シャトネラ・マリーナの保存株から分離されたフレキシバクターの増殖挙動

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Growth Response of *Flexibacter* sp. Isolated from the Stock Culture of *Chattonella marina*

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Keywords : *Chattonella marina*, *Flexibacter*, co-existent bacteria, vitamin B_{12}.

Abstract

Co-existent bacteria isolated from the stock culture of *Chattonella marina* on peptone-yeast extract (Z-C Ⅱ) agar plates were predominantly classified into four colony types. Among them, tiny pale pinkish colonies were composed mainly of a filamentous bacterium, which was identified as *Flexibacter* sp. on the basis of bacteriological characterization. The isolates of *Flexibacter* sp. formed very tiny circular colonies on Z-C Ⅱ agar, but they produced large thin rhizoidal colonies on yeast extract-enrichment solution (YES) agar. The agar media containing various kinds of peptones, sugars, organic acids or nucleic acid bases yielded very slow growth of bacterial colonies as compared with those on YES agar. The isolates were shown to have an essential vitamin B_{12} requirement for growth. Maximum growth yield of the isolates was observed at 26℃ and they could not grow above 40℃.

Chattonella marina is belonging to the class Raphidophyceae and well known to be a microalga causing red tide. One strain of *C. marina* have been isolated from Kagoshima Bay in which red tide occurred in June of 1982. The strain of *C. marina* have been given and maintained since then in our laboratory. The stock culture stored in our laboratory is not axenic and has several strains of co-existent bacteria. The non-axenic strain of *C. marina* is found to be alive in a batch culture for a longer period as compared with the axenic one. Therefore, the stock culture strain of *C. marina* is considered to have mutual relationships with co-existent bacteria for their growth.

In natural environments, many bacteria surrounding algal cells have been reported to be supplied with organic compounds including glycollate by microalgae, while microalgae are known to obtain growth factors such as vitamins produced by some bacteria[1-5].

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In this paper, we isolated a filamentous bacterium, identified as *Flexibacter* sp. from the stock culture of *C. marina* as one of the predominant bacteria and examined the growth response of the bacterial isolates in laboratory conditions with aim of elucidating the interactions between *C. marina* and co-existent bacteria.

Materials and Methods

Algal strain and cultural conditions

Chattonella marina strain was isolated from seawater of Kagoshima Bay in June of 1982 by Mr. Takayuki Aramaki (Kagoshima Prefectural Fisheries Experiment Station) and given to our laboratory. This is the original strain of NIES deposit No. 121[6]. The algal strain was cultured in ESP liquid medium[7] under illumination of 5,000–7,000 lx (12L:12D) at 23℃. An aliquot of the culture was transferred to new media (0.5 mℓ per 10 mℓ of ESP) every 20 days. The cell number of *C. marina* was counted in a Thoma cytometer chamber.

Isolation and culture conditions of bacteria

Bacterial strains were isolated from the culture of *C. marina* on Z-C II or YES agar plates incubated at 25℃. As described in a previous paper[8] Z-C II medium contains peptone (5 g/ℓ) and yeast extract (1 g/ℓ) as organic components and artificial seawater (ASW), while YES medium includes yeast extract (1 g/ℓ), enrichment seawater solution (ES) and natural seawater (NSW).

Characterization of bacterial isolates

Bacterial isolates were examined morphologically (colony form, cell form, motility and gram stain) and physiologically (oxidase, catalase, fermentation of glucose, salt requirement and hydrolyzation of starch, casein, gelatin, lecithin and DNA) by use of Z-C II medium as basal medium.

Bacterial growth on various agar media

Bacterial isolates were incubated on seawater agar media added with various organic components such as yeast extract, peptone, casamino acids or beef extract (2 g/ℓ), or vitamin B_{12}. Bacterial growth was estimated by measuring the colony diameter for 10–14 days. All data were expressed as the average of diameters of five representative colonies.

Effect of temperature on bacterial growth

A bacterial isolate was incubated in L-shape test tubes containing 10 mℓ of YES liquid medium by use of a temperature gradient incubator (Advantec Co., Model M-12). Turbidity of bacterial culture was determined during 7 days incubation by

use of a spectrophotometer (Tokyo Photo-Electric Co., ANA-77) at 540 nm.

Results

The change of bacterial colony counts in the algal culture

Fig. 1 shows the change of cell counts of *Chattonella marina* cultured in 10 mℓ of ESP medium under illumination. The algal cell count increased until it reached maximum level of 5.6 log No./mℓ at 20 days and then continued in the same level. Four types of bacterial colonies were mainly observed on Z-C II agar plates after the samples of *C. marina* culture were smeared on the plates and incubated for 6 days as shown in Fig. 2. Colony types, WBr-L, WGr-S, YO-S and WP-T represent large whitish brown, small whitish gray, small yellowish orange and tiny pink colonies, respectively. The representative strains of four colony types were isolated on Z-C II agar plates from the algal culture. On the base of bacteriological characterization, the

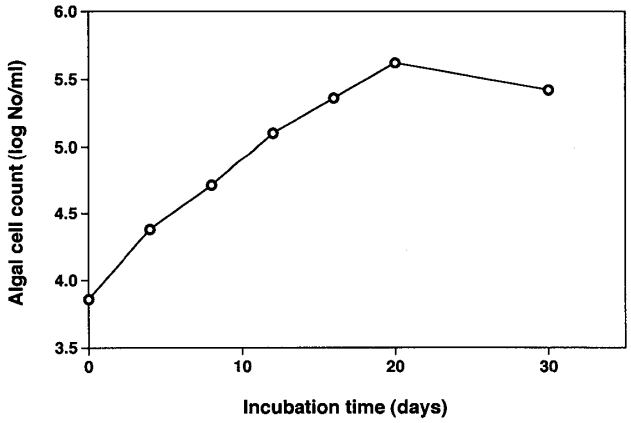

Fig. 1. The change of algal cell counts in *Chattonella marina* culture.

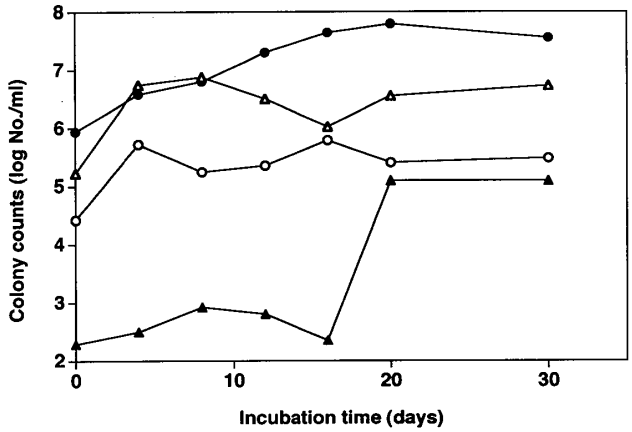

Fig. 2. The change of bacterial colony counts in *C. marina* culture.
●, WP-T colony; △, YO-S colony;
○, WBr-L colony; ▲, WGr-S colony.

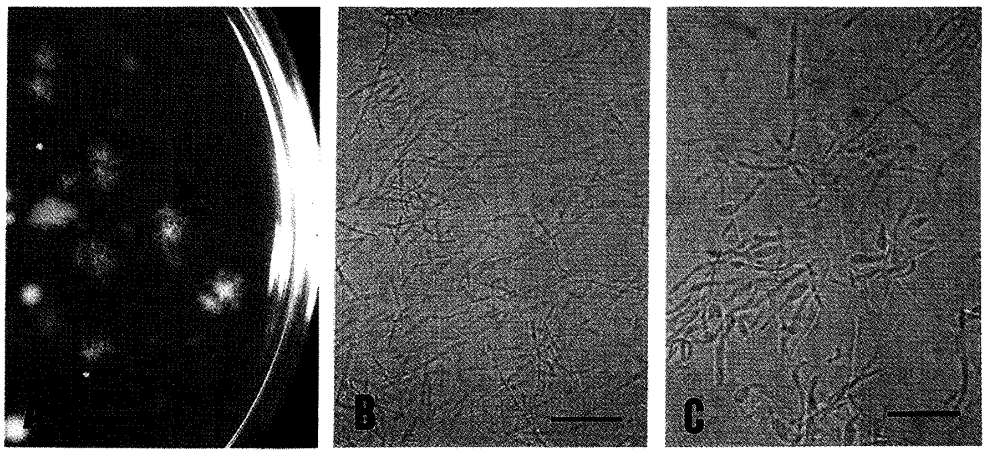

Fig. 3. Colony and cell morphology of *Flexibacter* sp.
A, colonies grown on a YES agar plate; **B**, cells grown on YES agar; **C**, cells grown on Z-C II agar.
Arrow indicates a rhizoidal colony. The length of a bar is 10 μm.

isolates from four colony types could be classified broadly into the genera groups, *Alteromonas-Pseudomonas*, *Moraxella-Acinetobacter*, *Flavobacterium* and *Flexibacter*, respectively. The isolates of *Flexibacter* sp. formed very tiny circular colonies of 1 to 2 mm on Z-C II agar but they produced large thin rhizoidal colonies on YES agar as illustrated in Fig. 3 (photograph A). The cell form of *Flexibacter* sp. was filamentous (5-15 μm in length) on the both media but the one part of filamentous structures was observed to swell to become a bleb-like form frequently on Z-C II agar (Fig. 3, photographs B and C). All isolates of *Flexibacter* sp. did not show hydrolyzing activity for various macromolecular substances including starch, casein, gelatin, lecithin and DNA.

Effect of enrichment solution on bacterial growth

Colony growth of a *Flexibacter* isolate was examined on agar media added with or without enrichment solution (ES) containing minerals, EDTA and vitamins. The diameter of bacterial colonies increased definitely during 10 days of incubation on agar media added with enrichment solution such as ZE-C II, ZE-C I and YES media, while the bacterial colony was found to grow at very slow rate on agar media without enrichment solution such as Z-C I and Z-C II media as demonstrated in Fig. 4.

Effect of vitamins on bacterial growth

The bacterial growth was examined on agar media with or without three kinds of vitamins. As shown in Fig. 5, vitamin B_1- or biotin-deficient media supported colony growth at the same level as that on YES agar medium. On the contrary, vitamin B_{12}-deficient media yielded very slow growth of bacterial colonies.

Fig. 6 demonstrates the colony growth of *Flexibacter* sp. on YS media containing

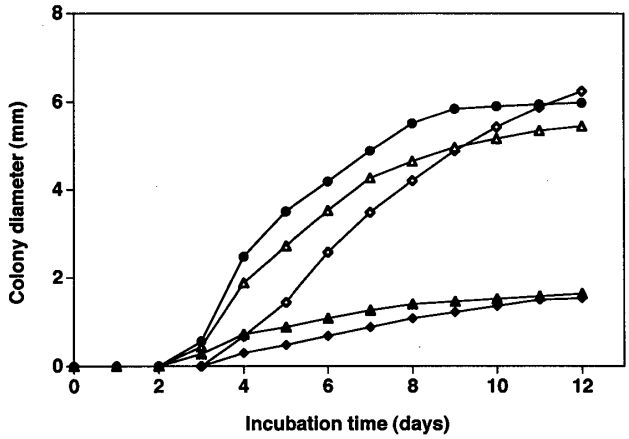

Fig. 4. Colony growth on media with or without enrichment solution.
●, YES agar; △, ZE-C I agar; ◇, ZE-C II agar;
▲, Z-C I agar; ◆, Z-C II agar.

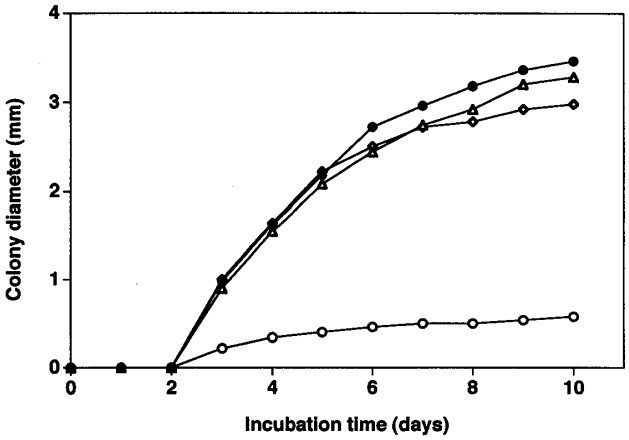

Fig. 5. Colony growth on media without vitamins.
●, YES agar; △, vitamin B_1-deficient YES agar;
◇, biotin-deficient YES agar; ○, vitamin B_{12}-deficient YES agar.

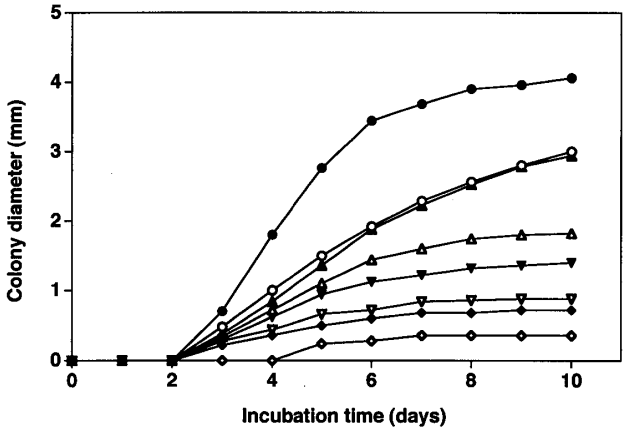

Fig. 6. Effect of vitamin B_{12} concentration on colony growth.
●, YES agar; ○, 4 μg/ℓ; ▲, 0.4 μg/ℓ; △, 0.08 μg/ℓ;
▼, 0.04 μg/ℓ; ▽, 0.008 μg/ℓ; ◆, 0.004 μg/ℓ; ◇, YS agar.

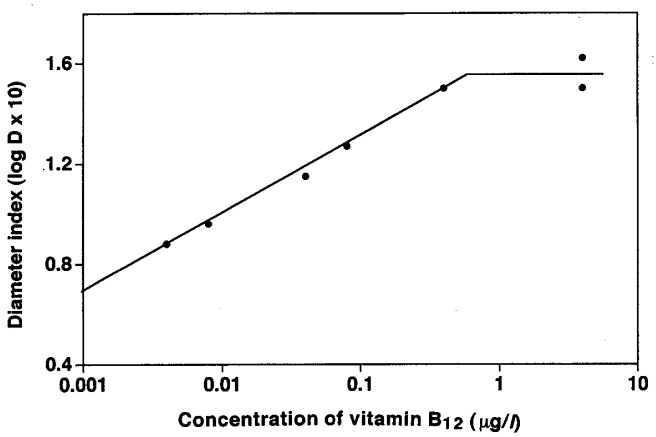

Fig. 7. Relationship between vitamin B_{12} concentration and maximum growth yield of *Flexibacter* sp.

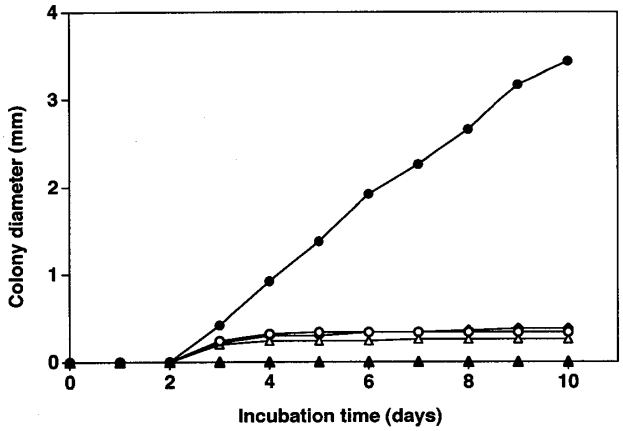

Fig. 8. Colony growth on media containing various peptones.
●, (B_{12})S + yeast extract agar; ◆, + Protease Peptone agar;
○, + Polypepton agar; △, + Trypticase Peptone agar;
▲, + Casamino acids agar.

various concentrations of vitamin B_{12}. Additionally, Fig. 7 shows plots of maximum growth yield (log maximum colony diameter × 10) to the concentration of vitamin B_{12}. Maximum growth yield of *Flexibacter* sp. gave linear relationship to the concentrations of vitamin B_{12} in a range of 0.004 to 0.4 $\mu g/\ell$.

Effect of organic compounds on colony growth

Various kinds of peptone caused very small colonies as compared with those on yeast extract media such as YES and YS + B_{12} media as shown in Fig. 8. Furthermore, various kinds of sugars, nucleic acid bases and organic acids were found to yield very slow growth of bacterial colonies in contrast with those on YES agar as illustrated in Fig. 9.

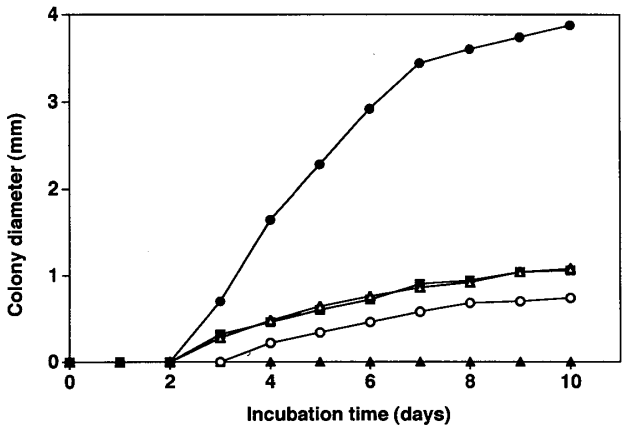

Fig. 9. Colony growth on media containing various sugars.
●, YES agar; ■, ES+starch agar;
△, +sucrose agar; ○, +glucose agar.

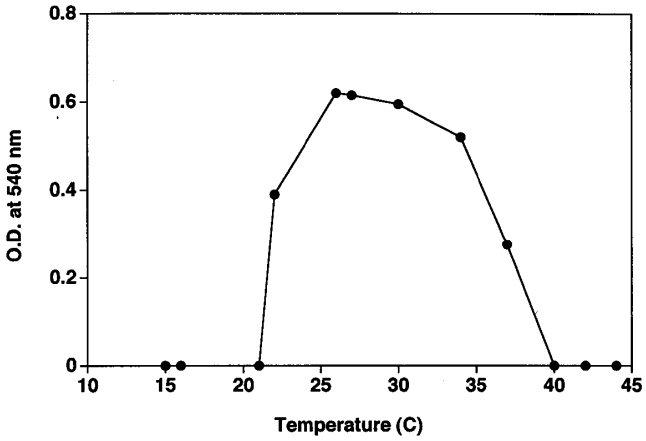

Fig. 10. Effect of temperature on bacterial growth in YES liquid medium.

Effect of temperature on colony growth

Turbidity of bacterial cultures was determined after L-shape test tubes containing 10 mℓ of the culture liquid were incubated for 6 days in a temperature gradient incubator. Maximum growth of the isolate was observed at 26℃ and it could not grow above 40℃ as shown in Fig. 10.

Discussion

There are 10^6–10^7/mℓ level of viable cells of bacteria in a stock culture of *Chattonella marina* isolated from Kagoshima Bay in June of 1982. Bacterial colonies grown on Z-C II agar plates were classified predominantly into four types, *i.e.*, large whitish brown (WBr-L), small yellowish orange (YO-S), small whitish gray (WGy-S) and tiny pale pink (WP-T). Tiny pale pinkish (WP-T) colonies were composed mainly of a filamentous bacterium, which was identified as the genus *Flexibacter* on the

basis of gram negative, non-fermentation of glucose, filamentous cell form (5–15 μm in length) and production of flexixanthin as cellular pigment[9-11]. The isolates of *Flexibacter* sp. were found to have an essential vitamin B_{12} requirement for growth. If the isolates were grown on vitamin B_{12}-deficient agar medium (Z-C II), the one part of filamentous structures swelled to become a bleb-like form. This phenomenon was considered to be caused by the interference of cell envelope biosynthesis by vitamin B_{12}-deficiency. The isolates of *Flexibacter* sp. required also low molecular substances of yeast extract for growth. Therefore, *Flexibacter* sp. would incorporate some organic compounds produced by *C. marina* and vitamin B_{12} containing in ESP liquid medium to grow. On the other hand, *C. marina* also requires vitamin B_{12} for growth. Consequently, *Flexibacter* sp. may compete *C. marina* to get vitamin B_{12} in ESP medium. Viable cells of axenic strain of *C. marina* decreased rapidly after 10 days of incubation as compared with those of non-axenic strain in ESP medium. Therefore, *Flexibacter* sp. is considered to supply some growth factors to *C. marina* or metabolize toxic products produced by *C. marina* itself in ESP medium. Further work is required to determine the relationship between *C. marina* and *Flexibacter* sp.

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