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Comparative Analysis of *Pseudomonas* Population in Oil-Contaminated Soils in Serbia and Plant-Pathogenic *Pseudomonas*

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ABSTRACT

Pseudomonas species are remarkable for their capacity to colonize almost all terrestrial and aquatic ecological niches. This genus includes species of ecological, economic and health-related importance. Although they are globally active in aerobic decomposition and biodegradation, *Pseudomonas* includes species pathogenic for humans, domestic animals and cultivated plants. The aim of this study was to identify members of the *Pseudomonas* species from oil-polluted soil, investigate their diversity and compare it to phytopatogenic strains isolated from host-plants near marked site.

Isolates were described phenotypically according to carbon assimilation, fluorescence on King B medium and susceptibility patterns to 5 different heavy metals. In addition, they were characterized genotypically using plasmid profile and fingerprints obtained with the $(GTG)_5$ primer.

We have observed high heterogeneity within the *Pseudomonas* strains collected from oil- contaminated soils. Phenotyping and $(GTG)_5$ pattern showed that similarities between strains ranged from 55% to 94%, with some strains showing high level of similarity with plant-pathogens.

Key words: *Pseudomonas*, polluted soil, heavy metals, BOX-PCR

INTRODUCTION

Pseudomonas sp. encompasses a group of saprophytes that colonize soil, water and plant surface environments. It is an obligate aerobe, except for some strains that can utilize NO_3 as an electron acceptor in place of O_2. Some of *Pseudomonas sp.* strains produce fluorescent pigments, particularly under conditions of low iron availability. *P. fluorescens* produces a soluble, greenish fluorescent pigment, while *P. aeruginosa* strains produce two types of soluble pigments: fluorescent pyoverdin and blue pyocyanin (Cody and Gross, 1987). Fluorescence on King B medium under ultraviolet light is helpful in early identification of *P. aeruginosa* colonies.

Pseudomonas has simple nutritional requirements and grows well in mineral salts media supplemented with any of a large number of carbon sources. *Pseudomonads* are noted for their metabolic diversity and are often isolated from enrichments designed to identify bacteria that partially or completely degrade pollutants such as styrene, TNT and polycyclic aromatic hydrocarbons (Timmis, 2002).

Pseudomonas is tolerant to a wide variety of environmental conditions, including temperature. It is resistant to high concentrations of salts, dyes, weak antiseptics, many commonly used antibiotics, and tolerant to heavy metal ions and metalloids (Silver, 1996). Although an excess of metals is generally toxic, some of them are essential in trace amounts (Cu, Mn, Zn, etc.). Microorganisms use a number of mechanisms to maintain the correct equilibrium, including the uptake, chelation and extrusion of metals (Robinson *et al.*, 2001, Cánovas et al., 2003). Various microbial species, including *Pseudomonas,* have been shown to be tolerant and relatively efficient in bioaccumulation of uranium, copper, lead, and other metal ions from polluted effluents, both as free-swimming or immobilized cells (Lovely et al., 1991).

The aim of this study was to isolate, identify and characterize *Pseudomonas* strains indigenous to oil-polluted soils in different locations in Serbia, and compare them to plant-pathogenic *Pseudomonas* strains isolated from the same locations.

METHODS

Indigenous *Pseudomonas sp.* were isolated from two different locations of polluted soils and labeled DZI and DmZI according to the location. Isolates from surfaces of different plants were labeled DBP. Isolates were tested for fluorescence on King B medium (Moragrega et al., 2003). Pathogenicity of isolates was tested following the protocol of Moragrega et al. (2003). Substrate utilization of crude oil and mineral oil was tested as described by Toledo et al., (2006). For susceptibility patterns to heavy metals, isolates were grown on NA with addition of 200µg/ml of Zn and Co, 50µg/ml of Mo, 20 µg/ml of Hg or 25µg/ml of Cd. Plasmid profiles were obtained by method of Wheatcroft and Williams (1981). rep-PCR analysis using BOX type $(GTG)_5$ primer was performed as recommended by de Bruijn (1992). Similarity was estimated by means of the simple matching coefficient (*S*SM) and clustering was based on the unweighted pair group arithmetic average-linkage algorithm using STATISTICA 5 software .

RESULTS andSCUSION

We have examined *Pseudomonas* species indigenously growing on two locations with soil polluted with petrol and mineral oil. We have been able to isolate 27 different bacterial isolates from polluted soil, 6 of which were identified as *Pseudomonas* sp (DZI2, DZI3, DZI5, DmZI1, DmZI4, DmZI6). Three plant-pathogenic *Pseudomonas* strains were isolated from plant leaves near one of the locations (DBP1, DBP2, DBP3). Pathogenicity of the isolates was confirmed as described in Methods.

Phenotypic analysis of the strains was performed as described in Methods, and the results are summarized in Table 1. All strains except DmZI1 and DZI2 were fluorescent on King B medium.

Isolates	Fluorescence on King B medium	Plasmid number	Heavy metals (µg/ml)					Substrate utilization (0,5%)	
			Hg 20	Mo 50	Zn 200	Co 200	Cd 25	Crude Oil	Mineral Oil
DmZI1	-	1	±	+	+	+	+	+	+
DZI2	-	1	-	±	+	-	±	±	+
DZI3	+	nd	+	+	+	+	+	+	+
DmZI4	+++	2	+	+	+	+	+	+	+
DZI5	++	2	+	+	+	+	+	+	+
DmZI6	+++	1	+	+	+	+	+	+	+
DBP1	+	1	+	+	+	+	+	+	+
DBP2	++	1	±	+	+	+	+	+	+
DBP3	+	nd	±	-	-	±	-	-	+

Table1. Phenotypic analysis and plasmid profiles of investigated *Pseudomonas* isolates. Fluorescence on King B medium, plasmid number, heavy metal tolerance and substrate utilization of *Pseudomonas sp*. isolates. n.d.-not detected

Strain DBP3 showed high sensitivity to investigated heavy metals, strain DZI2 was moderately sensitive, and the rest of the strains were highly tolerant to investigated concentrations of Mo, Zn, Co and Cd. High tolerance to heavy metals was previously reported for *Pseudomonas* strains. Nakahara et al. (1977) tested 787 clinical *Pseudomonas* isolates on four metals (Hg, Cd, As, and Pb), and showed that 99.8% were tolerant to metals, with most (99.5%) showing multiple tolerance.

Strains DmZI1, DmZI4, DmZI6, DZI3, DZI5, DBP1and DBP2 were tolerant to 200µg/ml of Zn and Co, which is higher than the concentration reported in a similar study (100 mg/l of Cu, Pb, Cd, Zn, Malekzadeh et al. 1996). In addition, investigated strains showed resistance to 50µg/ml of Mo, 20 µg/ml of Hg and 25µg/ml of Cd. Multiple resistance to investigated heavy metals is probably regulated by metal-dependent members of COG0789 (Permina et al., 2006), that include mercury detoxification (MerR), resistance to zinc (ZntR), copper (CueR and HmrR), cadmium (CadR) and a number of other toxic metals (Rouch et al., 1997, Brown et al., 2003, Hobman et al., 2005)

Seven of the nine examined strains were able to grow on both mineral oil and crude oil as only source of carbon, while strain DZI2 grew well on mineral, but only poorly on crude oil. Phytopathogenic

strain DBP3 grew well on mineral oil, but showed no growth on crude oil. *Pseudomonas* growth in the presence of different PAHs was previously reported by Hubert et al., (1999), Baldwin et al (2000), Barathi et al., (2001) and Toledo et al. (2006).

Genotypic analysis was performed by plasmid profile and rep-PCR. Plasmid profile analysis placed examined strains in 3 groups: strains DZI3 and DBP3 had no plasmids, DmZI4 and DZI5 had two plasmids, and the other five strains had one plasmid in their plasmid profile (Table 1).

Rep-PCR (BOX) pattern obtained with $(GTG)_5$ primer (Figure 1) revealed highest level of similarity between DZI3 and DBP1 (94%) (Figure 2). Similarity of these two strains with DBP3 was 83.5%, same as the similarity between DmZI4 and DZI5. The rest of investigated strains showed less than 67% of similarity in BOX patterns.

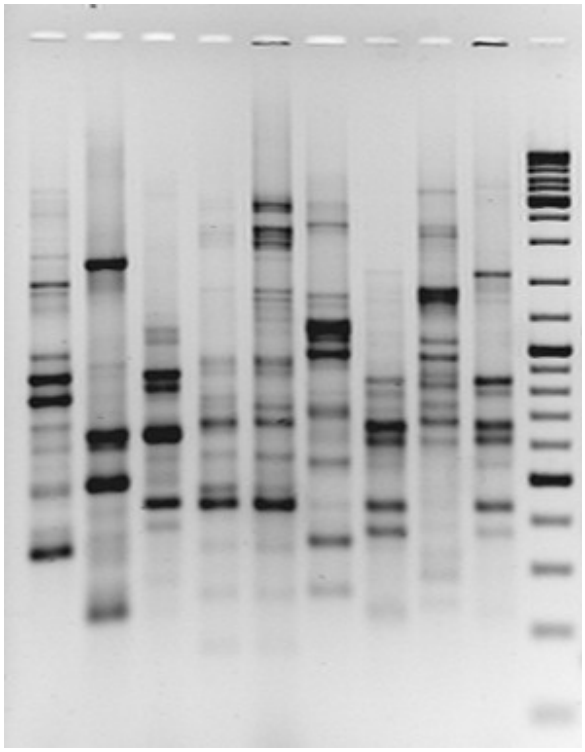

Figure 1. Rep-PCR (BOX) profiles of investigated *Pseudomonas* isolates. Lane 1-9: isolates DmZI1, DZI2, DZI3, DmZI4, DZI5, DmZI6, DBP1, DBP2, DBP3 respectively. Lane 10: GeneRuler DNA Ladder mix SM0331 (Fermentas)

This study represents preliminary data on diversity of indigenous *Pseudomonas* strains in oil-polluted soils in different locations in Serbia. Investigation of microorganisms that indigenously live in polluted soils is of potential ecological and economic importance. Microorganisms that have high affinity for metals can be effective in sequestering heavy metals, and have been used to remove metals from polluted industrial and domestic effluent on a large scale (Silver, 1996). Further investigations will demonstrate the capabilities of *Pseudomonas* strains identified in this study in removing Zn, Co, Mo, Hg, Cd, and possibly other toxic metals from polluted sites.

REFERENCES

Baldwin, B.R., M.B. Mesarch and L. Nies. 2000. Broad substrate specificity of naphthalene and biphenyl utilizing bacteria. Appl. Microbiol. Biotechnol. 53: 748-753.

Barathi, S. and N. Vasudevan. 2001. Utilization of petroleum hydrocarbon by Pseudomonas fluorescens isolated from a petroleum contaminated soil. Environ. Int. 26:413-416.

de Brujin, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58:2180-2187.

Brown, N.L., J.V.,Stoyanov, S.P. Kidd and J.L. Hobman. 2003. The MerR family of transcriptional regulators. FEMS Microbiology Reviews 27:145-163.

Cánovas, D., I. Cases and V. de Lorenzo. 2003. Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. Environmental Microbiology doi: 10.1046/j.1462-2920.

Cody and Gross. 1997. Characterization of pyoverdin, the fluorescent siderophore produced by *Pseudomonas syringae* pv. *syringae*. Appl. Environ. Microbiol. 53(5):928-934.

Hobman, J.L., J. Wilkie and N.L. Brown. 2005. A design for life: prokaryotic metal-binding MerR family regulators. Biometals 5 (18):429-436.

Hubert, C., Y. Shen and G. Voordouw. 1999. Composition of toluene degrading microbial communities from soil at different concentrations of toluene. Appl. Environ. Microbiol. 65:3064–3070.

Lovely, D.R., E.J.P. Phillips, Y.A. Gorby and E.R. Landa. 1991. Microbial reduction of uranium. Nature 350:413-416.

Malekzadeh, F., A. Farazmand, H. Ghafourian, M. Shahamat, M. Levin, C. Grim and R.R. Colwell. Accumulation of heavy metals by bacterium isolated from electroplating effluent. 1996. Proceedings of the Biotechnology Risk Assessment Symposium June 23-25,. Ohawa, Canada, 388-398.

Moragrega, C.I., C. Llorente, C. Manaceau and E. Montesinos. 2003. Susceptibility of european pear cultivars to *Pseudomonas syringae* pv. *syringae* using immature fruit and detached leaf assays. Eur. J. Plant Pathology 109:319-326.

Nakahara, H., T. Ishikawa, Y. Sarai, I.Kondo, H. Kozukue and S. Silver. 1977. Linkage of mercury, cadmium, and arsenate and drug resistance in clinical isolates of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 33:4

Permina, E.A., A.E. Kazakov, O.V. Kalinina and M.S. Gelfand. 2006. Comparative genomics of regulation of heavy metal resistance in Eubacteria BMC Microbiology 6:49doi:10.1186/1471-2180-6-49.

Robinson, N.J., S.K. Whitehall, and J.S. Cavet. 2001. Microbial metallothioneins. Adv. Microb. Physiol. 44: 183–213.

Rouch, D.A. and N.L. Brown. 1997. Copper-inducible transcriptional regulation at two promoters in the Escherichia coli copper resistance determinant pco. Microbiology 143(4):1191-202.

Silver, S. 1996. Bacterial resistances to toxic metal ions – a review. Gene 179: 9–19.

Timmis, K.N. 2002. Pseudomonas putida: a cosmopolitan opportunist par excellence. Environ. Microbiol. 4: 779–781.

Toledo F.L., C. Calvo, B. Rodelas and J. Gonzales-Lopez. 2006. Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Syst. Appl. Microbiol. 29: 244-252.

Wheatcroft, R. and Williams, C. 1981. Extraction of Whole Plasmid DNA for Agarose Gel Electrophoresis, J.Gen. Microbiol. 124:433-437.

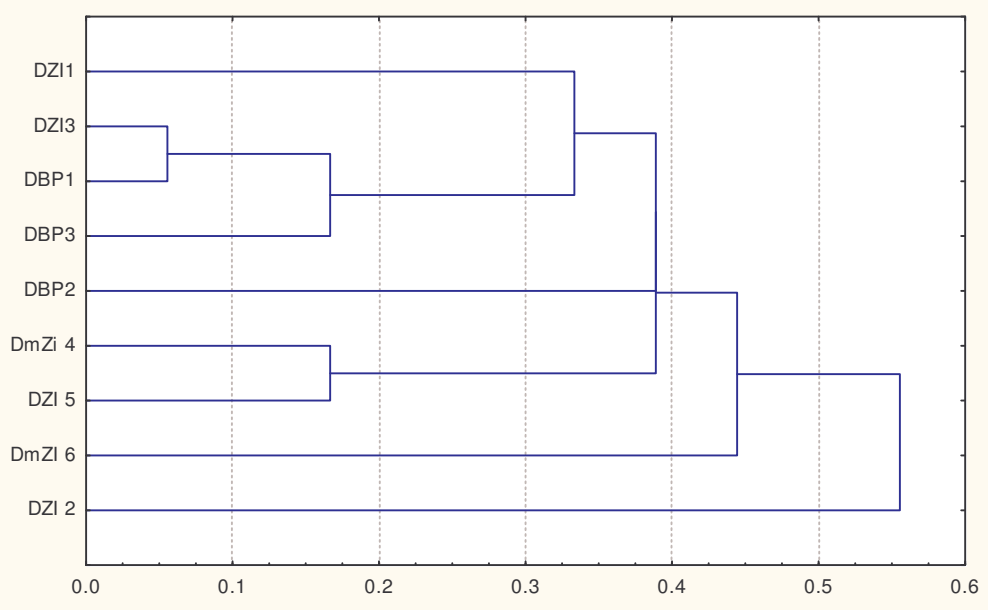

Figure 2. Tree Diagram for *Pseudomonas sp.* isolates rep-PCR: BOX analysis with $(GTG)_5$ primer. X-axis: percent disagreement.