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Presence of previously undescribed bacterial taxa in non-axenic *Chlorella* cultures

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We determined the bacterial community profile in non-axenic cultures of *Chlorella* (Chlorophyceae, Chlorophyta) isolated from soil. The bacterial composition at the phylum level was different from that of whole soil bacteria, but it was similar to that reported for non-axenic cultures of marine microalgae such as diatoms (Bacillariophyceae, Heterokontophyta). Expected novel bacteria, i.e. those which do not have close relatives among described species, were maintained in the cultures, and these bacteria were chiefly composed of members of the phylum Bacteroidetes. They may have been 'as-yet-uncultured' but in practice unintentionally been cultured in microalgal cultures. They could serve as good bioresources in various fields of biological and ecological studies.

Key Words——*Bacteroidetes*; *Chlorella*; microalgae; nonculturable bacteria; soil; uncultured bacteria

Introduction

It has been reported that 'nonculturable bacteria; i.e. those entering into viable but nonculturable (VBNC) states or unable to survive on artificial media, comprise a major fraction of the total observable bacteria in soil (Casida, 1965; Fægri et al., 1977; Olsen and Bakken, 1987; Torsvik et al., 1996). Although culture-independent techniques to analyze bacterial diversity or to obtain useful genes from various environments have been developed recently (e.g. Daniel, 2004; Muyzer, 1999), the importance of a culture-based approach has also been illustrated (e.g. Okibe et al., 2003; Schramm et al., 1998). Bacteria whose culture methods have been established can also enter into a VBNC state (Gauthier, 2000), or one bacterial species may include both culturable and nonculturable populations. Therefore, nonculturable bacterial populations do not always belong to novel species. However, it is expected that there are a number of novel and as-yet-uncultured bacteria in soil. Establishment of culture methods of such bacteria would provide not only new bioresourses but also information on their ecological functions.

Isolation of microalgae from environments is frequently accompanied by bacterial contamination, and microalgal cultures are sometimes maintained without purification. There are some reports on the relationship between bacteria and aquatic microalgae in such cultures. Watanabe et al. (2005) isolated one strain each of four bacterial species and one fungal species from a non-axenic culture of *Chlorella sorokiniana* (Chlorophyceae, Chlorophyta), and detected a growth-promoting effect of a bacterial and the fungal strain on

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the alga. A symbiotic relationship between bacteria and microalgae in artificial media has also been reported. Croft et al. (2005) isolated a vitamin B_{12}-producing bacterium, *Halomonas* sp., from a non-axenic culture of *Amphidinium operculatum* (Dinophyta), and succeeded in co-culturing the bacterium with each of the two vitamin B_{12} auxotrophic algae *Porphyridium purpureum* (Rhodophyta) and *A. operculatum* in a mineral medium without organic carbon sources. This indicated that *Halomonas* sp. provided vitamin B_{12} for the alga in return for the products of photosynthesis. Microalgae also may have some effects on the growth of bacteria. It is known that microalgae secrete organic compounds that are utilized by bacteria (Chröst, 1983; Fukami, 1991; Hellebust, 1974). It was also reported that certain microalgal extracellular substances have growth-promoting or growth-inhibiting effects on bacteria (Safonova and Reisser, 2005).

Taking into account the above-mentioned associations between bacteria and aquatic microalgae, it should not be surprising if some relationship also exists between bacteria and microalgae in soil. Among as-yet-uncultured soil bacteria, there could be bacteria that require certain substances or symbiosis for their growth, and such bacteria could be included in alga-associated communities. Here, we report on the presence of unintentionally cultured bacteria in non-axenic cultures of *Chlorella* isolated from soil.

Materials and Methods

Isolation of microalgae. The top 3 cm of soil was collected from an experimental field in The University of Tokyo, Japan (Yayoi Campus). Ten grams of soil was added to 300 ml of C medium (Ichimura, 1971) and incubated for 3 weeks at 20°C under a 16:8 h L/D cycle with a photon flux density of approximately 30 μmol·m^{-2}·s^{-1} provided by daylight fluorescence lamps. Under a stereoscopic microscope, green-algal cells that had appeared in the media were isolated and rinsed by the micropipette-washing method (Hoshaw and Rosowski, 1973). Single cells were transferred to 10-ml tubes containing fresh C medium. Isolates were maintained as liquid cultures under the same condition as the above incubation. The cultures were passaged approximately once every 3 weeks, by transferring 0.5 ml of each culture to 10 ml of fresh medium. These cultures were expected to contain bacteria from soil in addition to *Chlorella*.

DNA preparation. DNA was extracted from seven cultures 1 month after isolation, and from one selected culture 1 year after isolation by the following method. One milliliter of each of the cultures was transferred into a 2-ml tube and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant liquid was decanted, following which 0.7 g of 0.1-mm zirconia/silica beads (BioSpec Products, OK, USA), 1 ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1% SDS], and 600 μl of chloroform were added to the tube. The cells were then disrupted by a FastPrep FP120 beadbeater (Funakoshi, Tokyo, Japan) at a power level of 5.0 for 45 s. After centrifugation at 15,500×*g* for 10 min at 20°C, 800 μl of the aqueous layer was transferred to a 1.5-ml tube. Then 80 μl of 3 M sodium acetate solution and 480 μl of 2-propanol were added to the tube and mixed well. After centrifugation at 19,000×*g* for 10 min at 4°C, the supernatant liquid was decanted and the pellet was rinsed with 70% ethanol and centrifuged. The pellet was air dried and resolved in 50 μl of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)].

One month after isolation in liquid cultures, cells were subcultured on a 1/10 nutrient broth (NB) agar plate [1.5 g/L Difco™ Bacto-agar (Becton & Dickinson, NJ, USA) and 0.8 g/L Difco™ Bacto Nutrient Broth (Becton & Dickinson)] and C agar plate [1.5 g/L Difco™ Bacto-agar (Becton & Dickinson) in C medium]. A tenth ml of each liquid culture was spread on each agar plate, and it was incubated in the dark at 30°C. Difco™ Bacto-agar (Becton & Dickinson) contains trace impurities, and it was confirmed through our study that some bacteria are capable of growing on C (an inorganic medium) agar plates without additional carbon sources. It was expected that bacteria that grew on 1/10 NB agar plates prefer rich media and those on C agar plates prefer low-nutrient media. After another month, all bacterial colonies cultured on each plate were harvested and suspended in 1 ml of 0.9% NaCl solution, and DNA was extracted from them by the method described above.

PCR-DGGE, excision and sequencing of DGGE bands, and phylogenetic analysis. PCR-denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene (16S rDNA) V3 region was carried out by the same methods as described previously (Otsuka et al., 2008).

DGGE band patterns from liquid cultures 1 month after isolation and agar cultures that originated from

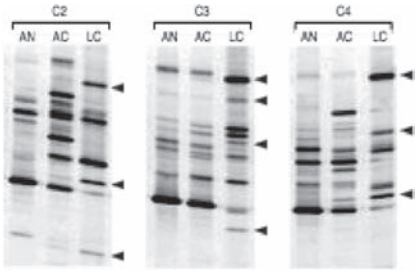

Fig. 1. Examples of PCR-DGGE banding patterns of the 16S rDNA V3 region derived from the *Chlorella* cultures C2, C3, and C4, with denaturing gradient of low to high from top to bottom.

LC represents patterns from non-axenic liquid cultures, and AN and AC represent those from 1/10 NB agar and C agar cultures, respectively. Bands indicated by arrows were excised and subjected to sequence analysis.

the liquid cultures were compared. It was expected that bands in the lanes for the agar cultures would be derived from culturable bacteria. In order to increase the chance to select bands from nonculturable bacteria, bands appearing only in the lanes for the liquid cultures were excised (Fig. 1). It was difficult to compare weak bands so that such bands were ignored in this analysis.

Almost all the DGGE bands including weak ones were excised from the selected liquid culture 1 year after isolation, in order to determine the total bacterial community profile in the culture.

Excision of bands and subsequent cloning and sequencing were carried out using the same methods as described previously (Otsuka et al., 2008). The sequences were compared with available sequences in the GenBank database by using the Blast program (Altschul et al., 1997). Based on the sequence similarities, the phyla and classes of bacteria providing the DGGE bands were estimated. When necessary, neighbor-joining trees (Saitou and Nei, 1987) were constructed using ClustalW version 1.8.3 (Thompson et al., 1994), and the validity of the estimation was confirmed.

Results and Discussion

We obtained seven cultures of green algae that were designated as C1, C2, C3, C4, C5, C6, and C7, respectively. They were single-celled, spherical in shape without flagella, and about 5 to 8 μm in diameter. During cultivation, autospore-forming in cells was observed. Based on these characteristics, they were identified as *Chlorella* spp. It would better be noted that these *Chlorella* are not necessarily specific to soil since there is a microalga such as *Chlorella vulgaris* that distributes both in soil and in freshwater (e.g. Fott and Nováková, 1969; Watanabe, 1977).

Figure 1 illustrates examples of the DGGE banding patterns derived from the cultures 1 month after isolation. A total of 25 bands were selected from the seven cultures and analyzed (Fig. 1), of which 18 bands corresponded to bacteria and the others to chloroplasts. The former were composed of 13 sequences with duplications, and the identities of the sequences are listed in Table 1. There is no criterion for the similarity value of the V3 region by which it can be ascertained whether or not the organism in question is closely related to described species. For reference, we examined the relationship of sequence similarities between the V3 region and nearly full-length 16S rDNA. From the data in DDBJ/EMBL/GenBank, we selected 142, 90, and 68 strains of the three bacteria, *Bacillus cereus*, *Burkholderia cepacia*, and *Pseudomonas fluorescens*, respectively showing similarities higher than 97% for nearly full-length 16S rDNA sequence within a species. Intra-specific similarities of the V3 regions of *B. cereus*, *B. cepacia*, and *P. fluorescens* had average values of 99.8%, 98.8%, and 98.5%, and minimum values of 97.5%, 96.4%, and 94.4%, respectively. With reference to these values, it is considered that at least six sequences, C1-C3-a, C1-C7-c, C2-d, C2-e, C2-C4-C6-f, and C7-l, whose similarities to the V3 regions of the closest type strains are not greater than 94%, may be from bacteria that have no close relatives within described bacterial species. Taking into account that there are cases where bacteria with high 16S rDNA similarities to one another are divided into multiple species based on phenotypic characteristics, the actual number of novel taxa at the species level would be higher. The expected novel bacteria providing the above six sequences were estimated to be members of the phyla Bacteroidetes and Planctomycetes. According to the results of Blast searches of the GenBank databese (Table 1), three of the six expected novel bacteria had close relatives detected from the environment but none among taxonomically described bacteria, which indicated the possibility that the three bacteria are not uncommon but have not so far been uncultured. Sequences C1-C3-a, C1-C5-b, C1-C7-c, and C2-C4-C6-f were detected from multiple algal cultures, indicating that certain bacteria bear some relation to at least a part of *Chlorella*.

Table 1. Identities of the selected DGGE bands from *Chlorella* cultures 1 month after isolation.

ID[a]	Phylum / Class[b]	Closest match		Closest type strain	
		Description [Accession number]	Similarity	Description [Accession number]	Similarity
C1-C3-a	*Planctomycetes* / *Planctomycetacia*	Uncultured bacterium [AM157644]	94	*Heliobacterium chlorum* ATCC35205^T [M11212]	76
C1-C5-b	*Proteobacteria* / *Alphaproteobacteria*	*Sphingomonas melonis* PG-224^T [AB055863]	100	*Sphingomonas melonis* PG-224^T [AB055863], etc.	100
C1-C7-c	*Bacteroidetes* / *Sphingobacteria*	Uncultured bacterium [EF662345]	98	*Roseivirga seohaensis* SW-152^T [AY739663]	84
C2-d	*Bacteroidetes* / *Sphingobacteria*	*Cyclobacterium marinum* DSM745 [AY533665]	87	*Capnocytophaga haemolytica* ATCC51501^T [X97247], etc.	79
C2-e	*Bacteroidetes* / *Flavobacteria*	Uncultured bacterium [AY989179], etc.	98	*Subsaximicrobium wynnwilliamsii* G#7^T [AY693997]	94
C2-C4-C6-f	*Bacteroidetes* / *Sphingobacteria*	Uncultured bacterium [AB104677], etc.	94	*Emticicia oligotrophica* GPTSA100-15^T [AY904352]	92
C3-g	*Proteobacteria* / *Alphaproteobacteria*	Uncultured bacterium [EF698525], etc.	100	*Rhodobacter blasticus* ATCC33485^T [D16429]	97
C4-h	*Proteobacteria* / *Alphaproteobacteria*	*Brevundimonas kwangchunensis* KSL-102^T [AY971368], etc.	98	*Brevundimonas kwangchunensis* KSL-102T [AY971368]	98
C5-i	*Bacteroidetes* / —	Uncultured bacterium [DQ232754], etc.	97	*Chryseobacterium shigense* GUM-Kaji^T [AB193101]	77
C6-j	*Proteobacteria* / *Alphaproteobacteria*	*Phyllobacterium leguminum* LMG22833^T [AY785323], etc.	99	*Phyllobacterium myrsinacearum* JCM7852^T [D12789], etc.	100
C7-k	*Bacteroidetes* / *Flavobacteria*	*Fluviicola taffensis* RW262^T [AF493694]	100	*Fluviicola taffensis* RW262^T [AF493694]	100
C7-l	*Bacteroidetes* / *Sphingobacteria*	Uncultured bacterium [EU133686]	98	*Flexibacter elegans* FX e 1^T [M58782]	84
C7-m	*Bacteroidetes* / *Sphingobacteria*	*Dyadobacter fermentans* NS114^T [AF137029], etc.	99	*Dyadobacter fermentans* NS114^T [AF137029]	99

[a] ID was assigned to each sequence but not to each DGGE band. C1, C2, C3, C4, C5, C6, and C7 represent the culture from which each sequence was detected. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB370151 (C2-C4-C6-f), AB370151 (C6-j), and AB370151 to AB370161 (the others).

[b] A minus sign "—" represents an unidentifiable taxon.

A total of 21 DGGE bands from culture C6 1 year after isolation were excised from the gel. There were cases where one band included more than one sequence, and a total of 24 sequences for bacteria were detected excluding those from chloroplasts. The taxonomic properties of the 24 sequences are presented in Table 2. Since the excision of DGGE bands was performed non-selectively, the number of detected sequences from culture C6 1 year after isolation was far greater than that for 1 month after isolation. Of the bacteria providing the 24 sequences, 15 belonged to the phylum Proteobacteria; six to Bacteroidetes; and one each to Actinobacteria, Verrucomicrobia, and an unidentifiable bacterium. With reference to the values mentioned above, it was expected that at least eight sequences, C6-8, C6-16, C6-17, C6-18, C6-19, C6-21, C6-22, and C6-24, were possibly from bacteria that have no close relatives within described bacterial species. These eight expected novel bacteria included one Proteobacteria, five Bacteroidetes, one Actinobacteria, and one 'unidentifiable' bacterium. The sequences C6-6 and C6-19 were also detected from culture C6 1 month after isolation (corresponding to the bands C6-j and C2-C4-C6-f, respectively), indicating that the

Table 2. Identities of the bacterial clones obtained from culture C6 1 year after isolation.

ID[a]	Phylum / Class[b]	Closest match		Closest type strain	
		Organism [Accession number]	Similarity	Organism [Accession number]	Similarity
C6-1	*Proteobacteria* / *Alphaproteobacteria*	*Afipia massiliensis* CCUG45153^T [AY029562], etc.	100	*Afipia massiliensis* CCUG45153^T [AY029562]	100
C6-2	*Proteobacteria* / *Alphaproteobacteria*	Uncultured bacterium [EF655648], etc.	98	*Ancylobacter polymorphus* DSM2457^T [AY211516]	97
C6-3	*Proteobacteria* / *Alphaproteobacteria*	*Caulobacter vibrioides* ATCC15252^T [AJ227756], etc.	100	*Caulobacter vibrioides* ATCC15252^T [AJ227756]	100
C6-4	*Proteobacteria* / *Alphaproteobacteria*	Uncultured bacterium [DQ837235], etc.	97	*Hyphomicrobium sulfonivorans* DSM13863^T [AF235089]	95
C6-5	*Proteobacteria* / *Alphaproteobacteria*	Unidentified bacterium [AF317761]	98	*Pedomicrobium australicum* IFAM ST1306^T [X97693]	97
C6-6	*Proteobacteria* / *Alphaproteobacteria*	*Phyllobacterium leguminum* LMG22833^T [AY785323], etc.	100	*Phyllobacterium leguminum* LMG22833^T [AY785323], etc.	100
C6-7	*Proteobacteria* / *Alphaproteobacteria*	Uncultured bacterium [AM884740], etc.	100	*Rhodobacter blasticus* ATCC33485^T [D16429]	97
C6-8	*Proteobacteria* / *Alphaproteobacteria*	*Azospirillum* sp. [EF434993], etc.	100	*Roseospira marina* CE2105^T [AJ298879], etc.	93
C6-9	*Proteobacteria* / *Alphaproteobacteria*	Uncultured bacterium [EF701553]	98	*Sphingomonas koreensis* JSS-26^T [AF131296]	97
C6-10	*Proteobacteria* / *Betaproteobacteria*	Uncultured bacterium [EF697165], etc.	100	*Curvibacter delicatus* LMG4328^T [AF078756], etc.	95
C6-11	*Proteobacteria* / *Betaproteobacteria*	Uncultured bacterium [EF072258], etc.	100	*Ralstonia mannitolilytica* LMG6866^T [AJ270258]	98
C6-12	*Proteobacteria* / *Betaproteobacteria*	Uncultured bacterium [EF516381], etc.	100	*Ramlibacter henchirensis* TMB834^T [AF439400]	96
C6-13	*Proteobacteria* / *Gammaproteobacteria*	*Lysobacter koreensis* Dae16^T [AB166878], etc.	98	*Lysobacter koreensis* Dae16^T [AB166878]	98
C6-14	*Proteobacteria* / *Gammaproteobacteria*	*Pseudomonas fragi* [AF094733], etc.	100	*Pseudomonas fragi* ATCC4973^T [AF094733]	100
C6-15	*Proteobacteria* / *Gammaproteobacteria*	*Pseudomonas migulae* [AY972434], etc.	100	*Pseudomonas viridiflava* LMG2352^T [Z76671]	98
C6-16	*Bacteroidetes* / *Bacteroidetes*	Uncultured bacteria [EF663490]	96	*Alkaliflexus imshenetskii* Z-7010^T [AJ784993]	84
C6-17	*Bacteroidetes* / —	Uncultured bacterium [AB205966], etc.	97	*Chryseobacterium shigense* GUM-Kaji^T [AB193101]	77
C6-18	*Bacteroidetes* / *Flavobacteria*	Uncultured bacterium [AM158366]	95	*Muricauda aquimarina* SW-63^T [AY445075]	88
C6-19	*Bacteroidetes* / *Sphingobacteria*	Uncultured bacterium [EF667913], etc.	94	*Emticicia oligotrophica* GPTSA100-15^T [AY904352]	92
C6-20	*Bacteroidetes* / *Sphingobacteria*	Uncultured bacterium [DQ676366], etc.	100	*Runella slithyformis* ATCC29530^T [M62786]	99
C6-21	*Bacteroidetes* / *Sphingobacteria*	Uncultured bacterium [EF378456]	97	*Sphingobacterium daejeonense* TR6-04^T [AB249372]	87
C6-22	*Actinobacteria* / *Actinobacteria*	Uncultured bacterium [AY235433]	94	*Solirubrobacter pauli* B33D1^T [AY039806]	91
C6-23	*"Verrucomicrobia"* / *Opitutae*	Uncultured bacterium [EF667689], etc.	99	*Opitutus terrae* PB90-1^T [AJ229235]	95
C6-24	— / —	Uncultured bacterium [EU135407]	93	*Nitrospira moscoviensis* DSM10035^T [X82558]	70

[a] ID was assigned to each sequence but not to each DGGE band. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB370127 to AB370150.

[b] A minus sign "—" represents an unidentifiable taxon.

bacteria with these sequences had a stable relationship with the alga in the culture.

Many bacteria closely related to already described species were also detected, such as members of the genera *Afipia*, *Ancylobacter*, *Brevundimonas*, *Caulobacter*, *Dyadobacter*, *Fluviicola*, *Lysobacter*, *Pedomicrobium*, *Phyllobacterium*, *Pseudomonas*, *Ralstonia*, *Rhodobacter*, *Runella*, and *Sphingomonas*. Members of these genera have been detected in and isolated from both soil and water except for the genus *Fluviicola,* which was described recently (O'Sullivan et al., 2005) and so far only one aquatic species of *Fluviicola* is known. Therefore, it is unclear whether or not the detected bacteria associate specifically with soil algae.

Janssen (2006) conducted a review on bacterial composition in soil, according to which Alphaproteobacteria, Acidobacteria, and Actinobacteria are frequently abundant in soils and members of Bacteroidetes are generally less abundant. In the present study, however, alga-associated bacteria that were originally isolated from soil had a different composition. In addition, a relatively high ratio of taxa of the Flavobacteria-Sphingobacteria (FS) group of the phylum Bacteroidetes in soil may belong to described and named genera (Janssen, 2006). On the other hand, in the present study, only three out of the 10 detected taxa of the FS group possibly belong to or are closely related to described and named genera (*Dyadobacter*, *Fluviicola*, and *Runella*). These results indicated that the bacterial community in algal cultures does not represent whole soil bacteria but is hemi-selectively composed of alga-associated bacteria. In addition, it should be noted that, although soil was used as the isolation source, it is at present unclear whether the detected bacteria are active in soil or not, since the *Chlorella* isolates were isolated from the liquid culture after 3 weeks' incubation.

Grossart et al. (2005) examined bacterial dynamics in cultures of two marine diatoms (Bacillariophyceae, Heterokontophyta), and reported that the diatom-associated bacteria mainly belonged to the FS group. Sapp et al. (2007) also examined bacterial profiles in diatom and dinoflagellate (Dinophyta) cultures isolated from marine water, and reported that members of Alphaproteobacteria, Gammaproteobacteria, and the FS group were predominant in the cultures. Among the bacteria in culture C6 that were detected 1 year after isolation, the number of taxa of Alphaproteobacteria, Gammaproteobacteria, and the FS group comprised approximately 38%, 13%, and 17% of the total number of taxa, respectively. This result indicated the possibility that the phylum/class-level composition of microalga-associated bacteria is common to or similar among microalgae less associated to the algal taxonomic positions. The present results support those of Sapp et al. (2007), which state that the compositions of the bacterial communities are not necessarily species specific for microalgae.

In the present study, expected novel bacteria were found to be present and co-cultured in non-axenic cultures of *Chlorella* isolated from soil. At least a fraction of these bacteria were expected to be as-yet-uncultured, based on low similarity values of the 16S rDNA V3 region to already described species. It can be said that they are 'unintentionally cultured bacteria.' With regard to cultures of such bacteria stably maintained with algae, they could serve as good bioresources in various fields of biological and ecological studies.

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