Growth Promotion of *Pavlova viridis* by Bacteria Isolated from the Microalga

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The marine microalga *Pavlova viridis* can grow fast and has the ability to accumulate essential nutrients for culturing marine animals, such as EPA and DHA, and it has been used as food for raring larval fish and prawn. The symbiotic relationship between the flagellate microalga *Pavlova viridis* and its associated bacteria was investigated. An axenic culture of *P. viridis* was obtained by repeated treatment of the microalga with an antibiotic cocktail. The axenic status was confirmed after sub-culturing three times in a sterile f/2 medium without an antibiotic. The axenic alga was then co-inoculated with five bacteria, arbitrarily designated as I1 - I5, isolated from the alga to test the growth promotion of the algae. All bacterial strains promoted the growth of *P. viridis*, and bacterial isolate I3 was the most effective among the five bacteria tested. The cell number of *P. viridis* in the co-culture with I3 was significantly higher than that of the control culture. A sequence analysis of the 16S rRNA gene isolated from I3 revealed a 97% nucleotide sequence similarity to that of *Citrobacter* sp. The growth of strain I3 was also significantly enhanced by co-culturing with *P. viridis*, indicating a symbiotic relationship between the microalga and its associated bacterium. The association between the microalga and bacterium was confirmed by scanning electron microscopy.

Key words : Aaxenic, *Citrobacter* sp., coculture, *Pavlova viridis*, symbiosis

Introduction

Microalgae are microscopic unicellular organisms found typically in marine and fresh waters, and they usually coexist with bacteria [18]. They are primary producers that affect the nutrient cycles in aquaculture ecosystems [8]. Microalgae are indispensable natural food sources in aquaculture for all growth stages of bivalve, crustacean, and fish species, and they also serve as food for zooplankton to continuously support the food web. Microalgae provide energy and nutrients to marine organisms that need those for growth and development. Because they possess well-balanced nutrient contents, they have been used for the enrichment of nutrients in zooplanktons before these are fed to juvenile fish, and are very important in the culture of diverse aquatic animals [3, 4, 26].

Microalgae are used for various other purposes as well.

For example, they are good sources of highly valuable bioactive compounds, polyunsaturated fatty acid, antioxidants, and pharmaceuticals [13]. Algae are also known for their conspicuous color pigments. They produce not only chlorophyll, the photosynthetic pigment, but also phycobiliproteins and carotenoids that are used to protect skin from damage due to sunlight, as well natural food colorants with applications in, e.g., cosmetics [16, 22].

The marine microalga *Pavlova viridis* used in this study can grow quickly, and owing to its high contents of polyunsaturated fatty acids, EPA, and DHA, it is used as food for rearing larval fish and prawn [19]. It has the ability to accumulate essential nutrients for culturing marine animals [5].

The isolation and culture of microalgae is often accompanied by bacterial contamination. Axenic algal cultures are essential for physiological, chemical, molecular, or taxonomic studies and the determination of zooplankton food preference and algal histories [23]. As some aquatic bacteria can inhibit algal growth or cause lysis of algal cells, establishing axenic algal cultures is important to protect the algae from such algicidal action [8]. A few methods are used for the establishment of axenic microalgae cultures, although obtaining an axenic culture from a highly contaminated algal

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culture is an arduous job [6, 7]. Treatment with antibiotics is the most common approach to attain axenicity of microalgae from bacteria [7].

Many bacteria live on the cell surface of microalgae and they have diverse effects on the growth of the host algae. The relationships between microalgae and bacteria vary depending on the species and environmental conditions [18]. Bacteria associated with microalgae may have positive or negative impacts on the growth of microalgae. Many reports have demonstrated that aquatic bacteria can cause algal cells to lyse, or exert inhibitory effects on the growth of microalgae via detrimental chemicals produced by the bacteria [2, 12, 18]. In addition to these suppressive actions, influential or stimulatory effects between bacteria and microalgae have also been reported [10, 21, 24]. Watanabe et al. [27] Watanabe et al. [27] reported a strong positive effect on the growth of the microalga *Chlorella sorokiniana* when cocultured with the bacterium *Microbacterium trichotecenolyticum*. Moreover, Riquelme et al. [21] Riquelme et al. [21] reported the fastest growth of the alga *Asterionella glacialis* with *Pseudomonas* sp.; they assumed that the bacteria produce chemicals like vitamins and lipoprotein, which promote the growth of microalgae. Also, eight bacterial strains separated from *Chlorella ellipsoidea* had growth-promoting effects in coculture with the microalga [20].

In the current study, we isolated and characterized bacteria associated with *P. viridis* and studied their interactions by coculturing the axenic alga with and without the isolated bacteria.

Materials and Methods

Microalga culture and identification

The marine microalga *P. viridis* was obtained from the Korea Marine Microalgae Culture Center, Pukyong National University, Busan, Korea. The alga was cultured in f/2 medium [14, 15] at 20°C under a 16:8 hr light:dark cycle with a light intensity of 30 μmol m^{-2} s^{-1} [1]. For the genetic identification of host alga, genomic DNA was isolated according to a previously described method (http://www.gbiogene.com). The 18S rRNA gene of the alga was amplified by PCR (HS Prime Taq premix 2×; Genet Bio, Cheonan-si, Korea) using primers 512F (5′-ATTCCAGCTCCAATA GCG-3′) and 978R (5′-GACTACGATGGTATCTAATC-3′). The PCR conditions were as follows: 94°C for 2 min, followed by 35 cycles at 94°C for 2 min, 52°C for 45 s, and 72°C for 1 min, followed

by a final extension at 72°C for 10 min. The PCR products were visualized in a 1% agarose gel. The amplified DNA fragments were purified from agarose gels using a Gel SV kit (Gene All, Seoul, Korea) and were sequenced using 18S rRNA gene primers (512F/978R; G&C Bio, Daejon, Korea). Homology analysis was carried out using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Isolation of bacteria

To obtain bacterial isolates from the original algal culture, 100-μl algal cultures of *P. viridis* were spread on Luria‑Bertani (LB) agar plates and incubated at 37°C for 72 hr. Colonies showing different morphology and color were streaked again on LB plates and incubated at 37°C for 72 hr. After three passages on LB plates, glycerol stocks of single colonies were prepared in LB broth containing 20% glycerol and then stored at -80°C for further study.

Establishing an axenic culture of *P. viridis*

The isolated bacteria were tested against the five antibiotics listed in Table 1. LB agar plates with different concentrations of antibiotic were prepared and single colonies were inoculated and incubated at 37°C for 72 hr. Antibiotic susceptibility was identified by the growth of bacterial colonies. An antibiotic cocktail was prepared based upon the antibiotic resistance for the cleaning of *P. viridis*. An axenic culture of *P. viridis* was established by following the method described by Hong et al. [17] Hong et al. [17]. Well-grown alga in f/2 medium was transferred to f/2 medium with antibiotic cocktail. After 8-10 days, the alga was transferred again to fresh f/2 medium containing the same antibiotic cocktail. Axenic status was verified through spreading the culture on LB agar plates followed by incubation at 37°C for 7 days. These procedures were repeated until no bacterial growth occurred.

Identification of bacterial isolates

The taxonomic identification of bacterial isolates was performed by sequence analysis of the 16S rRNA gene. An overnight culture of 3-5 ml was centrifuged at 13,000 ×g for 1 min, after which the pellet was suspended in 500 μl of extraction buffer [400 mM NaCl, 20 mM Trizma Base, 5 mM EDTA (pH 8.0), 1% SDS]. Next, 50 μl of 20% SDS and 500 μl of phenol:chloroform:isoamylalcohol (25:24:1) was added to the suspension. The mixture was vortexed for 1 min and then centrifuged at 13,000 rpm for 5 min. The supernatant

was transferred to a new tube and 0.1 volumes of 3 M so-
dium acetate and 0.6 volumes of isopropanol were added
to the tube and mixed gently by inverting, followed by cen-
trifugation at 13,000 rpm for 20 min. Finally, the dried DNA
was dissolved in 50 μl of dH$_2$O. The 16S rRNA gene from
bacteria was amplified by PCR (HS Prime Taq premix 2◇;
Genet Bio) using 27F (5′-AGAGTT TGATCCTGGCTCAG-3′)
and 1492R (5′-GGTTACCTTGTTA CGACTT-3′) [24] primers
under the same PCR conditions as described above. The PCR
product was sequenced using 16S rRNA gene primers (27F
and 1492R). Homology analysis was carried out using
EzTaxon (http://www.ezbiocloud.net/).

Culture of bacterial isolate I3

Suitable growth conditions for bacterial strain I3 were de-
termined by optimizing the temperature, NaCl concen-
tration, and pH. For each comparison, a 40-μl aliquot of bac-
terial isolate I3 culture freshly grown at 37°C overnight in
LB broth was used to inoculate 4 ml of LB broth. The opti-
mum temperature was determined by inoculating a 40-μl ali-
quot of bacterial isolate I3 culture to 4 ml of LB broth and
incubating the culture at 4°C, 20°C, 25°C, 30°C, 37°C, or 45°C
with shaking at 150 rpm for 24 hr. The optimum NaCl con-
centration for bacterial growth was examined by preparing
LB broth containing 0%, 1%, 2%, 3%, 4%, 5%, 7%, and 10%
NaCl and inoculating 4 ml of broth with a 40-μl aliquot of
bacterial isolate I3 culture, followed by incubation at 37°C
with shaking at 150 rpm for 24 hr. The optimum pH was
examined by preparing LB broth containing 1% NaCl with
the pH adjusted to 3, 4, 5, 6, 7, 8, 9, or 10; then 4 ml of
broth was inoculated with a 40-μl aliquot of bacterial isolate
I3 culture and incubated at 37°C with shaking at 150 rpm
for 24 hr. Three replications were made for each temperature.
Bacterial growth at each temperature was monitored after
24 hr by measuring the absorbance at 600 nm using a spec-
trophotometer (MBA 2000; Perkin-Elmer, Wellesley, MA,
USA). Means were determined from three replicates.

Algal growth assays with five bacterial strains

To determine the growth promoting effect of the bacteria,
100 ml of f/2 medium was inoculated with axenic *P. viridis*
and cultured for 7 days at 20°C as described above. Five
bacterial isolates were inoculated separately, with each in-
oculum consisting of 4 ml cultured in LB broth overnight
at 37°C. The f/2 medium (100 ml) was inoculated with 1
ml of algal culture containing 7.08×10^4 cells/ml plus 1 ml

each of the five bacterial suspensions that had been adjusted
to an optical density of 0.5 or 1 ml of LB broth as the control.
Cultures were prepared in triplicate and incubated at 20°C
for 10 days, and the growth of *P. viridis* was monitored daily
by counting the cells using a hemocytometer.

Algal growth with bacteria-free filtrate

The bacterial filtrate was prepared by growing the I3 iso-
late in 25 ml of LB broth for 3 days at 37°C and 150 rpm.
The culture was centrifuged at 4,000 × *g* for 10 min, and
filtered twice through 0.2-μm polycarbonate filters (Minisart,
Goettingen, Germany). Then, a 1-ml aliquot of this bac-
teria-free filtrate or LB broth as a negative control was added
to 100 ml of f/2 medium containing 7.02×10^4 algal cells/ml
prepared as described above; the mixtures were incubated
at 20°C for 8 days. Each culture was prepared in triplicate
and the algal cells were counted daily for up to 8 days using
a hemocytometer.

For comparative assays of algal growth with different
amount of filtrates, 1, 2.5, 5.0, and 10.0 ml of bacteria-free
filtrates of I3 were added to 98, 96.5, 94, and 89 ml of f/2
medium, respectively, containing 7.02×10^4 algal cells/ml.
The same amounts of LB broth were used as controls with-
out bacteria-free filtrate. The cells were cultured at 20°C and
the cells were counted after 5 days using a hemocytometer.

Growth promotion of the alga and bacteria in coculture

The inoculums of *P. viridis* and bacterial isolate I3 were
prepared as described above. Bacterial suspension (1 ml; op-
tical density 0.5) and 1 ml of axenic *P. viridis* bearing initial
cells of 7.06×10^4 were inoculated to 100 ml of fresh f/2 me-
dium as a coculture treatment. Controls were prepared sim-
ilarly using the bacteria alone or alga alone. All cultures
were prepared in triplicate. Three replicates of 100-μl sam-
ples were taken each day from the *P. viridis* single culture
and coculture. Samples were serially diluted and algal cells
were counted using a hemocytometer. For the bacterial sin-
gle culture and bacteria‑algal cocultures, 100-μl samples
were taken daily and serially diluted by 10^4‑10^5 using f/2
medium; then 100 μl of each dilution was spread on LB agar
plates and incubated at 37°C for 3 days to count the colony
forming units (CFU) of bacteria.

Scanning electron microscopy (SEM) method

The association of the bacteria and algae in coculture was

verified by SEM observation. One milliliter of 2% osmium tetroxide was added to 14 ml of overnight coculture for fixation and then stored at 4°C overnight. The cells were collected by low-speed centrifugation at 800 ◊ g for 20 min, and the supernatant was discarded. The cells were resuspended with 15 ml of distilled water and poured onto a 0.2-µm cellulose acetate membrane filter of 47 mm diameter (Toyo Roshi Kaisha, Tokyo, Japan) connected to a vacuum through a bottle-top filter (Thermo Scientific, Billerica, MA, USA). After rinsing with 1 ml of distilled water, the membrane was removed, folded sample-side-in, and placed in a 1.8-ml microcentrifuge tube. The cells were sequentially dehydrated with 1.5 ml of 20%, 30%, 50%, 70%, 80%, 85%, 90%, 95%, and 100% ethanol for 30 min each. After two more dehydration steps at 100% ethanol for 30 min, the cells were stored overnight in 100% ethanol. After replacing the ethanol with isoamyl alcohol twice for 1 hr, the samples were dried at critical point, coated with gold‐palladium, and observed under SEM (JSM-6490LV; JEOL, Tokyo, Japan).

Results

Identification of the host

To identify the host alga, genomic DNA was prepared from the pure culture of the microalga and used for PCR amplification. A DNA fragment of 490 bp was obtained and its sequence was determined without cloning. Homology analysis was carried out from the GenBank database using BLAST. The results indicated 99% nucleotide sequence identity to that of the 18S rRNA sequence of *P. viridis* (GenBank accession number HQ877913).

Isolation and identification of bacteria

Five bacterial isolates were obtained from the microalga *P. viridis* and arbitrarily designated I1‐I5. Genomic DNA was prepared from each bacterial isolate for amplification of the partial 16S rRNA (~1,450 bp). The sequences were determined and compared with sequences in the database using EzTaxon. The 16S rRNA sequence of bacterial strains I1‐I5 showed 99.950%, 99.034%, 99.69%, 99.217%, and 98.825% sequence identity to that of *Kocuria marina* (AY 211385), *Staphylococcus cohnii* (D83361), *Citrobacter freundii* (ANAV01000046), *Micrococcus yunnanensis (Fj214355)*, and *Staphylococcus haemolyticus* (L37600), respectively.

Antibiotic resistance test and cleaning of microalga

To prepare an antibiotic cocktail to remove bacteria from the alga, five different antibiotics (60 µg/ml ampicillin, 75 µg/ml chloramphenicol, 30 µg/ml kanamycin, 30 µg/ml streptomycin, and 30 µg/ml tetracycline) were tested separately against the isolated bacteria. Bacterial strains I2, I3, and I4 were susceptible to all antibiotics at the above concentrations. Bacterial isolate I1 demonstrated susceptibility to all antibiotics except ampicillin. Bacterial isolate I5 was susceptible to all antibiotics except streptomycin. Therefore, a working stock of antibiotics containing 2.5 mg/ml of chloramphenicol, 2.0 mg/ml ampicillin, 1 mg/ml of kanamycin, streptomycin, and tetracycline was prepared to achieve the appropriate final concentrations, and this was used to remove these bacteria from the host, *P. viridis*. The bacterial flora in the microalga prior to antibiotic treatment was 8.1×10^5 cells/ml. However, no bacterial colony was observed when the algal culture was spread on LB plates after 10 days of culture in f/2 medium containing the antibiotic cocktail. The antibiotic treatment was repeated two more times to confirm that the culture of the microalga was axenic at least in current bacterial culture conditions

Test for growth promotion of *P. viridis* by isolated bacteria

Each of the five bacterial isolates was inoculated with *P. viridis* containing 7.08×10^4 cells/ml in f/2 medium. All bacterial isolates significantly increased the growth of *P. viridis* over 10 days, although the stimulatory effect varied among isolates. Bacterial isolate I3 showed the highest growth promotion activity among the five isolates tested (Fig. 1). The growth of *P. viridis* increased by approximately twofold compared to that of the control culture by the most effective bacterial isolate (I3), whereas the other bacterial isolates increased the growth of the microalga by about 0.5‐1.5-fold.

Growth and molecular characteristics of bacterium I3, *Citrobacter* sp.

Isolate I3 whose 16S rRNA gene showed 99.69% sequence similarity to that of *Citrobacter freundii* also formed a close cluster with this species among other *Citrobacter* species in an UPGMA phylogenetic tree (Fig. 2). Therefore, this isolate was designated as *Citrobactor* sp. without further biochemical test for species identification. The isolate 3 appeared on LB medium as a translucent colony with a smooth edge. The bacterium showed slow growth at 4°C and the
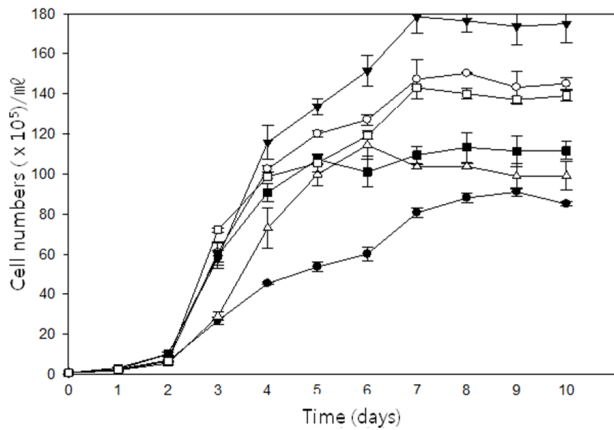
Fig. 1. Growth promotion of *P. viridis* by five bacterial isolates. Axenic *P. viridis* was cocultured with five different bacterial isolates, I1 (—□—), I2 (—○—), I3 (—▼—), I4 (—△—), and I5 (—■—). Data shown represent the means ± SD from three replicates.

Fig. 3. Growth promotion of the *bacterial isolate I3* and microalga *P. viridis* in coculture. The growth of bacteria in single culture (—●—) and in coculture (—○—), and the growth of *P. viridis* in single culture (—△—) and *in* coculture (—▼—) are *represented as* the means ± SD from three replicates.

growth rate increased with rising temperature from 25°C to 30°C. The optimum temperature for growth was 37°C and the growth declined drastically with further increases of temperature. Considerable growth was observed at 0% NaCl and the fastest growth was achieved at 1% NaCl; it decreased gradually with further increases of NaCl concentration and very little growth was recorded at 10% NaCl. Slow growth was detected at pH 3, but this increased progressively with increasing pH; the fastest growth rate was reached at pH 7.0, followed by a gradual decline with further increases in pH, and only slight growth was observed at pH 10.
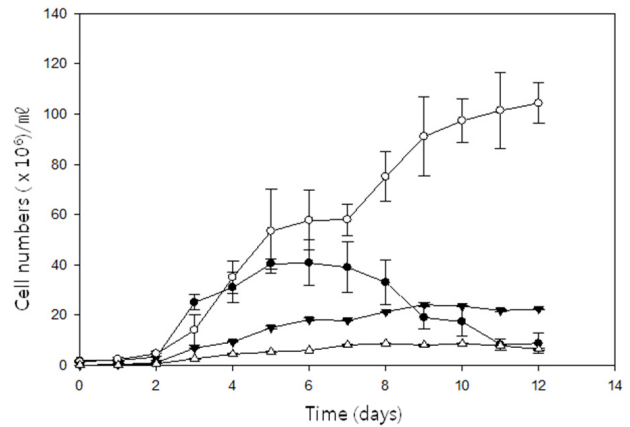
Growth promotion of bacteria and the alga in coculture

The growth of bacteria I3 (*Citrobacter* sp.) and the host *P. viridis* in coculture was measured to investigate their symbiotic relationship. Initially, the lag phase of bacterial growth was almost the same in coculture and in single culture. However, the growth of bacteria in coculture was much faster than that in single culture at the end of the culture period (Fig. 3). The exponential growth phase was also longer than that in single culture. A significant difference was observed between the two conditions. Similarly, bacterial isolate I3 markedly promoted the growth of *P. viridis* in coculture, and the exponential growth of *P. viridis* lasted longer in coculture than in single culture (Fig. 3).
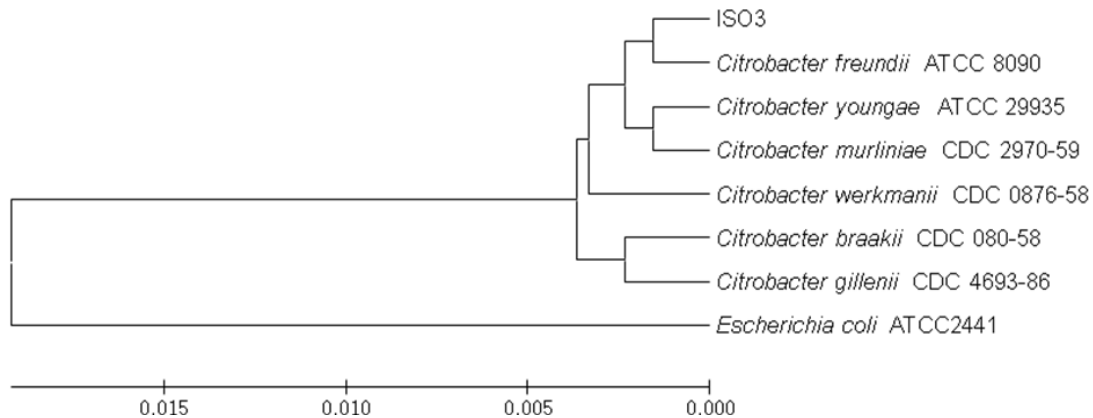

Fig. 2. An UPGMA phylogenetic tree constructed based on the 16S rRNA gene of the isolate 3. *Citrobacter* species showing the high sequence similarity in the analysis using Eztaxon were included and *Escherichia coli* was included as an outgroup. The result is from 1,000 bootstraps.

Growth promotion of the alga with bacteria-free Filtrate

The bacteria-free filtrate of bacteria I3 was mixed with a pure culture of *P. viridis* and the effect on algal growth was investigated for 8 days. There was no bacterial growth when the filtrate was added to fresh LB and incubated at 37°C indicating the sterility of the filtrate. Significant growth enhancement was observed in the algal culture containing bacteria-free filtrate compared to alga in f/2 medium containing same amount of LB broth. Increased algal growth was observed with increasing the filtrate concentration, but no significant growth difference was observed between 5% and 10% filtrate (Fig. 4). The growth curves for *P. viridis* in f/2 medium containing LB or 10% bacterial filtrate are depicted in Fig. 5. At the beginning, no difference in growth was observed between the two cultures. However, after 2 days, the microalga cultured with filtrate displayed faster growth with a longer period of exponential growth. After 8 days, the cell number in the f/2 medium containing 10% of bacterial filtrate was twice that observed in the same medium containing LB broth (Fig. 5). There was no bacteria growth in the filtrate after incubation of 5ml aliquots of the filtrate for 8 days

Observation by SEM

The intimate relationship between bacterial isolate I3 and *P. viridis* was observed under SEM. The average size of the alga cultured under two different conditions was similar
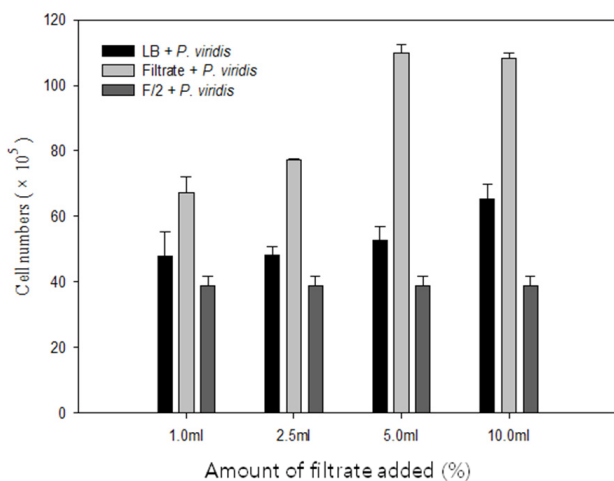

Fig. 4. Growth promotion of *P. viridis* with different amounts of bacteria-free filtrate from bacterial isolate *I3*. Data represent the means ± SD from three replicates. Black box: LB + *P. viridis*; gray box: filtrate + *P. viridis*; dark gray: f/2 + *P. viridis*.
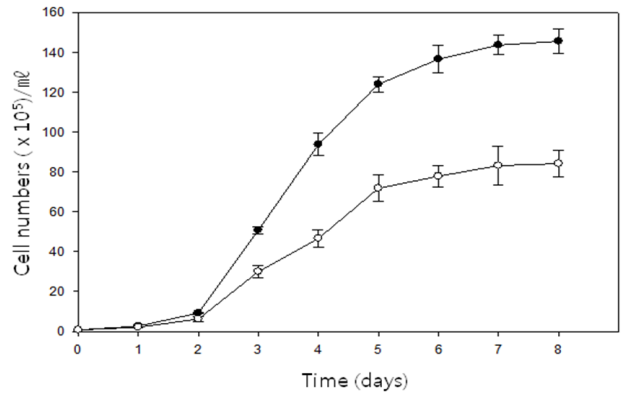

Fig. 5. Comparison of the growth of microalga *P. viridis* in pure culture (—○—) and in the presence of 10% bacteria-free filtrate from bacterial isolate I3 (—●—). Data represent the means ± SD from three replicates.

with an average diameter of 2.8 μm. Bacterial isolate I3 exhibited direct adhesion to the surface of alga cells (Fig. 6). The size of the bacteria on the algal surface was about 3.5×0.7 μm. Other than the presence of bacteria on the surface of the alga, no morphological difference existed between the alga in coculture and that in single culture.

Discussion

Microalgae and bacteria are the most influential organisms with respect to numbers in all aquatic environments and together control nutrient cycling [8]. Many bacteria are associated with microalgae and they may have progressive or suppressive effects on the growth of host algae. The interactions between microalgae and bacteria are variable, depending on the species and environmental conditions [18, 21].

Riquelme et al. [21] Riquelme et al. [21] reported that bacterial strains of *Flavobacterium* sp. inoculated in axenic culture of the marine diatom *A. glacialis* successfully promoted the growth of microalga. They also reported that the naturally occurring bacterial strain *Pseudomonas* sp. promoted the growth of microalga, whereas *Vibrio* sp. did not affect algal growth. In another report, several bacterial strains isolated from the benthic diatom *Nitzschia* sp. were cocultured with the diatom, and among many bacterial strains, only *Alcaligenes* sp. showed significant growth-promoting effects on *Nitzschia* sp. [12]. Furthermore, the marine bacterium *Flavobacterium* sp. increased the growth of the diatom *Chaetoceros gracilis* but was algicidal toward the microalga *Gymnodinium mikimotoi*. Unexpectedly, this bacterium has no
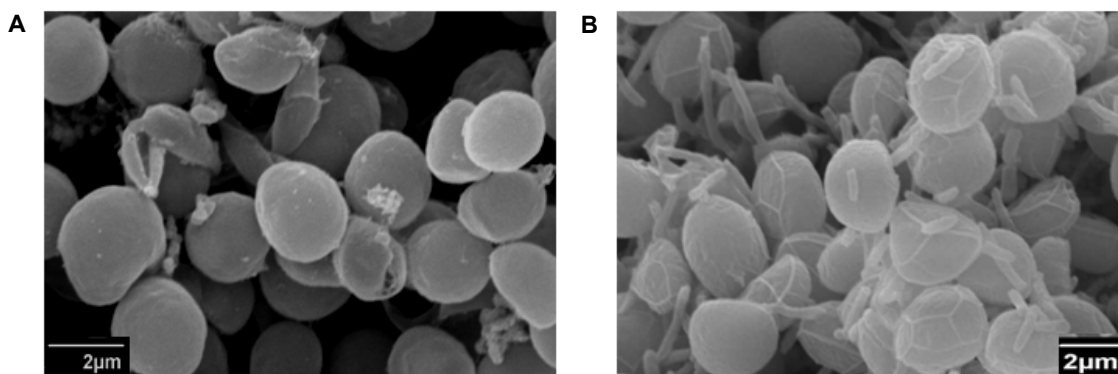
Fig. 6. Scanning electron microscope pictures of *P. viridis* in pure culture (A) and in coculture with bacterial isolate I3. Scale bar: 2 μm.

obvious effects on the growth of *Isochrysis galbana* and *Pavlova lutheri* [24]. All of these results implied that the interactions between bacteria and microalgae are species-specific [12, 18].

In the current study, five bacterial isolates were obtained from a culture of *P. viridis* and these isolates were subsequently cocultured with the cleaned microalga. Each of the bacterial isolates promoted the growth of *P. viridis*, which exhibited positive effects on the growth of the associated bacteria.

The most effective bacterial isolate, I3, was identified as *Citrobacter* sp. by sequence analysis of its 16S rRNA gene. Coculture with a bacteria-free filtrate from this isolate also revealed a stimulating effect on the growth of the microalga, and this effect was dose-dependent. These results are indicative of a bacteria‐algae association. Bacteria are known to produce metabolites such as vitamins that play an important role in the growth of microalgae [21]. Many microalgae require extra nutrients such as biotin, thiamine, and cobalamin as growth factors [9]. One survey reported that 171 of the 326 algal species examined required exogenous vitamin B12 for their growth. Microalgae collect vitamin B12 from associated bacteria, implying symbiotic interactions [9].

Nutrients are regenerated by heterotrophic activity through microorganisms in the environment. Heterotrophic demineralization supplies continuous nutrients and algae also produce some organic compounds to contribute to the smooth running of the nutrient cycle. This phenomenon is critical in a symbiotic relationship [8, 28]. Croft et al. [9] Croft *et al.* [9] separated *Halomonas* sp., a vitamin B12-producing bacterium, from a non-axenic culture of *Amphidinium operculatum* and cocultured it with two auxotrophic vitamin B12-requiring algae, *Porphyridium purpureum* and *A. oper-*

culatum, in a mineral medium without organic carbon sources. They found that vitamin B12 was supplied by *Halomonas* sp. to the algae in exchange for the products of photosynthesis and that this interaction was symbiotic.

The results of our coculture experiment between *P. viridis* and *Citrobacter* sp. indicated the promotion of growth of both the microalga and bacterial strain. The growth of the bacteria in coculture was much faster than that of the single culture at the end of the culture period, and the growth of *P. viridis* was also faster in coculture than in single culture. The bacteria-free filtrate of bacterial strain I3 also promoted the growth of the microalga. These results suggest that the alga produces some organic compounds that increase the growth of the bacterium, and that the bacterial strain produces some metabolites that promote the growth of the microalga, indicating that these two organisms have developed a symbiotic relationship and future study for the identification of the substances involved in the interaction will clarify the relationships. Because the interactions between algae and bacteria occur in an aquatic environment where growth-promoting metabolites can diffuse quickly, close contact is required between the two organisms. Such close association was confirmed by SEM observation, which demonstrated the growth of bacteria on the algal surface. Direct attachment of symbiotic bacteria on the surface of microalga and indirect attachment on the sheath produced by the microalga has been observed in other microalga‐bacteria combinations [28].

The findings of this study indicated a close symbiotic relationship between the microalga *P. viridis*, which is important in aquaculture, and the bacterium *Citrobacter* sp., which was isolated from the microalga. This information can be useful in the mass culture of *P. viridis* for increasing the microalgal

population for potential use in aquaculture.

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초록 : 파블로바 비리디스로부터 분리한 세균에 의한 미세조류의 생장 촉진

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해양 미세조류인 파블로바 비리디스는 빨리 자라며, DHA, EPA와 같은 해양생물을 키우는데 필수적인 영양요소를 축적하는 능력을 가지고 있어 어류와 새우류 치어 사육에 이용되어 왔다. 본 연구에서는 파블로바 비리디스와 이 미세조류의 표면에 붙어서는 세균과의 공생적 상호작용을 연구하였다. 무균의 파블로바 비리디스 균주는 항생제 혼합액을 포함하는 액체배지에 반복 배양함으로써 얻어졌다. 무균상태는 항생제를 포함하지 않는 배지에 3번 계대 배양한 후 확인하였다. 무균상태의 조류는 이 조류로부터 분리되었으며, 임의로 I1 - I5로 명명한 세균과 혼합배양하면서 조류의 성장 촉진 효과를 조사하였다. 모든 세균이 파블로바 비리디스의 생장을 촉진하였으며, 그 중 I3로 명명한 세균이 5가지 세균 중 가장 효과가 좋았다. 혼합배양 상태에서 파블로바 비리디스의 세포 수는 대조구에 비하여 유의하게 많았다. I3의 16S rRNA 유전자에 대한 염기서열 분석 결과 시트로박터 종의 그것과 97%의 염기서열 상동성을 보였다. I3을 파블로바 비리디스와 혼합배양할 경우 I3의 성장도 유의하게 증가하였으며, 이것은 조류와 그 표면에 부착하여 살아가는 세균들 사이에 공생관계가 존재한다는 것을 제시한다. 미세조류와 세균과의 공생관계는 전자현미경적 관찰을 이용하여 확인하였다.