Mechanism of an Early Lysis by Fatty Acids from Axenic *Phormidium tenue* (Musty Odor-Producing Cyanobacterium) and Its Growth Prolongation by Bacteria

Naoki Yamada, Nobutoshi Murakami, Norihisa Kawamura, and Jinsaku Sakakibara

Aichi Prefectural Institute of Public Health, 7-6 Nagare, Tsujimachi, Kita-ku, Nagoya 462, Japan, and Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467, Japan.

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We have previously demonstrated that bacteria-containing *Phormidium tenue*, a cyanobacterium which produces musty odor 2-methylisoborneol, grew beyond 8 weeks, whereas axenic alga perished suddenly between the 3rd week and the 4th week while being cultured in the laboratory. This mechanism was investigated. It is assumed that when algal cells grow beyond a certain level, the supply of CO₂ becomes inadequate and results in the rapid lysis of axenic alga. At that time, inhibitory substances liberated from algal cells kill the surviving alga. Since the process occurs continuously, this alga is finally annihilated. On the other hand, since inhibitory substances are metabolized or degraded by bacteria coexistent with alga, bacteria-containing *P. tenue* maintains growth for a long time. The growth-inhibitory substance was found to be unsaturated free fatty acids.

Keywords cyanobacterium; *Phormidium tenue*; axenic; algal-bacterial interaction; autolysis; unsaturated free fatty acid

An earthy-musty odor, which occurs in approximately 50% of source-water reservoirs in Japan, has become a severe problem in the management of public water supplies. This unpleasant odor proved to be due to geosmin and 2-methylisoborneol (2-MIB), which are secondary metabolites produced by blue-green algae (cyanobacteria), particularly of the planktonic algae, and by the actinomycetes. An extraordinary increase in these aquatic microorganisms occurred frequently in eutrophic lakes and reservoirs.

In previous papers, we reported the isolation of bacteria-free *Phormidium tenue* (*P. tenue*), a cyanobacterium which produces 2-MIB. Also, we discussed growth characteristics such as optimal pH, temperature, light intensity and 2-MIB production in axenic and bacteria-containing *P. tenue*. Interestingly, the growth of bacteria-containing *P. tenue* was sustained for a considerable time, but axenic alga perished suddenly after a certain time. This observation suggested that bacteria was advantageous for sustaining the growth of *P. tenue*. Based on previous experimental observations, three possible mechanisms were considered: 1. Waste products from alga were metabolized and/or removed by bacteria. 2. Bacteria produce a growth-maintaining substance for alga. 3. The symbiosis between alga and bacteria consisted of a CO₂-O₂ exchange. In particular, CO₂ supplied by the bacteria may have facilitated the growth of alga.

In the present study, we examined these possibilities.

MATERIALS AND METHODS

**Alga Employed** Axenic alga employed throughout these studies was obtained from unialgal *P. tenue* by the capillary pipette-washing method. Unialgal *P. tenue* was originally isolated from the moat around the Nagoya Castle (Aichi Prefecture) in 1981 and was maintained in CT-medium.

**Medium and Growth Conditions** The medium used for *P. tenue* was CT-medium as shown in Table I. Algal cultures (100 ml in a 200 ml Erlenmeyer flask capped with a silicon rubber-stopper) were grown at 24 ± 1°C with continuous illumination at 1000 lux by 20 W cool-white fluorescent lamps at flask level. The initial density of alga was 1.0 µg for chlorophyll a (Chl.a) amounts. During the course of the experiment, the stationary flasks were swirled gently once a day. These culture conditions were used unless otherwise specifically mentioned. Growth of algal culture was followed by Chl.a measurement as described by Parsons & Strickland. Algal cultures were checked for bacteria contamination in parallel with Chl.a analysis. Culture data was determined for the three flasks and the mean value was used.

**Effect of Exchange of Freshly Prepared Medium on the Growth of Axenic *P. tenue*** After cultivation for 3 weeks to attain the maximum growth, the culture supernatant was removed by centrifugation at 10000 rpm for 20 min. Freshly prepared medium was then added and cultivation was continued.

**Effect of Bacterial Products on the Growth of *P. tenue***

### TABLE I. Composition of CT-Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(NO₃)₂·4H₂O</td>
<td>150 mg</td>
</tr>
<tr>
<td>KNO₃</td>
<td>100 mg</td>
</tr>
<tr>
<td>β-Na₂glycerophosphate</td>
<td>50 mg</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>40 mg</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.1 µg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.1 µg</td>
</tr>
<tr>
<td>Thiamin-HCl</td>
<td>10 µg</td>
</tr>
<tr>
<td>PVI metals</td>
<td>3 ml</td>
</tr>
<tr>
<td>TAPS</td>
<td>400 mg</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Fill up to 11 with deionized water. a) PIV metals: FeCl₃, 6H₂O; 19.6 mg; MnCl₂·4H₂O; 3.6 mg; ZnSO₄·7H₂O; 2.2 mg; CoCl₂·6H₂O, 0.4 mg; Na₂MoO₄·2H₂O, 0.25 mg; Na₂EDTA·2H₂O, 100 mg in distilled water. b) TAPS. N-tris (hydroxymethyl) methyl-3-aminopropane sulfonic acid.

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Bacteria of genera *Flavobacterium* and *Micrococcus* isolated from unialgal *P. tenue* were grown on a PYG-medium containing 2.0 g proteose peptone (Difco), 1.0 g yeast extract (Difco) and 0.5 g glucose in 1 l of water, with shaking at 30°C for 5 d. These bacteria were harvested by high-speed centrifugation at 17000 × g and washed three times with distilled water. After washing, the bacteria were resuspended in 50 ml of distilled water and sonicated at 20 kHz, 100 W for 10 min and destroyed. An aliquot (ca. 1 × 10^11 cells) of the bacterial suspension was added to a 3-week-old culture of axenic *P. tenue* and cultivation was continued.

**Effect of the Addition of Glucose on the Growth of Bacteria-Containing and Axenic *P. tenue*** This experiment was carried out using CT-medium with K_2HPO_4 instead of sodium β-glycerophosphate for the exclusion of organic materials in the medium. Twenty ml of CT-medium were placed in 50 ml Erlenmeyer flasks. Axenic and bacteria-containing cultures of *P. tenue* were grown in the presence or absence of glucose.

**Effect of CO₂ on the Growth of *P. tenue*** In the growth of axenic *P. tenue* in laboratory cultures, atmospheric CO₂ may become one of the limiting factors. To test this possibility, an axenic culture was continuously aerated with sterilized air passed through a 0.2 μm membrane filter (Mirex FG-50, Millipore) at 80 ml/min from the 3rd week on.

In order to further confirm the enhancing effect of CO₂ in air on the growth of axenic *P. tenue*, CO₂-enriched air was supplied to the algal culture. The axenic culture was exposed to sterilized air containing 2% CO₂ which was passed through a membrane filter (Mirex FG-50, Millipore) at 100 ml/min for 10 min, twice a day from the 3rd week on.

**Effect of Exhausted Medium on the Growth of *P. tenue*** Exhausted media, which had nourished *P. tenue*, was filtered through a glass fiber filter paper (pore size, ca. 1.2 μm, Whatman GF/C) under vacuum. These media were newly enriched with adequate amounts of nutrients and trace metals according to Table I and adjusted to pH 8.0. Then, these media were sterilized by filtration using membrane filters (pore size, 0.2 μm, Millipore) and axenic *P. tenue* was inoculated into 100 ml of each medium.

Each exhausted medium in this experiment was as follows: Group A was the control and used newly prepared medium. Group B was the filtrate of a 5-week-old culture of axenic *P. tenue* which had already lysed and perished. Group C was the filtrate of a 3-week-old culture of axenic *P. tenue* which did not cause cell lysis. Group D was the filtrate of a 5-week-old culture of bacteria-containing unialgal *P. tenue* which did not cause cell lysis. Additionally, axenic *P. tenue* was inoculated into the autoclaved-exhausted medium (group E) and unialgal *P. tenue* was inoculated to Millipore-filtered exhausted medium (group F), and then cultivated. The exhausted media E and F were the filtrates of a 5-week-old culture of axenic *P. tenue* which had lysed and perished.

Further experiments were carried out as follows: Bacteria of genera *Flavobacterium* and *Micrococcus* (ca. 1 × 10^8 cells), which were isolated from unialgal *P. tenue*, were inoculated into Millipore-filtered exhausted medium after 5 weeks of culturing axenic *P. tenue*, respectively. These cultures were incubated at 30°C for 5 d on a reciprocal shaker at 80 exciton/min. The bacterial cultures were filtered through a glass fiber filter paper and nutrient elements were supplemented to be equal to the original CT-medium. These filtrates were sterilized through a membrane filter (Sterifil D, pore size, 0.2 μm, Millipore) and the axenic *P. tenue* was inoculated into 100 ml Erlenmeyer flasks containing 50 ml of each filtrate.

**Extraction and Identification of Algal Growth Inhibitor from the Lysed Culture of *P. tenue*** Lysed culture (3 l) of *P. tenue* was filtered through filter paper (Whatman GF/C). The filtrate was adjusted to pH 2—3 with 2 N HCl and was extracted twice with ethyl acetate. The extract was then washed with twice saturated NaCl solution and evaporated until dry under reduced pressure at 40°C. The extracted material was dissolved in 1.5 ml of methanol, and a 0.5 ml aliquot was subjected to bioassay. Another 1 ml aliquot of the extract was further purified by preparative thin-layer chromatography (TLC) on a Merck precoated Kieselgel 60F_254 with chloroform—methanol (10 : 1) as the developing solvent. Each band was scraped off, dissolved in CHCl₃—MeOH (1 : 1) and filtered through filter paper. The filtrate was evaporated, and the TLC fraction which showed growth inhibitory activity was then determined after methylation with diazomethane in gas-liquid chromatography—mass spectroscopy (GC-MS) measurements as described by our previous paper.

**Bioassay** Methanol solution of extract was sterilized by filtration using a membrane filter (pore size, 0.2 μm, Advantec). 200 μl of filtrate was added to a 50 ml Erlenmeyer flask containing 20 ml of *P. tenue* culture pre-cultivated for 10 d. The control was a culture to which only 200 μl of methanol was added. The extract in a 200 μl aliquot inoculated into a 20 ml culture corresponded to 20 times that in the original culture. The remaining extract was diluted with an equal volume of methanol. By two-fold serial dilution, the test cultures had extracts corresponding to 10, 2.5, 1.25 and 0.63 times that in the original culture, respectively.

The cultures were incubated for 3 d under conditions as described above, and the concentration of Chl.a was measured. They were bioassayed in duplicate.

**RESULTS AND DISCUSSION**

The interaction between blue-green algae and bacteria has been reported by several researchers. For example, Lang reported that alga-bacteria interaction was a mutually beneficial association, with bacteria producing CO₂ and algae producing O₂ during photosynthesis. Yagi et al. reported that associated bacteria affected floc formation of *Microcystis*.

**Effect of Exchange of the Freshly Prepared Medium on the Growth of Axenic *P. tenue*** When waste products from axenic alga exceed a certain level, the culture condition was aggravated, so that algal lysed and perished. Based on the above possibility, an experiment was carried out. The result is shown in Fig.1. In spite of the exchange of freshly prepared medium, algal cells rapidly lysed, similarly to the control. The exchange of the exhausted media for fresh
media neither prolonged the growth period nor increase the maximum growth.

It appears that the early and sudden lysis of axenic alga is not directly due to its own excreted waste products.

**Effect of Bacterial Products on the Growth of P. tenue**

In order to examine the possibility that prolongation of the growth period of alga is due to substances produced by bacteria coexistent with alga, a bacterial suspension was inoculated into a maximum growth 3-week-old axenic culture. The results shown in Fig. 2 indicate that both experimental groups rapidly perished, similar to the control, and prolongation of the growth period did not occur.

It appears that the bacteria do not produce substances which maintain or accelerate algal growth.

**Effect of the Addition of Glucose on the Growth of Bacteria-Containing and Axenic P. tenue**

It is likely that the symbiotic interaction consists of an interchange of CO₂ produced by bacteria and O₂ produced by algae. In particular, CO₂ is the limiting factor in the growth of culture in the laboratory. Figure 3 shows the results when bacteria-containing unialgal and axenic cultures of P. tenue were grown in the presence or absence of glucose. Glucose had no effect on the axenic alga, but it vigorously stimulated the growth of the association of bacteria and alga. The algal growth curve seemed to coincide with bacteria count.

This result suggests that bacteria assimilate added glucose and produce CO₂, which in turn accelerates algal photosynthesis. It appears that algal growth was accelerated significantly by culturing in a CO₂ atmosphere at the concentration produced by bacteria. Also, Lang has reported similar results for other cyanobacteria, genera Microcystis and Anabaena.\(^\text{12}\)

**Effect of CO₂ Gas on the Growth of P. tenue**

From the result in Fig. 3, since CO₂ gas produced by bacteria may be an important factor affecting the growth of P. tenue, an axenic culture in which maximum growth just before lysis was achieved was aerated with sterilized air from the 3rd week on. The result is shown in Fig. 4. Lysis of the alga was extensively delayed by an aeration, as compared with the control. CO₂ gas caused prolongation of the growth period of alga. In addition, an increase in CO₂ by aeration was accompanied by vigorous growth enhancement in the alga.

These results suggest that the early, sudden lysis of axenic alga was due to a deficiency in the supply of CO₂ gas in air passing through the silicon rubber-stopper.
Effect of Exhausted Medium on the Growth of *P. tenue*

If early lysis of alga is due to a simple deficiency in the supply of CO₂, the alga might not perish completely, but rather maintain a certain growth level. However, axenic alga died off early and completely. The results are shown in Fig. 5a. When axenic *P. tenue* was inoculated into the exhausted medium (group B) which had lysed, the alga could not support growth at all and the algal cells were not able to survive. In the case of the exhausted medium (group C and D) which did not cause cell lysis, alga grew similarly to the control (group A), followed by rapid lysis of the cells. The exhausted and autoclaved medium (group E) could not support growth at all.

Figure 5b shows that when unialgal *P. tenue* was inoculated into the membrane-filtrated medium of an exhausted axenic culture (group F), the algal cells grew similarly to the unialgal control (group G) and survived for longer than 8 weeks.

It was tested whether substances released into the lysed culture were metabolized by bacteria coexistent to unialgal *P. tenue*. The result in Fig. 5 indicates that the lysed culture pre-cultivated with both bacteria showed the same growth curves as the control. These attained maximum growth after 3 to 4 weeks and then the alga rapidly lysed and perished. These results indicate that when alga cells grow beyond a certain level, the supply of CO₂ tends to be deficient, as compared with the algal growth rate. At that time, lysis of axenic alga occurs. The inhibitory substances produced by alga are secreted into the culture, so that surviving alga are further attacked by inhibitory substances from the dead alga. This process occurs continuously and the alga is ultimately annihilated.

**Extraction and Identification of Algal Growth Inhibitor from the Lysed Culture of *P. tenue***

Approximately 10.7 mg of oily yellowish-white substance was obtained by ethyl acetate extraction from the lysed culture (3 l) after cultivating axenic *P. tenue* for 4 weeks.

This ethyl acetate extract was further purified by preparative TLC. As a result, six bands were obtained by TLC. Fraction 3 (Rf 0.6—0.5) exhibited potent inhibitory activity and was determined to be a mixture of free fatty acids by GC-MS measurement. The mixture of free fatty acids was found to consist mainly of myristic (C₁₄:0), palmitic (C₁₆:0), palmitoleic (C₁₆:1), cis-vaccenic (C₁₈:1), linoleic (C₁₈:2) and linolenic acid (C₁₈:3) in a ratio of 52:5:7:2:2:4:28. On the other hand, we reported that the composition of free fatty acids from *P. tenue* cells consisted of C₁₄:0, C₁₆:0, C₁₈:1, C₁₈:2 and C₁₈:3 in a ratio of 5:4:5:4:1:47:31. It was suggested that this difference in composition was due to the occurrence of fatty acids liberated from membrane lipids by esterase in the alga cells causing lysis.

**Bioassay** Table II shows the result of the algal growth inhibitory activity of ethyl acetate extract and TLC fractions from the lysed culture of *P. tenue*. The relative growth rate of alga was expressed as a percentage of the control.

A 2.5-fold or greater concentration culture of ethyl acetate extraction showed a strongly inhibitory effect on the growth of *P. tenue*, as compared with the control. A 1.25-fold concentration culture, which was approximately equal to the original culture, showed minimum growth inhibition, and its concentration was 4.5 ppm. In TLC...
Fig. 6. Effect of Exhausted Media Preincubated by Bacteria on the Growth of P. tenue

Control, ——; preincubated (Flavobacterium), ——Δ——; (Micrococcus), ———.

TABLE II. Growth-Inhibitory Activity of Extract from the Lysed Culture of P. tenue

<table>
<thead>
<tr>
<th>Concentration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ethyl acetate extract</th>
<th>TLC active fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ratios to original culture</td>
<td>ppm</td>
<td>Growth activity&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>72</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>—</td>
</tr>
<tr>
<td>2.5</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>1.25</td>
<td>4.5</td>
<td>+++</td>
</tr>
<tr>
<td>0.63</td>
<td>2.25</td>
<td>++++</td>
</tr>
<tr>
<td>0.31</td>
<td>1.13</td>
<td>++++</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>++++</td>
</tr>
</tbody>
</table>

<sup>a</sup> The numbers express the extract concentration ratios to the test media to that of the original culture.  
<sup>b</sup> ++ + + +, 100–80% of control growth; ++ + +, 80–51%; ++ +, 50–26%; ++, 25–11%; —, less than 10%.

fractions, Fr. 3 and 4 showed growth inhibitory activity. Fraction 3 exhibited potent inhibitory activity at concentrations up to 0.75 ppm, while Fr. 4 exhibited weak activity at 15 ppm.

In our previous report,<sup>8</sup> we demonstrated that unsaturated fatty acids such as linoleic and linolenic acids were potent growth inhibitors, while saturated fatty acids were inactive even at 100 ppm. The minimum growth inhibitory activity of these fatty acids occurred at a concentration of 0.5 ppm.

From the results of the above experiments, the growth-inhibitory substance from the lysed culture was found to be a mixture of some saturated and unsaturated fatty acids. These have been demonstrated by several researchers.<sup>16-17</sup> Recent reports have discussed allelopathic substances in aquatic plants.<sup>14-18</sup> It has often been suggested that algal extracellular metabolites play an important role in controlling succession in algal populations.

In conclusion, we suggest that the mechanism of the early lysis of axenic P. tenue and algal growth prolongation by bacteria was as follows: in the growth of P. tenue in laboratory cultures, atmospheric CO<sub>2</sub> gas may be one of the limiting factors. When the supplement of CO<sub>2</sub> gas became insufficient compared to the algal growth rate, algal lysis occurred. Free fatty acids liberate from the lysed cells, and then kill the surviving alga. This process occurs continuously until the alga is ultimately annihilated. On the other hand, since fatty acids are metabolized or decomposed by bacteria coexistent with alga, unialgal culture continued to grow for a long time.

From the present experiment, it is assumed that the enhancement of algal growth on CO<sub>2</sub> produced by bacteria and the decay by free fatty acids originating from algal lysis might be one of the factors affecting algal populations in lakes and ponds.

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