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Chemotaxonomic and Phylogenetic Study on the Oligotrophic Bacteria Isolated from Forest Soil

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Summary

Oligotrophic bacteria isolated from forest soil showed a specific community consisting of various taxonomic groups compared with those in other soil or aquatic habitats. Based on the cell shape, the isolates were divided into four groups: regular rod, curved/spiral rod, irregular rod, and prosthecate bacteria. The cellular fatty acids 60 oligotrophic isolates were analyzed. The 30 fatty acids which were identified or characterized are classified. At the dendrogram based on cellular fatty acid composition, four clusters(I -IV) were separated at a euclidian distance of about 50. Cluster 3 and 4-a strains were containing Q-8, these strains are accommodated in the Proteobacteria gamma and beta subdivision. The chemotaxonomic profiles of the cluster 4-a strains showed good agreement with those of the genus *Burkholderia*. Cluster 3 was characterized by the presence of branched-chain fatty acids, iso-C15:0, iso-C17:1, and iso-C17:0 as the major components. These chemotaxonomy suggested the close relationship of the isolates with *Xathomonas/Sterotrophomonas* group. Based on the 16S rDNA sequence analysis, the two representative strains(MH256 and MA828) of cluster 3 showed the close relation to genera, *Xathomonas/Sterotrophomonas,* but were not included in these genera. These strains were even further away from core *Xanthomonas*, and clearly were seen to branch outside the cluster formed by the *Sterotrophomonas maltophilia*. MH256 and MA828 16S rDNA sequence was different enough to put new genus on a separate branch.

The isolates with Q-10 were also studied. They are corresponded to the two large groups in Proteobacteria alpha subdivision. One was incorporated in the genus *Bradyrhizobium* cluster, which also includes *Agromonas*, a genus for oligotrophic bacteria. The strains of the other group showed high similarity to the genus Agrobacterium.

Introduction

The concept of oligotrophy and oligotrophic habitat was reviewed(6), and the term 'oligotrophic' is often used for those microorganisms which have the capability of growing in low-nutrient media(3). Criteria for oligotrophy have been discussed in several papers. Concentration of energy-yielding nutrients required by oligotrophic bacteria were considered to be in the order of 1 to 5 mgC l^{-1}(6) or 5 to 10 mgC l^{-1}(10). Recently, this concept has been modified by Japanese researchers to

include those bacterial isolated capable of growing on a medium containing less than 1 mg^{-1} of an organic carbon source (8).
Since Hattori & Hattori (2) used a 100-fold dilution of the full strength nutrient broth(DNB) to isolate soil bacteria, and found many isolates which were unable to grow on the full strength medium: such organisms were called DNB organisms. These bacteria may be a part of oligotrophic bacteria, although the term has not received any strict definition. We observed that oligotrophic bacteria, which could develop on the medium containing less then 1 mg of organic carbon per liter (16).

Oligotrophic bacteria are found in aquatic and terrestrial environments, and they may play an important role in decomposing organic matter and recycling nutrients in such low-nutrient environment as ocean, lakes or soil (1, 7). However, information on their physiology, biochemistry and taxonomy is still so far very limited (9).

In this study, I examined 60 strains of oligotrophic bacteria from forest soil at different layers(L, F, H and A layers) on the basis of chemotaxonomic and phenotypic characters, such as cellular fatty acids, quinone system, and DNA base composition. The 16S rDNA sequence (about 1500 bp) was determined for the isolates from each DNA-DNA hybridization groups.

Materials and Methods

Soil

The sampling site has been covered for centuries by natural forest. The soil profile at the sampling site was defined as follows: the upper portion(L layer) was covered with undecomposed, newly fallen leaves followed by partly resilient, brown leaves in older litter strata. Below the old litter(F layer) there was a 2-3 cm thick layer of dark brown or dark grey coloured recognizable plant debris and fecal pellets. The H layer was more or less sharp, dark grey or brownish coloured. Next to this an A layer of 15-20 cm was observed

Media

The following media to be use: nutrient broth(NB) composed of 10g each of peptone, beef extract, 5g of NaCl and 1,000 ml distilled water; and DNB medium, which is a 100-fold dilution of NB media.

Isolation of oligotrophic bacteria

Bacterial cells picked up from colonies on DNB plates and their growth on the NB and DNB media was tested. Isolates which grew only on the DNB medium were DNB organism. Organisms were also tested on a 10,000-fold dilution of the NB medium, because the NB medium used in our experiments contained 7550 mgC/liter, thus 10,000-fold dilution is assumed to contain approximately 1ppm. After 10 days of incubation growth was examined by plate counts on DNB medium, because the tubidity due to growth was slight. Cells grown on a 10,000-fold dilution of the NB medium were recultivated on a fresh one to

reconfirm their capability of growing on such a diluted medium. Based on the obove assumptions, the present investigation was limited to those organisms which were isolated on a 100-fold dilution of the NB medium, and were able to grow on a 10,000-fold dilution of the NB medium.

Phenotypic features

Morphological characterization of the living cells(form, size, arrangement, motility) was done by observing them under a phase contrast microscope. The following tests were performed: Hydrolysis of some macromolecular compounds, such as starch, gelatin, and casein, was determined by adding the substrate at the final concentration of 0.1%, 0.4% and 0.45%(W/V) to DNB medium, respectively, and observing the clear zones developing around the colonies after 5-7 days incubation at 20℃.

Cellular fatty acid and hydroxy fatty acid compositions

Cells cultivated in DNB medium at 28℃ for 4 days with shaking were usd to determine cellular fatty acid analysis. Fatty acid methyl esters(FAMEs) were extracted and purified (15). Profiles were obtained after gas chromatograph (Hewlett-packard model 5890) equipped with a 25m cross-linked 5% phenylmethyl -silicon capillary column (0.2 mm i.d.), and which a chromatograph-data processor

Quinone systems

Cells of reference strains that were cultivated aerobically for 6 days were used for the quinone analysis. Quinone systems were determined as described previously (14).

DNA manupulation and DNA base composition

DNA was extracted according to the procedure of Saito and Miura (11). To further purify DNA, extra chloroform/isoamyl alcohol extraction steps were added. Mean G+C contents(mol%) of DNA were determined by reversed-phase high-performance liquid chromatography as described by Tamaoka and Komakata (13).

DNA-DNA hybridizations

DNA-DNA hybridization was carried out at 55℃ by microplate-photobiotin method (4).

PCR amplification and sequence determination of 16S rDNA

Genomic DNA was isolated as described previously. Nearly complete 16S rRNA genes were amplified by PCR using a forward primer hybridizing at the complements of positions 8-27, and a reverse primer hybridizing at positions 1541-1525(*E.coli* 16S rRNA gene sequence numbering). PCR was carried out using perkin-Ekmer. 16S rDNA was sequenced by using Microseq 16S rRNA Gene Kit and PRISM 310 Genetic Analyzer(PE Applied Biosystems).

Phylogenetic analysis

All DNA sequences were deposited in the EMBL/GenBank database. They were compared with previously published 16S rDNA sequence. The Genbank database also to the FASTA software apperared to be related to oligotrophic isolates. Only well defined sequence with less than five undetermined nucleotides were used. Sequence were aligned using CLUSTA W 1.7 between position 98 and 1496 (E. coli numbering). Alignment was refined manually using SUNMASE algorithm. Phylogenetic trees were constructed using the neighbour-joining method of Sitou & Nei (12) with the PHYLO-WIN graphic tool. The tophology of this distance tree was tested by 1000 bootstrap resamplings of data.

Results and Discussion

Dilution effect of nutrient broth on the plate count

The average number of bacteria on the DNB medium was larger than that from the NB medium with all samples taken at different depth throughout the year. DNB-organisms exist aboundantly in bacterial population of rendzina forest soil throughout year. The number of colonies on NB plates became almost maximal within in a few days, but the number of colonies on DNB plates increased with incubation time following double or triple colony formation curves (5), and thus the final conts on the DNB medium were about 10-fold higher than those on the NB medium.

Isolation of oligotrops

Isolates from DNB plates were tested for their capability of growing on the NB medium and divided into two groups: NB and DNB organisms. About half of the isolates from the upper layers of the soil profile were DNB organisms. Among 393 DNB organisms, 203 isolates were selected as oligotrophs. A large percentage of such oligotrophs was isolated from the colonies which appeared after 600 h incubation time, whereas, only a few oligotrophic bacteria were isolated from the colonies formed before 600 h.

Grouping of 203 oligotrophic isolates

Based on the cell shape, isolates were divided into four groups: 1. Regular rods shaped organisms (53 isolates); 2. Curved and spiral shape organisms (30 isolates); 3. Irregular rods shaped organisms (66 isolates); and 4. Prosthecate organisms (54 isolates). I made further subdivision of these groups .

Physiological characteristics of the oligotrophic isolates

None of the oligotrophic isolates decomposed cellulose. Only very few isolates hydrolyzed macromolecular compounds, like starch (6% of the total number of isolates), gelatin(12% of the total of the isolates), and casein(10% of the total isolates). Sixty two perccent of the prosthecate bacteria (Group 4) utilized methanol; whereas small amounts of methanol were utilized by the other group of isolates. Only 10% of the oligotrophic isolates reduced nitrate to nitrite.

It is notable that the growth of organisms is remarkably affected by NaCl and peptone-meat extract mixture. Among 203 organisms tested, 82 organisms were strongly sensitive to 0.5% NaCl. The peptone-meat extract mixture was also inhibitory to DNB organisms. Ninety-one organisms did not show appreciable growth after 10 days of incubation in the presence of 0.5% each peptone and meat extract.

Grouping of oligotrophic isolates using cellular fatty acid profiles

Analysis of the cellular fatty acid composition is useful for the classification and identification of various bacterial genera. It is considered that cellular fatty acid analysis is suitable for differentiation and grouping of bacterial isolates. Sixty bacterial strains used are selected from the oligotrophic gram-negative bacteria. The cellular fatty acids 60 oligotrophc isolates were analyzed. The 30 fatty acids which were identified or characterized are classified.

At the dendrogram based on cellular fatty acid composition, four clusters(I - IV) were separated at a euclidian distance of about 50; 15 strains belonged to group I, 17 strains to group II, 4 strains to group III, and group IV included 24 strains. Group IV showed further subdivision 2.

All of the isolates contained C16:0, C18:0 and C18:1 were often the most abundant fatty acid methyl esters. Most isolates also contained C16:1 and iso-C10:1. Four discrete groups of strains were identified on the basis of types of hydroxy acids found, although these fatty acids usually accounted for less than 20% of the tatal peak area in a profile. These hydroxy fatty acids were of four types, 2-hydroxy, 3-hydroxy, iso-branched 2-hydroxy, and iso-branched 3-hydroxy.

Phylogenetic analysis

The strains representing cluster 4a contained C16:0 and C18:0 acids predominantly. 3OH-C14:0 and 3OH-C16:0 acids were also found as the characteristic components, Their quinone system is Q-8. Seven strains of this cluster were divided into three DNA homology groups. They are accommodated in the cluster of the genus *Burkholderia* in the Proteobacteria gamma subdivision. The chemotaxonomic profiles of the isolates showed good agreement with those of the genus *Burkholderia*.

Cluster 3 was characterized by the presence of branched-chain fatty acids, iso-C15:0, iso-C17:1, and iso-C17:0 as the major components. The strains also possessed Q-8 These chemotaxonomy suggested the close relationship of the isolates with *Xathomonas/Sterotrophomonas* group. Based on the 16S rDNA sequence analysis, the two representative strains(MH256 and MA828) of cluster 3 showed the close relation to genera, *Xathomonas/Sterotrophomonas,* but were not included in these genera. These strains were even further away from core *Xanthomonas*, and clearly were seen to branch outside the cluster formed by the *Sterotrophomonas maltophilia.* MH256 and MA828 16S rDNA sequence was different enough to put new genus on a separate branch.

In addition to these Q-8 containing strains, the isolates with Q-10 were also studied. They are corresponded to the two large groups in Proteobacteria alpha subdivision. One was incorporated in the genus *Bradyrhizobium* cluster, which

also includes *Agromonas*, a genus for oligotrophic bacteria. The strains of the other group showed high similarity to the genus Agrobacterium.

The oligotrophic bacteria studied here showed diversity and indicated the close relationship to established genera of non-oligotrophic bacteria. The results of this study will be a great help for elucidation of oligotrophy to compare the two types of nutrition-utilization.

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