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DETECTION OF GENES FROM SOIL BACTERIA WITH ROLE IN THE ORGANIC NITROGEN AND PHOSPHORUS MOBILIZATION

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ABSTRACT. The main aim of our study is the development of bacterial biopreparates based on organic nitrogen and phosphorus mobilizing microorganisms. As an important step of bacterial strain selection, in this study, we performed the detection of functional genes (*apr, npr*) that play role in the organic nitrogen mobilization. The amplified *apr* genes were controlled using DNA sequence analysis.

Keywords: *soil bacteria, nitrogen mobilization, apr, npr gene detection*

INTRODUCTION

In the living systems most of the organic nitrogen is built-in proteins and nucleic acids. A large proportion (40%) of the soil nitrogen content is bounded in proteinaceus compounds like enzymes, structural proteins, glycoproteins, peptides and amino acids [1]. These protein residues with organic nitrogen content are prone to mineralization processes.

When an organism dies, its proteins are attacked by the proteases of soil bacteria to produce polypeptides and amino acids. Proteases mediate the first steps of mineralization that often are rate-limiting for the nitrogen cycle. Soil proteases are derived mainly from heterotrophic soil bacteria. Władyka and coworkers divided the proteases depending on the location of reaction they catalyse, into endopeptidases (peptidases that hydrolyse peptide bonds in the inner regions of peptide chains) and exopeptidases (with activity directed to the amino- or carboxyl-termini of proteins) [5].

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The following step in the mineralization process of the organic nitrogen iscalled ammonification, through which the organic compounds are transformed to ammonia. In addition to the ammonification of amino acids, other compounds such as nucleic acids, urea, and uric acid go through the ammonification process, due to the microbial extracellular enzyme activity [2]. There are numerous bacterial species that were previously described as having a decisive role in the nitrogen mineralization: *Bacillus cereus, B. megaterium, B. subtilis, Serratia marcescens, Achromobacter* spp., *Flavobacterium* spp., *Mycobacterium* spp., *Nocardia* spp., *Bactoderma* spp. [2, 3, 4].

Proteases, based on their catalytic mechanism were divided into seven groups. Depending on the amino acids that serve as nucleophilic amino acid for the active site of the enzyme the following protease groups were distinguished: aspartic acid proteases, cysteine proteases, glutamic-acid proteases, metalloproteinases, serine proteases, threonine proteases, and assigned group without any particular catalytic type. It is presently known that extracellular peptidases of fungal origin are mostly cysteine and aspartic peptidases, while those of bacterial origin are alkaline metallopeptidases (Apr), neutral metallopeptidases (Npr) and serine peptidases (Sub) [6, 7, 8]. Singh and coworkers [12] described the presence of the alkaline protease enzymes in salt-tolerant alkaliphilic actinomycetes.

Bacterial proteases were studied by Bach and coworkers [6] in several bacterial strains. The *sub* and *npr* genes, coding serine peptidases and neutral metallopeptidases were mainly found in *Bacillus* species, whereas *apr* genes coding alkaline metallopeptidases in *Pseudomonas fluorescens* biotypes and also in *Flavobacterium – Cytophaga* strains [6, 7, 9].

The main aim of the present study was to detect bacterial peptidase (*apr* and *npr*) genes from the genome of 37 bacterial strains isolated from Harghita County mountainous regions [10] in order to select bacterial strains with important role in nitrogen mineralization.

RESULTS AND DISCUSSION

In this study, the detection of alkaline metalloproteinase (*apr*) and neutral metalloproteinase (*npr*) genes of the 37 bacterial strains (Table 1.) was performed using degenerated primer pairs. The annealing temperatures of the primer pairs were optimised using as controls the *Pseudomonas fluorescens* ATCC 13525 strain for *apr* and the *Bacillus cereus* ATCC 14579 strain for *npr* gene detection.

A gradient polymerase chain reaction (PCR) was performed in order to determine the optimal annealing temperature of primers setting a 9ºC gradient from 49ºC to 58ºC. As it is shown on Figure 1, the amplicons resulted from the *apr* gene amplification are varying with temperature. At lower temperatures the

products are slightly visible, whereas the amount of amplification product is increasing between the temperature ranges from 49 to 55.7ºC, and is decreasing with the further increase in temperature. Between the temperatures set from 52.9ºC to 58ºC by-products were generated (not only a single band detected). The optimal annealing temperature chosen for *apr* gene amplification was 57.3ºC because on this temperature the ratio between the expected PCR product and the by-product was considered most adequate. The amplification of the *npr* gene was observed only on 52.9ºC (Figure 1.) for the *Bacillus cereus* ATCC 14579 strain. The *npr* primer pair gave a 233 bp amplicon and some additional unspecific bands with the lowered temperature was observed for *Bacillus cereus DSMZ 3101*, *Bacillus cereus NCBI M38910* strains at 53ºC in case of Bach and coworkers study[6, 9].

Table 1. The bacterial strains used in this work and the results of the proteinase gene amplifications (+ – positive results, - – negative results)

Bacterial strain No	Isolated from	Species affiliation	*apr* gene	*npr* gene
1BS	soil – Borsáros	*Pseudomonas* sp.	+	-
2BR	rhizosphere of *Carex* sp. – Borsáros		-	-
11BS	soil – Borsáros		+	-
12BS	soil – Borsáros		+	-
13BS	soil – Borsáros		+	-
19BS	soil – Borsáros		+	-
20BS	soil – Borsáros		+	-
2BS	soil – Borsáros	*Delftia lacustris*	+	-
3BS	soil – Borsáros		+	-
4BS	soil – Borsáros		+	-
6BS	soil – Borsáros		+	-
8BS	soil – Borsáros		+	-
17BS	soil – Borsáros		+	-
1BR	rhizosphere of *Carex* sp. – Borsáros		+	-
3BR	rhizosphere of *Carex* sp. – Borsáros	*Bacillus cereus*	+	-
14BS	soil – Borsáros		+	-
16BS	soil – Borsáros		-	-
21BS	soil – Borsáros		-	-
4BR	rhizosphere of *Carex* sp. – Borsáros	*Serratia plymuthica*	+	-
5BS	soil – Borsáros		-	-
9BS	soil – Borsáros		+	-
10BS	soil – Borsáros		+	-
15BS	soil – Borsáros		+	-
18BS	soil – Borsáros		+	-

Bacterial strain No	Isolated from	Species affiliation	*apr* gene	*npr* gene
1CNS	not fertilized soil – Cristuru Secuiesc	*Acinetobacter lwoffii*	-	-
2CNS	not fertilized soil – Cristuru Secuiesc		-	-
1CZR	rhizosphere of *Zea mays* – Cristuru Secuiesc		-	-
2CZR	rhizosphere of *Zea mays* – Cristuru Secuiesc		-	-
3CZR	rhizosphere of *Zea mays* – Cristuru Secuiesc		-	-
4CZR	rhizosphere of *Zea mays* – Cristuru Secuiesc		-	-
7BS	soil – Borsáros	*Bacillus fordii*	+	-
3CCR	rhizosphere of *Carex* sp. – Cristuru Secuiesc	*Erwinia cypripedii*	+	-
5CZR	rhizosphere of *Zea mays* – Cristuru Secuiesc	*Pseudomonas fluorescens*	+	-
6CZR	rhizosphere of *Zea mays* – Cristuru Secuiesc	*Pseudomonas fluorescens*	+	-
1CCR	rhizosphere of *Carex* sp. – Cristuru Secuiesc	*Pseudomonas jessenii*	+	-
2CCR	rhizosphere of *Carex* sp. – Cristuru Secuiesc		-	-
4CCR	rhizosphere of *Carex* sp. – Cristuru Secuiesc		+	-

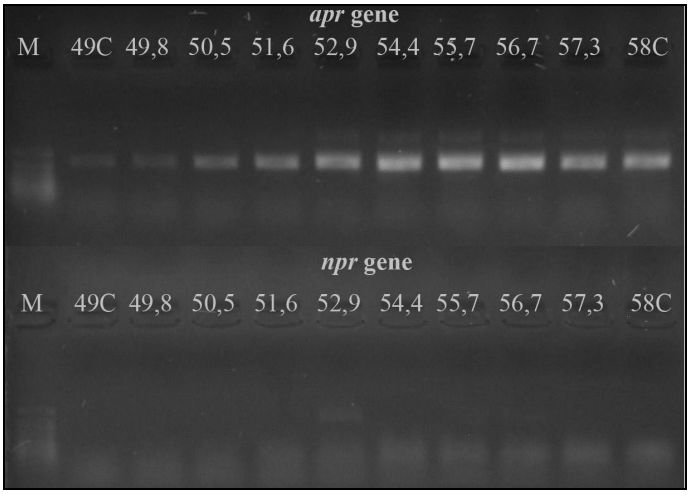

Figure 1. The products of *apr* and *npr* gene amplification of control strains by gradient PCR.

The presence of *apr* gene was detected in the genome of the studied strains, giving a 194 base pair (bp) product, compared to the positive control *Pseudomonas fluorescens* ATCC 13525 (Figure 2.).

In several isolates (1BR, 3BS, 4BS, 6BS and 8BS) additional multiple unspecific bands were observed (Figure 2.), due to the degenerated nature of the used primers. The sequence analysis of these isolates showed the closest homology with the *Delftia lacustris* DMS 21246$^{(T)}$ strain (unpublished data). According to the *Gene Bank* data the genome of *Delftia sp.* strains also encodes protease enzymes. Using the *NCBI Protein Blast* program the similarities between the amino acid sequences of *Pseudomonas fluorescens* and *Delftia* metalloproteinase were analyzed *in silico.* A difference in size of *Pseudomonas fluorescens* and *Delftia* metalloproteinases were observed, the amino acid sequences for *Delftia* metalloproteinase were found to be longer.

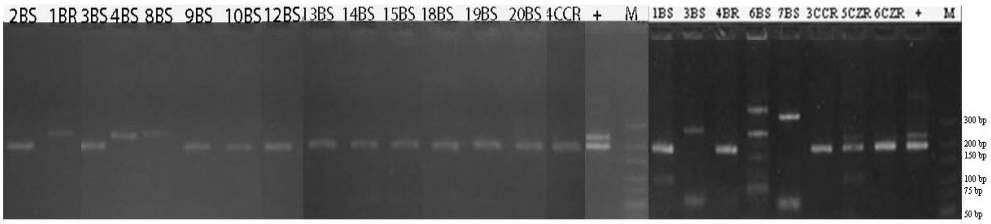

Figure 2. The amplified *apr* gene products separated by agarose gel electrophoresis (M - GeneON LowRange DNA Ladder, + positive control, 2BS, etc. - bacterial strain Nos). The 194 bp bands represent the target *apr* gene. In some cases additional unspecific bands appeared.

The *npr* gene failed to amplify from the samples, which implies that the genome of the isolates not likely contains genes homologous to the *npr* amplified by the primers.

Table 2. Sequences showing significant homology with our isolated *apr* gene sequences retrieved from the *NCBI Nucleotide Collection* database.

Strain	**Description**	**Similarity**
Erwinia cypripedii strain 3CCR	*Pseudomonas fluorescens aprA* gene, *aprI* gene, *aprD* gene and *aprE* gene (partial), strain M114	95%
	Pseudomonas fluorescens Pf0-1, metalloprotease	91%
	Pseudomonas fluorescens No.114 gene for metalloproteinase	91%
	Pseudomonas fluorescens CIP7325 *AprX* gene	91%
	Pseudomonas fluorescens strain CHA0 metalloproteinase (*aprA*)	90%
Pseudomonas fluorescens	*Pseudomonas fluorescens* genes for ABC exporter operon, complete cds (coding region)	92%

Strain	Description	Similarity
strain 6CZR	*Pseudomonas fluorescens* SBW25, metalloproteinase	90%
	Pseudomonas fluorescens CIP7325 *AprX* gene	89%
	Pseudomonas tolaasii eprA, eprI, eprD, eprE, eprF genes	88%
	Pseudomonas fluorescens strain TSS extracellular metalloproteinase (*aprX*) gene	88%

The amplified genes for two strains (3CCR and 6CZR) were further analyzed using DNA sequence determinations. The sequences of the PCR products showed similarities to metalloproteinase enzyme sequences isolated from different *Pseudomonas* strains (Table 2.). Thus it can be concluded that the amplified sequences encode alkaline metalloproteinase enzyme genes.

CONCLUSIONS

During the work the *apr* gene was positively amplified for 26 bacterial strains (Table 1.) using degenerate primers designed for amplification of metalloproteinase enzymes by Bach and coworkers [6,9]. The isolated bacterial strains that produce alkaline metalloproteinase enzymes possibly participate in the mobilization of nitrogen from organic sources. The *npr* gene failed to amplify from the samples, which implies that the genome of the isolates not likely contains genes homologous to the tested ones. The amplified *apr* genes were sequenced in case of two strains, and showed acceptable similarity to *Pseudomonas fluorescens* metalloproteinase genes (Table 2.).

The bacterial strains that gave positive results for the analysed functional gene (*apr,* alkaline metalloproteinase) have an important role in nitrogen mineralization and can be selected as potential plant growth promoting bacteria.

EXPERIMENTAL SECTION

Bacterial strains and growth medium

The bacterial strains were isolated from soil and rhizosphere of *Carex* sp. and *Zea mays* from Cristuru Secuiesc region; from soil and rhizosphere of *Carex* sp. fromBorsáros raised bog natural reserve. The used isolatory medium was King's B agar, containing 20 g/L proteose peptone, 10 ml/L glycerol, 1.5 g/L K_2HPO_4, 1.5 g/L $MgSO_4.7H_2O$, 18 g/L agar, pH = 7.2.

Cell lysis and DNA purification

Genomic DNA was obtained using the NaOH-based DNA extraction method. In 1.5 ml Eppendorf-tubes 25 µl 0.5 M NaOH solution was distributed and a loopful of bacteria suspended in it thorough vortexing and incubated at

room temperature for 15 minutes. After that 25 µl 1 M Tris solution (pH 8) and 300 µl ultra-pure H_20 was added, and centrifuged for 30 seconds at maximum speed to remove the cell debris. The supernatants were used as DNA samples, which were stored at -20ºC.

Polymerase Chain Reaction

For the detection of the *apr* and *npr* genes using PCR the following primer pairs were selected: FP *aprI* (5'-TAYGGBTTCAAYTCCAAYAC-3'), RP *aprII* (5'-VGCGATSGAMACRTTRCC-3'), FP *nprI* (5'-GTDGAY GCHCAYTAYTAYGC-3') and RP *nprII* (5'-ACMGCATGBGTYADYTCATG-3'). The gradient PCR was performed setting a gradient from 49ºC to 58ºC. The reaction mixture in a 25 µl final volume contained the following for each reaction: 2.5 µl 5x PCR buffer, 5 µl dNTP, 2 µl 25mM $MgCl_2$, 1 µl of each primer (FP *aprI* and RP *aprII* or FP *nprI* and RP *nprII*) and 1 µl *Taq polymerase* (2U/µl). The used temperature profile for the PCR was the following: preheating at 98°C for 5 minute for denaturation of DNA and activation of polymerase, 40 cycles with denaturation at 95ºC for 30 seconds, annealing at 49…58ºC for 30 seconds, extension at 72ºC for 30 seconds and a final extension at 72ºC for 10 minutes. The results of amplification were analysed using agarose gel electrophoresis (2% agarose gel, 80 V, 20 minutes, GeneOn 10bp LowRange DNA-Ladder). Further PCR reactions for selected bacterial strains were performed on the optimum annealing temperatures. Amplicons were analysed using agarose gel electrophoresis (1,8% agarose [Cambrex MetaPhor] in TBE buffer, 70 V, 60 minutes, GeneOn 10bp LowRange DNA-Ladder).

Amplicon sequencing

The purification of the DNA was made using *PCR-MTM CleanUp System* (*Viogene,* Sijhih, Taiwan) according to the manufacturer's description. After purification the products were visualized using agarose gel electrophoresis (2% agarose gel, 80 V, 20 minutes, GeneOn 10bp LowRange DNA-Ladder). The sequencing reaction was realised using *AmpliTaq® FS Big Dye ™ Terminator* sequencing kit (Applied Biosystems). The reaction was performed for two bacterial strains (3CCR, 6CZR). The reaction mixture contained 2.25 µl 5x Big Dye buffer, 1.5 µl Big Dye, 1 µl forward primer, 4.5 µl DNA, 5.75 µl dH_2O in a 15 µl final volume. The amplification program was the following: 28 cycle with denaturation at 96ºC for 10 seconds, annealing at 50ºC for 5 seconds and extension at 60ºC for 4 minutes. The products were precipitated using a premix of: 62.5 µl absolute ethanol, 19.5 µl dH_2O and 3 µl 3M sodium acetate, for 15 µl PCR products, followed by a 15 minutes incubation at room temperature and a centrifugation on 14000 rpm, 4ºC for 20 minutes and supernatant removal. This step was repeated for each sample adding an amount of 250 µl 70% ethanol. After precipitation, the DNA fragments were dried using vacuum centrifugation

for 20 minutes. Sequence data were obtained using an ABI 310 Automated Genetic Analyser. The sequences were manually checked using the *CHROMAS 2.33* (Technelysium Pty Ltd) software. The sequences were compared with those of the *NCBI* (National Center for Biotechnology Information) Gene Bank public database. Alignments were carried out by the *NCBI Blast* program and the sequences showing highest similarity were aligned by *MEGA4* (Molecular Evolutionary Genetics Analysis) program.

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