Applied Soil Ecology 108 (2016) 315–324

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Bioprospecting of facultatively oligotrophic bacteria from non-rhizospheric soils

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ARTICLE INFO

Article history:
Received 21 May 2016
Received in revised form 2 September 2016
Accepted 5 September 2016
Available online xxx

Keywords:
Facultatively oligotrophic bacteria
Methylotrophic
Agarolytic
Photoheterotrophic
Nitrogen fixation
Phosphate solubilization

ABSTRACT

Conventional cultivation method relies on the use of nutrient-rich medium, which might limit the exploration of oligotrophic bacteria from soil ecosystems. In the present study attempts were made to isolate oligotrophic bacteria from non-rhizospheric samples collected from mountain roadside, beach, bay and wetland using 1000-fold diluted nutrient agar. Phylogenetic analysis and biogeographic studies of these isolates were performed based on their 16S rDNA sequences. Besides, tests for the utilization of methanol or agar as their carbon source (methylotrophic or agarolytic), light as their energy source (photoheterotrophic), fixation of nitrogen or solubilization of tricalcium phosphate were conducted. A total of 74 isolates with facultatively oligotrophic behavior were obtained, since they can proliferate on both 1000-fold diluted and conventional nutrient agar. 16S rDNA sequence analyses assigned them mainly to class Actinobacteria and α-Proteobacteria. All these isolates belonged to 24 genera which encompassed 35 species, demonstrating that these facultatively oligotrophic bacteria occupied a wide range of bacterial lineages. Fifty-seven out of 74 isolates formed colonies on methanol-containing agar, while only 21 showed better growth in methanol-containing medium. Nine isolates were verified to utilize agar instead of methanol for growth. A total of 4 isolates showed photoheterotrophic trait and the *pufLM* gene was successfully PCR amplified and sequenced. The methylotrophic or photoheterotrophic behaviors might provide advantages for them to inhabit oligotrophic environments. Members affiliated with many genera were first demonstrated to fix nitrogen or solubilize phosphate, and isolates with profound activities have potentials to be developed as bioinoculants used to promote plant growth. In addition, the use of diluted nutrient agar helped to explore several unrecognized species from non-rhizospheric soils. All these isolates may provide opportunities for studying their oligotrophic growth, metabolism and interaction with plants in a near future.

1. Introduction

The heterogeneous soils in natural ecosystems harbor a great diversity of microorganisms, but only small minority are cultivated. Traditional cultivation method using nutrient rich medium easily enriches fast grower such as copiotrophic, Gram-positive and spore-forming bacteria (Kasahara and Hattori, 1991). The use of 100-fold diluted nutrient broth medium not only allowed the isolation of both copiotrophic and oligotrophic bacteria, but highlights the ecological significance of oligotrophic isolates (Gorlach et al., 1994; Hashimoto et al., 2009). Vartoukian et al. (2010) also discussed the possible reasons for "unculturability" of a variety of microorganisms in nature and suggested to use dilute nutrient media particularly suited for the growth of bacteria adapted to oligotrophic condition. Based on phylogenetic analysis Mitsui et al. (1997) reported that isolates belonging to the Cytophaga/Flexibacter/Bacteroides division, the Proteobacteria α-subdivision, β-subdivision and the Firmicutes high G+C division, except for *Arthrobacter*-related strains, were oligotrophic bacteria. Bacteria affiliated with phyla Acidobacteria, Actinobacteria and α-Proteobacteria have been further categorized as oligotrophs, while Bacteroidetes and β-Proteobacteria exhibited copiotrophic attributes (Fierer et al., 2007; Kéki et al., 2013). By using a low organic carbon agar medium and repeating transfer, Senechkin et al. (2010) concluded that the vast majority of strains belonging to the trophic group of microorganisms adapted to a

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http://dx.doi.org/10.1016/j.apsoil.2016.09.004
0929-1393/

"broad range" of carbon concentrations, including well-known and widespread bacterial genera.

Methylotrophs are those microorganisms able to grow at the expense of reduced carbon compounds containing one or more carbon atoms but containing no carbon–carbon bonds (Anthony, 1982). They have been shown to distribute in environments such as oligotrophic marine waters, lake sediments, soils, plant roots and phyllosphere which contained methanol as a prominent C1 source (Chistoserdova et al., 2009; Dourado et al., 2015; Ramachandran and Walsh, 2015; Vorholt, 2012). Aerobic anoxygenic phototrophic bacteria (AAnPB) are facultative photoheterotrophs which are capable of oxidizing organic carbon to support their chemotrophic growth, while light seems to be used as a supplementary source of energy (Yurkov and Beatty, 1998). They use organic substrates as the main source of energy, reducing power and carbon, but are capable of photosynthetic light utilization when organic carbon is scarce. AAnPB are globally distributed in the euphotic zone or coastal regions and appear to be critical to the cycling of both organic and inorganic carbon in the ocean (Kolber et al., 2001; Ritchie and Johnson, 2012). Aside from cyanobacteria and proteorhodopsin-containing bacteria, AAnPB which taxonomically belong to various subgroups of α-, β-, and γ-Proteobacteria serve as the third most numerous group of phototrophic prokaryotes in the oligotrophic ocean (Koblížek, 2015; Zheng et al., 2015).

Non-rhizospheric environments which are free of plant roots differ from rhizosphere, the hot spot with higher turnover rate of microbial biomass and enhanced evapotranspiration rate (Cheng, 2009). Limitation of available substrates in root-free soils leads to the selection of microbes with slower growth rates and more efficient metabolism, and microorganisms in the rhizosphere and root-free soil exhibited different carbon use efficiency dynamics (Blagodatskaya et al., 2007, 2014). However, our knowledge of the microbiology of the oligotrophic environment has historically lagged behind our knowledge of the microbiology of the rhizosphere, an environment rich in organic substances (Vorholt, 2012). There were only few studies dealt with the diversity of oligotrophic bacteria in soil ecosystems (Uksa et al., 2015).

To fulfill the exploration and application of oligotrophic bacteria from non-rhizospheric soils, the present study was undertaken to obtain isolates from both terrestrial and aquatic environments using diluted nutrient agar. All these isolates were subjected to phylogenetic and biogeographic studies. Tests for these isolates to grow under various nutritional conditions, fix nitrogen or solubilize tricalcium phosphate were conducted to screen candidates with plant growth promoting potential.

2. Materials and methods

2.1. Soil sampling and bacterial isolation

In the present work attempts were made to collect soils from nutrient-limited terrestrial environment (mountain roadside in Miaoli County) and coastal areas (Tongxiao beach in Miaoli County, Baisha bay in Pingtung County and Gaomei wetland in Taichung City), which are quite different from rhizospheric soils. Red soils are classical soils found in northern part of Taiwan. In general they are naturally low in pH value and contain less amount of organic matter if not used for agricultural purpose. Tongxiao beach, Baisha bay and Gaomei wetland are famous places in Taiwan due to their special geographic landforms. Samples collected from these soils received intermittent water flow with diluted nutrients. The oligotrophic nature of these samples provided opportunities for obtaining oligotrophic bacteria, although the isolations were performed from four diverse ecosystems which may give different ecological meanings. Soil was collected with a shovel, put into zipper bags and brought to laboratory. After serial dilutions samples were plated on 1000-fold diluted nutrient broth (Difco, BD, USA) agar or 1000-fold diluted nutrient broth agar containing 3% NaCl. Colonies obtained on diluted nutrient agar were transferred and maintained on conventional nutrient agar at 30 °C. Bacterial cultures were preserved in nutrient broth containing 30% glycerol at −80 °C.

2.2. Bacterial identification and phylogenetic analysis

Genomic DNA was isolated using UltraClean Microbial Genomic DNA Isolation Kit (MO BIO Laboratories, Inc., USA). 16S rDNA was amplified by PCR with bacterial universal primers (Edwards et al., 1989) and purified using DNA Clean & Concentrator Kit (ZYMO RESEARCH CORP., USA). Cycle sequencing and determination of the nucleotide sequence of PCR product were performed at Genomics BioSci&Tech Ltd., Taiwan. Bacterial identification was carried out by comparing the 16S rDNA sequences of isolates and their most closely-related type strains using EZTaxon Identify function. Sequences were aligned using the ClustalX program version 2 (Larkin et al., 2007) and distances and clustering with the neighbor-joining method were performed using the software package MEGA version 6 (Tamura et al., 2013). Bootstrap values based on 1000 replications were listed as percentages at the branching points. Besides, based on 16S rDNA sequence, isolates were subjected to biogeographic studies through comparing with their most closely-related environmental strains retrieved from NCBI GenBank database.

2.3. Methanol utilization, mxaF gene amplification and agar utilization

Colonies grown on nutrient agar were picked and suspended in phosphate buffer saline (l^{-1} distilled water: NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g) to remove residual nutrient from the former medium. Cultures were streaked on methanol-containing agar or inoculated in methanol-containing mineral salt medium (DSMZ Medium125). 1.5% of bacteriological agar powder (HiMedia Laboratories Pvt. Ltd., India) was added when methanol-containing agar was prepared. After 72 h of cultivation, the growth of isolates on methanol-containing agar was recorded as revealed by the formation of colonies. Bacterial growth in methanol-containing mineral salt medium was compared with that in mineral salt medium without methanol supplement to demonstrate their abilities to utilize methanol. For few representatives, their growths in methanol-containing mineral salt medium during 240 h of cultivation were monitored. These isolates were also subjected to the analysis of gene encoding for methanol dehydrogenase alpha subunit. The *mxaF* gene was amplified by PCR with primers *mxa* f1003 and *mxa* r1561 (McDonald and Murrell, 1997). The PCR was performed in a final volume of 25 μl containing 0.625 units TakaRa ExTaq DNA polymerase, 1X Ex Taq buffer, 0.2 mM dNTP, 2 mM $MgCl_2$, 20 pmol each primer and 20–40 ng extracted DNA. Cycling conditions for PCR of *mxaF* gene were: initial denaturation for 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C, with a final extension of 7 min at 72 °C. The amplified *mxaF* gene was separated, purified and cycle sequenced using primer mxaF1003 to determine the nucleotide sequence.

Water agar which was prepared by distilled water and 2.0% of bacteriological agar powder was used to screen agar utilizers. Fresh colonies grown on nutrient agar were picked and streaked on water agar. After 120 h of cultivation agar plates were stained with Lugol's solution, and the formation of clear zone around colonies demonstrated their agarolytic activity (Saraswathi et al., 2011). For some isolates with prominently clear zone around colonies, the