論 文 の 内 容 の 要 旨

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論 文 題 目

Isolation, identification, and functional analysis of bacteria in green microalgal-bacterial consortia

（微細緑藻–細菌共存系を構成する細菌の分離、同定、および機能解析）

1. Introduction

Microorganisms play an enormous role in the underlying processes of material cycle on earth especially in the transfer of energy through natural ecosystems and in the cycling of bio-chemically important elements. In natural ecosystem, microorganisms are found in a variety of forms of consortium with a wide range of associations from parasitism to symbiosis. However, there have been only a small number of studies that succeeded in revealing some aspects of associations between microorganisms.

In soil ecosystems, the interactions of bacteria and plants have been well studied especially on those in the rhizosphere, the area in the soil where microorganisms are influenced by plant roots. The concept of the "phycosphere" is a pelagic analogy to the rhizosphere. The phycosphere is the zone surrounding an algal cell within which microorganisms are influenced by algal products. Not a small number of microalgae are living in soil, and the phycosphere in soil may represent a good microhabitat for soil bacteria. Studies on the associations between green microalgae (the phylum *Chlorophyta*, kingdom *Plantae*) and bacteria are considered to be of great value, because the form is not only a microorganism-microorganism association but, at the same time, may also be a primitive plant-microorganisms association. However, not much information

on these associations has been accumulated to date. Furthermore, there is limited information available on which bacteria are living in the green algal phycosphere.

Under the above circumstance, bacteria were isolated from green microalgal-bacterial consortia and identified in this study. Then the effects of the isolates on the growth of green microalgae were examined. In addition, genes involved in vitamin B_{12} (cobalamin) synthesis of a selected isolate were identified, and the transcriptional activity of one of the genes of the isolate co-cultivated with a cobalamin-requiring green microalga was examined, since cobalamin has been reported to be a key of some marine algal-bacterial associations.

2. Composition of cultivable bacteria in green algal-bacterial consortia

Chlorella spp. (*Chlorophyta*) are one group of the representative soil algae. Three non-axenic strains of *Chlorella* spp., C2, C6, and CSB-227, were used as algal-bacterial consortia in this study. *Chlorella* sp. C2 and C6 were originally isolated from soil and had been maintained without purification. *Chlorella vulgaris* CSB-227 was established by inoculating soil into an axenic strain *C. vulgaris* NIES-227 (NIES, National Institute fro Environmental Studies). Twenty-three bacterial strains were isolated from these three consortia, and identified as members of the genera *Caulobacter*, *Brevundimonas*, *Polaromonas Variovorax, Ensifer, Shinella, Aminobacter, Microbacterium, Bacillus,* and *Emticicia*, based on the neighbor-joining trees constructed with almost full length of 16S rRNA gene sequences. Several taxonomic properties placed the strains belonging to the genera *Caulobacter* and *Variovorax* as novel species, for which scientific names, *Caulobacter aquatilis* and *Variovorax aquatilis* were proposed.

3. Effect of co-cultivation with bacteria on algal growth

One strain each of nine bacterial genera was selected and individually co-cultivated with each of the two strains of microalgae, *C. vulgaris* NIES-227 and *Monomastix minuta* NIES-255 (*Chlorophyta*), to examine the effects of the bacteria on the growth of the algae. Prior to this experiment, *M. minuta* NIES-255 was screened in this study as a vitamin B_{12} -requiring green alga from axenic green algal collections maintained in the Microbial Culture Collection of NIES. *Brevundomonas* C2e1 statistically significantly increased the culture lifetime of *C. vulgaris* NIES 227, and decreased the die-off rate of *M. minuta* NIES-255. *Bacillus* strain CSBb decreased the die-off rate of *M. minuta* NIES-255 but did not affect on the growth of *C. vulgaris* NIES-227.

On the other hand, the culture lifetime of *M. minuta* NIES-255 was statistically significantly decreased when co-cultivated with the *Caulobacter* C2a1, *Aminobacter* C6b, *Ensifer* CSBa, and *Variovorax* C6d. The latter two strains also statistically significantly decreased the lifetime of *C. vulgaris* NIES-227. Other strains showed no effect on the growth of the two algae.

Among the above results, the effect of *Brevundimona*s C2e1 on the growth of *C. vulgaris* NIES-227 was prominent. Therefore, another strain of *Brevundimonas*, DC2a-G2, isolated by Ueda (2010, master's thesis) from another algal-bacterial consortium was added, and the effect of *Brevundimona*s C2e1 and DC2a-G2 on the growth of five strains of *Chlorella*, *Chlorella sorokiniana* NIES-2167, *C. sorokiniana* NIES-2168, *C. vulgaris* NIES-2170, *Chlorella* sp. NIES-217, and *Chlorella elkhatiense* NIES-2250, was examined. As the results, *Brevundimona*s C2e1 statistically significantly increased the culture lifetime of the *C. vulgaris* NIES-2170, decreased the culture lifetime of *C. sorokiniana* NIES-2168, inhibited the growth of *C. sorokiniana* NIES-2167, and showed no effect on the growth of *Chlorella* sp. NIES-2171 and *C. elkhatiense* NIES-2250. Another strain, *Brevundimona*s DC2a-G2, statistically significantly inhibited the growth of *C. sorokiniana* NIES-2167 and showed no effect on the growth of the other four algae.

4. Identification and transcriptional activity of cobalamin biosynthesis genes of *Ensifer* CSBa

In an inorganic medium without cobalamin, each of the above nine strains of bacteria and cobalamin-requiring *M. minuta* NIES-255 were co-cultivated, for the purpose of screening bacteria that synthesis and release cobalamin to the alga. Three strains, *Ensifer* CSBa, *Bacillus* CSBb, and *Aminobacter* C6b, enabled *M. minuta* NIES-255 to grow, indicating that these three bacteria supplied cobalamin to the alga, although the growth rate of the alga co-cultivated with *Aminobacter* C6b was very small. It was surprising that out of nine bacterial strains isolated from cobalamin-independent algae, three could synthesize cobalamin. Among the three, *Ensifer* CSBa was subjected to a further study, because the genus *Ensifer* includes populations that have a symbiotic association with higher plants, and *Ensifer* bacteria have been repeatedly detected from green algal-bacterial consortia in previous studies.

Draft genome analysis was done for *Ensifer* CSBa, and the genes involved in cobalamin synthesis (*cob* genes) were identified. It was revealed that *Ensifer* CSBa harbored 22 putative *cob* genes in total, and each of them were homologous to those found in a cobalamin- synthesizing bacterium, *Pseudomonas denitrificans* SC510 Rif and/or SBL27 Rif. Based on the composition of the *cob* genes, it was suggested that *Ensifer* CSBa

synthesizes cobalamin by the oxygen-dependent (aerobic) pathway as *P. denitrificans* SC510 Rif and/or SBL27 Rif does. There are two pathways in the biosynthesis of cobalamin; one is aerobic and another is anaerobic. The anaerobic pathway has been well studied in *Salmonella typhimurium, Bacillus megaterium* and *Propionibacterium freudenreichii*, but the whole gene set involved in the aerobic pathway has so far been revealed only in *P. denitrificans* SC510 Rif and/or SBL27 Rif.

Based on the DNA sequence of one of the *cob* genes, *cobT*, specific PCR primers for the gene were designed. Real-time quantitative reverse transcription PCR analysis was performed targeting *cobT* of *Ensifer* CSBa co-cultivated with *M. minuta* NIES-255 in an inorganic medium with and without cobalamin. It was revealed that the presence/absence of cobalamin in the medium did not affect the transcriptional activity of *cobT* of *Ensifer* CSBa co-cultivated with a cobalamin-requiring alga, *M. minuta* NIES-255.

5. Conclusion

In this study, the cultivable bacterial composition of green microalgal-bacterial consortia were identified, and two novel bacterial species names were taxonomically proposed. The bacterial isolates showed culture lifetime-increasing, culture die-off rate-decreasing, or growth inhibiting effects on microalgae, depending on the combination of a bacterial and an algal strain. Totally 22 putative genes involved in the aerobic pathway of cobalamin synthesis (*cob* genes) were identified with an isolate, *Ensifer* CSBa. The presence/absence of cobalamin in the medium did not affect the transcriptional activity of *cobT* of *Ensifer* CSBa co-cultivated with a cobalamin-requiring alga. It seems that keys to the green microalgal-bacterial associations are still more complex. Further studies are necessary to reveal the mechanisms with compounds produced by algae/bacteria other than cobalamin as the targets.