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THE APPLICATION OF PCR REACTION FOR IDENTIFICATION
OF MHB BACTERIA SPECIES

ANNA ZĄBKIEWICZ, MAGDALENA MYGA-NOWAK,
KATARZYNA BANDURSKA, JUSTYNA PACZYŃSKA, ANNA SZYBECKA,
PIOTR KRUPA

Częstochowa Jan Długosz University, Institute of Chemistry, Environmental Protection and Biotechnology,
Department of Microbiology and Biotechnology
Al. Armii Krajowej 13/15, 42-200 Częstochowa, Poland

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Abstract: This study characterizes mycorrhiza helper bacteria (MHB) from selected unpolluted locations as well as subjected to industrial emissions. To determine the species of bacteria isolated from the roots of ectomycorrhizal pine and birch, a method based on the sequence analysis of a 16S rRNA gene was used. The isolated bacteria were initially characterized by available biochemical methods and phenotypic observation. On the selected bacteria representatives isolation of DNA was performed, on which the PCR reaction was carried out. In this way amplified samples were automatically sequenced and the obtained results were compared to public databases. Among the isolated bacteria *Pseudomonas fluorescens* SBW25 and *Burkholderia xenovorans* LB400 species were dominant.

INTRODUCTION

Soil is probably the most complex ecosystem in which many relationships between plants and microorganisms exist, the most important ability is to form symbiotic associations with plants in the form of rhizosphere and mycorrhiza [3, 10]. Rhizosphere is a dynamic environment, rich in carbon and energy source that supports the activity of bacteria [15]. A similar situation occurs in the layer of soil directly associated with mycorrhizal roots for which Linderman (1988) [19] suggested the name “mycorrhizosphere”. Organisms inhabiting enriched rhizosphere are plant pathogens, opportunistic commensals, saprophytes, and associated with roots “co-workers” as well as endosymbiotes [19, 1].

Mycorrhiza for a long time was treated as a symbiosis between plant roots and a specific soil fungi. However, reports from recent years indicate that in the wild mycorrhizas are accompanied by numerous populations of bacteria, which together with specific mycorrhizal fungi form consortia that interact simultaneously on mycobiote and fitobiote. Therefore, bacteria participate in the formation and functioning of the

mycorrhizal symbiosis [11]. Studies on bacteria exhibiting a beneficial effect on mycorrhiza led to the concept of mycorrhiza helper bacteria (MHB) [14].

The aim of this study was to isolate bacteria from ectomycorrhizas of Scots Pine (*Pinus sylvestris* L.) and silver birch (*Betula pendula* Roth) growing in environmentally clean as well as degraded by industry areas and to identify the species of bacteria.

This was done by:

- Isolation, multiplication and by obtaining pure cultures of bacteria from the roots of mycorrhizal;
- Isolation of the genomic DNA of the bacteria;
- Amplifying the 16S rRNA gene using the polymerase chain reaction (PCR);
- Automatic sequencing;
- Identification of bacteria species examined based on the analysis of DNA sequences in the National Center for Biotechnology Information (NCBI) database and www.pseudomonas.com.

EXPERIMENTAL PROCEDURE

Isolation of bacteria from mycorrhizal roots

The samples were collected from four research stations. The two stations treated as a control and found to be ecologically clean were the city of Olsztyn, which lies in the buffer zone of landscape park “Stawki” and the town of Piasek, lying near the reservation “Falcons Mountain”. The other two were located in industrial areas (areas of Częstochowa Steelworks and Zinc and Lead works in the town of Miasteczko Śląskie). Samples of mycorrhizal roots of pine and birch were taken from comparable age afforestation growing on fresh mixed coniferous forest habitats.

In accordance to the methodology, which was presented by Krupa (2004) [18], the derived mycorrhizal roots were purified from the remaining soil in the laboratory and shaken in sterile saline. Next, they were surface inoculated in Petri dishes with agar nutrient. The plates were incubated at a temperature of about 25°C. The colonies were grouped according to morphology, growth and color (according to the methodology of Poole et al., 2001) [20]. Single representatives from each group were streaked to obtain pure cultures.

Initial identification of bacteria

Cell morphology was assessed by Gram stain method, which allowed the bacteria of the respondents predict the bacilli Gram-positive and Gram-negative bacteria most often typed as MHB. Particular attention was paid to the genus *Pseudomonas*. The distinction was made between other Gram-negative rods belonging to the family *Enterobacteriaceae* and *Spirillaceae* to exclude the impact of pollutants, including fecal ones. For the phenotypic classification of the bacteria according to the recommendations given by the Burbianka and Pliszka (1977) and includes features such as: cell morphology, mobility, the ability to produce oxidase, type of growth on Hugh-Leifsona medium and production of fluorescent pigments on the King’s B medium [8].

Isolation of genomic DNA from bacteria

Among colonies, stored as library collection on nutrient agar, there were selected representatives of different morphology, growth rate and color of the colony, which

were transferred to tubes with nutrient broth. Genomic DNA was isolated from 24-hour cultures of particular bacteria. PCR reaction was carried out for bacterial 16S rRNA gene in accordance with the procedure described in [25]. In amplification, the Biometra® thermocycler was used whose work program was presented by [25]. Analysis of the PCR product was on a 2% agarose gel containing ethidium bromide at 0.5 mg/ml in a buffer at a voltage 1xTAE 7 V/cm. Purified amplicons were sequenced, and then the results were analyzed.

RESULTS

Morphological diversity of mycorrhiza

The presence of mycorrhiza was confirmed in the roots of the trees growing in industrial as well as in unpolluted areas. On the surfaces of researched roots various morphotypes of mycorrhizas, often within a single root, were found. For research samples were taken from the root tip of ectomycorrhizas having different colors and morphology. There was no characterization in symbioses with fungal partners.

Compared to the control areas (Piasek and Olsztyn), the presence of mycorrhizal roots in industrial areas was lower. They occur most frequently in birch roots in the area of Olsztyn, and the least collected were from birch tree roots growing near the Częstochowa Steelworks. A reflection of this is the number of bacteria isolates obtained from those locations.

Microbiological analysis

Morphological tests allowed to assign all acquired strains of bacteria into two groups: fluorescent and non-fluorescent coccobacilli. Using Gram stain method, 50% isolates of Gram negative were obtained, 25% of which produced fluorescein on King's B medium. All Gram negative isolates were rod-shaped bacteria but different in size. Some rods took more spherical than elongated form.

Molecular analysis

Using software Chromas fluorograms were read. Forward and reverse sequences were made into a single contig. The obtained sequences were compared with sequences publicly available in the NCBI database, by using the BLAST program the memberships of bacteria strains was determined. Detailed analysis was performed with help of *Pseudomonas* Genome Database (www.pseudomonas.com) and *Burkholderia* Genome Database (www.burkholderia.com). It was possible to determine the membership of each species isolates with use of the BLAST software. The results are shown in Table 1.

Out of the isolates from industrial areas subjected to sequencing 30% belong to the genus *Burkholderia* sp. The species were identified as *Burkholderia xenovorans* LB400. The bacteria of this type were in mycorrhizal birch roots growing near the Częstochowa Steelworks and pine tree roots in the tested area adjacent with Zinc and Leadworks in Miasteczko Śląskie. 60% of these isolates showed belonging to the genus *Pseudomonas* sp. and all received high (over 99%) degree of sequence similarity to the *Pseudomonas fluorescens* SBW25 species. Bacteria belonging to this species also showed no specificity for the host plant and were found on all surfaces except the birch roots

Table 1: The results of molecular analysis of selected bacteria strains.

The research station: location, tree species	Recognized bacteria species	The degree of compliance [%]
Częstochowa Steelworks		
Pine	<i>Pseudomonas fluorescens</i> SBW25	98,72
	<i>Pseudomonas fluorescens</i> SBW25	99,15
Birch	<i>Burkholderia xenovorans</i> LB400	97,95
Miasteczko Śląskie Zinc and Lead Steelworks		
Pine	<i>Burkholderia xenovorans</i> LB400	98,29
	<i>Pseudomonas fluorescens</i> SBW25	99,05
Birch	<i>Pseudomonas fluorescens</i> SBW25	99,01
Olsztyn		
Birch	<i>Pseudomonas fluorescens</i> PfO1	98,16
	<i>Bacillus cereus</i> AH521	99,00
	<i>Pseudomonas fluorescens</i> PfO1	97,67
	<i>Pseudomonas putida</i> GB1	98,71
	<i>Pseudomonas fluorescens</i> PfO1	98,71
Piasek		
Pine	<i>Pseudomonas fluorescens</i> SBW25	95,83

growing near the Częstochowa Steelworks. Among the bacteria with known 16S rRNA gene sequence located in ecologically clean areas the representatives of the genus *Pseudomonas* sp were dominant. Bacteria of this type were also found on the mycorrhizal roots of pine and birch. From the roots of birch growing on the tested area of Olsztyn, Gram-positive bacilli have been isolated, by analyzing the nucleotide sequence, and identified as *Bacillus cereus* AH521.

DISCUSSION

Methodology

Methodology used in this research (microbiological, molecular, bioinformatical) allowed for the characterization of bacterial species associated with mycorrhiza. Bacteria isolated from soil were associated with ectomycorrhizal mantles of the studied trees. But we cannot definitively determine whether the bacteria belong to MHB, more relevant in this case seems to be the term “bacteria accompanying mycorrhiza”.

Molecular method that was used for the identification of bacteria is based on the analysis of the 16S rRNA gene. Particles of 16S rRNA are characterized by highly conserved regions, which helps the analysis. They also have regions of high variability, making it possible to define the evolutionary distance of given organisms. To assign bacteria to the species, the differences in their sequences cannot exceed 3% [22].

It should be noted that Taq polymerase used in the experiment has a lack of repairing activity function from 3' → 5', and therefore it is a cause of 1 error on 250 present nucleotides. When the length of 16S rRNA particles is about 1500 nt, it can cause approximately 0.4% difference in nucleotide sequence. Gene 16S rRNA libraries may not represent a complete picture of the bacterial population. Species diversity is very high, therefore libraries containing less than 400 sequences cannot fully characterize the bacteria in a given sample. The disadvantage of the method based on the analysis of the 16S rRNA gene may be that some of the sequences contained in the sample may be derived from a contaminated DNA and a sample will not show the studied bacteria. However, the method based on the comparison of the available sequences of the 16S rRNA gene to gene libraries is very useful and widely used due to its simplicity and allows to pre-determine structure of the bacterial population in the soil [17]. In this paper, a high degree of sequence conformity was achieved in each case, more than 97.6%. It can be concluded that the isolates were correctly identified.

The study did not estimate the quantitative values of bacteria. Determination of the quantity of bacteria by the plate culture technique method may not provide reliable results since in the sample there may be micro – organisms not growing in the laboratory, or which cannot be cultured from time to time, called viable but nonculturable (VBNC). It is estimated that 90–99% soil microorganisms belong to the VBNC [9]. On the one hand, it may mean that cultured MHB represent only a small fraction of all bacteria from the rhizosphere, on the other hand, we can say that the MHB represent the dominant bacterial group capable of being cultivated among groups characterized to date [21].

Characteristics of species composition

Bacteria isolated from ecologically clean areas characterized by higher growth in the culture medium. Colonies of bacteria belonging to the same species, but from different areas differed in size and growth rate. Smaller and slower growing colonies derived from industrial areas.

Comparative analysis of the 16S rRNA gene sequences of bacteria from polluted industrial areas have revealed the presence of two types of bacteria: *Burkholderia* sp. and *Pseudomonas* sp.

Gram negative bacteria, with no endospores, straight rods of the genus *Burkholderia* were found on mycorrhizal birch tree roots growing in the areas known for industrial emissions. The membership of the *Burkholderia* genus to MHB is reported by several authors [20, 1, 2, 16]. In particular, attention deserves Poole et al. (2001), who isolated this type of bacteria from symbiotic connection of *Pinus sylvestris* – *Lactarius rufus*. Bacteria of the genus *Burkholderia* were also found in symbiosis with arbuscular mycorrhizal fungi [20, 6].

The most of the identified bacteria from industrial areas belong to the genus *Pseudomonas* sp. The analysis of the 16S rRNA gene sequence allowed for the affiliation of the species to the *Pseudomonas fluorescens* SBW25 with almost 99% of the compatibility of the sequence.

In the rhizosphere *Pseudomonas* sp. is a dominant genus [15]. Among the species found in the soil most frequently mentioned are *P. fluorescens* [13], *P. monteilii*, *P. resinovans* [12] and *P. aeruginosa* [4]. Frey Klett et al. (1997) [13] suggested

that *Pseudomonas fluorescens* promotes mycorrhizal symbiosis survival by increasing living time of mycorrhizal fungus in the soil during the presymbiotic growth, although the research of Brule et al. (2001) [7] confirmed this theory only in adverse growth conditions. Literature data also confirm that the species of the genus *Pseudomonas* sp. are capable of supporting mycorrhization in different tree species such as *Acacia holosericea* [12] and *Pinus sylvestris* [23] in the presence of different fungal symbiotic partners, both ectomycorrhizal (eg. *Pisolithus alba* [23]) and endomycorrhizal – *Glomus intraradices* [11].

Also in this study the species *Pseudomonas fluorescens* was present at the same time on the mycorrhizal roots of pine and birch. Its presence was demonstrated in all research areas. Furthermore, it accounted for about 67% of all species in industrial areas identified by sequencing.

The dominance of *Pseudomonas* sp. in mycorrhizal symbiosis is confirmed by the research of Wrótniak and Dahm (2001) [24], who isolated this type of bacteria from fruiting bodies of ectomycorrhizal fungi in excess of 80% of all isolates. Also Izumi et al. (2007) [16] using molecular methods confirmed that 77% of the obtained clones were closely related to *Pseudomonas* sp. and *Burkholderia* spp.

Comparing the presence of certain bacteria in the areas of research and control, the dominance of bacteria of the genus *Pseudomonas* can be seen, regardless of the location. The species belonging to this type (SBW25 and PfO1) are considered to be closely related [25]. Other species were found only in industrial (*Burkholderia*) or ecologically clean (*Bacillus*) areas, but one cannot rule out their deployment in different environments, as the selection of isolates subjected to sequencing was based on the basic phenotypic and biochemical tests. Moreover, at the stage of obtaining pure cultures many of them proved to be unable to re-grow in culture conditions.

The affiliation of bacteria of the genus *Bacillus* with MHB was confirmed by Bending et al. (2002) [23]. The authors have found this type of bacteria in symbiosis with *Suillus luteus* – *Pinus sylvestris* and proved that they have the ability to more than double the degree of mycorrhization by stimulating the growth of the roots of the first order.

CONCLUSION

The described methodology allowed for the isolation of bacteria from the mycorrhizal roots of pine and birch trees growing in ecologically unpolluted areas (control) and areas subjected to industrial emissions. In these areas, the presence of bacteria in mycorrhiza was confirmed. Preliminary microbiological studies revealed that the predominant form of bacteria present on the mycorrhizal roots are Gram-negative rods, often producing a pigment exhibiting fluorescence under UV light. Molecular methods based on comparative analysis of the 16S rRNA gene sequence with sequences available in online databases have proven to be simple and useful methods of determining species affiliation of studied bacteria. Within the selected isolates *Pseudomonas fluorescens* SBW25 was an outnumbered strain. Other bacteria belonged to the species *Burkholderia xenovorans* LB400. The studies provide a basis for further characterization of mycorrhizosphere and occurring interactions.

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ZASTOSOWANIE REAKCJI PCR DO IDENTYFIKACJI GATUNKOWEJ BAKTERII MHB

W pracy scharakteryzowano bakterie wspomagające mikoryzę pochodzące z wybranych terenów ekologicznie czystych oraz z terenów poddanych emisji przemysłowej.

Przedstawiono wykorzystanie metody opartej na analizie sekwencji genu 16S rRNA do określenia przynależności gatunkowej bakterii wyizolowanych z korzeni ektomikoryzowych sosny i brzozy. Wyizolowane bakterie zostały wstępnie scharakteryzowane przy pomocy dostępnych metod biochemicznych i obserwacji fenotypowej. Dla wybranych przedstawicieli dokonano izolacji DNA, względem którego przeprowadzono reakcję PCR. Powielone w ten sposób próbki automatycznie zsekwencjonowano, a uzyskane sekwencje porównywano w ogólnodostępnych bazach danych. Wśród wyizolowanych bakterii dominowały gatunki *Pseudomonas fluorescens* SBW25 oraz *Burkholderia xenovorans* LB400.