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Isolation and molecular characterization of non-*Azotobacter* bacteria using *Azotobacter* isolation protocols from pastures in the south of Türkiye

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Abstract

Soil is a biodiversity-rich ecosystem. Nitrogen-fixing *Azotobacter* bacteria, an important component of this ecosystem, are frequently isolated using various methods. The aim of the present study was to perform partial molecular characterization of non-*Azotobacter* isolates derived during two *Azotobacter* isolation protocols, as well as to determine which bacteria can be obtained using this method. A total of 800 isolates were acquired from soil samples collected from various pastures in Antalya province of Turkey, with 92 of them being molecularly characterized. These isolates were clustered through RFLP analysis of 16S rRNA gene and the DNA sequences of the isolates representing different groups were performed. According to these results, the bacteria belonging to various genera (*Agrobacterium*, *Phyllobacterium*, *Variovorax*, *Acinetobacter*, *Pseudomonas*, *Agromyces*, and *Arthrobacter*) were identified. The results show that similar bacteria could be obtained through two isolation protocols used in this study. However, more diverse bacteria were encountered on the Brown-medium than on soil-past.

Keywords: *Azotobacter*, isolate, soil, 16S rDNA

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Türkiye'nin güneyindeki meralardan *Azotobacter* izolasyon protokolleri kullanılarak *Azotobacter* olmayan bakterilerin izolasyonu ve moleküler karakterizasyonu

Özet

Toprak, biyolojik çeşitlilik açısından zengin bir ekosistemdir. Bu ekosistemin önemli bir bileşeni olan azot bağlayan *Azotobacter* bakterileri çeşitli yöntemlerle sıklıkla izole edilir. Bu çalışmanın amacı, iki *Azotobacter* izolasyon protokolü sırasında elde edilen *Azotobacter* olmayan izolatların kısmi moleküler karakterizasyonunu gerçekleştirmek ve bu yöntemle hangi bakterilerin elde edilebileceğini belirlemektir. Türkiye'nin Antalya ilindeki çeşitli meralardan toplanan toprak örneklerinden toplam 800 izolat elde edilmiş ve bunlardan 92'si moleküler olarak karakterize edilmiştir. İzolatlar, 16S rRNA genlerinin RFLP analizi ile gruplandırılmış ve farklı grupları temsil eden izolatların DNA dizileri gerçekleştirilmiştir. Bu sonuçlara göre çeşitli cinslere (*Agrobacterium*, *Phyllobacterium*, *Variovorax*, *Acinetobacter*, *Pseudomonas*, *Agromyces* ve *Arthrobacter*) ait bakteriler tespit edilmiştir. Sonuçlar, bu çalışmada kullanılan iki izolasyon protokolü ile benzer bakterilerin elde edilebileceğini göstermektedir. Bununla birlikte, Brown Besi ortamında Soil-Past'a göre daha çeşitli bakteriler tespit edilmiştir.

Anahtar kelimeler: *Azotobacter*, izolat, toprak, 16S rDNA

1. Introduction

Soil is an ecosystem having great biodiversity, including many diverse organisms belonging to numerous groups on the soil's surface and underground. A gram of soil has been estimated to contain several thousand species of bacteria [1]. Bacteria, a significant part of this enormous diversity, play a variety of roles in elemental cycles and

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biochemical reactions [2], and also they establish unique interactions with the other organisms in the soil. Nitrogen, one of the basic elements of living organisms, is essential for the survival of all organisms. Earth's atmosphere contains almost 78 % nitrogen [3]. But many organisms are not able to use atmospheric nitrogen. The fixation of nitrogen, one of the most significant processes on Earth, from the atmosphere to soil or other ecosystems, is performed by only prokaryotes [4]. Bacteria (such as *Rhizobium*, *Azotobacter*, and *Cyanobacteria*) having the ability of nitrogen fixation can take place in different phylogenetic groups [5]. These bacteria can convert atmospheric nitrogen (N_2) to ammonia, which is essential for plant growth and supports the nutrition of plants. Therefore, agricultural activities utilize nitrogen-fixing bacteria for enhancing plant crops. Isolation of nitrogen-fixing bacteria such as *Azotobacter* is very significant for their diverse contributions to plant growth and ecosystems [6, 7]. Since it has been shown that bacteria, can grow in microbial cultures, corresponding to less than 1% of the entire microbial diversity [8, 9] we know that cultivation methods are relatively limited. On the other hand, it may be an obligation to isolate some bacteria for the purpose of utilizing them or investigating their properties. The *Azotobacter* genus is a Gram-negative free-living nitrogen-fixing bacteria that has been commonly studied for a long time. There are various isolation media such as Winogradsky, Brown, and Burk [10] to isolate them. But diverse bacteria may be encountered in these enhancement and semi-selective media. This study aims to identify and characterize bacteria growing in the media used for the isolation of *Azotobacter*.

2. Materyal ve yöntem

2.1.Sampling Site and Sample Collection

Rhizosphere and bulk soil samples were collected from four pastures (Elmalı-Eymir, Manavgat-Demirciler, İbradı-Eynif and Akseki-Çimi) Antalya in Turkey. The rhizosphere soil was picked up using a shovel that took a profile of the soil together with the plant roots. Bulk soil was taken from 10 cm depth of top soil. All samples were transported to the laboratory in a cool ice chest on the same day and stored at +4 °C for a maximum of one night until the isolation step. The rhizosphere soil taken with the plant root was removed from the part of the plant root under septic conditions in the laboratory. All soil samples were sieved with a mesh size of 4 mm.

2.2. Isolation of Bacteria

In the study, two distinct culturing methods were utilized for the isolation of bacteria. Firstly, a 10 g soil sample from each sample was placed in a 250 mL erlenmayer flask containing 90 mL of sterilized 0.85% (w-v) NaCl solution and glass beads and shaken at 100 rpm for 15 minutes at room temperature [11]. For soil suspensions, 10-2-10-5 dilutions were spread on the selective-Brown medium (pH 6.8-7) of *Azotobacter* and incubated at 29–30 °C for 5–9 days [12, 10]. The mucoid colonies on plates at 10-2-10-5 dilutions were transmitted to TSA (Triptic Soy Agar) medium. Second, in a sterilized plate, 30-50 g of soil samples were completely mixed with mannitol solution (1 %) and the surface was smoothed [13]. Plates were incubated for 5-9 days at 29-30 °C. Isolates from these plates were transferred to nitrogen-free Brown medium. All isolates, from both isolation methods, were tested by the KOH method for Gram properties [14], and then they were grown on the selective LG medium [10] of *Azotobacter*. The bacteria with yellow pigment growing on LG medium were chosen and characterized by molecular methods.

2.3. DNA Extraction and PCR analysis

Two or three bacterial colonies from TSA medium were suspended in 500 µL double sterilized water, then precipitated by centrifuge. The precipitate was suspended in 100 µL water and mixed with 100 µL of 10 mM Tris-HCl (pH, 8.2). The suspension was treated with 1 mg-ml proteinaz K and incubated at 55 °C for 2 hours [13]. The supernatant obtained was stored at -20 °C. The 16S rRNA gene was amplified using universal bacterial primers 27f and 1495r by PCR [15]. PCR was performed in a 25 µL mix with 0.5 U GoTaq of DNA polymerase enzyme and 2 µL of DNA sample.

2.4. RFLP (Restriction Fragment Length Polimorfizm) and Electrophoresis

Three different restriction enzymes (Alu I, Rsa I and Hha I) were used according to manufacturer's protocol (Promega) for RFLP analysis of 16S rRNA gene. The 16S rRNA gene PCR products were mixed with 10 U of enzyme, BSA, and NaCl and incubated at 37 °C for 12 hours. The PCR products and the fragments of 16S rDNA from RFLP were run on 0.5 % and 2.5 % agarose gels, respectively, using TBE buffer. Gels were stained with Ethidium Bromide and viewed using a UV transilluminator.

2.5. Analysis of RFLP and DNA Sequences

A matrix was constituted using the profile of the DNA bands from RFLP of 16S rDNA according to the absence or presence of DNA bands. For clustering of all isolates, the matrix was performed with UPGMA (Unweighted Pairgroup Method with Aritmetic Average) analysis by Bioedite 5 and PHYLIP 3.66 programs [16, 17]. The distance between groups was accounted for according to Nei ve Li'e [18]. The dendrogram of clustering was obtained using the Treeview 1.66 program. DNA sequences were conducted for isolates representing different groups using Macrogen sequencing service (Macrogen Ltd., Seoul, South Korea). DNA sequences were compared with nBlast analysis [19]. All the 16S rRNA gene sequences reported in this paper have been deposited in the NCBI GenBank database.

3. Results

A total of 800 isolates were derived using two distinct nitrogen-free media (Brown and soil-past) from various pasture soils in Turkey. The 92 isolates were chosen depending on the characteristics of pigment and growth on the media. For molecular characterization of these isolates, the RFLP method was performed using the 16S rRNA gene. All isolates were clustered using UPGMA analysis according to RFLP results (Figure 1). According to the cluster diagram, isolates were separated into at least seven major groups and the substitution of branches was clustered into subgroups. The 16S rDNA of 11 isolates representing separate groups was sequenced and identified partially (Table 1). The results of 16S rDNA sequences show that some isolates of these groups correspond to Variovorax, Phyllobacterium, Acinetobacter, Agromyces, Agrobacterium, Arthrobacter and Pseudomonas. The majority of isolates (88%) were recovered by Brown-medium isolation, while only a few isolates were acquired through soil-past isolation. (Figure 1).

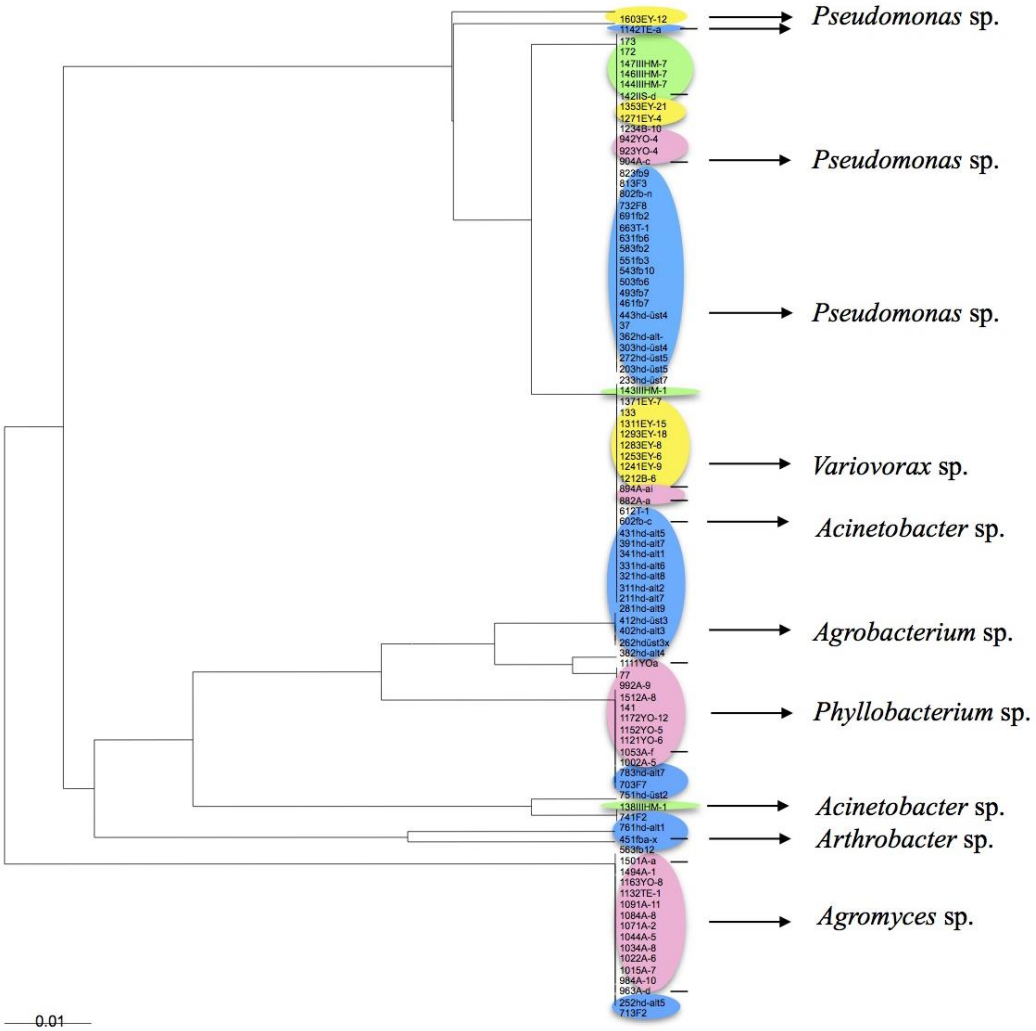

Figure 1. Dendrogram generated using UPGMA cluster analysis. - : isolates from soil past isolation methods, Others were isolated from Brown medium. Colors point distinct pastures, Blue: Çimi; Yellow: Eynif; Pink: Eymir; Green: Demirciler. Accession numbers for 16S rDNA sequences; OP686576 (1hdalt-5), OP686577 (2TE-a), OP686578 (3EY-12), OP686579 (3hdust-4), OP686580 (4A-c), OP686581 (IIIHM-1), OP686582 (1EY9), OP686583 (1fbax), OP686585 (2hdalt3), OP686586 (2YO12) and OP696653 (4A-c)

Table 1. Blast results for 16S rDNA sequences from isolates

NCBI accession numbers	identification	isolate	similarity (%)
JN989304.1	*Agromyces* sp. G4342	1A2	99
CP002417.1	*Variovorax paradoxus* EPS	1EY-9	99
NR_026233.1	*Arthrobacter aurescens* strain DSM	1fbax	99
GQ169811	*Agrobacterium tumefaciens*	2hdalt3	100
NR_043192.1	*Phyllobacterium ifriqiyense*	2YO12	99
AJ633632.1	*Acinetobacter calcoaceticus*	1hdalt-5	99
NR_025228.1	*Pseudomonas koreensis*	2TE-a	99
NR_025228.1	*Pseudomonas koreensis*	3EY-12	99
NR_025228.1	*Pseudomonas* sp.	3hdust-4	99
MK883099.1	*Pseudomonas* sp.	4A-c	93
X81661.1	*Acinetobacter calcoaceticus*	IIIHM-1	99

4. Conclusions and discussion

The present study shows that many diverse bacteria can be isolated from the selective media practically. No doubt, if more restriction enzymes in RFLP analysis had been used, the groups of isolates in cluster analysis would have been branched more. Thus, that could have provided further enhanced the diversification of bacteria. Despite the fact that two isolation procedures were used in the study, most isolates were derived from Brown-medium. As a result, it appears that isolating a specific bacteria using Brown-medium may be more difficult.

It seems that numerous *Pseudomonas* were recovered from Brown-medium. The cluster diagram shows that *Pseudomonas* was also isolated from all pastures. Since *Azotobacter* and *Pseudomonas* are closely related [20, 21], it is not surprising that many and diverse *Pseudomonas* are obtained using these isolation steps. *Acinetobacter* and *Pseudomonas* bacteria could be commonly isolated together from different environments [22, 23]. Regarding the isolates from the present study, we do not completely know whether they are nitrogen-fixing bacteria. But, somehow, these isolates were able to grow on nitrogen-free media. Some strains of *Phyllobacterium* and *Acinetobacter* have been reported to fix atmospheric nitrogen [24, 25]. Because of the abundance of organic molecules, growth factors for *Variovorax* are not necessary [26]. As a result, *Variovorax* isolates may have had a chance to thrive on the Brown-medium that was utilized. On the other hand, it is remarkably significant to identify these bacteria in the Ibrad-Eynif pasture sampled here because Finkel et al. [27] reported that a single genus, *Variovorax* is efficient for maintaining plant root growth in a complex microbiome, and cor bacteria of *Variovorax* in plant-microbe interactions. *Arthrobacter* and *Agromyces* are Gram-positive bacteria, and although Gram-negative isolates were chosen using the KOH test in the present study, the reason we encountered them might have been a false negative. The KOH test is a simple, rapid, and reliable method [28], but some bacteria may give a false negative KOH reaction [29].

Culture-independent methods are becoming more common and help us comprehend the microbial diversity in various ecosystems [30, 31]. However, the ability to isolate and grow the bacteria is a significant step toward utilizing them and learning their features. It seems that the bacteria isolated in this study are able to grow in a medium containing specific nutrition sources. Consequently, this research also aids in our comprehension of the efficiency of the nutrient media employed in the isolation of bacteria. Microbial populations in soil play a central role in the productivity and health of terrestrial ecosystems [32], and affect their ecosystems due to their various metabolic activities. Assuming that each of these bacteria may have a specific function in the soil ecosystem, determining the presence of these bacteria may contribute to our understanding of pasture soils in the future.

It is exceedingly difficult to isolate a specific bacteria from soil that has an enormous diversity of microorganisms using cultural methods. In general, many studies focus on the relevant microorganism when they use cultural methods for the isolation of a particular microorganism. But, the priority of the present study is to determine what kinds of microorganisms can grow on the nitrogen-free media used for the isolation of *Azotobacter*. Moreover, this study shows, albeit partially, the diversity of soil and various bacterial groups occurring in the different pasture soils.

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Conflict of Interest

The author declares no conflict of interest for current research article.

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