-

ganic carbon (TOC) and the amount dissolved (DOC) and the protozoan biomass carbon. The sample for TOC measurement was obtained by disrupting samples in the vial with 10 ml of deionized water and 1 ml of orthophosphoric acid. The sample for DOC measurement was obtained as follows: the culture in the vial with 10 ml of deionized water was passed through a 0.45 µm-pore-sized durapore membrane filter (HVLP, Millipore Corp.), and the filtrate was added to 1 ml of orthophosphoric acid. The amount of organic carbon in samples was measured using a non-dispersive infrared gas analyzer with 1.0 g of potassium peroxodisulfate and a modified oxidation vessel¹⁸). Glass beads did not affect the measurement of organic carbon.

The protozoan biomass in terms of carbon was measured as follows: $B_c = N \times V \times G_s \times D_w \times C_c$, where B_c is protozoan biomass carbon, N is mean cell number ($n=4$), V is mean cell volume (3370 µm³, $n=82$), G_s is cell specific gravity (1.1 g/cm³)⁸), D_w is ratio of dry weight (0.2), and C_c is carbon content per dry weight (0.5).

Estimation of rates of glucose consumption and CO₂ evolution in the steady state

The rate of glucose consumption per unit bacterial biomass was the rate of glucose consumption per vial divided by bacterial biomass. The rate of glucose consumption per vial was the rate of glucose addition (50 µg C/vial/day), because no remaining glucose in the culture was detected. Bacterial biomass was calculated from biovolume, cell specific gravity (1.1 g/cm³), ratio of dry weight (0.25), and carbon content per dry weight (0.45). Bacterial biovolume was estimated by a direct count method using phenolic anilin blue (PAB)^{7,16}.

The rate of CO₂ evolution per vial was the difference between the rate of glucose consumption per vial and the rate of DOC excretion and/or the rate of excretion of particulate waste products by the protozoa. The DOC excretion rate was amount of DOC in the culture divided by the incubation period. The rate of excretion of particulate waste products by the protozoa was calculated as the amount of particulate waste products in the culture divided by the incubation period.

Statistical analyses

Analysis of variance (ANOVA) was used to test the effect of the pore size of glass beads on the mean size of the protozoa in the culture with three different diameters of glass beads at 40-day-incubation. Scheffe's multiple range test was then used to find which cultures were significantly different. All analyses were performed using SPSS® Base

System Release 6.13J for Windows (SPSS Japan Inc.).

Results

In the culture without glass beads, when the protozoa was absent, the bacterial biomass was almost constant, 420–470 $\mu\text{g C/vial}$, during the 50-day-incubation period (Fig. 1 (a)). On the other hand, when present, the bacterial biomass decreased, reaching a steady state (110–180 $\mu\text{g C/vial}$) after 26 days incubation. After a transient increase, the protozoan biomass reached a steady state (5–12 $\mu\text{g C/vial}$) after 26 days incubation (Fig. 1 (b)).

In the culture with glass beads (diameter, 70 μm), when the protozoa was absent, the bacterial biomass increased gradually during the 40-day-incubation period, then reached a steady state (620–630 $\mu\text{g C/vial}$) (Fig. 2 (a)). On the other hand, when present, the bacterial biomass decreased and reached a steady state (84–170 $\mu\text{g C/vial}$) after 26 days incubation (Fig. 2 (a)). After a transient increase, the protozoan biomass reached a steady state (14–16 $\mu\text{g C/vial}$) after 26 days incubation (Fig. 2 (b)).

Fig. 1. Change in bacterial biomass (a) when the protozoa was absent (open circle) or present (close circle), and change in protozoan biomass (b) in 1.5 ml cultures in 50 ml vials without glass beads. Bacterial biomass included particulate waste products excreted by protozoa when present. Glucose was added at a rate of 50 $\mu\text{g C/vial/day}$. Each value of bacterial biomass and protozoan biomass is the mean of three and four measurements, respectively. Bars indicate the range of minimum and maximum values.

Table 1. Amount ($\mu\text{g C/vial}$) of bacterial biomass^a at 40 days incubation with different inoculations of bacterial biomass in culture with glass beads.^b

Protozoa	Initial bacterial biomass ($\mu\text{g C/vial}$)		
	160	470	710
Absent	590 (550–630)	630 (590–670)	660 (610–710)
Present	200 (190–230)	100 (70–130)	150 (110–190)

^a Bacterial biomass included particulate waste products excreted by protozoa if present.

^b Each value is the mean of three measurements. Minimum and maximum values are shown in parentheses. The diameter of the glass beads was 70 μm .

The effect of inoculum size on the bacterial biomass at 40 days incubation in the culture with glass beads was evaluated (Table 1). Irrespective of size, the bacterial biomass was almost the same, 590–660 and 100–200 $\mu\text{g C/vial}$, when the protozoa was absent and present, respectively.

Direct counting at 40 days incubation revealed that when

Fig. 2. Change in bacterial biomass (a) when the protozoa was absent (open circle) or present (close circle), and change in protozoan biomass (b) in 1.5 ml cultures in 50 ml vials with glass beads. Bacterial biomass included particulate waste products excreted by protozoa when present. Glucose was added at a rate of 50 $\mu\text{g C/vial/day}$. The diameter of the glass beads was 70 μm . Each value of bacterial biomass and protozoan biomass is the mean of three and four measurements, respectively. Bars indicate the range of minimum and maximum values.

Table 2. Amount ($\mu\text{g C/vial}$) of bacterial biomass and particulate waste products excreted by protozoa at 40 days incubation when protozoa was present in culture without or with glass beads.^a

Glass beads	Bacterial biomass	Amount of particulate waste products
Without	26	46
With	34	35

^a The diameter of the glass beads was 70 μm .

the protozoa was present, the bacterial biomass consisted of not only bacterial cells but also particulate waste products excreted by the protozoa. In the culture without glass beads, the bacterial biomass and the amount of particulate waste products was 26 and 46 $\mu\text{g C/vial}$, respectively (Table 2). In the culture with glass beads, this was 34 and 35 $\mu\text{g C/ml}$, respectively. When the protozoa was absent, the bacterial biomass in the culture without and with glass beads was almost the same with POC of 450 and 650 $\mu\text{g C/vial}$, respectively (data not shown).

The carbon balance in each culture at 40 days incubation was determined (Fig. 3). When the protozoa was absent, the bacterial biomass in the culture without and with glass beads was 17 and 19 times higher than when the protozoa was present. The rates of glucose consumption and CO_2 evolution were similar among the cultures. When the protozoa was absent, the rate of glucose consumption per unit bacterial biomass was 0.11 and 0.077 $\mu\text{g glucose-C}/\mu\text{g biomass-C/day}$ in the culture without and with glass beads, respectively. While, when the protozoa was present, the corresponding values were 1.9 and 1.5 $\mu\text{g glucose-C}/\mu\text{g biomass-C/day}$, respectively.

The effect that the size of the glass beads had on the bacterial biomass at 40 days incubation was evaluated (Table 3). When the protozoa was absent, the bacterial biomass in the culture without glass beads was the smallest of all. Irrespective of the diameter of the beads, the bacterial biomass was almost the same, 630–670 $\mu\text{g C/vial}$. When the protozoa was present, irrespective of the presence or diameter

Fig. 3. Carbon balance showing successive intake and transfer of carbon at 40 days incubation when the protozoa was absent (a, c) or present (b, d) in the culture without (a, b) or with (c, d) glass beads. The area of a circle shows biomass ($\mu\text{g C/vial}$) respectively, and the arrow shows the rate of intake and transfer ($\mu\text{g C/vial/day}$). The diameter of the glass beads was 70 μm .

(except 50 μm) of glass beads, the bacterial biomass was almost the same at 100–160 $\mu\text{g C/vial}$, while it was the greatest, at 300 $\mu\text{g C/vial}$, when the diameter of the beads was 50 μm .

Assuming that the glass beads in the vial were as cubic closest packed structure, the pore size of glass beads was calculated (Table 4). Data from ANOVA performed on the long length of the protozoa showed no significant difference among cultures, while a significant difference in short length was observed ($P < 0.01$). When the smaller glass beads were used, the short length of the protozoa was

Table 3. Amount ($\mu\text{g C/vial}$) of bacterial biomass^a at 40 days incubation when the protozoa was absent or present in the culture without or with glass beads of three different diameters.^b

Protozoa	Without glass beads	Diameter of glass beads added		
		50 μm	70 μm	100 μm
Absent	450 (440–460)	670 (640–700)	630 (590–670)	640 (580–700)
Present	160 (120–200)	300 (280–330)	100 (70–130)	140 (100–180)

^a Bacterial biomass included particulate waste products excreted by protozoa when present.

^b Each value is the mean of three measurements. Minimum and maximum values are shown in parentheses.

Table 4. Relationship between pore size of glass beads and mean size of protozoa at 40 days incubation.

Diameter of glass beads (μm)	Pore size of glass beads ¹⁾ (μm)	Mean size ($\mu\text{m}\pm\text{SE}$) of protozoa			Density of protozoa ²⁾ ($\times 10^4$ cell/vial)
		Long length	Short length	n	
50	7.7	23 $\pm$ 0.9	12 $\pm$ 0.6 a ³⁾	15	1.6 $\pm$ 0.18
70	11	26 $\pm$ 0.7	14 $\pm$ 0.4 ab ³⁾	25	3.3 $\pm$ 0.37
100	15	26 $\pm$ 0.9	16 $\pm$ 0.5 b ³⁾	22	3.0 $\pm$ 0.26
Without glass beads	—	27 $\pm$ 1.1	15 $\pm$ 0.5 b ³⁾	20	1.2 $\pm$ 0.42

¹⁾ Diameter of maximum inscribed sphere between glass beads in cubic closest packed structure.

²⁾ The mean of four measurements ($\pm\text{SE}$).

³⁾ Values followed by different letters are significantly different among the cultures (Scheffe's multiple range test; $P<0.01$).

smaller. The number of protozoa was smallest when the glass beads were 50 μm in diameter. In the culture without glass beads, the size of the protozoa was almost the same as in the culture with glass beads except for the 50 μm glass beads. The number of protozoan was smaller in the culture without than with glass beads.

Discussion

Our study revealed that, (i) protozoan predation decreased the bacterial biomass, (ii) the presence of glass beads increased the bacterial biomass, and (iii) neither protozoan predation nor glass beads affected the rate of glucose consumption per vial or CO_2 evolution per vial. These results indicated that protozoan predation increased glucose consumption per unit bacterial biomass, while the presence of glass beads decreased it.

The rate of CO_2 evolution per unit bacterial biomass was estimated only when the protozoa was absent, and was 0.10 and 0.069 $\mu\text{g CO}_2\text{-C}/\mu\text{g biomass-C/day}$ in the culture without and with glass beads, respectively. These values are similar to the rate of CO_2 evolution per unit microbial biomass in arable soil reported to be 0.02–0.06¹³⁾, 0.075¹⁵⁾, or 0.042¹⁷⁾ $\mu\text{g CO}_2\text{-C}/\mu\text{g biomass-C/day}$. The rate of CO_2 evolution per unit bacterial biomass when the protozoa was present was not estimated directly. Assuming that the yield coefficient of the bacterium at the expense of glucose is 0.5 and ignoring the rate of DOC excretion, the rate at which CO_2 evolves is estimated at 25 $\mu\text{g CO}_2\text{-C/day}$. Then, the estimated rate per unit bacterial biomass is 0.96 and 0.73 $\mu\text{g CO}_2\text{-C}/\mu\text{g biomass-C/day}$ in the culture without and with glass beads, respectively. These values are 10 times higher than when the protozoa was absent.

From the increase in the rates of glucose consumption and CO_2 evolution per unit bacterial biomass when the protozoa was present, it is considered that the specific

metabolic activity of the bacteria was enhanced by protozoan predation as other studies^{1,11,12)} have reported. This enhancement was apparently not caused by the decrease in bacterial biomass due to protozoan predation. Assuming that the yield coefficient of the bacterium at the expense of glucose is 0.5, bacterial biomass is produced at a rate of 25 $\mu\text{g biomass-C/day}$. If the value was summed with the amount of bacterial biomass when the protozoa was present, the theoretical amount of bacterial biomass assuming the absence of the protozoa was obtained, i.e., 51 and 59 $\mu\text{g C/vial}$ in the culture without and with glass beads, respectively. Because these values are much lower than when the protozoa was absent, the enhancement of the rate of glucose consumption per unit bacterial biomass was not due to a decrease of bacterial biomass.

The larger amounts of bacterial biomass in the culture with glass beads suggested that the maintenance requirement per unit bacterial biomass was smaller than in the culture without glass beads. In the steady state when the protozoa was absent, the rate of glucose consumption per unit bacterial biomass of 50 $\mu\text{g glucose-C}/\mu\text{g biomass-C/day}$ was considered to be equal to the maintenance requirement per unit bacterial biomass. While, in the steady state when the protozoa was present, the rate of bacterial consumption by the protozoa was equal to the rate of bacterial production, and the value was considered to be equal to the maintenance requirement of the protozoa. The larger amounts of protozoan biomass in the culture with glass beads suggested that the maintenance requirement per unit protozoan biomass was smaller than in the culture without glass beads, or the yield coefficient of the bacterium at the expense of glucose was larger.

When the protozoa was present, the bacterial biomass was highest in the culture in which the diameter of glass beads was 50 μm (Table 3). In the culture with 50 μm beads, the pore size was calculated at 7.7 μm , which was

much smaller than the size of the protozoa (Table 4). We considered that the small size of the liquid phase impeded the motility of the protozoa, and so some bacterial cells avoided protozoan predation. In a soil environment, the location of bacteria in small pores (<6 µm) provides significant protection from protozoan predation²⁴). If we had used smaller (<50 µm) glass beads, the bacterial biomass might have become much larger even when the protozoa was present because of significant protection from predation, and the rate of glucose consumption per unit bacterial biomass would be expected to be similar to that when the protozoa was absent.

Although we used a simple culture system, we obtained basic information on the effects of protozoan predation and particles on the carbon balance especially in soil ecosystems in which protozoa or particles are major components. Our next focus in this field is to evaluate the effects of a more complex culture system, which contains bacterial species or fungi, on the bacterial or microbial biomass, bacterial activity, or carbon balance. Studies to evaluate the effects of particles are also needed.

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