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Short Communication

Cocultivated bacteria can increase or decrease the culture lifetime of *Chlorella vulgaris*

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Microalgae and other microorganisms exhibit a wide range of associations ranging from symbiosis to parasitism (Cole, 1982). The concept of the "phycosphere" was introduced as a pelagic analogy to the rhizosphere (Bell and Mitchell, 1972), and the selection of bacterial species within the phycosphere was suggested (Bell, 1983). The validity of this suggestion was demonstrated later by some authors. For instance, bacteria chiefly belonging to the phyla *Proteobacteria* and/or *Bacteroidetes* were reported to be predominant in the bacterial community associated with microalgae or cocultivated with microalgae (Grossart et al., 2005; Otsuka et al., 2008a; Sapp et al., 2007; Ueda et al., 2010). These findings suggest the existence of interactions between microalgae and bacteria selected in the phycosphere.

There are several reports on interactions between bacteria and microalgae. Watanabe et al. (2005) isolated four bacterial strains from a nonaxenic culture of *Chlorella sorokiniana*, and detected a growth-promoting effect of one of the strains on the alga. Croft et al. (2005) isolated a vitamin B_{12}-producing bacterium, *Halomonas* sp., from a nonaxenic culture of *Amphidinium operculatum* (*Dinophyta*), and succeeded in coculturing the bacterium with each of the two vitamin B_{12}-requiring auxotrophic algae *Porphyridium purpureum* (*Rhodophyta*) and *A. operculatum* in a mineral medium without organic carbon sources. This indicates that *Halomonas* sp. supplies vitamin B_{12} for the algae in return for the products of photosynthesis. It has also been reported that eight bacterial strains isolated from a nonaxenic culture of *Chlorella ellipsoidea* promote algal growth (Park et al., 2008). However, these studies are only a few examples highlighting the interactions between algae and bacteria.

In the present study, we isolated bacteria from consortia of *Chlorella* algae and bacteria, and analyzed the phylogenetic properties of the isolates. Using these isolates, we examined the effect of these bacteria on the growth of *Chlorella vulgaris* strain NIES-227 in order to expand our knowledge of the interactions between algae and bacteria.

Nonaxenic strains of *Chlorella* sp., C2 and C6, were isolated from soil in November 2005 in our previous study (Otsuka et al., 2008a). A consortium of *C. vulgaris* NIES-227 and bacteria inoculated from the soil was established in November 2007 by Ueda et al. (2010), and was referred to previously as the "mixed culture" and in the present study as "culture CSB." Until their isolation in December 2008, the three cultures C2, C6, and CSB were cultivated in liquid C medium (Ichimura, 1971) under a 16 : 8 light/dark cycle with a

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light intensity of about 10−20 μmol photons m^{-2} s^{-1} at 25°C in the light and 20°C in the dark, and maintained by serial subcultivation once every 4 weeks.

The three cultures were diluted 10^5-fold and spread onto agar plates of 10-fold diluted Nutrient Broth (Difco, Detroit, MI, USA) (1/10 NB). The plates were incubated at 26°C for about 2 months. Bacterial colonies formed on the plates were isolated to cover all types of colonies depending on the morphological characteristics such as shape, color, and shine with two or three colonies from one type. Each strain was purified and maintained by subcultivation on the 1/10 NB plates.

Crude DNA was extracted from bacteria according to Ashida et al. (2010), and used as the template for subsequent PCR. The almost full-length 16S rRNA gene (16S rDNA) was amplified by PCR under the same reaction conditions as described by Saito et al. (2008), with Biotaq DNA polymerase (Bioline, London, UK) and a thermal cycler GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The amplified products were purified by MicroSpin S-400 HR columns (GE Healthcare Biosciences, Oslo, Norway) and directly sequenced as described previously (Saito et al., 2008).

Based on the sequences, the type strains of species phylogenetically closely related to the isolated strains were searched using the SeqMatch program (Cole et al., 2007). The phylogenetic analyses of the isolated strains and the closely related species were performed using Clustal W version 2.0 (Larkin et al., 2007) as described previously (Otsuka et al., 2008b), and the strains were identified at the genus level.

One strain was selected from each of those genera. Three to seven days after the last subcultivation, two to five colonies of each selected strain were harvested from the agar plates (the number of colonies depended on the size), and transferred to 1 ml of C liquid medium. The cell suspension was centrifuged at 5,000 $\times g$ for 15 min and resuspended in 1 ml of C liquid medium. This was repeated two times to remove the culture broth.

Two milliliters of an axenic culture of *C. vulgaris* NIES-227 1 to 2 weeks after the last subcultivation was transferred to 90 ml of sterile C liquid medium in a 200-ml glass flask capped with a Silicon Plug (AZ One, Osaka, Japan). The bacterial suspensions prepared as described above were then inoculated individually into the C liquid medium in the flasks. This coculture was prepared in triplicate for each bacterial strain. In addition, three control cultures of the alga without bacterial inoculation were prepared.

The cocultures and control cultures were cultivated under the same conditions for the alga for 8 weeks as described above. Once every week, the culture fluid was mixed thoroughly and 500 μl was collected. The fluid was centrifuged at 5,000 $\times g$ at 4°C for 15 min to collect the algal cells. The cells were resuspended in 1 ml of 95% ethanol (Wako Pure Chemical Industries, Osaka, Japan) used as a solvent for chlorophylls, and kept at 4°C for 20−24 h. After centrifugation at 5,000 $\times g$ at 4°C for 10 min, the supernatant containing chlorophylls was harvested to remove the algal cells.

The increase in the amount of chlorophylls approximates that of algal cells (Aruga, 1979). Therefore, the absorption of light by the extracted chlorophylls was used to measure algal growth according to the method of Huang and Cong (2007). Briefly, light absorption at a wavelength of 665 nm (A_{665}) and 750 nm (A_{750}) was measured using the UV/VIS spectrophotometer V-550 (JASCO, Tokyo, Japan), and the difference between A_{665} and A_{750} values was calculated. All measurements were carried out in triplicate. Analysis of variance combined with Tukey's honestly significant difference test was performed using R program ver. 2.8.1 (Venables et al., 2008) to examine significant differences ($\alpha < 0.05$) in algal growth with and without each bacterial strain.

The colony forming unit (CFU) of each bacterial strain cocultivated with the alga was used to measure bacterial growth. Culture fluid (100 μl of 10^3- and 10^4-fold diluted) was spread onto a 1/10 NB agar plate in triplicate for each coculture, and the plates were incubated at 26°C for 3 to 7 days depending on strains. CFUs on each plate were counted.

In our preliminary tests, bacteria were isolated from the cocultures using 1/10 and 1/100 NB agar plates, and all the isolates obtained from the 1/100 NB plates were also able to grow on the 1/10 NB plates; many of them grew faster on the 1/10 NB plates than on the 1/100 NB plates. Therefore, only 1/10 NB was used in the present study. Extended incubation times may enable us to isolate slow-growing species of bacteria (Janssen et al., 2002), but we could not obtain such bacteria. Almost no colony formation was observed after 1 month of cultivation, and the late-appearing colonies showed fast growth after isolation.

Eight, six, and seven strains were isolated from the cultures C2, C6, and CSB, respectively. Based on the

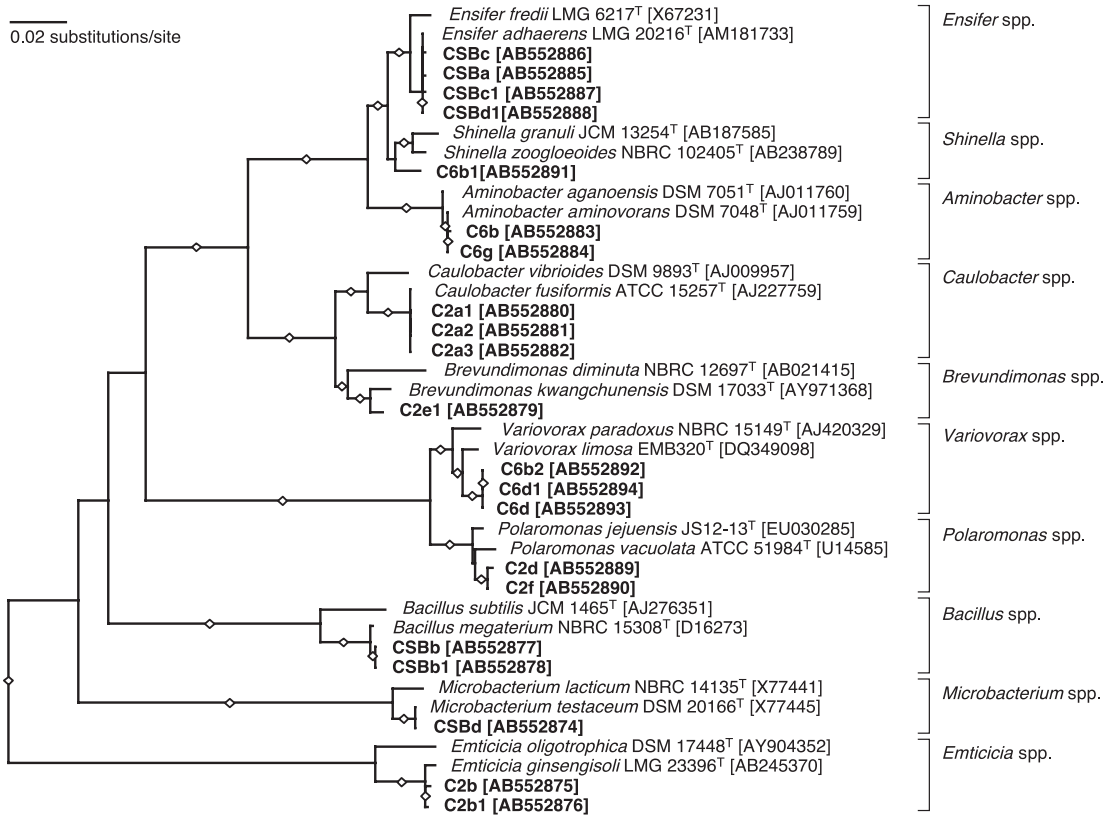

Fig. 1. Neighbor-joining tree based on the nearly full length of 16S rRNA gene sequences of the bacteria isolated in the present study and their phylogenetically close species.

The names of the taxonomic units beginning with C2, C6, and CSB denote their source cultures *Chlorella* sp. C2, *Chlorella* sp. C6, and *C. vulgaris* CSB, respectively. A node with greater than 70% bootstrap value is marked with an open rhombus.

neighbor-joining tree (Fig. 1), the isolated strains were identified as bacteria belonging to the genera *Microbacterium*, *Bacillus*, *Brevundimonas*, *Caulobacter*, *Aminobacter*, *Ensifer*, *Polaromonas*, *Shinella*, *Variovorax*, and *Emticicia*. The strain(s) belonging to one genus was/were isolated from only one of the cocultures.

The composition of bacteria in the cultures C6 and CSB was analyzed by a PCR-based method described previously (Otsuka et al., 2008a; Ueda et al., 2010), and only some of the bacteria detected previously were isolated in the present study. At least two explanations are possible: (i) these bacteria are not readily cultivable under the cultivation conditions of the present study, (ii) bacterial composition in the cultures changed between the previous analyses and present isolation. On the other hand, bacteria such as those belonging to the genus *Bacillus* were not detected previously but isolated in the present study. This may also be explained by the change in bacterial composition. However, another reason is possible. It has been estimated that a sequence comprising 0.1−1% or more of the total DNA can be detected by DGGE (Gelsomino et al., 1999). Therefore, it is possible that these bacteria comprised too small a proportion of the total bacteria (including the uncultivable ones) to be detected by DGGE; however, they comprised a detectable proportion of the cultivable bacteria on the 1/10 NB plates.

The genus *Shinella* has been taxonomically defined as exhibiting flocculent growth in liquid media (An et al., 2006), possibly because of which the cells of *C. vulgaris* NIES-227 cocultivated with *Shinella* sp. strain C6b1 aggregated in the flocculation and could not be used for the absorption measurement by the procedure described above. Therefore, algal growth cocultivated with each of the other nine strains was measured. As shown in Fig. 2, the nine bacterial strains grew with the alga, but none of them statistically significantly promoted or inhibited algal growth (vs. control) until 5 weeks after the start of cultivation. The bac-

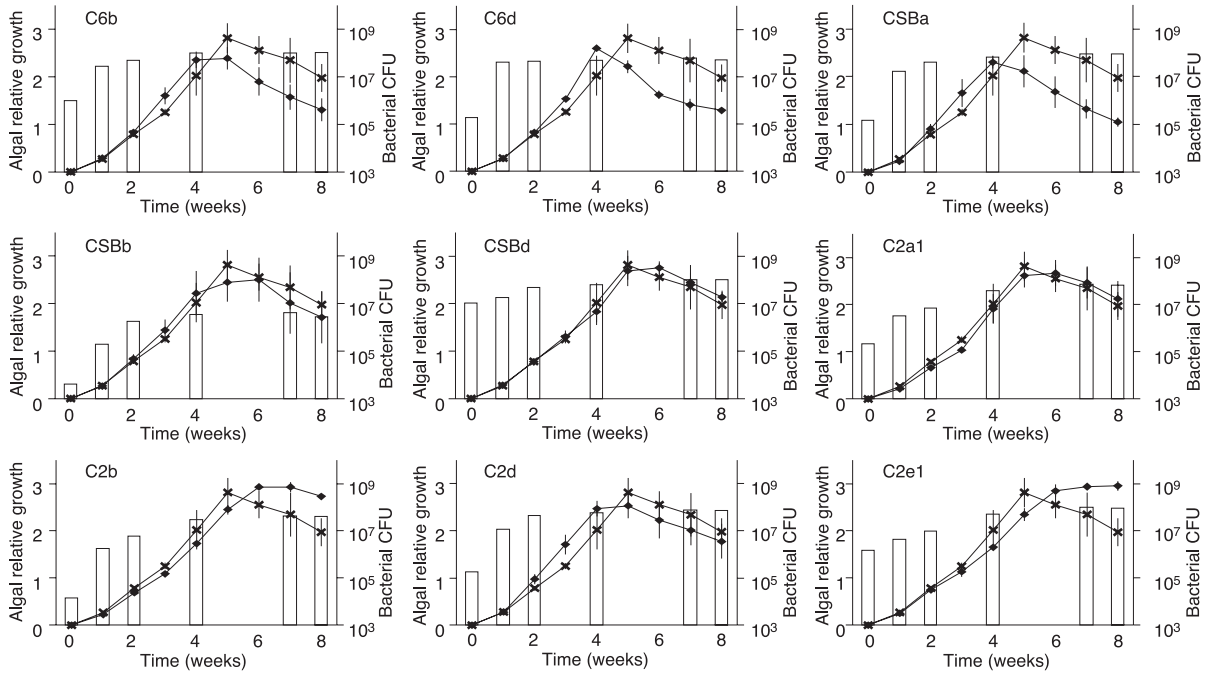

Fig. 2. The relative growth of *C. vulgaris* NIES-227 represented by the light absorption of chlorophylls 0 to 8 weeks after the start of cultivation (line graphs), and the colony formation units (CFUs) of the bacteria cocultivated measured after 0, 1, 2, 4, 7, and 8 weeks (bar graphs).

The closed rhombus denotes relative algal growth with each of the bacterial strains *Aminobacter* sp. C6b, *Variovorax* sp. C6d. *Ensifer* sp. CSBa, *Bacillus* sp. CSBb, *Microbacterium* sp. CSBd, *Caulobacter* sp. C2a1, *Emticicia* sp. C2b, *Polaromonas* sp. C2d, and *Brevundimonas* sp. C2e1, and the cross denotes the algal growth without bacteria (the control). The control data in the nine graphs are the same. Error bars of the line graphs represent standard deviations. Bar graphs have no error bars because the CFUs were calculated based on the sum of the colony numbers that appeared on the three plates for each strain.

terial strains isolated and examined by Park et al. (2008) increased the growth rate of *C. ellipsoidea* by up to three times of that of the control during the logarithmic phase, while in the present study, the growth rate of *C. vulgaris* NIES-227 cocultivated with any one of the nine bacterial strains was very similar to that of the control during the logarithmic phase. In addition, the species difference in the bacteria as well as the presence/absence of the bacteria did not statistically significantly affect the maximum growth values of the alga. However, the light absorption of chlorophylls was significantly high ($\alpha < 0.05$) for the alga cocultivated with *Brevundimonas* sp. strain C2e1 after 8 weeks (Fig. 2). Algal growth without bacteria (the control) reached its peak at 5 weeks of cocultivation and then started decreasing, but algal growth with strain C2e1 reached its peak (or nearly the peak) at the sixth week and showed no decrease by the eighth week. It is possible that strain C2e1 increased the culture lifetime of the alga rather than promoting growth. Park et al. (2008) reported that among eight bacterial strains promoting the growth of *C. ellipsoidea*, a strain of *Brevundimonas* sp. significantly enhanced algal growth. It is therefore suggested that *Brevundimonas* bacteria might have a common function to assist the growth of *Chlorella* spp. On the other hand, the light absorption of chlorophylls was statistically significantly low ($\alpha < 0.05$) for the *C. vulgaris* NIES-227 cocultivated with the *Variovorax* sp. strain C6d after 6 and 7 weeks, and the *Ensifer* sp. strain CSBa after 6 to 8 weeks (Fig. 2). From a viewpoint similar to that above, the strains C6d and CSBa decreased the culture lifetime of the alga rather than inhibited algal growth.

Although some algae produce antibiotic compounds (Ohta et al., 1993), *C. vulgaris* NIES-227 seemed to show no antibiotic activity against the bacterial strains examined (Fig. 2). Judging from the increase in CFU, the nine strains of bacteria reached their growth plateau by or about after 4 weeks. Interestingly, bacteria maintained their maximum values of CFU after algal

growth started decreasing. The bacteria may not only have utilized the organic substances produced and released by living algal cells but also degraded algal residues.

Park et al. (2008) found the growth-promoting effect of all the eight isolated strains on *C. ellipsoidea*, while we found only one strain, *Brevundimonas* sp. C2e1, that increased the culture lifetime of *C. vulgaris* NIES-227. To the best of our knowledge, however, the lifetime increasing effect concerning the algal–bacterial interactions has not been reported. In addition, the presence of bacteria that may decrease the culture lifetime of the alga was indicated. Algicidal bacteria are attracting increasing interest for their use in the inhibition of harmful algae such as red tide-forming algae (Pei et al., 2007). The results obtained in the present study would be a key not only for understanding the ecology of these organisms but also to their use as biological control agents. The culture lifetime increasing and decreasing effects of the bacteria on *C. vulgaris* NIES-227 would be some examples of the aspects of interactions among microorganisms. Further study is required to reveal more such interactions in detail.

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