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Community composition of bacteria co-cultivated with microalgae in non-axenic algal cultures

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We examined the community compositions of bacteria in three non-axenic microalgal cultures, *Chlorella saccharophila* NIES-640, *Ulothrix variabilis* NIES-329 and *Volvox aureus* NIES-864, which had been maintained by subculturing without purification in the Microbial Culture Collection at the National Institute for Environmental Studies, Japan. Based on the similarity of the partial sequence of 16S rRNA gene, it was estimated that many species of bacteria in the cultures of *U. variabilis* NIES-329 and *V. aureus* NIES-864 are as-yet undescribed, in contrast to those in the *C. saccharophila* NIES-640 culture. The present and previous studies have indicated that some bacteria detected in the cultures might be associated with algae and/or can be hemi-selectively enriched with algae (e.g. *Phyllobacterium* bacteria and *Chlorella* algae). The present study indicates the potential usefulness of non-axenic cultures of algae as materials for studies on algal-bacterial associations.

Key words: algal-bacterial association, bacteria, community composition, culture collection, microalgae

Isolation of microalgae is frequently accompanied by bacterial contamination. Non-axenic algal cultures often need to be purified because physiological, chemical, molecular or taxonomic studies on microalgae usually require axenic cultures (e.g. Stanier *et al.*, 1971; Watanabe *et al.*, 1998). Some aquatic bacteria have been known to cause lysis of algal cells (Cole, 1982), and the removal of such algicidal action is another important reason for purification. In a minority of cases, non-axenic algal cultures can be used for studies of symbiosis and interaction between algae and bacteria. For instance, Watanabe *et al.* (2005) investigated the associations between a strain of the alga *Chlorella solrokiniana* IAM C-212 (NIES-2169) and its extracellular symbionts (that is, in a strict sense, contaminating microorganisms isolated from the algal culture), and found a growth-promoting effect of a bacterial and a fungal isolate on the alga. In the present study, we examined bacterial community compositions in non-axenic algal cultures maintained in a culture collection, in order to evaluate their potential usefulness as materials for studies on algal-bacterial associations.

Three non-axenic cultures of green microalgae, *Chlorella saccharophila* NIES-640, *Ulothrix variabilis* NIES-329 and *Volvox aureus* NIES-864, were obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (MCC-NIES), Tsukuba, Japan. They were originally isolated in 1987 in Hokkaido, Japan, in 1984 in Ibaraki, Japan, and in 1997 in Brandenburg, Germany, respectively. They had been maintained in the MCC-NIES without purification or cryopreservation before their use in the present study (M. Erata, personal communication).

C. saccharophila NIES-640 and *U. variabilis* NIES-329 were cultivated in C medium (Ichimura, 1971), and *V. aureus* NIES-864 was cultivated in AF-6 medium (Kato, 1982), under a 16:8 light/dark cycle with a light intensity of approximately 20 μmol photons m^{-2} sec^{-1} at about 25℃ in light and 20℃ in dark. DNA extraction, polymerase chain reaction amplification of the V3 region of the 16S rRNA gene (16S rDNA) and denaturing gradient gel electrophoresis (DGGE) of the amplicons, followed by cloning and sequencing of the excised DGGE bands, were performed as described previously (Otsuka *et al.*, 2008a, 2008b). A BLAST search was conducted for homologies to the partial sequences of 16S rDNA obtained (Altschul *et al.*, 1997), and phylogenetic analysis was performed, whenever necessary, as described previously (Otsuka *et al.*, 2008a, 2008b).

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All visible DGGE bands were excised from the gel, and 10, three and six sequences of bacterial 16S rDNA (V3 region) were detected from *C. saccharophila* NIES-640, *U. variabilis* NIES-329 and *V. aureus* NIES-864, respectively. Table 1 shows the estimated phyla/classes of the bacteria detected and the closest described species. It is known that the described species with sequence similarity values close to 100% of the detected bacteria (Table 1) (e.g. *Phyllobacterium*, *Bosea*, and so on) and/or their close relatives are generally distributed in water as well as in soil.

Based on previous studies (e.g. Grossart *et al.*, 2005; Otsuka *et al.*, 2008a; Sapp *et al.*, 2007), the frequency of bacteria belonging to the classes *Flavobacteria/Sphingobacteria* is expected to be high, whereas that of bacteria belonging to the class *Betaproteobacteria* would be low. This held true for the bacteria in the *U. variabilis* NIES-329 culture, but not for those in the *C. saccharophila* NIES-640 and *V. aureus* NIES-864 cultures in the present study (Table 1). The phenomenon of one sequence

Table 1 Identities of the bacteria detected in non-axenic algal cultures at the phylum/class level and the closest described species

Sequence ID[a]	Estimated phylum / class	Closest described species represented by type strain[b] Name [accession number in DDBJ/EMBL/GenBank]	%similarity
640A	*Bacteroidetes / Flavobacteria*	*Flavobacterium limicola* NBRC 103156^T [AB075230]	96.8
640B	*Proteobacteria* / Alphaproteobacteria	*Phyllobacterium myrsinacearum* JCM 7852^T [D12789] and some other bacteria within the order Rhizobiales	100
640C*	*Proteobacteria* / Alphaproteobacteria	*Bosea eneae* CIP 106338^T [AF288300] and three other bacteria within the genus *Bosea*	98.5
640D	*Proteobacteria* / Alphaproteobacteria	*Afipia massiliensis* CIP 107022^T [AY029562]	100
640E*	*Proteobacteria / Betaproteobacteria*	*Comamonas testosteroni* ATCC 11996^T [M11224]	100
640F	*Proteobacteria / Betaproteobacteria*	*Janthinobacterium lividum* DSM 1522^T [Y08846]	100
640G*	*Proteobacteria / Betaproteobacteria*	*Janthinobacterium agaricidamnosum* JCM 21444^T [Y08845]	98.9
640H	*Proteobacteria / Gammaproteobacteria*	*Acinetobacter radioresistens* DSM 6976^T [X81666]	95.7
640I	*Proteobacteria / Gammaproteobacteria*	*Acinetobacter lwoffii* DSM 2403^T [X81665]	96.3
640J	*Proteobacteria / Gammaproteobacteria*	*Pseudomonas mandelii* CIP 105273^T [AF058286] and two other bacteria within the genus *Pseudomonas*	100
329A	*Bacteroidetes / Sphingobacteria*	*Thermonema rossianum* DSM 10300^T [Y08956]	89.2
329B*	*Proteobacteria / Gammaproteobacteria*	*Escherichia fergusonii* ATCC 35469^T [AF530475] and *Shigella dysenteriae* ATCC 13313^T [X96966]	100
329C*	*Proteobacteria* / —	*Maricaulis virginensis* CIP 107438^T [AJ301667]	76.3
864A	*Proteobacteria / Alphaproteobacteria*	*Sphingomonas ursincola* DSM 9006^T [Y10677] and some other bacteria within the order Sphingomonadales	99.3
864B	*Proteobacteria / Betaproteobacteria*	*Curvibacter lanceolatus* ATCC 14669^T [AB021390]	92.6
864C	*Proteobacteria / Betaproteobacteria*	*Hylemonella gracilis* ATCC 19624^T [AF078753]	91.9
864D	*Proteobacteria / Gammaproteobacteria*	*Acinetobacter johnsonii* DSM 6963^T [X81663]	100
864E	*Proteobacteria* / —	*Polaromonas naphthalenivorans* DSM 15660^T [AY166684]	70.0
864F	*Actinobacteria / Actinobacteria*	*Sanguibacter suarezii* NBRC 16159^T [X79452]	92.9

[a] 640, 329, and 864 in sequence IDs represent algal cultures *Chlorella saccharophila* NIES-640, *Ulothrix variabilis* NIES-329, and *Volvox aureus* NIES-864, respectively, from which each sequence was detected. Accession numbers in DDBL/EMBL/GenNbank for the sequences 640A to 640J, 329A to 329C, and 864A to 864F are AB480717 to AB480726, AB480727 to AB480729, and AB480730 to AB480735, in serial, respectively. The five sequences with asterisks were included as very minor fractions in DGGE bands (comprising one clone each out of five clones derived from a DGGE band).

[b] There are cases where more than one type strains shared the same sequence of the V3 region of 16S rDNA.

with multiple DGGE bands has been reported (e.g. Otsuka *et al.,* 2008b). Additionally, in this study all three DGGE bands from the *U. variabilis* NIES-329 sample, except for that from the chloroplasts, were represented by only one sequence 329A. Two out of the three DGGE bands yielded the sequences, 329B or 329C, each comprising only one of five clones derived from each band. Therefore, bacteria harbouring the sequence 329A, which were estimated to belong to the class *Sphingobacteria*, comprised the majority of bacteria in the *U. variabilis* NIES-329 culture.

Each of the ten bacterial sequences detected from the *C. saccharophila* NIES-640 culture showed more than 95% similarity to that of its closest type strain(s), seven of which showed more than 98% similarity (Table 1). These results indicate that many of the bacteria present in the culture are closely related to previously described bacteria, in contrast to the results from our previous study (Otsuka *et al.,* 2008a). On the other hand, many bacteria detected in the *U. variabilis* NIES-329 and *V. aureus* NIES-864 cultures showed little relation to previously described bacteria. It is possible that *U. variabilis* NIES-329 and *V. aureus* NIES-864 may be key to cultivating those bacteria that have not yet been cultured.

The sequences 640B and 640D detected in the *C. saccharophila* NIES-640 culture were the same as those detected in a non-axenic culture of *Chlorella* sp. (culture C6) isolated from soil (Otsuka *et al.,* 2008a). The sequence 640B was estimated to be from bacteria belonging to the genus *Phyllobacterium* or one of the closely related genera, and the sequence 640D was from bacteria belonging to the genus *Afipia* (Table 1). The two bacteria harbouring these sequences may have an association with *Chlorella* spp. This idea is partly supported by a previous report that *Phyllobacterium myrsinacearum* has a close association with *Chlorella vulgaris* (Gonzalez-Bashan *et al.,* 2000).

In addition, five out of nine alphaproteobacterial sequences detected from the *Chlorella* sp. C6 culture in our previous study (Otsuka *et al.,* 2008a) and all three alphaproteobacterial sequences detected in the *C. saccharophila* NIES-640 culture in the present study were estimated to belong to the order *Rhizobiales*. *Chlorella*-associated *P. myrsinacearum* (Gonzalez-Bashan *et al.,* 2000) also belongs to this order. It is therefore possible that bacteria belonging to *Rhizobiales* could potentially associate with *Chlorella* spp., or could be hemi-selectively enriched in the alga. Watanabe *et al.* (2008) determined the contents of low molecular-mass carbohydrates, amino acids, and so forth, in the culture broth of *C. sorokiniana* IAM C-212 under photoautotrophic conditions, and established an artificial medium imitating the nutritional conditions surrounding the algal strain. Using that medium, they isolated 14 strains of bacteria belonging to the class *Alphaproteobacteria*, and five of them were estimated to belong or be closely related to the order *Rhizobiales*. The results obtained by Watanabe *et al.* (2008) illustrated the high proportion of *Rhizobiales* bacteria in alga-associated populations. However, it must be noted that *Rhizobiales* bacteria have been isolated from algae other than *Chlorella*; for instance, *Ochrobacterium* sp. was isolated from brown algae samples (Zhou *et al.,* 2008).

Bacteria harbouring the sequence 864A were estimated to belong to the genus *Sphingomonas* sensu lato or a closely related genus. This group of bacteria have been repeatedly isolated from various algae samples, including *Chlorella*, *Thalassiosira*, *Skeletonema*, and so on. (Otsuka *et al.,* 2008a; Sapp *et al.,* 2007; Watanabe *et al.,* 2005). We also detected bacteria that were estimated to be members of the genus *Acinetobacter* (864D) from the *V. aureus* NIES-864 culture and those phylogenetically related to this genus (640H and 640I) from the *C. saccharophila* NIES-640 culture. Burmølle *et al.* (2006) have isolated an *Acinetobacter* strain from another alga, *Ulva*. Therefore, bacteria related to *Sphingomonas* or *Acinetobacter* may associate with a variety of algae with less specificity. Borde *et al.* (2003) reported photosynthesis-enhanced biodegradation of phenol and phenanthrene by microcosms of *Chlorella-Acinetobacter* and *Chlorella-Sphingomonas*, respectively. It is interesting that the bacteria that were reported to have associations with the alga *Chlorella* and which have been isolated from various algae were also detected in non-axenic algal cultures in a culture collection.

Based on the results presented here, the potential usefulness of non-axenic cultures of microalgae in a culture collection as a material for studies on algal-bacterial associations was indicated. However, no care has been taken to achieve the stability of the algal-bacterial associations, since algal cultures in culture collections are maintained mostly as algal

resources. Some cases may require specific techniques to maintain the associations in culture.

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微細藻類の非無菌培養株に共存して培養される細菌の群集組成

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MCC-NIES コレクション（国立環境研究所）において非無菌の状態で継代培養されてきた 3 株の微細緑藻 *Chlorella saccharophila* NIES-640，*Ulothrix variabilis* NIES-329，*Volvox aureus* NIES-864 について，それらの株と共存して培養されている細菌の組成を解析した．16S rRNA 遺伝子の部分塩基配列の相同性に基づき，*U. variabilis* NIES-329 および *V. aureus* NIES-864 の培養株には，既知の種と近縁ではない *Bacteroidetes* 門や *Proteobacteria* 門，*Actinobacteria* 門に属する細菌が多く共存し，*C. saccharophila* NIES-640 の培養株には，既知の種と近縁な *Bacteroidetes* 門や *Proteobacteria* 門に属する細菌が多く共存することが示された．本研究と過去の報告とから，例えば *Phyllobacterium* 属細菌と *Chlorella* 属緑藻のように，一部の細菌が藻類との間に何らかの関係を持っている，または藻類の培養株に半選択的に集積される可能性が示唆された．本研究により，藻類－細菌相互作用の研究材料としての，非無菌の藻類培養株の潜在的な有用性が示された．

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