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Effect of Filamentous Bacteria on the Growth of *Chattonella marina*

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Abstract

A filamentous bacterium (strain F193-3), which forms small whitish-pink colony, was isolated from the stock culture of *Chattonella marina*. The addition of the filamentous bacterium to the axenic culture of *C. marina* resulted in the increase of algal cell yield and extension of the growth period. Vitamin B_{12} in the axenic culture was exhausted for 15 days of incubation, while its concentration increased from 3.8 to 5.8 ng/m*l* in the algal culture with the filamentous bacterium. These findings indicated that vitamin B_{12} was produced by the filamentous bacterium and that it was effective for the growth of *C. marina*.

Microalgae transform inorganic nutrients into the organic matters through photosynthesis in marine ecosystems. Some organic substances such as glycolate which are excreted into the water from algal cells, are probably utilized by bacteria shortly after excretion.[1] On the other hand, microalgae require some essential nutrients including vitamins besides carbon dioxide and minerals.[2] In the previous paper,[3] we reported that the stock culture of a red-tide alga, *Chattonella marina*, harbored several bacteria and that the viability of algal cells improved in the algal culture with these bacteria.

This algae-bacteria relationship may be based on the capability of algae to produce organic compounds and oxygen which are utilized by bacteria. In turn, bacteria supply nutrients such as vitamins, amino acids, or other metabolites that are required for the growth of algae. Some bacteria have been isolated from fresh water, sea water, suspended matters and coastal marsh muds; they provide vitamins including vitamin B_{12} in freshwater and marine environments.[4-7] The vitamin production and utilization by microalgae were also observed by many workers.[8-10]

In this paper, we compared the growth of *Chattonella marina* with or without a filamentous bacterium isolated from the stock culture of *Chattonella marina* in order to evaluate the effect of co-existent bacteria on algal growth.

Materials and Methods

Organisms

An axenic strain of *Chattonella marina* was prepared and a filamentous bacterium F193-3 was isolated from the stock culture of *C. marina*, as previously described.[3] *Euglena gracilis* strain z was obtained through the courtesy of Dr. Toshitaka Nishijima of Kochi University. Stock cultures of *Euglena* were transferred every week and incubated at 30℃ under 3,000-4,000 lx of fluorescent lamps in the stock culture medium containing the basal medium as described in Table 1 with 15 g sucrose, 2.5 g agar, 2.0 g Tryptone and 50 ng vitamin B_{12} per litre.

Scanning Electron Microscope

Bacterial strains were cultured in a L-shape test tube containing a 10 m*l* ZE-CI medium at 25℃ for 3 days. Preparation of sample for scanning electron

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Table 1 Composition of *Euglena gracilis z* strain basal medium (double strength)

Ingredient*1	Amount (g/l)	Ingredient of metals 45*2	Amount (g/l)
KH_2PO_4	0.60	$FeSO_4(NH_4)SO_4 \cdot 6H_2O$	14.000
$MgSO_4 \cdot 7H_2O$	0.80	$ZnSO_4 \cdot 7H_2O$	4.400
L-Glutamic acid	6.00	$MnSO_4 \cdot H_2O$	1.550
$CaCO_3$	0.16	$CuSO_4 \cdot 5H_2O$	0.310
DL-Aspartic acid	4.00	$CoSO_4 \cdot 7H_2O$	0.480
DL-Malic acid	2.00	H_3BO_3	0.570
Glycine	5.00	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.640
NH_4HCO_3	1.24	$NaVO_3 \cdot 4H_2O$	0.038
Succinic acid	0.94		
Metals 45*2	0.44		
Thiamin HCl	(1.2mg)		
Sucrose	30.00		

*1 A half amount of dry mixture was solved in 1 *l* of deionized and distilled water.
*2 Dry mixture of metals 45.

microscopic observation was carried out by a modified method of Maeda *et al.*[11] The appropriate bacterial suspension was fixed with 1% of glutaraldehyde and later concentrated onto a 0.2μm Nucleopore filter under moderate vacuum. Bacterial cells on the filter were dehydrated stepwise using a series of ethanol solutions. Successively ethanol was substituted by *t*-butyl alcohol, and the dehydrated samples were kept in cold storage. The filters were dried by means of a *t*-Bu Freeze Dryer (VFD-21) and then coated by gold with an ion coater (EIKO IB-3). Bacterial samples were observed and photographed on a scanning electron microscope (Hitachi, Model S-4100 H).

Epifluorescence Microscopy

The appropriate bacterial suspension was fixed with 1% glutaraldehyde, and then added with 10 drops of 4', 6-diamidino-2-phenylindole (DAPI) at a final concentration of 0.5μg/m*l*. Samples were mixed well and stored in cold storage for 20 min. Bacterial cells were concentrated onto a 0.2μm Nucleopore filter under moderate vacuum. The filters soaked with low fluorescence immersion oil were mounted on a glass slide and examined immediately by an epifluorescence microscope (Olympus, BH-2) with an ultraviolet excitation filter and photographed.

Vitamin Requirements of Algal Cells

Axenic cultures of *C. marina* were maintained in ESS media in the same condition as described in the previous paper.[3] All glasswares were acid-cleaned, rinsed thoroughly, and baked at 160℃ for 60 min. Prior to any experiments, axenic algal cells were starved for vitamins by culturing in the vitamin-free ESS medium for 7 days. Vitamin requirement tests were done in the vitamin-free ESS media which were enriched with vitamin B_{12}, biotin, or thiamin at 4, 2, or 200 ng/m*l*, respectively. The concentrations of vitamins were chosen based on the Provasoli's enrichment solution (ESP).[12]

Preparation of Samples for Vitamin B_{12} Bioassay

Prior to any experiments, axenic algal cells were starved for vitamins by culturing in the vitamin-free ESS medium for 7 days. Then, 500μ*l* of the algal cell culture was added to 9.5 m*l* of ESS medium without the addition of bacterial cells or with the addition of 0.5 m*l* logarithmic phase culture of a filamentous bacterium F193-3. Before assay of concentration of vitamin B_{12}, culture supernatants were obtained by centrifuging the axenic algal cultures or mixed cultures at 8,000 rpm for 20 min. The supernatant solutions were first filtered through a glass fiber filter (GF/C) and then by a Millipore filter (0.45μm), after which the filtrates were freeze-stored. The supernatants obtained were diluted appropriately. Two and a half m*l* of the double-strength basal medium was added to 2 m*l* of each dilution sample and 0.5 m*l* of DDW was added to give a total volume of 5 m*l*, then measured the vitamin B_{12} concentration by a bioassay method for *Euglena gracilis*.[13-14]

Bioassay for Vitamin B_{12}

A half m*l* of the stock culture of *Euglena gracilis* strain z was added to 5 m*l* of a pre-incubation medium containing the basal medium with 15 g sucrose and 80 ng vitamin B_{12} per litre. After 2-6 days of incubation, a green colored culture was selected and transferred into a sterile tube for centrifugation at 3,000 rpm for 10 min. Using aseptic technique, the supernatant was decanted and the algal cells were suspended in 10 m*l* of the sterile pre-incubation medium (vitamin B_{12} free). The algal cells should be washed in this manner three times to reduce B_{12} carry-over. The washed cells were suspended at an appropriate dilution of optical density at 430 nm of wave length to 0.10-0.15, and transferred one drop by a sterile micro-syringe to 5 m*l* of test sample solutions or vitamin B_{12} standard solutions, then incubated at 30℃ under dark condition. After 7 days of incubation, the test tubes were agitated uniformly and optical density of the test cultures was determined at 430 nm using a spectrophotometer.

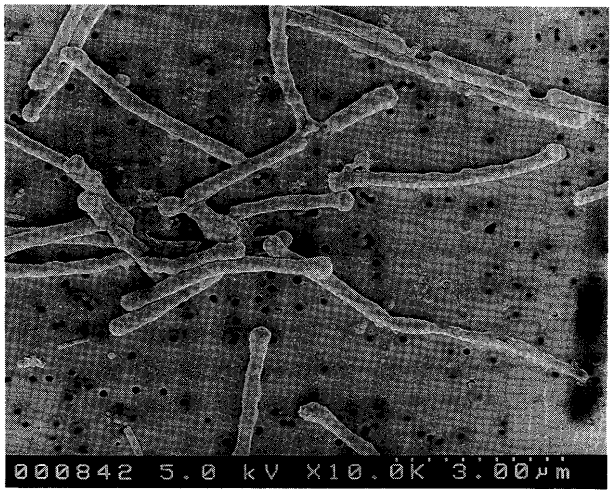

Fig. 1 Scanning electron micrograph of a filamentous bacterium F193-3.

Results

Microscopic Observations

A scanning electron micrograph and an epifluoresence micrograph of filamentous bacterium F193-3 are shown in Figs. 1 and 2. Filamentous bacterial cells are shown to form multicellular rods, which are 0.8-1.2㎛ wide and 4-15㎛ long. As shown in an electron micrograph, both end cells of bacterial filaments are considerably different from other not-end cells in cell length and the rounded cell-end form.

Vitamin Requirements

For determining vitamin requirement, the vitamin starved axenic algal cells of *Chattonella marina* were cultured in ESS media containing vitamins and in a vitamin-free medium as the control. As demonstrated in Fig. 3, algal cells completely died within 3 days of

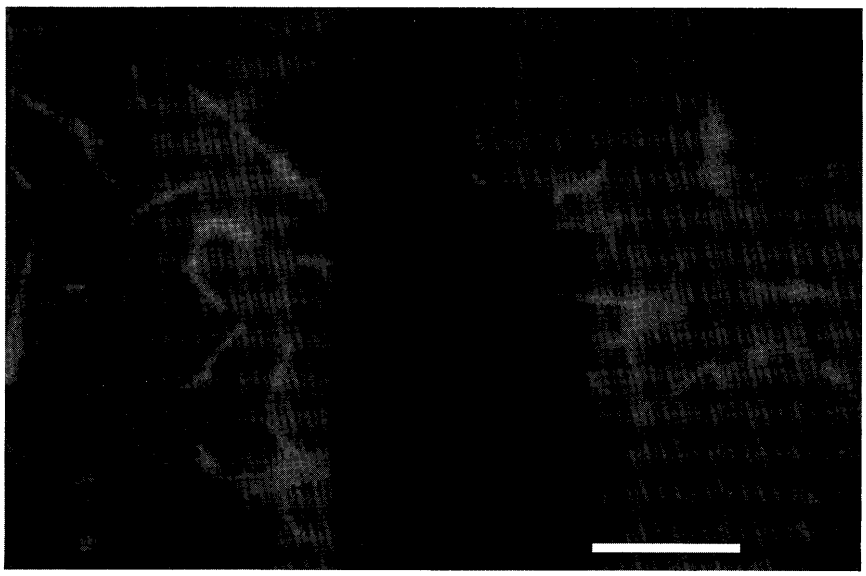

Fig. 2 Epifluorescence micrograph of a filamentous bacterium F193-3 stained by DAPI. The bar indicates 15㎛.

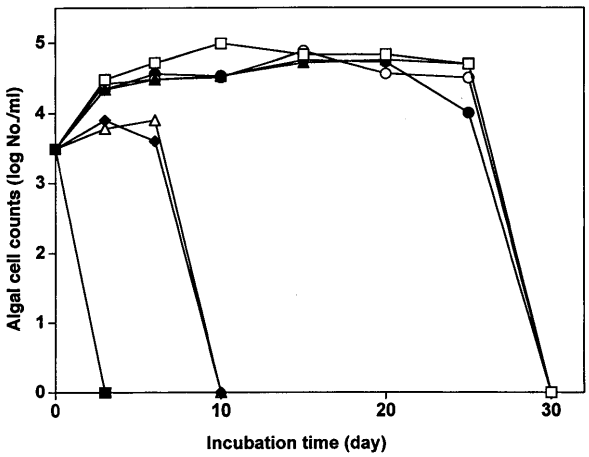

Fig. 3 The growth of axenic starved cells of *C. marina* in ESS media containing various vitamins.
■, ESS vitamin-free medium; ◆, ESS medium added with biotin (2 ng/m*l*); △, with biotin (2 ng/m*l*) and thiamin (200 ng/m*l*); ●, with vitamin B_{12} (4 ng/m*l*); ○, with thiamin (200 ng/m*l*) and B_{12} (4 ng/m*l*); ▲, with biotin (2 ng/m*l*) and B_{12} (4 ng/m*l*); □, ESP containing B_{12} (4 ng/m*l*), thiamin (200 ng/m*l*), and biotin (2 ng/m*l*);

incubation in the vitamin-free medium and within 10 days in the media with biotin or biotin and thiamin. However, the algal cells cultured in vitamin B_{12} containing media were maintained for 25 days and completely died after 30 days of incubation. These findings indicate that vitamin B_{12} is an essential nutrient among the B group vitamins for the growth of *Chattonella marina*.

Algal Growth with Vitamin B_{12} in Mixed Culture

Growth curves of *C. marina* in an axenic culture and in a mixed culture with a filamentous bacterium (strain F193-3), as well as vitamin B_{12} concentration profiles in the algal cultures are shown in Fig. 4. The vitamin B_{12} concentration in the axenic and mixed cultures was calculated based on the calibration curve as shown in Fig. 5, in which OD values of the cultures of *E. gracilis* in the basal media with each concentration of vitamin B_{12} were plotted against concentrations of vitamin B_{12} (pg/5 m*l*).

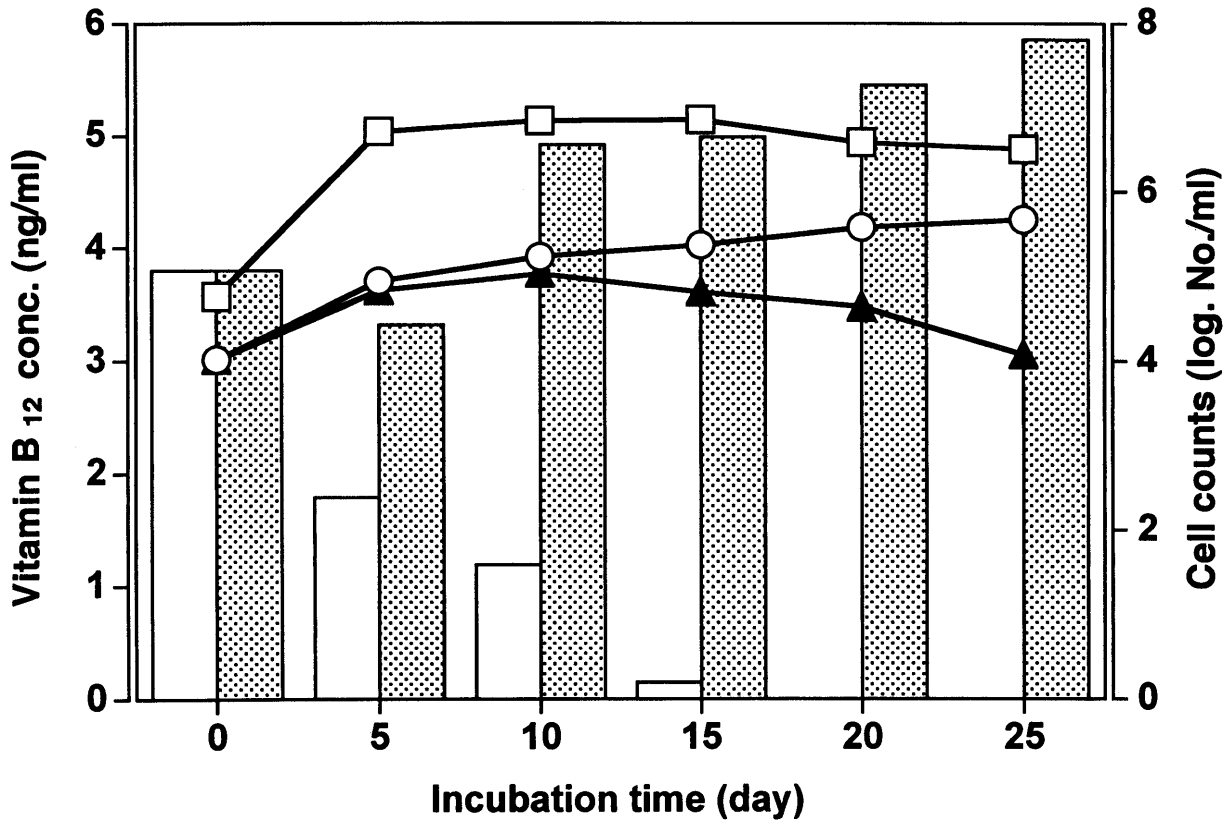

Fig. 4 Algal growth and vitamin B_{12} concentration in axenic and mixed cultures.
White columns and patterned columns show vitamin B_{12} concentrations in the axenic culture and mixed culture of *C. marina*, respectively. ○, algal cell count in the mixed culture with F193-3; ▲, algal cell count in the axenic culture; □, colony count of F193-3 in the mixed culture.

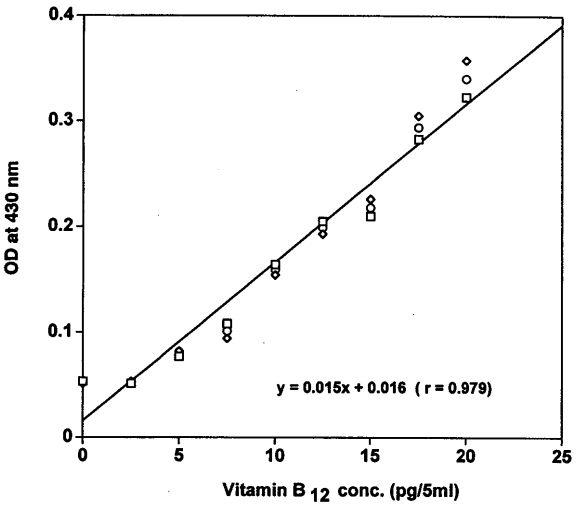

Fig. 5 Calibaration curve for vitamin B_{12} bioassay using *Euglena gracilis* strain z.

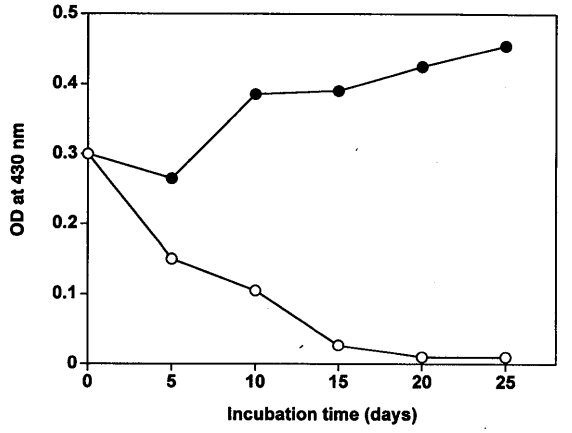

Fig. 6 Growth response of *Euglena gracilis* strain z for 7 days of incubation in the cuture filtrates of axenic (○) and mixed (●) cultures of *C. marina* with F193-3 obtained at different incubation times.

The vitamin B_{12} concentration in the axenic culture of *C. marina* was closely correlated with the algal growth. The algal cell count rose from 4.1 log No./m*l* at initial time to 5.1 log No./m*l* at maximum for 10 days of incubation, and while vitamin B_{12} concentration decreased from 3.8 ng/m*l* to 1.2 ng/m*l*. Thereafter, the algal cells decreased as vitamin B_{12} in the culture dropped to the lower levels. This observation indicated that vitamin B_{12} in the culture was consumed by algal cells for maintaining their growth.

Different outcome on the algal growth and vitamin B_{12} concentrations occured when algal cells were cultured in a mixed culture with the filamentous bacterium. After commenced by 4.1 log No./m*l*, the algal cell number in the mixed culture gradually increased to about 5.7 log No./m*l* for 25 days of incubation. In addition, the total viable count of the filamentous bacterium also increased from 4.8 log No./m*l* to 6.9 log No./m*l* for 15 days of incubation and then was maintained at 6.5 log No./m*l* until 25 days incubation. As the growth of the bacterium was enhanced, the concentration of vitamin B_{12} in the culture was slightly dropped from 3.8 ng/m*l* of initial concentration to 3.3 ng/m*l* and then increased to 5.8 ng/m*l* for 25 days of incubation.

Optical density of the culture of *Euglena gracilis* used for the bioassay of vitamin B_{12} decreased in the culture filtrate from an axenic culture of *Chattonella marina*, while it increased for the culture filtrate of non-axenic algal culture with the filamentous bacterium as shown in Fig. 6. The results obtained in this study indicated that vitamin B_{12} was produced by the bacterium F193-3, and it was effective for the growth of *C. marina*.

Discussion

Qualitative and quantitative studies on vitamin B_{12} in the fresh water and sea water environments have been carried out by a number of investigators. Vitamin B_{12}-active compounds have been shown to be essential or stimulatory for the growth of various marine microorganisms, especially microalgae. Carlucci[14] reported that *Dunaliella teriolecta* produced vitamin B_{12} but not other 4 microalgae. It was reported by Nishijima and Hata[15] that *Chattonella antiqua* required essentially vitamin B_{12} among the B group vitamins. On the other hand, Starr *et al.*[16] demonstrated that 63% marine bacteria tested had produced vitamin B_{12} activity. Yu *et al.*[17] described that several bacteria were isolated from culture tanks of the rotifer *Brachionus plicatilis* and that they played an important role as vitamin B_{12} supplier for the rotifer growth.

In this study, it was pointed out that among the B group vitamins, vitamin B_{12} is an essential nutrient for the growth of *C. marina*. Axenic algal cells

continued to grow for a longer time in the media containing vitamin B_{12} than in the media without vitamin B_{12}. The high concentations of vitamin B_{12} in the algal culture medium gave the maximum growth rate and cell yield for algal growth, whereas the limitation of vitamin B_{12} generally suppressed the growth rate and cell yield. This result showed that vitamin B_{12} in the culture medium was utilized by axenic algal cells during incubation to increase the cell density up to 6 log No./m*l*. The adequate concentrations of vitamin B_{12} caused the algal cells to look apparently active during logarithmic growth phase. *C. marina* required vitamin B_{12} essentially for its cell growth and division. Therefore, this compound must be supplied by other microorganisms for *C. marina* cells to grow in the culture after it was exhausted.

Filamentous bacteria were isolated from non-axenic culture of *C. marina* and were tentatively identified as *Flexibacter* spp., as described in the previous paper.[3] Filamentous bacteria formed multicellular rods and small, whitish-pink and rough colonies. The addition of these bacteria to the culture of the axenic algal strain resulted in the increase of algal cell yield and extension of algal growth as compared with the axenic culture. It was expected that the algal cells utilized vitamin B_{12} added initially to the culture medium for early incubation period until co-existent bacteria began to grow at the maximum rate to produce vitamin B_{12} vigorously.

The same filamentous bacterial species were also isolated as one of the predominant bacteria from the culture of *H. akashiwo*, which was isolated from the bloom occurred in April, 1995 in Kagoshima Bay, and the culture water of *Tetraselmis* sp., which was cultured as the food for Kuruma prawn in an aquaculture facility, Kagoshima Prefecture.

These results suggest that certain co-existent bacteria including filamentous bacteria provide some essential nutrients such as vitamin B_{12} to give an advantage for the growth of various microalgae.

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