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MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF *PSEUDOMONAS PUTIDA* ISOLATED FROM BOTTLED UNCARBONATED MINERAL DRINKING WATER

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Abstract - *Pseudomonas putida* belongs to a group of opportunistic pathogens that can cause disease in people with weakened or damaged immune systems. Some strains have medical significance, and for most ingestion is not the primary route of infection. If water used by predisposed subjects is contaminated by *P. putida*, they may become ill. The aim of this work was the biochemical and molecular characterization of strain ST3 of *P. putida* isolated from non-carbonated bottled drinking water from Jakov Do 4 on Mt. Vlasina. Characterization of *P. putida* was performed to assess the risk to human health of the indigenous strains present in the water. Biochemical characterization of strains was performed using the manual identification system ID 32 GN (BioMérieux). Identification was obtained using the database identification software ATB System (Bio-Mérieux). Molecular characterization was performed by PCR amplification and 16S rDNA "thermal cycling sequencing". Biochemical identification of the strain ST3 was accurate (Id = 99.8%). Comparing the sequences obtained for strain ST3 with NCBI gene bank sequences for 16S rRNA, the highest similarity of our strain (96% identity) with a strain of *P. putida*, designated as *biotype A* (gi|18076625|emb|AJ308311.1|.PPU308311) isolated in New Zealand, was obtained. While comparison with the NCBI collection of all deposited sequences showed that the 16S rRNA gene sequence of strain ST3 has very high homology, it is not identical, indicating indirectly that strain ST3 is an indigenous strain.

Key words: Pseudomonas putida, 16S rRNA gene, drinking water

INTRODUCTION

The genus *Pseudomonas* is the most heterogeneous and ecologically significant group of known bacteria. Some species of *Pseudomonas* are opportunistic pathogens that primarily cause nosocomial infections (Brady et al., 1998). *Pseudomonas fluorescens, Pseudomonas putida*, and *Pseudomonas fragi* are the most frequently found species; however, distribution of the species in water and the food ecosystem remains relatively unknown (González et al., 1987; Arnaut-Roller et al., 1999).

Pseudomonas putida is a nonfermenting Gram-negative rod belonging to rRNA group I of the genus *Pseudomonas*. Certain strains of *P. putida* have medical significance, and for most of them ingestion is not the primary route of infection. Infections caused by *P. putida* are rare and are mostly reported in immunocompromised patients (Manfredi et al., 2000), such as newborns (Ladhani et al., 1998), neutropenic and cancer patients (Anaissie et al., 1987; Martino et al., 1996). However, a few cases of *P. putida* bacteremia in adult patients have been reported (Yoshino et al., 2011). Cases of infection by this bacterium have been

recorded even in U.S. Marines who fought in Iraq and Afghanistan (Carpenter et al., 2008).

Because of their ability to metabolize a wide range of biogenic and xenobiotic compounds, members of this species are able to colonize several niches, including soil, freshwater and the surfaces of living organisms (Ramos-Díaz et al., 1998).

In spite of the common occurrence of pseudomonads and the numerous papers about members of this group, their identification and classification is not easy. Due to lack of modern standardization methods, many studies of the ecologically specialized (commonly occurring, saprophytic, usually fluorescent) pseudomonads appear to have been hampered by the absence of detailed characterization (John-Brooks et al., 1925; Clara et al., 1934). For example, the description of the genus *Pseudomonas* shown in the latest edition of Bergey's Manual of Determinative Bacteriology is significantly revised from previous editions, so that many species that inhabit soil and water remain poorly characterized (Rhodes et al., 1959).

P. putida strain ST3 was isolated from a source that is used for bottling water. Water sources that are exploited for bottling are located on the mountain Vlasina, southeast Serbia, at an altitude of about 1500 m at 42^0 38' 14" north latitude and 22^0 16' 35" east longitude.

Having in mind the capacity of the spring, the consumption of drinking water and the fact that the predisposition *P. putida* can cause various infections, we performed a biochemical and molecular characterization of the strain ST3.

MATERIALS AND METHODS

Isolation of pure cultures

Isolation of the strain *Pseudomonas putida* ST3 was performed by the membrane filtration technique (MF); 100 ml of bottled drinking water was filtered through a membrane filter disk of 0.2 μm (manufacturer PALL-Gelman). After filtration, the filter disk was incubated on Cetrimide Agar (Merck S.r.l.) for 48 h at 35^0C. Most strains of *P. putida* are capable of growing on selective medium Cetrimide agar (Lambe et al., 1972). Pure cultures were obtained by multiple subsequent dilutions using inoculation loop streaking on Nutrient agar to obtain single colonies.

Biochemical characterization of isolates

The preliminary identification of isolated strains was done using the identification system ID 32 GN (Croize et al., 1987). The ID 32 GN system is an automatic identification system for Gram-negative rods using standardized and miniaturized assimilation tests with a specially adapted database. The reading of results is automatic by the ATB Expression instrument; the reader records the color of each tube and transmits the data to the computer. The computer transforms this information into an 11-digit numerical profile and indicates the identification of the strain.

DNA extraction

For the isolation of bacterial DNA, overnight cultures were used. After centrifugation, the supernatant was discarded and the bacterial pellet was resuspended in 400 μl of Solution E1 (50 mM Tris i 10 mM EDTA) adjusted to pH 8 (Better et al., 1983). Nucleic acids were released from the cells with 250 μl of 2% aqueous solution of N-lauryl sarcosine. Then 200 μl of pronase solution (final concentration 5 mg/ml) was added, mixed well and incubated for 30 min at 37^0C, followed by the addition of 150 μl neutral phenol, mixing by vigorous vortexing and centrifugation at maximal speed. This step was repeated until a clean supernatant was obtained. Precipitation of DNA was done by adding 1/10 of sodium acetate 3M and 0.6 of isopropanol in the aqueous phase. After centrifugation, the pellet of DNA was washed with 500 μl of 75% ethanol and dried in vacuum evaporators. The dried pellet was resuspended in 50 μl of water containing RN*ase* A and incubated for 15 min at 37^0C. Isolated DNA was stored at -20^0C prior to use.

The quantity and quality of isolated DNA was checked by agarose gel electrophoresis (Agarose Low EEO) in a 1x TAE buffer at a constant voltage of 5 V/cm. The standard DNA Ladder Mix (Fermentas) was used to compare the size and intensity of the DNA fragments. Visualization of DNA was performed by adding ethidium bromide (0.5 μg /ml) to the gel and exposing in a UV illuminator.

Amplification of 16S rDNA by PCR

For the amplification of 16S rDNA, Hot Master Taq polymerase was used which is most effective at 68^0C. The 16S rDNA gene fragments were amplified by PCR using the primers BAC16S1 (5'-GTT TGA TCC TGG CTC AG-3') and BAC16S2 (5'-GAC GGG CGG TGT GTA CAA-3').

The amplification of 16S rDNA was done by PCR touchdown. The following thermocycling program was used: 3 min initial denaturation at 95^0C; amplification in two phases: first, a PCR program of 15 subsequent cycles of 40 s denaturation at 95^0C, 1 min annealing at 60^0C; 2.5 min extension at 68^0C; second PCR program of 10 cycles of 40 s denaturation at 95^0C, 1 min annealing at 45^0C; 2.5 min extension at 68^0C; the final extension step was 10 min at 68^0C.

DNA sequencing

The sequencing of amplified DNA was performed by the thermal cycling sequencing method. The termination method involves a random incorporation of 2',3'-dideoxinucleotide triphosphate (ddNTP).

Prior to sequencing, amplified DNA was first purified using the QIAquick PCR Purification Kit (QIAGEN-GmbH). After that, the primer BAC16S was added to the purified DNA and the DNA mix was dried. The sequencing of the samples was done by the CRIBI Sequencing Service at the University of Padua, Italy.

Database searches for similar sequences were performed using the BLAST program at the National Center for Biotechnology Information (Altschul et al., 1997).

Nucleotide sequence accession number

The nucleotide sequences for 16S rDNA of *Pseudomonas putida* ST3 was submitted to the EMBL GenBank under accession number HE585248.

RESULTS

Physical and chemical characteristics water

The spring water from which *Pseudomonas putida* ST3 was isolated is characterized by a uniform flow (0.3 l/s), low mineralization (< 50 mg/l), pH=6.4 and low temperature (5.8^0C) (Tasić et al., 2012). According to the chemical characteristics, the water belongs to the class of hydrocarbonic waters with complexed cationic composition. Tests for total α and β activities and gamaspectroscometric analysis showed that the radioactivity of water was within permissible limits (Tasić, 2008).

General characteristics of isolate Pseudomonas putida ST3

Using exhaustion streaking techniques on the nutrient agar, a pure culture of strain ST3 was obtained that expressed a specific smell. Strain ST3 showed good growth on nutrient agar at temperatures between 22^0C and 37^0C. No growth was observed at 41^0C. The strain produced fluorescent pigments. Motility of bacterial cells was detected on M medium (BioMérieux). Gram staining showed that strain ST3 has Gram-negative rods, with an average size of 0.5 x 3 μm without capsules. Inclusions of poly-β-hydroxybutyrate (PHB) were not detected. The cells were motile, with oxidative metabolism and positive catalase and oxidase reactions

Biochemical analysis

The strain ST3 had the biochemical profile number 40172073033 (ID 32 GN system, BioMérieux , France). Using the database identification software

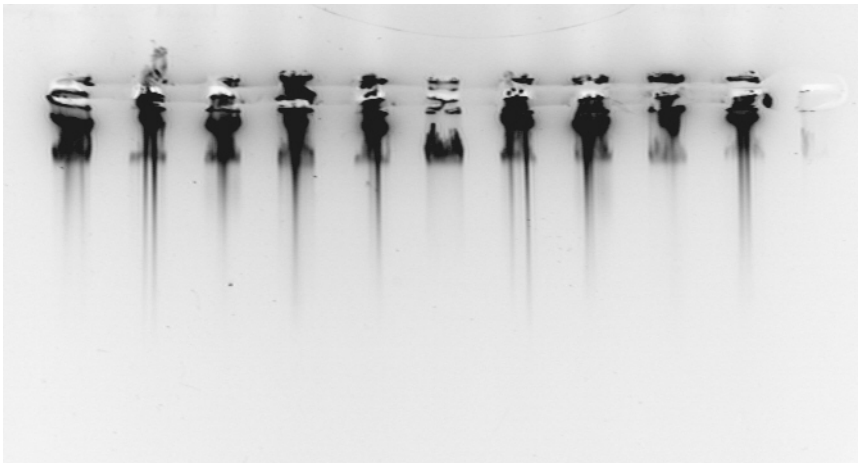

Fig. 1. Agarose gel electrophoresis of chromosomal DNA of strain ST3

ATB System (Bio-Mérieux), the strain ST3 was classified as *Pseudomonas putida.* The percentage of identification (Id%) was 99.8 (Tasić et al., 2008).

Positive biochemical tests of the isolated strain ST3 were obtained for D-ribose, itaconate, acetate, lactate, alanine, D-glucose, L-arabinose, propionate, caprate, valerate, citrate, 2-ketogluconate, 3-hydroxybutirate and L-serine.

Negative tests were obtained for rhamnose, N-acetyl-glucosamine, inositol, D-sucrose, maltose, suberate, malonate, mannitol, salicin, D-melibiose, L-fucose, D-sorbitol, histidine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate and 4-hydroxy-benzoate.

Out of 32 tests, typical biochemical reactions were obtained in 29 cases (14 positive and 15 negative). Atypical reactions were for itaconate (+, 10%), histidine (-, 96%) and 4-hydroxy-benzoate (-, 96%). Results of conventional biochemical tests were the same or equivalent to those obtained by the ID 32 GN system.

Molecular characterization of Pseudomonas putida ST3

Using universal primers for bacterial 16S rDNA and chromosomal DNA of strain ST2 in PCR reaction, a DNA molecule with an expected size of about 1 300 bp was obtained. The quality of chromosomal DNA was analyzed on an agarose gel; it was found that the isolated DNA was of satisfactory quality, without degradation (Fig. 1).

Electrophoresis of amplified DNA on 1% agarose gel showed that the PCR reaction was highly specific (without nonspecific products) (Fig. 2).

Using one sequencing reaction, a sequence of 1 147 nucleotides was obtained.

DISCUSSION

Because of the unclear results obtained by the biochemical determination of isolate ST3, we decided to do perform a molecular characterization. The method used here was the sequencing of the gene for 16S rRNA. The sequence of 16S rDNA of isolate ST3 from oligomineral bottled drinking water of 1 147 nucleotides was compared with the sequences in the NCBI gene bank data for 16S rRNA, and the highest similarity (96% identity) of strain ST3 with the strain *Pseudomonas putida*, designated as *biotype A* (gi|18076625|emb|AJ308311.1|.PPU308311), was obtained. From the results of DNA alignments of the 16S rDNA sequence of strain ST3 and the genes for 16S rDNA deposited in all DNA databases, it was possible to conclude that the isolate from bottled oligomineral drinking water is *Pseudomonas putida* (Tasić et al., 2009).

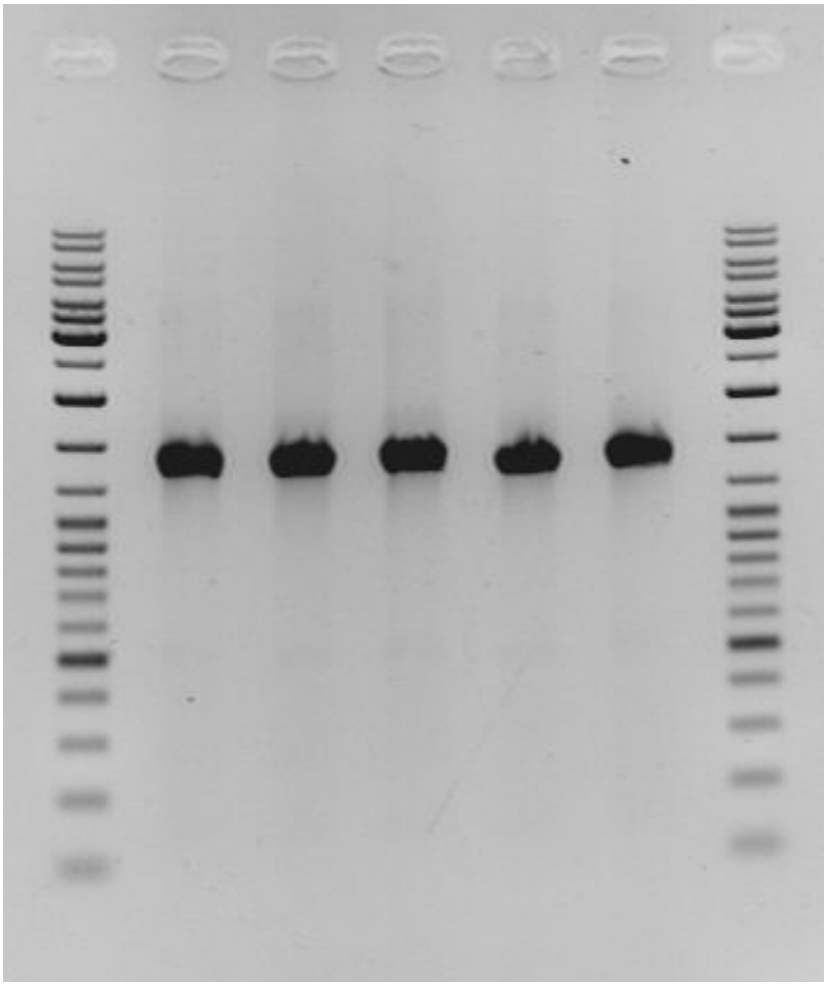

Fig. 2. Agarose gel electrophoresis of amplified DNA of 16S rDNA of the *Pseudomonas putida* ST3 on 1% agarose gel. (first and last line contains marker, 2-6 *Pseudomonas putida*; DNA marker contains fragments of following sizes (from botton to the top): 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1031 bp, 1200 bp, 1500 bp, 2000 bp, 2500 bp, 3000 bp, 3500 bp, 4000 bp, 5000 bp, 6000 bp, 8000 bp and 10000 bp.

The spring water, from which the ST3 strain of *P. putida* was isolated, is characterized by very low mineralization to which this strain adapted.

Having in mind that the identity of 16S rRNA genes between these two strains was not 100%, it can be assumed that strain ST3 is an autochthonous isolate. *Pseudomonas putida* strain ST3 represents 1.61% of total strains of Gram-negative aerobic bacilli isolated from sources on Mt. Vlasina (Tasić, 2008). *Pseudomonas putida* belongs to a heterogeneous group of Gram-negative opportunistic bacteria whose presence in drinking water, in a legal sense, is not allowed. During bacteriological examination of drinking water, *Pseudomonas putida* can be easily mistaken for a nonpathogenic species of so-called aerobic mesophilic bacteria. Biochemical characterization has, in addition to its scientific importance, is with considerable practical importance for a proper risk assessment in human health.

The classification of the *Pseudomonas* genus by specific physiological and biochemical characteristics is useful, but is not enough to distinguish all

Pseudomonas species. Our results confirm that the correct identification and characterization of the *Pseudomonas* species of group 1 can only be achieved by combining culture, biochemical and molecular tests. In this study, differences between the results of biochemical and molecular tests were observed.

In the case described herein, the molecular method of identification was very successful. Which of the applied methods for identification will yield more successful results of identification depends on the isolated bacteria. We propose the use of both biochemical and molecular genetics methods.

Due to the presence of *P. putida* in the water at the Jakov Do 4 source, the source has been removed from the system of exploitation.

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