ANALYSIS OF PHYLOGENETIC VARIATION THE DOMINANT MICROORGANISMS SPECIES OF *Pseudomonas fluorescens* REVEALED FROM MUSHROOMS

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Abstract. The aim of research was to determine the taxonomic position of dominant microorganisms in the fruit body of mushroom of the representatives of the genera *Pseudomonas* based on phylogenetic analysis of the nucleotide sequence of the 16S rRNA gene. Identification the bacteria they used the analysis of the nucleotide sequence of 16S rRNA gene. Bacterial DNA was extracted from the suspension of bacterial cells using GeneJet Genomic DNA Purification Kit (Thermo Scientific), amplification of the 16S rRNA gene was performed with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R (5'-GGTTACCTTGTTACGACTT-3'). The taxonomic position is determined by dominant microorganisms in the mushroom based on phylogenetic analysis of the nucleotide sequence of the 16S rRNA gene. The nucleotide sequence of a fragment of the gene 16S rRNA of the mentioned above strains was registered in the international database GenBank (NCBI) with number TI-2019 (3). Obtained amplicon in size of ~1500 BP. The purified PCK-product was sequenced in two directions using a set of reagents «BigDye Terminator v 3.1 Cycle Sequencing Kit». Analysis of the isolated strains of *Pseudomonas fluorescens* or the similarity of the nucleotide sequences of the 16S rRNA gene revealed 99% similarity with sequences of typical representatives of the species concerned. Promising strains of *Pseudomonas fluorescens* can be successfully introduced in the metagenome of aboriginal groups of the substations as biological agents of microbial preparations. They can provide metabolic functions of biological systems of the *Agaricus bisporus*, and be practically valuable agents of bioprotector action, induction of systemic resistance of plants against bacterial pathogens.

Key words: sequencing; 16S rRNA; *Pseudomonas fluorescens*; phylogenetic identification.

INTRODUCTION

The genus *Pseudomonas* is a data and complex heterogeneous group of organisms belonging to the family *Pseudomonanaceae*. They contain approximately 211 described species and more than 56 of which are reclassified to another genus. These genera have permanently undergone continuous taxonomic regrouping by improvements in methodologies identification of species. Organisms previously classified within the genus *Pseudomonas* (rRNA homology groups I-V) are now divided among the genera *Pseudomonas, Burkholderia, Ralstonia, Comamonas, Delftia, Hyrodenophaga, Brevundimonas, Stenotrophomonas, and Xanthomonas* [2, 19]. Many identified stamps have no designated species. Commercial identification test systems do not provide definitive speciation of many of the clinically significant. In agriculture and mushroom farm situations where precise identification is important for determining optimal measures, prognosis, and appropriate infection control intervention [4].

As a result, increasing environmental problems are leading to a decrease in the resistance of mushroom grown in industrial conditions to abiotic and biotic factors. Therefore, recently the development of the mushroom industry has focused on studying the ways of transmission of fungal, bacterial, and viral diseases and creating an methodology which capable of responding quickly to stress and further regulation [5].

In particular, scientists emphasize that one of the reasons for the rapid spread of diseases is the low-quality processing of mushroom growing chambers, which are often infected with pathogens [14, 18, 25].

It should be noted that the diagnosis of viral rather than bacterial infections by molecular biological methods is more common in Ukraine. The development of highly accurate and effective test systems for the identification of mixed bacterial infections of champignons will make it possible to provide express diagnostics and successful cultivation of mushrooms [12, 21-24].

MATERIAL AND METHODS

Identification of *Pseudomonas* isolates by the PCR method described by H. I. Lee is carried out using a set of primers (Pt-1A, Pt-1D1). This is a specific aspect of the detection of these bacteria. The reference strain *Pseudomonas tolaasii* CFBP 2068T is used as a positive control. The resulting sequences were assembled using Pregap4 from the Staden software package [9, 20, 23, 24].

In 2000, Korean scientists developed a method of multiplex PCR analysis for the detection of *Pseudomonas tolaasii* and *Pseudomonas agarici* using PTOF/PTOR and PAGF/R23-1R primer sets [4, 30].

It is worth noting that the named method is successfully used in world practice. Iranian scientists identified strains of *P. tolaasii* and *P. reactans* in different centers of mushroom cultivation using the above-mentioned method. Similar tests were also conducted in Serbia. Pathogenic samples of *P. tolaasii* (forty six samples) were isolated by REP, ERIC-PCR analysis, and Southern blotting. Scientists also determined their genotypic diversity. This helped to identify among the studied isolates those that cause the brown color of spotted mushroom disease and differ both phenotypically and genotypically [1] .

We have selected to analyze the fruiting bodies with disease symptoms. The fruiting bodies that didn`t have symptoms according to a visual assessment and electron microscopic analysis [5, 8-11, 17] were as control.

Isolation nucleic acids

These methodologies were carried out the detection of total and double-stranded RNAs in *Basidiomycetes* [6, 27].

Molecular biological method of extraction total DNA, cDNA, reverse transcription of PCR, amplification, sequencing of viral cDNA, and dsRNA were performed according to the method described previously [2]. Biotechnological (obtaining and subcultivation of samples of mycelium *in vitro* using electrophoresis in agarose and polyacrylamide gels (PAGE) [27], determining the hydrogen index (pH) of the nutrient medium mycological (a measurement of growth).

Bacterial DNA was isolated from a suspension of bacterial cells using the GeneJet Genomic DNA Purification Kit (ThermoScientific), according to the manufacturer's protocol. Amplification of the 16S rRNA gene was performed with primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-CGGTTACCTTGTTACGACTT-3') at the following temperature: 95°C, 2 min.; 30 cycles – 95°C, 30 sec.; 55°C, 45 sec.; 72°C, 90 sec.; final elongation 72°C, 7 min. The 25 µL PCR mixture contained 12.5 µL of 2x DreamTaq PCR Master Mix (ThermoScientific), 30 µmol of each primer and 50 ng of DNA. PCR was performed on an amplifier Mastercycler Personal 5332 (Eppendorf, Germany). PCR products were separated in a 1.7% agarose gel containing 0.01% ethidium bromide. The results were visualized in UV light. The resulting amplicon measuring ~ 1500 bp cut from the gel and purified using the GeneJet PCR Purification Kit (ThermoScientific). The DNA concentration was determined on a DS-11FX + spectrophotometer (DeNovix, USA). The purified PCR product was sequenced in two directions on a Genetic Analyzer 3130 (Applied Biosystems, USA) using the BigDye Terminator v 3.1 Cycle Sequencing Kit.

The resulting nucleotide sequence was compared with GenBank database data using the NCBI Blast program (http://www.ncbi.nlm.nih.gov/blast). Phylogenetic analysis, alignment of nucleotide sequences of 16S rDNA of representatives of different species of the genera *Bacillus* and *Phyllobacterium* was performed using the program MEGA 10 [3, 6, 10]. The dendrogram of phylogenetic relationships was constructed using the Neighbor Joining method using a two-parameter Kimura model based on 1000 replicates of bootstrap analysis. The 16S rRNA gene sequences of the reference cultures of bacteria of the genus *Pseudomonas* were used from the GenBank database [12, 13, 27, 28].

Statistical analysis of data

Statistical analysis of data has been carried out with the help of statistical and other computer software.

RESULTS

We performed a bioinformative analysis to create primers specific to the nucleotide components of *Pseudomonas* on the mushroom. The first stage was the screening of conservative diseases of the genes that encode the disease protein of the corresponding pathogen. We screened it using genetic data (GenBank) [20]. Based on general data of known nucleotide components of microbial genomes, strictly specific conservative nucleotide components were identified. They can be used as matrices for oligonucleotide primers in the process of synthesis of specific fragments of nucleic acids.

Our monitoring of bacterial diseases on mushroom farms and fresh produce markets in Ukraine revealed the presence of brown spot symptoms [16, 26]. We noted different degrees of the brown coloring of *A. bisporus* fruiting bodies (Figures 1, 2).

Figure 1. The presence of brown spots on the fruiting bodies of the *A. bisporus* mushroom

Figure 2. Brown mushroom fruit bodies of *A. bisporus*

In so far as the research after extraction, more than one hundred bacterial isolates were obtained from symptomatic samples, thirty-seven fluorescent, Gram-negative isolates from different sources and locations were selected for pathogenicity tests. Bacterial isolates showed different degrees of color change and tissue

degradation on the fruiting bodies of mushrooms, varying from light to dark brown (Fig. 3) [17, 18].

Thirty-six bacterial isolates caused sunken brown lesions on tissue blocks of *A. bisporus* carp after 72 h. This indicates that they are caused by the reference strain *Pseudomonas fluorescens*. The rest of the isolates showed a light brown surface color on both tissue blocks and sporophores, like the reference strain *Pseudomonas agarici*.

In figure 1 deep brown lesions on the *A. bisporus* peduncles induced by *Pseudomonas fluorescens* can be seen.

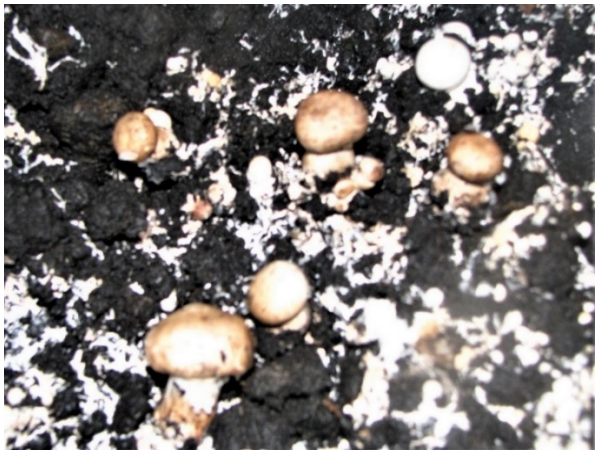

Figure 3. Changes in color and degradation of tissues on mushrooms of *A. bisporus*

DISCUSSION

According to the studied biochemical properties, all studied bacterial isolates be divided into two groups. Those exhibiting LOPAT characteristics (series of defining tests: L, levan production; O, oxidase production; P, pectinolytic activity; A, arginine dihydrolase production; and T, tobacco hypersensitivity). They also had the following biochemical properties: catalase-positive, esculin hydrolysis negative, Tween 80 hydrolysis positive, casein hydrolysis positive; negative hydrolysis of gelatin and reduction of nitrates. They used mannitol, erythritol, sorbitol, inositol, and trialose as carbon sources, but did not use sucrose, arabinose, or D (−) tartrate. The obtained results of the analysis demonstrate the characteristics of *Pseudomonas fluorescens*. Identification was confirmed in PCR tests.

The identity of these seven species was confirmed by analysis of 16S rDNA components as *Pseudomonas agarici* (deposited in NCBI GenBank under accession numbers: TI-2019(3).

The results of this experiment showed that *Pseudomonas fluorescens* was a predominant bacterium associated with the symptoms of brown color change on *A. bisporus*. However, this is not unexpected, given that *Pseudomonas fluorescens* is recognized as the most common pathogen that causes significant fungus losses in the cultivation process. Despite that recent studies have shown that the brown spots of *A. bisporus* caused by *Pseudomonas fluorescens* is a complex disease. Because some other species of pseudomadades also have the ability to cause stain symptoms with different levels of color change (Fig. 4) [29].

Moreover, the pathogenicity tests in our study also showed a difference in the change in the color of the inoculated fungus of the mushroom caps, indicating that some other fluorescent pseudomonads participated in the expression of symptoms of the disease.

Further monitoring of fungal products in the sale of markets did not show repeated release of this bacterium. As for the economic impact of *Pseudomonas fluorescens* as a predominant bacterial pathogen in the mushroom farms of Ukraine, this depends mostly on the phytosanitary measures taken in the cultivation chambers. Although small manufacturers are still prevalent, they are ready to introduce and use strict sanitary measures and new products in mushroom cultivation techniques.

The next step was to carry out and apply our data from the strains received to the Genbank fig. 5.

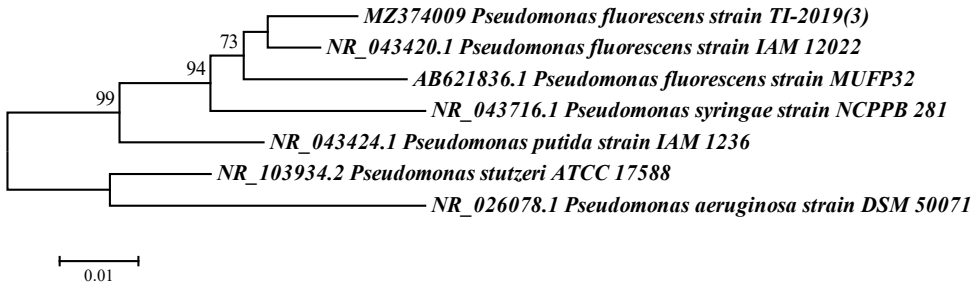

Figure 4. Results of phylogenetic analysis of PHLF regulatory protein bacteria TI-2019 (3) in the form of dendrogram of the studied isolates

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1   gcgctggcgg caggtccaac acatgcaagt cgagcggtag cagagaagct tgcttctctt
61  gacagcggcg gacgggtgag taatgcctag gaatctgcct ggtagtgggg gataacgttc
121 ggaaacggac gctaataccg catacgtcct acgggagaaa gcaggggacc ttcgggcctc
181 gcgctatcag atgagcctag gtcggattag ctagttggtg gggtaatggc tcaccaaggc
241 gacgatccgt aactggtctg agaggatgat cagtcacact ggaactgaga cacggtccag
301 actcctacgg gaggcagcag tggggaatat tggacaatgg gcgcaagcct gatccagcca
361 tgccgcgtgt gtgaagaagg tcttcgggtt gtaaagcact ttaagttggg aggaagggca
421 ttaacctaat acgttagtgt tttgacgtta ccgacagaat aagcaccggc taactctgtg
481 ccagcagccg cggtaataca gagggtgcaa gcgttaatcg gaattactgg gcgtaaagcg
541 cgcgtaggtg gtttgttaag
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Figure 5. Fragments of nucleotide sequences of *Pseudomonas fluorescens* TI-2019 (3) gene 16S ribosomal RNA (560 NP Linear DNA)

Nucleotide sequences from the genetic bank were chosen by accident, but so as to cover as a possible area of distribution of pathogens of mushrooms in each case. The selection of primers is designed in such a way that the annealing temperature of all oligonucleotides was within +50-55 °C [10].

The taxonomic position is determined by dominant microorganisms in the fruitiest bodies of mushroom based on phylogenetic analysis of the nucleotide sequence of the 16S rRNA gene. The nucleotide sequence of a fragment of the gene 16S rRNA of the mentioned above strains was registered in the international database GenBank (NCBI) with numbers: TI-2019 (3). Obtained amplicon in size of ~1500 BP was cut out from the gel and purified using GeneJet PCR Purification Kit (Thermo Scientific). The DNAconcentration was determined on a spectrophotometer DS11FX+ (DeNovix, USA). The purified PCK-product was sequenced in two directions on the device 3130 «Genetic Analyzer» (Applied Biosystems, USA) using a set of reagents «BigDye Terminator v 3.1 Cycle Sequencing Kit».

From an ecological point of view, the distribution of microbial species in agroecosystems is of particular interest, primarily because it is microorganisms that carry out the primary transformation of organic substances, being as a result an important element in the food chain of flora and fauna in closed agricultural systems (growing cameras). Therefore, we carried out the corresponding analysis.

Thus, to understand the biochemical processes of revealing the mechanisms of interaction in the substrate–microorganisms–mushroom system, the main task of microbiology is an objective, comprehensive assessment of the qualitative composition and functional orientation of microbial coenoses formed under the influence of various agricultural measures.

Of particular Zaki Saati-Santamaría, and Ezequiel Peral-Aranega indicated [33] that *Pseudomonas* represents a very important bacterial genus that inhabits many environments and plays either prejudicial or beneficial roles for higher hosts. However, there are many *Pseudomonas* species which are too divergent to the rest of the genus. This may interfere in the correct development of biological and ecological studies in which *Pseudomonas* are involved. Thus, is very important to study the correct taxonomic placement of *Pseudomonas* species. Based on the study of their genomes and some evolutionary-based methodologies, we can to propose the description of new genera and many reclassifications of species previously included in *Pseudomonas*.

According point of Wargo and LaBauve [31], summing up that analysis of the isolated strains of *Pseudomonas fluorescens* for the similarity of the nucleotide sequences of the 16S rRNA gene revealed 99% similarity with sequences of typical representatives of the species concerned. Promising strains of *Pseudomonas fluorescens* can be successfully introduced in the metagenome of aboriginal groups of the substations as biological agents of microbial preparations. It is important to continue research in this direction.

Thanks to the powerful development of molecular biological methods of analysis [32], which allow revealing the real taxonomic diversity of the components of the soil microbial biome, regardless of the possibility of their cultivation on nutrient media, the microbiology of the soil and mushroom substrate has gained access to a comprehensive study of the microbiota in its entirety.

As a result of the application for the evaluation of the genetic resources of microorganisms modern methods of isolation of total DNA, screening of libraries of cloned genes, genetic fingerprinting, whole-genome sequencing, it is possible to obtain unique data on the composition, structure and, accordingly, functions of soil microbial communities.

Looking through the last scientific results of our research field some scientists discovered that a symbiotical relationship exists between biological objects and microorganisms wherein the microbes help the plants in nitrogen acquisition, water uptake, and survival during stress [14]. According to estimates, rhizobia contribute to 50% of the biological nitrogen fixation on 11 earth [35]. Various functions performed by beneficial soil microorganisms include accumulation and cycling of organic compounds, stimulation of nutrient mineralization, and production of plant growth hormones. Species, such as *Pseudomonas fluorescens, P. putida, P. aeruginosa, Bacillus subtilis*, and other *Bacillus spp.,* are widely used for the commercial production of PGPR. Various fermentation technologies have been used to formulate potential PGPR isolates using organic and inorganic carriers. Thus we can see importance and useful properties of species *Pseudomonas*.

The creation of modern pyrosequencing platforms and rapid development of bioinformatics determined the formation of a new direction in the study of microbial systems - metagenomics.

At the same time, all molecular approaches available for structure evaluation and functional diversity of microbial communities have their own taxonomic and methodological advantages and limitations. Since there is still no clear idea about the species the diversity of microorganisms, so it is impossible to choose a method for its determination, which was would be the best and provide full access to genetic and functional diversity of complex soil microbial communities. For a comprehensive assessment should use a combination of several methods.

According to our estimates extracting stamps selected provide metabolic functions of biological systems of the *Agaricus bisporus*, and be practically valuable agents of bioprotector action, induction of systemic resistance of plants against bacterial pathogens.

The experiment was the detection and identification of *Pseudomonas fluorescens* in eleven places in seven mushroom farms in Ukraine. Most markets have been infected with *P. fluorescens*, which indicates that this pathogen was the main cause of bacterial diseases in the mushroom farms of Ukraine over the last 10 years [8, 11, 18, 19, 30].

The creation of a database remains relevant and important for Ukraine metagenomic diversity and taxonomic composition of prokaryotes of spent mushroom substrate according and finding indicator species in development of bioindication regarding the impact of agrotechnical measures.

Accordingly, the «polyphasic» methodology is centered on morphological and biochemical data complemented with molecular techniques data. The combination of the classical approach together with 16S rRNA genes, molecular fingerprinting techniques, and/or other molecular markers is considered an extremely important foundation for the identification and classification of microbes. The microbiota thriving in the rhizosphere, the thin layer of soil surrounding plant roots, plays a critical role in plants adaptation to the environment [36]. In our case mushroom it is generally accepted that bacteria through various mechanisms can acquire genetic information from the surrounding environment. Moreover, recombination frequencies and mutation rates tend to increase under stressful conditions. Rates of evolutionary change may therefore be enhanced in adverse environments. So, from this work we can understend how important is a community of *Pseudomonas* bacteria, especially dominant strains, who have worked out the stress resistance mechanism of a specific plant in specific environmental conditions for many years.

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