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Ectosymbiotic Role of Food Bacteria for *Paramecium*: Bacterial Detoxification of *Paramecia*-Killing Toxin Contained in Wheat Grass Powder

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ABSTRACT—Bacterized plant infusion is a popular culture medium for *Paramecium,* using *Klebsiella pneumoniae* for the bacterium and Wheat Grass Powder (WGP) for the plant. It has been thought that WGP plays a role in the growth of bacteria, which in turn serve as the direct food for paramecia. However, we found that bacteria suspended in saline solution were unable to support the growth of paramecia. WGP including no bacteria was able to support neither the growth nor the survival of paramecia; instead, it killed paramecia. The killing effect of the WGP-derived substance(s), estimated to be of molecular weight less than 1,000, was abolished when bacteria were once grown in the WGP and then eliminated, suggesting that bacteria might change the toxic substance into an inactive form. This inactivation of the toxic substance may be caused either by metabolization inside of the bacteria or by neutralization by means of bacteria-derived substance outside of the bacteria. The second alternative is likely, because paramecia were able to survive and grow in the WGP medium containing a sufficient amount of dead bacteria killed by formalin or kanamycin. Dead bacteria killed by autoclaving were ineffective, probably because bacterial contents were lost. These findings revealed an ectosymbiotic role of bacteria; they confer benefits upon paramecia not only as food but also as machinery to detoxicate a plant toxin.

INTRODUCTION

Culture medium is fundamental to any kind of microbiological experiment. Experimental results may be different if biological materials prepared in different culture media are used. For example, the experimental outcomes in studies of *Paramecium tetraurelia* regarding the cellular and ciliary components of phospholipids, fatty acids and sterols (Andrews and Nelson, 1979; Hennessey *et al.,* 1983; Kaneshiro *et al*., 1979; Rhoads and Kaneshiro, 1979), the growth rate, the final cell density, the accumulation of macronuclear DNA fragments (Holmes and Holmes, 1986), and the clonal life span (Fukushima *et al*., 1990) were different when chemically defined medium or axenic medium was used instead of bacterized plant infusions. The clonal life span of *P. tetraurelia* was reduced when the bacterized plant infusions prepared using a reduced concentration of the plant (Wheat Grass Powder, WGP) were used (Takagi *et al*., 1987). Human diploid fibroblasts, which normally have a limited division potential became able to divide indefinitely if serum in the standard culture medium was replaced with certain growth factors (Hayashi and Sato, 1976).

For the growth of *Paramecium*, two-component culture medium, composed of plants (WGP, lettuce) and bacteria (*Klebsiella pneumoniae*), has been most frequently used. It has been taken for granted that in the two-component culture medium, the plant infusion plays the role of allowing the growth of the bacteria, which in turn serve as the direct food for paramecia (Sonneborn, 1950, 1970). However, the role of each component in the growth of paramecia has not been examined critically, except for cases in which nutritional requirements were studied in axenic or chemically defined medium (Nerad and Daggett, 1992; Soldo, 1992; Van Wagtendonk, 1974).

In our laboratory, *Paramecium* has usually been cultured in WGP medium inoculated with *K. pneumoniae* 2 days before use. The density of *K. pneumoniae* increases from ca. 10^3 cells/ml to ca. 10^8 cells/ml during the 2 days in WGP medium. The present study addresses the simple question of whether *Paramecium* can be grown on bacteria alone. The negative answer that we found to this question led us to further studies giving fresh insight into the ectosymbiotic role of bacteria for *Paramecium*.

MATERIALS AND METHODS

Cells and maintenance of stock cultures

Wild-type stock 51 (mating type VII) of *Paramecium tetraurelia* was generally used. (When referred to as paramecia, we mean this stock of *P. tetraurelia* cells unless otherwise stated.) Other stocks used were G3 (mating type O) of *P. caudatum*, stock YM26 (mating type III) of *P. multimicronucleatum* collected from the wild by Mr. Y.

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Murakami of Ishinomaki Sensyu University, stock G312 (mating type undecided) of *P. bursaria* and stock 4B-1 (mating type unknown) of *P. trichium* provided by Dr. T. Takahashi of Hiroshima University.

The stock cultures were maintained in test tubes covered with stainless steel caps at 17°C, replacing half of the culture with an equal volume of fresh culture medium every 3 weeks. For starting experiments, cells from the stock culture were washed as described (Sonneborn, 1970) and grown as clones.

Culture medium

The standard culture medium was 0.5% phosphate-buffered WGP (Wheat Grass Powder; Pines International, Inc., USA) medium inoculated with *Klebsiella pneumoniae* 2 days before use (designated 2×WGP; Takagi *et al*., 1987). WGP is identical to Cerophyl, and *K. pneumoniae* is identical to *Aerobacter aerogenes* in former reports. We describe the method (basically after Sonneborn, 1970) in detail because the culture medium is critical in this study. WGP in double-distilled water (5 g/l) was boiled for 5 min. After cooling, WGP was removed by filtration through filter paper. The infusion contained in the remaining wet WGP-paste was squeezed out through gauze folded successively eight times. $Na_2HPO_4 \cdot 12H_2O$ (0.5 g /l) was added, and the resulting infusion was bottled in flasks capped with silicone sponge closures (Sigma), and autoclaved for 20 min at a pressure of 1.2 atm. The WGP infusion was inoculated with bacteria at about 10^3 cells/ml and incubated at 26°C for 2 days to grow the bacteria to the saturation density of about 10^8 cells/ml. The bacterized WGP medium was adjusted to pH 6.8 with NaOH before inoculating with *Paramecium*. When various concentrations of WGP ranging from 6xWGP to 1/64xWGP were used, we first prepared 6×WGP (15 g/l) diluted in autoclaved 0.05% $Na_2HPO_4 \cdot 12H_2O$.

Lettuce juice medium was prepared as follows (basically after Miyake, 1968). Fresh lettuce leaves were washed and immersed in boiling distilled water for a few seconds to inactivate enzymes. The leaves were treated with the juicer repeatedly, squeezed out through gauze folded successively eight times and filtered through filter paper. The juice was stored in a freezer (–25°C). After thawing, the juice was diluted to 2% with double-distilled water, buffered by sodium phosphate (2 mM $Na_2HPO_4 \cdot 2H_2O + Na_2HPO_4 \cdot 12H_2O$, pH 6.8), and autoclaved. The bacterized lettuce medium, usually containing a saturation density of about 10^8 bacteria/ml, was adjusted to pH 6.8 with NaOH before inoculating with *Paramecium.*

Bacteria

K. pneumoniae was grown on agar slants (25 g/l bacto agar, 0.2 g/l glucose, 5 g/l yeast extract) in a test tube capped with a silicone sponge closure (Sigma) at 26°C. Other bacteria used were *Escherichia coli* (Tübingen-3, TF1002 and TF1003) provided by Dr. H. Tanabe of Kinki University, which were grown on LB plates containing 50 μg/ml ampicillin. Tübingen-3 (Neo^{r}I) contains ampicillin- and neomycin-resistance genes on a plasmid and a kanamycin-resistance gene in its chromosomal DNA. TF1002 (Neo^{s}II) contains the ampicillin-resistance gene but no neomycin-resistance gene on a plasmid and no kanamycin-resistance gene in its chromosomal DNA. TF1003 (Neo^{r}II) contains the ampicillin- and neomycin-resistance genes on a plasmid but no kanamycin-resistantce gene in its chromosomal DNA.

Bacteria from a slant were collected in 2 ml of distilled water that had been sterilized by autoclaving, and then were sedimented by centrifugation at 10,000 rpm (8,600 g) for 10 min. The harvested bacteria were resuspended in 0.5 ml of sterilized distilled water. The bacterial concentration of this suspension was 10^{10} cells/ml.

To eliminate bacteria from the bacterized 2×WGP, the culture was centrifuged for 15 min at 10,000 rpm and then filtered with DISMIC-25cs (Toyo Roshi Kaisha, Ltd.).

To prepare dead bacteria, a final concentration of 1 mg/ml kanamycin or 20% formalin was added to the suspension containing 10^{10} bacteria/ml. After 24 hr, the suspensions were centrifuged. Killed bacteria were washed three times in 2 ml of sterile distilled water and were resuspended in 0.5 ml of sterile distilled water. This suspension was checked for the absence of living bacteria by colony formation.

Bacteria-free paramecia

Bacteria are found growing in autoclaved WGP medium after *Paramecium* cells are introduced, because bacteria are brought in with the paramecia. Paramecia may live closely associated with bacteria so that they are not separable unless antibiotics are used. We therefore treated an early stationary phase culture of paramecia with 50 μg/ml kanamycin for 24 hr at 26°C. At this concentration of kanamycin, there were no detectable effects on paramecia, in terms of the rate of cell division in isolation cultures, the saturation cell density in mass cultures, and the rate of food vacuole formation.

Survival test of paramecia

Five bacteria-free paramecia were placed in each well of 3-well depression slides, each containing 0.5 ml of test medium. A set of 9 wells were prepared to obtain a single data point for experiments to examine the rate of death (or % survival) in each test medium. For daily check of survival, for example, 3 sets (27 wells of 9 depression slides) were prepared, and each set was monitored daily for 3 days. For hourly check of survival, the necessary numbers of sets were prepared depending on the length of time of the observations. All cultures were incubated in a moist chamber at 26°C. In the experiments in which bacteria-free WGP inoculated with kanamycin-treated paramecia was used, the absence of living bacteria was checked by colony formation.

Saline solution

SMBIII (Miyake, 1981) was used as physiological balanced solution (1.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.05 mM $MgSO_4$, 2 mM Na-phosphate buffer pH 6.8, 2×10^{-3} mM EDTA) for both paramecia and bacteria. Dryl's solution (2 mM sodium citrate, 1 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM $CaCl_2$) (Dryl, 1959) was also used in some experiments. Bacteria can grow in SMBIII to ~10^6 cells/ml.

Ultrafiltration

2×WGP was subjected to ultrafiltration using a Diaflow membrane YM10 (Amicon) with a nominal cut-off molecular weight of 10,000. The resulting fraction with a molecular weight of less than 10,000 was subjected to ultrafiltration using a Diaflow membrane YM1 (Amicon) with a nominal cut-off molecular weight of 1,000. Thus, 2×WGP was separated into three fractions of molecular weight <1,000, 1,000~10,000 and >10,000. Each fraction was supplemented with double-distilled water containing 0.05% $Na_2HPO_4 \cdot 12H_2O$ in order to achieve the original volume.

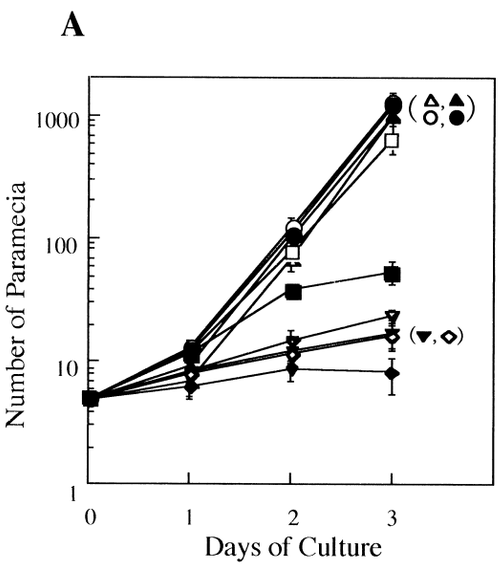
RESULTS

Growth of paramecia in bacterized WGP medium with different buffers, different bacteria and different concentrations of WGP

The bacterized 2×WGP medium contains buffering chemicals, WGP-extracts and bacteria. We examined the effects of each of these components on the growth of paramecia.

WGP was dissolved in (a) 0.05% $Na_2HPO_4 \cdot 12H_2O$, or (b) SMBIII, or (c) Dryl's solution, or (d) 10 mM Tris-HCl pH 6.8, or (e) double-distilled water, and then inoculated with *K. pneumoniae*. The standard is (a). Paramecia grew well in all of the media with similar growth kinetics (data not shown).

WGP was dissolved in 0.05% $Na_2HPO_4 \cdot 12H_2O$, and then inoculated with (a) *K. pneumoniae*, or (b) *Escherichia coli*, Tübingen-3, or (c) *E. coli*, TF1002, or (d) *E. coli*, TF1003. The



B

Symbols	Concentration of WGP	Saturation density of bacteria (cells/ml)
△	6x	1.3×10^8
▲	4x	1.3×10^8
○	2x (5 g/l)	1.1×10^8
●	1x	7.6×10^7
□	1/2x	6.1×10^7
■	1/4x	2.5×10^7
▽	1/8x	1.7×10^7
▼	1/16x	1.2×10^7
◇	1/32x	7.0×10^6
◆	1/64x	3.8×10^6

Fig. 1. Growth kinetics of paramecia (A) and saturation density of bacteria (B) in bacterized WGP medium at various concentrations of WGP. Symbols for the concentrations are the same in A and B. Each point is the mean±SD (n=9).

standard is (a). Paramecia grew similarly in all of the media, although there was a time-lag before starting exponential growth in *Escherichia* medium when transferred from *Klebsiella* medium (data not shown).

WGP of 10 different concentrations ranging from 6× to 1/64× (the standard is 2×) was dissolved in 0.05% $Na_2HPO_4 \cdot 12H_2O$ and inoculated with *K. pneumoniae*. Two days later, bacterial density was counted in each medium and then 5 paramecia were introduced into each medium to study their growth kinetics. The growth was vigorous in 6×, 4×, 2× and 1× medium, weak in 1/8×, 1/16×, 1/32× and 1/64× medium, and intermediate in 1/2× and 1/4× medium (Fig. 1A). The saturation density of bacteria decreased correspondingly as the WGP concentration decreased (Fig. 1B); in vigorously growing cultures, bacterial density was on the order of 10^8 cells/ml.

Altogether, it was concluded that at least for short-term experiments, as in this case, different kinds of buffers and bacteria were similarly effective for the growth of paramecia. However, the concentration of WGP and density of bacteria were crucial for the growth of paramecia.

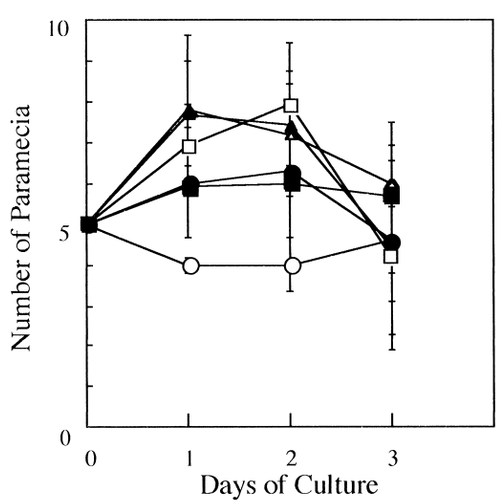


Fig. 2. Fate of 5 paramecia when they were placed in SMBIII including various concentrations of bacteria. Bacterial concentrations (cells/ml) were 10^9 (filled circles), 10^8 (open triangles), 10^7 (filled triangles), 10^6 (open squares), 10^5 (filled squares), and zero (open circles). Each point is a mean±SD (n=9).

Bacteria alone can not support the growth of paramecia

The results in the previous section support the traditional idea that WGP is used for the growth of the bacteria and that a sufficient number of bacteria (on the order of 10^8 cells/ml) is necessary for the growth of paramecia. To test this idea, we concentrated bacteria, suspended them in saline solution (SMBIII) at various densities, and introduced 5 paramecia into each suspension. Paramecia could survive, but were unable to divide more than 2 times even in the medium containing bacteria at 10^9 cells/ml (Fig. 2); they began to grow if WGP was supplied in that medium (data not shown). We conclude, therefore, that the growth of paramecia can not be supported by bacteria alone, but requires a nutritional supply from WGP.

Paramecia die in WGP medium containing no bacteria

To answer the question if WGP alone can support the growth of paramecia, we placed paramecia at early stationary phase in WGP medium containing no bacteria. We have tried to wash out bacteria by repeatedly transferring paramecia into sterilized SMBIII before introducing them into WGP medium. It was, however, impossible to eliminate bacteria completely. Bacteria that escaped from washing grew finally to 10^6 cells/ml in SMBIII and to 10^8 cells/ml in WGP medium. We therefore used kanamycin. The treatment of paramecia

with 50 μg/ml kanamycin for 24 hr was found sufficient both to eliminate bacteria and to keep paramecia uninjured: bacterial elimination was confirmed by colony counting on agar plates; the normality of paramecia was confirmed by obtaining identical results between treated and untreated cells in comparisons of the fission rate in isolation cultures, final cell density in mass cultures, and the rate of feeding activity (data not shown).

Five paramecia that were pretreated with kanamycin were placed in each well of depression slides containing 10 different concentrations of WGP ranging from 6× to 1/64× as in Fig. 1, and the number of surviving cells was counted for 3 days (Fig. 3A). Paramecia that were placed in 6×WGP–1/4×WGP died out within the period of observation, while those that were placed in 1/8×WGP–1/64×WGP remained alive, at least partly (Fig. 3A). These results strongly suggest that some substance(s) that is toxic to paramecia is contained in WGP. In 2×WGP containing no bacteria, paramecia began to die after about 7 hr of incubation, while in 2×WGP with bacteria, paramecia began to divide at this time (Fig. 3B).

To estimate the molecular weight of the toxic substance, 2×WGP was separated by ultrafiltration using two membranes into three fractions of molecular weights less than 1,000, from 1,000 to 10,000, and more than 10,000. Paramecia died out on the first day in the fraction of molecular weight less than 1,000, but survived for 3 days in the other two fractions, although the number of living cells decreased (Fig. 3C). It is supposed, therefore, that some substance(s) with molecular weight less than 1,000 is the main component of the toxic substance(s).

We then examined whether the above findings can be extended to species other than *P. tetraurelia* and to a plant

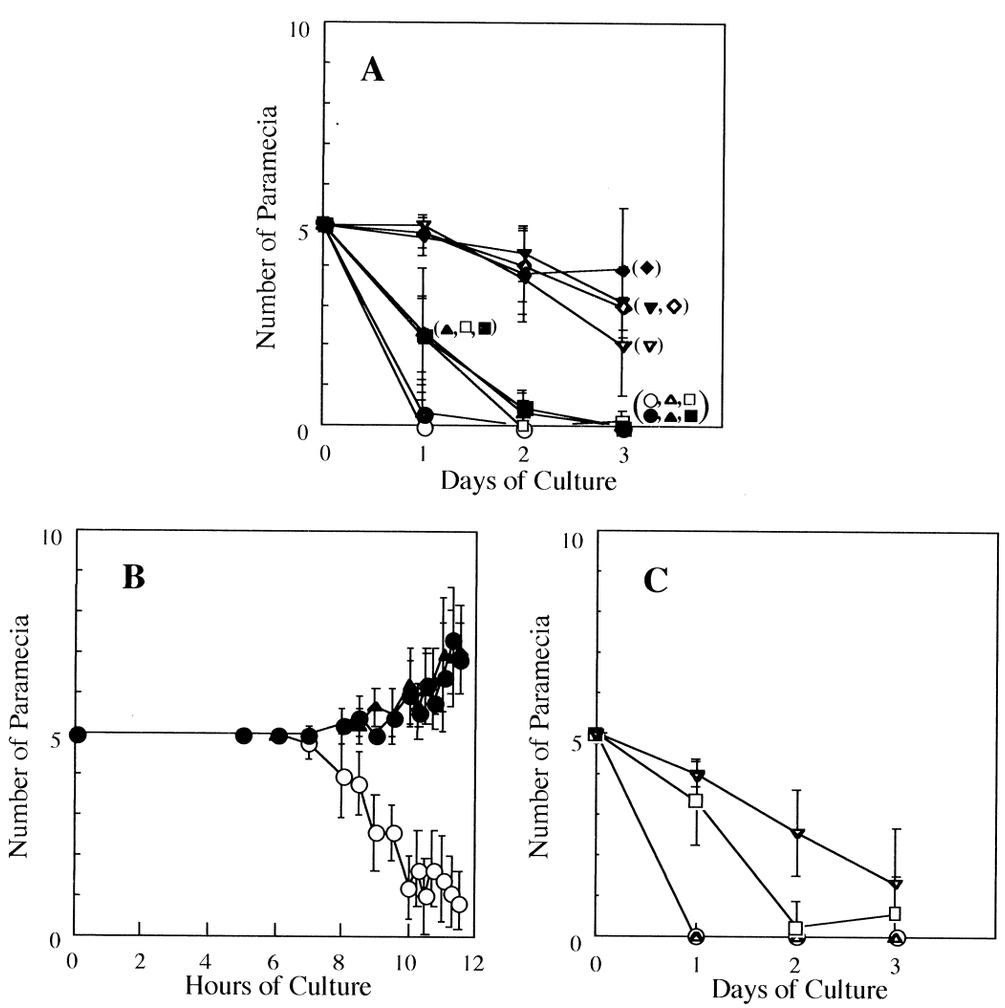


Fig. 3. Fate of 5 paramecia placed in WGP medium of various concentrations. A: Cells were placed in WGP medium of various concentrations in which bacteria were not inoculated, and their survival was monitored for 3 days. Symbols are the same as in Fig. 1. B: Cells were placed in 2×WGP (open circles) or in bacterized 2×WGP (filled circles: kanamycin-pretreated cells; filled triangles: cells not treated with kanamycin), and their survival was monitored for 12 hr. C: Cells were placed in 2×WGP (open circles) and in the ultrafiltrated fractions of 2×WGP with molecular weight of less than 1,000 (open triangles), from 1,000 to 10,000 (open squares), or more than 10,000 (open reversed triangles), and their survival was monitored for 3 days. Each point is the mean±SD (n=9).

other than WGP. First we studied how cells of *P. caudatum*, *P. multimicronucleatum*, *P. bursaria* and *P. trichium* grow or die in 2×WGP with and without bacteria. The results were similar to those with *P. tetraurelia*; every species grew in bacterized 2×WGP, but died out before long in 2×WGP in which bacteria were not inoculated (Fig. 4). We next studied how paramecia grow in lettuce juice with and without bacteria. The results were again similar to those with WGP; paramecia grew in bacterized lettuce juice at the concentrations of 0.5%, 1%, 2% and 4%, but died out at 8% (Fig. 5A), while in bacteria-free lettuce juice they died out faster as the concentration of the lettuce juice was increased (Fig. 5B).

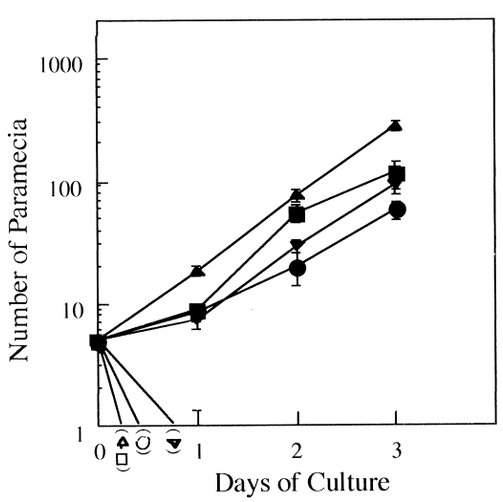


Fig. 4. Fate of 5 cells of 4 species of *Paramecium* placed in 2×WGP in which bacteria were inoculated (filled symbols) or not inoculated (open symbols). Circles: *P. caudatum*, triangles: *P. multimicronucleatum*, squares: *P. bursaria*, reversed triangles: *P. trichium*. Each point is the mean±SD (n=9).

Toxic substance(s) contained in WGP is eliminated by bacteria

Some toxic substance(s) is contained in 2×WGP, but paramecia can grow in 2×WGP in which bacteria are inoculated 2 days before use. It is therefore likely that the toxic substance is eliminated during the 2 days in which bacteria grew to the saturation density. To test this hypothesis, we conducted the following experiments.

The bacteria were eliminated from the 2-day-old bacterized 2×WGP by centrifugation and filtration, and paramecia were placed in this bacteria-free 2×WGP. Paramecia survived in this medium, but did not grow. Paramecia were able to grow if bacteria were inoculated again. Bacteria grew to 10^8 cells/ml in this medium (Fig. 6). Similar experiments were conducted by using 2xWGP in which bacteria had been incubated for 1, 2, 3, 4 and 5 days and then eliminated. The results were similar in all of the experiments: paramecia survived but did not grow (data not shown). These results show that the toxic substance in 2×WGP is eliminated by bacteria in a day or more, and support the conclusion in the previous section showing that paramecia require both bacteria and WGP for growth.

Do paramecia survive or die if they are placed in 2×WGP immediately after bacteria are inoculated? To answer this question, paramecia were placed in 2×WGP immediately after bacteria of various densities ranging from 10^3 to 10^8 cells/ml were inoculated. In 2×WGP in which bacteria were suspended at 10^3 and 10^4 cells/ml, paramecia died out within the period of observation; in medium containing 10^5 bacteria/ml, paramecia began to grow on the second day; in medium containing 10^6, 10^7 or 10^8 bacteria/ml, paramecia began to grow on the first day (Fig. 7A). This rapid detoxification by a sufficient amount of bacteria suggests the elimination of the WGP-toxin by adsorption on bacterial cell surface or by neutralization by means of bacteria-derived substances, rather than by metabolization inside of the bacteria.

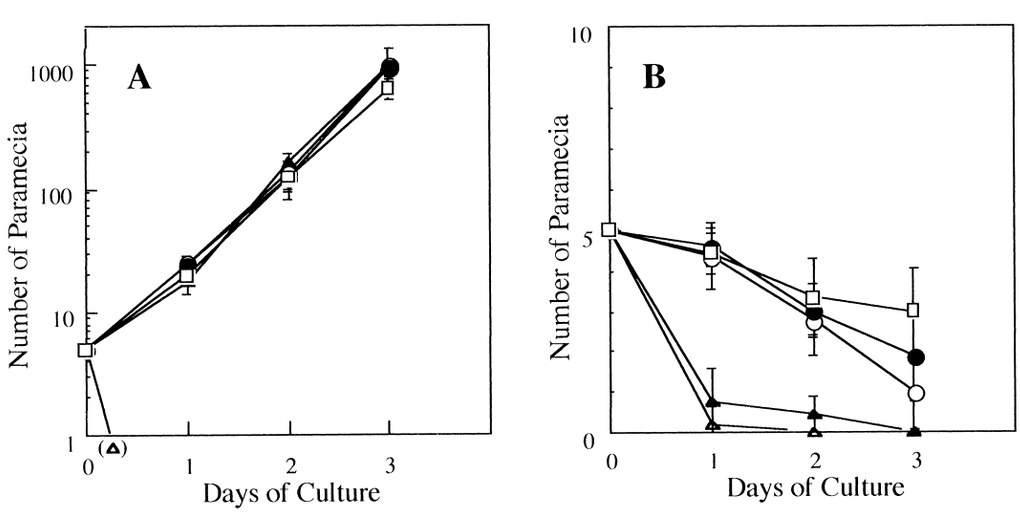


Fig. 5. Fate of 5 paramecia placed in lettuce juice of various concentrations in which bacteria were inoculated (A) or not inoculated (B). The concentrations of lettuce juice were 8% (open triangles), 4% (filled triangles), 2% (open circles), 1% (filled circles) and 0.5% (open squares). Each point is the mean±SD (n=9).

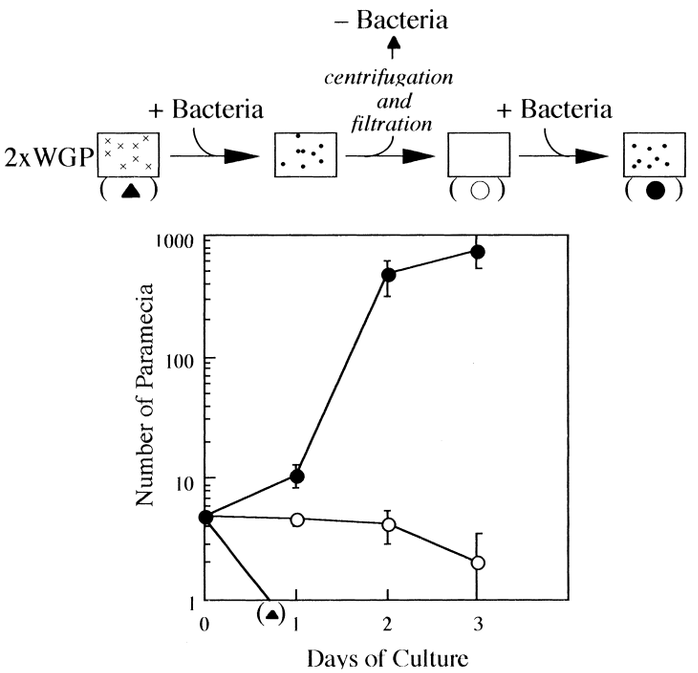


Fig. 6. Fate of 5 paramecia placed in 2×WGP before bacterial inoculation (closed triangle), or in 2×WGP in which bacteria were once grown and then eliminated by centrifugation followed by filtration (open circles), or in that same medium to which the eliminated bacteria were inoculated again (filled circles). Above the figure, the experimental procedure is illustrated with Xes for the supposed toxic substances in 2×WGP and dots for grown bacteria. Each point is the mean±SD (n=9).

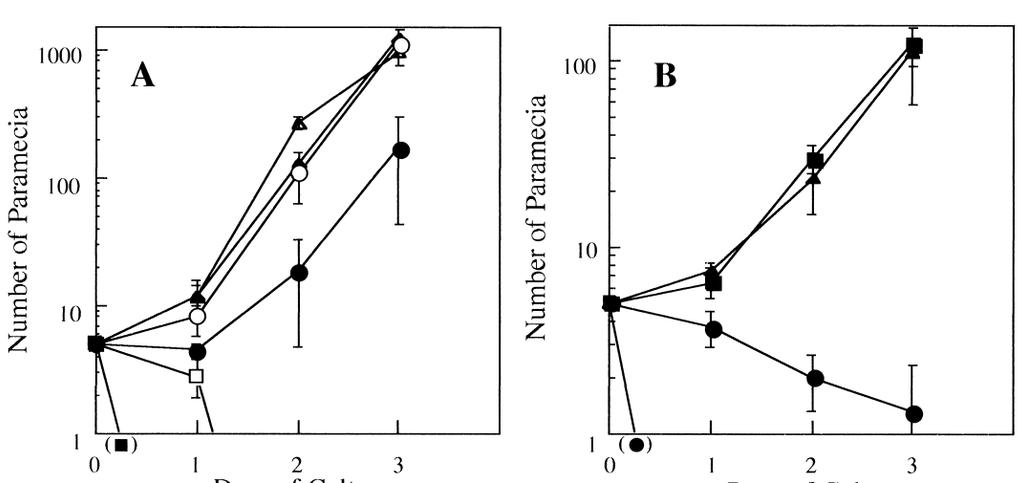


Fig. 7. Fate of 5 paramecia placed in 2×WGP immediately after different numbers of bacteria were suspended. A: Living bacteria were suspended at 10^8 (open triangles), 10^7 (filled triangles), 10^6 (open circles), 10^5 (filled circles), 10^4 (open squares) and 10^3 (filled squares) cells/ml. B: Killed bacteria were suspended at 10^8 cells/ml. Bacteria were killed by 20% formalin (filled squares), 1 mg/ml kanamycin (filled triangles) or autoclaving (filled circles). Each point is the mean±SD (n=9).

To test further this possibility, we performed similar experiments using dead bacteria. Bacteria were killed by formalin, kanamycin or autoclaving. We confirmed, by colony formation, that bacteria were not alive. As shown in Fig. 7B, paramecia grew in 2×WGP in which bacteria killed by formalin or kanamycin were suspended at the density of 10^8 cells/ml, while they tended to be reduced in cell number and to die out early or late in 2×WGP, in which bacteria killed by autoclaving were suspended at the density of 10^8 cells/ml. Bacterial cell contents may remain almost intact when killed by formalin or kanamycin, but may have been lost when killed by autoclaving. Because bacteria killed by autoclaving were countable under microscope, they had not been broken to pieces but only partly destroyed. The elimination of the WGP-

toxin may, therefore, be caused by neutralization by means of bacteria-derived substance rather than by adsorption on the bacterial cell surface, although the latter possibility can not be completely ruled out.

DISCUSSION

Paramecia, except for autotrophic species, are usually found in nature in water that is rich in organic substances and thus also rich in bacteria. Many kinds of combinations of plants (leaves and grains) and microorganisms (bacteria, yeast and algae) have been used for the cultivation of paramecia, although they differ greatly in suitability (Sonneborn, 1950, 1970). In our laboratory, phosphate-buffered WGP in which *Klebsiella pneumoniae* have been grown has long been used. Our stock of *P. tetraurelia* that has long been cultured in this medium was able to grow similarly in the medium prepared with different buffering solutions and with *E. coli* instead of *K. pneumoniae*. However, paramecia were unable to grow if WGP of low concentrations that resulted in a low density of bacteria was used (Fig. 1). This result supports the traditional interpretation of the role of plant infusion and bacteria as the food for paramecia: plant infusion is used for the growth of bacteria, and bacteria are used for the growth of paramecia (Sonneborn, 1950, 1970).

We found that this was not true, because neither as many as 10^9 bacteria/ml suspended in saline solution nor WGP without bacteria was able to support the growth of paramecia (Figs. 2, 3); both WGP and bacteria are necessary for the growth of paramecia. It was surprising that paramecia were not only unable to grow but were also killed in WGP containing no bacteria (Fig. 3). WGP apparently contains some toxic substance(s) as well as essential nutrients for paramecia. Similar substances are also contained in lettuce (Fig. 5). The key substance is of molecular weight less than 1,000 (Fig. 3C), although we can not exclude the possibility of involvement of additional components.

The most important finding in this study is that the toxic substance is changed to an inactive form by bacteria. Paramecia were killed in 2×WGP containing no bacteria, but survived in 2×WGP in which bacteria were once grown to 10^8 cells/ml in 2 days and then eliminated (Fig. 6). Interestingly, paramecia not only survived but also grew in 2×WGP in which 10^8 bacteria/ml killed by formalin or kanamycin were suspended (Fig. 7B). Since dead bacteria can not metabolize the WGP-toxin, the toxin may be eliminated either by adsorption on bacterial cell surface or by neutralization by some substance(s) released from killed bacteria. Bacteria killed by autoclaving looked intact in that they were countable under microscope and yet they were ineffective (Fig. 7B), suggesting that the detoxification by neutralization rather than by adsorption appears more likely. It may be that bacteria killed by autoclaving might have lost their cell contents more than those killed by formalin or kanamycin, or that the bacteria-derived substance could be a polypeptide and thus would have been inactivated by heat during autoclaving.

It also remains open whether the WGP-toxin is neutralized before or after it is incorporated into paramecia. This is related to the question whether the WGP-toxin acts externally on the cell surface of paramecia or acts internally after it is incorporated into food vacuoles. The result in Fig. 3B showing that paramecia began to die 7 hr after isolation into 2×WGP appears to support the internal action, although not decisive. Conjugating paramecia lacking the ability to form food vacuoles (Mikami, 1988) may be useful to examine this problem.

The results revealed in this study lead us to an understanding of the unexpected role of bacteria in the growth of paramecia. Bacteria play a critical role in saving the lives of paramecia not only by providing food resources but also by eliminating toxic components of food plants. Also in a physical sense, paramecia are living so closely with bacteria that bacteria can not be completely washed away from paramecia by passing repeatedly through sterilized saline-solution unless antibiotics are used. We thus understand that food bacteria living on and around paramecia may be regarded as ectosymbionts for host paramecia.

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