

LIMITED AROMATIC PATHWAY GENES DIVERSITY AMONGST AROMATIC COMPOUND DEGRADING SOIL BACTERIAL ISOLATES

Gordana MINOVSKA, Tanja NARANČIĆ, Mina MANDIĆ, Lidija ŠENEROVIĆ, Branka VASILJEVIĆ and Jasmina NIKODINOVIĆ-RUNIĆ

Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

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Identification and characterization of novel genes belonging to microbial aromatic biodegradation pathway is of great importance as they have been proven versatile biocatalysts. In this study, the selection of 19 environmental bacterial isolates capable to degrade a wide range of aromatic compounds has been screened for the presence of five genes from the lower and the upper aromatic biodegradation pathway using PCR methodology. In the case of 4-oxalocrotonate tautomerase and toluene dioxygenases, although present in the most of environmental isolates, very limited diversity of the genes has been encountered. Highly conserved sequences of these genes in environmental samples revealed high homology with gene sequences of the characterised corresponding genes from *Pseudomonas putida* species. The screen using degenerate primers based on known catechol- and naphthalene dioxygenases sequences resulted in a limited number of amplified fragments. Only two catechol 2,3-dioxygenase from two *Bacillus* isolates were amplified and showed no significant similarities with dioxygenases from characterized organisms, but 80-90% identities with partial catechol 2,3-dioxygenase sequences from uncultured organisms. Potentially three novel catechol 1,2-dioxygenases were identified from *Bacillus* sp. TN102, *Gordonia* sp. TN103 and *Rhodococcus* sp. TN112. Highly homologous tautomerase and toluene dioxygenases amongst

Corresponding author: Jasmina Nikodinović-Runić, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, P.O.Box 23, 11010 Belgrade, Serbia
Telephone: +381 11 3976034, bFax: +381 11 3975808, E-mail: jasmina.nikodinovic@gmail.com
jasmina.nikodinovic@imgge.bg.ac.rs

environmental samples isolated from the contaminated environment suggested horizontal gene transfer while limited success in PCR detection of the other three genes indicates that these isolates may still be a source of novel genes.

Key words: Biodegradation, aromatic compounds, 4-oxalocrotonate tautomerase

INTRODUCTION

Aromatic hydrocarbon-polluted sites represent a long-term source of pollution and pose a severe risk to environmental health. In addition, these sites are rich source of microorganisms that have the metabolic pathways for their biodegradation (Fig. 1) (ANDREONI and GIANFREDA, 2007; NARANCIC *et al.*, 2012A). Microbial degradation of these pollutants is regarded as a promising approach to clean up contaminated sediments (ANDREONI and GIANFREDA, 2007). Genes for the degradation of many contaminants are often plasmid encoded (DIGIOVANNI *et al.*, 1996; TOP and SPRINGAEL, 2003). It has been established that plasmid DNA plays an important role in genetic adaptation as it represents a highly mobile form of DNA which can be transferred via conjugation or transformation and can confer novel advantageous phenotypes, including hydrocarbon-oxidizing ability, to recipient organisms (LEAHY and COLWELL, 1990). More recently, microbial oxygenases, especially ones from the aromatic degradation pathways, are receiving growing attention due to their applicability in biocatalysis (HOLLMANN *et al.*, 2011; NOLAN and O'CONNOR, 2008).

It has been recognized that one of the most challenging reactions in organic synthesis is the regiospecific hydroxylation of the aromatic ring (ULLRICH and HOFRICHTER, 2007). The ring-hydroxylating dioxygenases of prokaryotes are nonheme iron enzymes that convert aromatic compounds to dihydrodiols in the presence of dioxygen and NADH. The ability of oxygenases and oxidases to regiospecifically hydroxylate the aromatic ring or side chain substituent of an aromatic substrate presents opportunities for biocatalysis to synthesize compounds such as dihydrodiols and catechols that are otherwise difficult to synthesize (BURTON, 2003; NOLAN and O'CONNOR, 2008). Furthermore, enzyme mediated oxidations operating under mild conditions with usually high selectivity are increasingly resorted to as more environmentally suitable option (HOLLMANN *et al.*, 2011).

The new addition to green-chemistry tool-box is 4-oxalocrotonate tautomerase (4-OT; TAUT), encoded by *xylH* gene. As a part of aromatic compounds degradation pathway in *Pseudomonas putida* mt-2 it naturally catalyzes the conversion of 2-hydroxyhexa-2,4-dienedioate into 2-oxo-3-hexendioate (HARAYAMA *et al.*, 1989). This particular enzyme has recently been suggested as an important bridging link between organo- and biocatalysis (ZANDVOORT *et al.*, 2012a). Due to its promiscuous activity, 4-OT was described to catalyze Michael-type addition of acetaldehyde to α -nitrostyrene, the isomerisation of *cis*-nitrostyrene to *trans*-nitrostyrene as well as aldol condensation and dehydration activities with good enantioselectivity (ZANDVOORT *et al.*, 2012a; ZANDVOORT *et al.*, 2012b). This ability makes this enzyme exceptionally desirable target for further development into applicable biocatalyst.

The aim of this work was to identify potentially novel genes for biocatalysis, preferably novel 4-oxalocrotonate tautomerase and dioxygenases amongst different bacterial isolates with the established ability to degrade 15 different aromatic compounds (NARANCIC *et al.*, 2012a; NARANCIC *et al.*, 2012b). These strains were isolated from the sediment exposed to petrochemical industry effluents and were reasoned to be great potential source for novel

variants of aromatic pathway genes as they were mostly Gram-positive species that are usually not known as aromatic degraders. Firstly, we have screened for the potentially novel 4-OT genes belonging to lower aromatic degradation pathway and followed by the screen for oxygenase genes (C23O, C12O, NAPH and TOD) from the upper aromatic degradation pathway (Fig. 1).

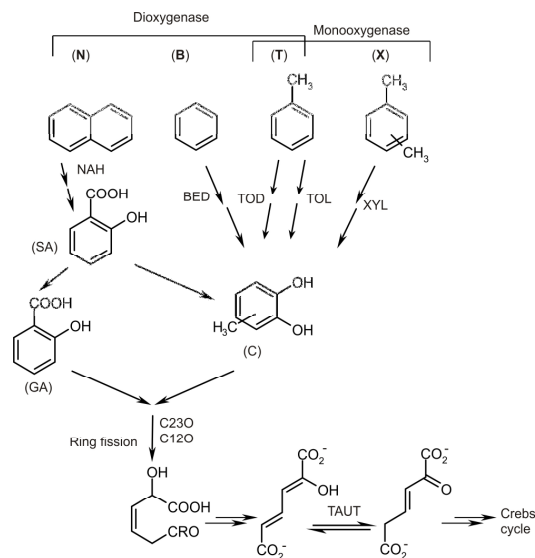


Figure 1. Schematic presentation of the microbial aerobic degradation of aromatic compounds: naphthalene (N), benzene (B), toluene (T) and xylenes (X). Naphthalene is initially oxygenated by naphthalene dioxygenase (NAH) and further degraded to yield salicylate (SA). SA is metabolised either through gentisic acid (GA) or through catechol (C). Similarly, monoaromatic compounds are initially oxygenated via dioxygenases (BED and TOD) or monooxygenases (TOL and XYL) to yield catechols. Ring fission is carried out by catechol dioxygenases (C23O). Further in the degradation pathway, the enzyme that converts 2-hydroxycrotonate to 2-oxo-3-hexanedionate is called 4-oxalocrotonate tautomerase (TAUT or 4-OT).

MATERIALS AND METHODS

Reagents

All chemicals, antibiotics and other salts were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA). KAPA Taq DNA polymerase and 100 bp H3 RTU DNA ladder were obtained from Nippon Genetics (Dueren, Germany) while O`Gene 100 bp DNA ladder was obtained from Fermentas (Burlington, Canada). T4 DNA ligase and pGEMT-Easy cloning kit were purchased from Promega (Madison, WI, USA). Oligonucleotide primers were obtained from Invitrogen (Darmstadt, Germany). The QIAprep spin plasmid mini-prep kit and QIAEX II gel purification kit were purchased from QIAGEN (Hilden, Germany). Tryptic soy broth, yeast

extract and other media components were purchased from Becton Dickinson (Sparks, MD, USA).

Microorganisms and culture conditions

All 19 TN bacterial strains were previously isolated and characterized as aromatic carbon compounds degrading bacteria from the sediment collected from the Tamis river banks in close proximity to petrochemical industry effluents collector (NARANCIC *et al.*, 2012a). Isolates have been taxonomically identified and sequences for 16S rRNA genes were deposited in GenBank under Accession numbers: JN800323-JN800341.

Pseudomonas putida KT2440 (ATCC 47054) and *Pseudomonas aeruginosa* PAO1 (ATCC 47085) strains were from the American Type Culture Collection (ATCC), while *Pseudomonas putida* mt-2 (NCIMB10432), toluene and *m*- and *p*-xylene degrader, was obtained from NCIMB culture collection (Aberdeen, Scotland, UK) and used as the source of *xylH* gene (WORSEY and WILLIAMS, 1975).

All strains were maintained in mineral salts medium (MSM) containing $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$ (9 g l⁻¹), KH_2PO_4 (1.5 g l⁻¹), NH_4Cl (1 g l⁻¹), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (0.2 g l⁻¹), CaCl_2 (0.002 g l⁻¹), trace element solution (1 ml l⁻¹) (SCHLEGEL *et al.*, 1961) and glucose (10 g l⁻¹) as a carbon source. Strains were grown at 30°C for 5 days on the orbital shaker at 180 rpm. When necessary, the medium was solidified by adding 1.5% (w/v) bacteriological agar Becton Dickinson (Sparks, MD, USA).

Escherichia coli DH5 α (Invitrogen, Darmstadt, Germany) was used for subcloning and was maintained in Luria-Bertani (LB) complex medium (SAMBROOK *et al.*, 1989). Ampicillin (50 $\mu\text{g ml}^{-1}$) was routinely used to select and maintain ampicillin resistance (amp^R).

Isolation of genomic DNA and PCR based screening for genes involved in the aromatic compounds degradation pathway

Total genomic DNA from pure bacterial cultures was isolated according to the procedure previously developed for *Streptomyces* strains (NIKODINOVIC *et al.*, 2003) and was applied to all bacterial strains without modifications. DNA concentration was quantified spectrophotometrically ($A_{260/280}$; NanoVue, GE Healthcare, Buckinghamshire, UK).

4-Oxalocrotonate tautomerase and core regions of naphthalene dioxygenase and toluene dioxygenase were amplified using previously described primers (Table 1) and using total gDNA as template (400- 500 ng). Reaction volume was 50 μl with 2 mM dNTPs, 2.5 mM MgCl_2 and 3% (v/v) DMSO in final concentration. PCR was done on a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA) using the following method: 95°C for 3 min; 30 cycles of 95°C for 30s, 49°C for 40s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

Core regions of catechol 2,3-dioxygenase and catechol 1,2-dioxygenase were amplified using step down PCR program whereby the initial DNA denaturation cycle of 95°C for 3 min was followed by 10 cycles of 95°C for 30s, 61°C for 30s, 15 cycles of 95°C for 30s, 55°C for 30s, 15 cycles 95°C for 30s, 51°C for 30s, and 72°C for 1 min; and a final extension at 72°C for 10 min (ROUX and HECKER, 1997).

Amplified PCR fragments were resolved using a 0.9% (w/v) agarose gel and visualized using ethidium bromide staining. Appropriate fragments were cut and purified from the gel using QIAEX II gel purification kit. Fragments of interest were ligated to the pGEM-T Easy vector cloning system to generate constructs suitable for sequencing. Constructs were sequenced

on an Applied Biosystems 3130 Genetic Analyser (Foster City, USA) using universal M13f/M13r primers (YANISCH-PERRON *et al.*, 1985). Sequence homologues were identified by the BLASTN algorithm (ALTSCHUL *et al.*, 1997).

Table 1. Oligonucleotide primers used for PCR detection of genes from the microbial aerobic degradation pathway of aromatic compounds.

Primer	Target gene	Sequence (5' – 3')	Reference
TAUT-F	4-Oxalocrotonate tautomerase	ATACATATGCCTATTGCCAGATC	(POELARENDS <i>et al.</i> , 2006)
TAUT-R		CATGGATCCTCAGCGTCTGACCTTGCT	
C23Of	Catechol 2,3-dioxygenase	AAGAGGCATGGGGGCGCACCGGTTCGA TCA	(SEI <i>et al.</i> , 1999)
C23Or		CCAGCAAACACCTCGTTGCGGTTGCC	
C12Of	Catechol 1,2-dioxygenase	GARTGGCCNYTNTTYTNGAYGTN	(AN <i>et al.</i> , 2001)
C12Or		NARRTGNARRTGNGCNGGNCCCA	
NAH-F	Naphthalene dioxygenase	CAAAARCACCTGATTYATGG	(BALDWIN <i>et al.</i> , 2003)
NAH-R		AYRCGRGSGACTTCTTTCAA	
TOD-F	Toluene dioxygenase	ACCGATGARGAYCTGTACC	(BALDWIN <i>et al.</i> , 2003)
TOD-R		CTTCGGTCMAGTAGCTGGTG	

RESULTS AND DISCUSSION

Screen for novel 4-OT genes amongst carbon degraders

Aromatic hydrocarbon degrading microorganisms have been isolated from the various environments, mostly from the polluted sites, but they are widespread in the marine and freshwater habitats (SEO *et al.*, 2009). From Tamis river sediments that were affected by the proximity of a petrochemical industrial site 19 bacterial isolates were characterized to be able to degrade and utilize 15 different aromatic compounds as sole source of carbon and energy (NARANCIC *et al.*, 2012a). These isolates included Gram-positive *Rhodococcus* sp. (6 isolates), *Bacillus* sp. (6 isolates), *Gordonia* sp. (2 isolates), *Arthrobacter* sp. (2 isolates), *Staphylococcus* sp. (1 isolate), and two representatives of Gram-negative representatives - *Pseudomonas* sp. TN301 and *Acinetobacter* sp. TN302. As all of the isolates had the ability to utilize *p*- and *o*-xylene as a carbon source, we have decided to screen them for the presence of 4-OT gene, using primers designed to amplify the whole *xylH* 200 bp fragment from *P. putida* mt-2 (Table 1). Indeed, in addition to control strain *P. putida* mt-2, the fragment of correct size was amplified in

15 out of 19 isolates (Fig. 2). The PCR products were not observed in two *Rhodococcus* sp. strains (namely TN105 and TN113) in *Gordonia* sp. TN108 and *Bacillus* sp. TN303. In addition, no PCR product was observed when gDNA from *P. putida* KT2440 was used as template and unspecific fragments of less than 100 bp and approximately 600 bp were observed when gDNA from *P. putida* PAO1 was used as template (Fig. 2). PCR fragments from TN isolates were successfully cloned in pGEM-T Easy vector and sequenced. Sequencing and analysis revealed 98-100% identity with *xylH* gene from *P. putida* mt-2 (data not shown; obtained sequences supplied as on-line Supplemental Information).

Having in mind that 4-OT is one of the enzymes with the smallest subunits (each subunit has only 62 amino acids) with six identical subunits arranged into three dimmers, it is likely that the slight differences in amino acid sequence could easily result in the novel overall conformation of the active site neighbourhood and affect the protein activity. Therefore we have carried out the screen for potentially novel 4-OT genes with likelihood of novel promiscuous activities. However, all detected fragments were highly conserved and identical to already described 4-OT from *P. putida* mt-2 (HARAYAMA *et al.*, 1989).

4-OT is encoded by *xylH* gene that is found on pWW0 or TOL plasmid which is the best characterised catabolic plasmid encoding all the enzymes responsible for toluene degradation, as well as degradation of *m*- and *p*-xylene and related compounds to acetaldehyde and pyruvate (CERDAN *et al.*, 1994). This plasmid of 116.5 kb was originally described in *Pseudomonas putida* (*arvilla*) mt-2, with numerous TOL plasmids described since with most encoding similar biochemical pathways and regions of high DNA sequence homology to pWW0 (WILLIAMS *et al.*, 2002). Detailed sequence analysis of this plasmid (GenBank accession number AJ344068) has revealed its complex evolution and that multiple transposition and recombination events have made a major contribution to its present structure (GREATED *et al.*, 2002; WILLIAMS *et al.*, 2002). TOL plasmids have generally been associated with Gram-negative *Pseudomonas* and related species (ANDREONI and GIANFREDA, 2007; WACKETT, 2003). In addition, 'chromosomal location' of TOL plasmid was confirmed by conjugation experiments in *Pseudomonas putida* MW1000 with 56 kb fragment conferring the ability to grow on toluene in this strain found to be almost identical to homologous sequence of pWW0 (SINCLAIR *et al.*, 1986).

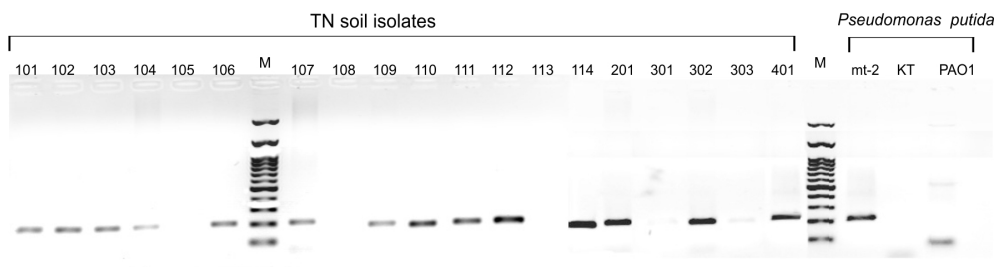


Figure 2. PCR detection of 4-OT gene (192 bp) in 19 aromatic compounds degrading bacterial isolates and three *Pseudomonas* species. M; 100 bp Nippon Genetics H3 RTU DNA ladder with the heaviest band of 500 bp.

PCR screen for catechol dioxygenases

Having established highly conserved 4-OT sequence amongst representatives of 7 different bacterial genera, indicating potential horizontal gene transfer via one of TOL plasmids, we have decided to assess diversity of catechol dioxygenases as enzymes further up in the aerobic metabolic pathway of aromatic compounds (Fig. 1). Catechol undergoes ring fission that can take place between the two hydroxyl groups, involving action of catechol 1,2-dioxygenase (C12O), or proximal to one of the two hydroxyl groups, involving action of catechol 2,3-dioxygenase (C23O). They are widely distributed in nature, involved in variety of other pathways and exhibit high diversity (ELTIS and BOLIN, 1996; VAILLANCOURT *et al.*, 2006). On the other hand, they are also biotechnologically important enzymes with C23O (EC 1.13.11.2) mostly used for the biotreatment of wastewaters (GUZIK *et al.*, 2009) and C12O (EC 1.13.11.1) in production of adipic acid, a raw material for manufacture of resins, pharmaceuticals and agrochemicals (DINARDO *et al.*, 2009; YOSHIKAWA *et al.*, 1993).

The PCR screen for catechol dioxygenases was performed by using primers designed for the detection of core regions of catechol 2,3-dioxygenase in bacterial populations capable of degrading aromatic compounds via catechol cleavage pathways (SEI *et al.*, 1999). PCR fragment of the expected size of 380 bp was successfully amplified in *P. putida* mt-2 and *P. putida* KT2440. Sequencing of the products revealed expected 100% identical sequence with corresponding sequence of *xylE* from *P. putida* mt-2 strain (GenBank accession number M64747) while sequence obtained for the fragment amplified from KT2440 indicated unspecific priming (data not shown). Meanwhile, the correct size fragment was amplified in only two TN isolates, namely *Rhodococcus* sp. TN101 and *Rhodococcus* sp. TN112 (Fig. 3A). However, sequencing data indicated unspecific binding to transcriptional regulators with the highest homology *Rhodococcus erythropolis* PR4 gene sequence for putative TetR family transcriptional regulator (GenBank accession number AP008957, nucleotide region 2286389-2286958) (SEKINE *et al.*, 2006). Interestingly, two PCR products that appeared shorter in length of approximately 300 bp, amplified using the gDNA from two *Bacillus* strains (TN107 and TN303, the 7th and 18th line in the gel after the marker; Fig. 3A) upon sequencing and comparison to the GenBank database showed no significant similarities with C23O dioxygenases from characterized organisms, but between 80-90% identities with partial C23O sequences from uncultured organisms (GenBank accession number AB286741.1) obtained in an effort of functional gene analysis and characterization of the microbial community degrading nonylphenol originating from sewage sludge (Tadashi Shoji, unpublished work). However, the two sequences shared 99% nucleotide sequence identity, again indicating possible gene transfer events amongst TN isolates (Fig. 4). PCR fragment of the correct size of 280 bp was successfully amplified in four TN isolates and both *Pseudomonas putida* strains (Fig. 3B) using step down PCR methodology due to high degree of redundancy in C12Of/r sequence (Table 1). However, sequencing data revealed that only PCR product obtained when gDNA of *Pseudomonas* sp. TN301 was used as template had significant similarity (95% homology) with core region of catechol 1,2-dioxygenase *catA* gene from *Pseudomonas putida* F1 (ZYLSTRA and GIBSON, 1989). Sequence of PCR products from *Bacillus* sp. TN102, *Gordonia* sp. TN103 and *Rhodococcus* sp. TN112 had no significant similarity with available sequences of known bacterial catechol dioxygenases.

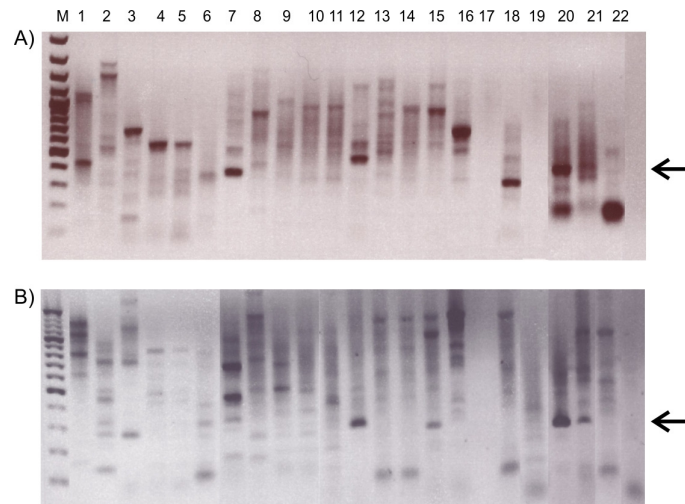


Figure 3. PCR screen for catechol dioxygenases: A) catechol 2,3-dioxygenase (C23O) and B) catechol 1,2-dioxygenase (C12O). Arrows are pointing to the product of the expected size. M; Fermentas O^oGene 100 bp DNA ladder.

	Score	Expect	Identities	Gaps	Strand
	610 bits(330)	2e-179	340/345(99%)	0/345(0%)	Plus/Plus
TN107	3	GAGGCATGGGGGCGCACCGGTTTCGATCATGCCTTTCCTTTATGGTCCGCATGTGGATCAGG	62		
TN303	1	GAGGCATGGGGGCGCACCGGTTTCGATCATGCCTTTCCTTTATGGTCCGCATGTGGATCAGG	60		
TN107	63	TGATGCATTTCTTCGTGGAAGCGCTGGATTTTCAGCCTAGCGGAACAAGTGATGATGCGGG	122		
TN303	61	TGATGCATTTCTTCGTGGAAGCGCTGGATTTTCAGCCTAGCGGAACAAGTGATGATGCGGG	120		
TN107	123	ACGTTTTGCTGGCGAICTGGTTGCTTCGCGCAACAAGGCGCAGCATACCGCCTTTCGTCA	182		
TN303	121	ACGTTTTGCTGGCGAICTGGTTGCTTCGCGCAACAAGGCGCAGCATACCGCCTTTCGTCA	180		
TN107	183	AGCACCCCGAGCCGGGCAAGCTGCATCACATCTCCTTCTTCCTGGAGTCTTGGCAGCAGC	242		
TN303	181	AGCACCCCGAGCCGGGCAAGCTGCATCACATCTCCTTCTTCCTGGAGTCTTGGCAGCAGC	240		
TN107	243	TCGCCCCGCGGCGGAGCATCGCCCCCTACACATCTCGCTGGATATCGGGCCGACTC	302		
TN303	241	TCGCCCCGCGGCGGAGCATCGCCCCCTACACATCTCGCTGGATATCGGGCCGACTC	300		

Figure 4. Nucleotide alignment of two core regions of catechol 2,3-dioxygenase (C23O) genes obtained by PCR amplification from *Bacillus* sp. TN107 and *Bacillus* sp. TN303 strains.

The correct size fragment was amplified when each gDNA was used as template from both *Pseudomonas putida* strains and the sequence revealed 98-100% identity with PP_3713 (GenBank accession number NC_002947, nucleotide region 4235833-4236768) which is 936 bp catechol 1,2-dioxygenase gene from *Pseudomonas putida* KT2440 (NELSON *et al.*, 2002).

Generally, the aromatic ring-cleaving oxygenases are well studied in terms of the distribution, evolution and reaction mechanisms (VAILLANCOURT *et al.*, 2006). The alignment of 35 different extradiol dioxygenases revealed only nine conserved residues of which, three were metal ligands (ELTIS and BOLIN, 1996). Therefore, they do not appear to be of highly conserved sequence and it seems that they have evolved in accordance to substrate specificity. More recent findings have implicated them in catabolic pathways that do not necessarily involve aromatic compounds (FETZNER, 2012). Genes encoding catechol dioxygenase could be both plasmid and chromosomally encoded. The most of the characterized catechol dioxygenases are encoded by genes located on plasmids as in *Pseudomonas* sp. HV3, *Pseudomonas* sp. EST1001, *Pseudomonas putida* (TOL plasmid), *Planococcus* sp. S5, *Rhodococcus rhodochrous* (pTC1 plasmid) (CANDIDUS *et al.*, 1994; CERDAN *et al.*, 1994; HUPERT-KOCUREK *et al.*, 2012; YRJALA *et al.*, 1994). On the other hand, in *Pseudomonas putida* PaW85, *Pseudomonas* sp. KL28, *Comamonas testosteroni* TA 441 and *Alcaligenes eutrophus* CH34 catechol dioxygenases are found to be encoded chromosomally (ARAI *et al.*, 2000; JEONG *et al.*, 2003). In versatile aromatic degrader *Cupriavidus necator* JMP134 these genes are more or less equally distributed between chromosomes 1 and 2 indicating probable evolution from ancestral plasmids (LYKIDIS *et al.*, 2010). Although both C23O and C12O catechol dioxygenases have been identified in members of all 7 different genera that TN isolates belonged to using PCR screening approach we were able to detect and confirm only three catechol dioxygenases amongst TN isolates indicating that these aromatic-degrading isolates may possess catechol dioxygenase genes with low sequence homology with those previously published.

PCR based detection of NAPH and TOD dioxygenases

With limited number of catechol dioxygenases detected amongst TN strains, we have expanded the diversity assessment to enzymes belonging to upper aromatic aerobic degradation pathway and included naphthalene and toluene dioxygenase (NAPH and TOD, Fig. 1). Both of these enzymes are versatile biocatalysts (BOYD *et al.*, 2001; BOYD *et al.*, 2006; NOLAN and O'CONNOR, 2008).

Naphthalene dioxygenase specific primers used in this study (Table 1) target the N.2.A subfamily of naphthalene dioxygenases with high sequence identity to *nahAc* from *Pseudomonas putida* G7 (GeneBank accession number M83949) and were expected to amplify fragment of 377 bp (BALDWIN *et al.*, 2003). As expected these primers did not amplify any fragments in three control *Pseudomonas* strains (Fig. 5A), while under tested reaction conditions discrete PCR fragments of 800 bp, 600 bp, 300 bp were amplified using gDNA from *Gordonia* sp. TN108, *Bacillus* sp. TN109, *Pseudomonas* sp. TN301 respectively. In addition, two PCR fragments of 200 bp and 600 bp were amplified using gDNA from *Acinetobacter* sp. TN302 (Fig. 5A). Sequencing of these fragments revealed unspecific binding of these primers with none of the fragments exhibiting any similarity to known naphthalene dioxygenases. For example the discrete fragment obtained when gDNA from *Bacillus* sp. TN109 was used as template showed 98% homology with surfactin synthetase from *Bacillus subtilis* (data not shown). Naphthalene degradation in *Bacillus thermoleovorans* Hamburg 2 strain with dioxygenation at the 2,3 position was reported previously which differs from the usual pathway of naphthalene degradation in mesophilic bacteria (ANNWEILER *et al.*, 2000). Indeed, NDOs were shown to have a relaxed substrate specificity and could catalyze the dioxygenation of many related 2- and 3-ring aromatic and hydroaromatic (benzocyclic) compounds to their respective cis-diols (BOYD *et al.*, 2001).

The genes encoding the enzymes required for the catabolism of naphthalene are often carried on plasmids, such as the NAH plasmids (YEN and GUNSALUS, 1982). However, some organisms, such as *Pseudomonas stutzeri* AN10, carry these genes on their chromosome (BOSCH *et al.*, 1999).

On the contrary, the expected PCR fragment size of 757 bp was successfully amplified in 10 out of 19 TN isolates, and was not amplified in the three control *Pseudomonas* strains (Fig. 5B). Sequencing of these fragments[#] revealed high 87-99% homology with known *todC1* gene from *Pseudomonas putida* F1 strain (GenBank accession number J04996; ZYLSTRA and GIBSON, 1989). TOD can also be encoded chromosomally such as in *P. putida* F1 and *P. mendocina* KR1, while it is often found on toluene degradation plasmids (HOAKI and SUZUKI, 2001; ROMINE *et al.*, 1999). From our data of highly conserved sequences of TOD genes amongst TN isolates, events of horizontal gene transfer via plasmid could be anticipated.

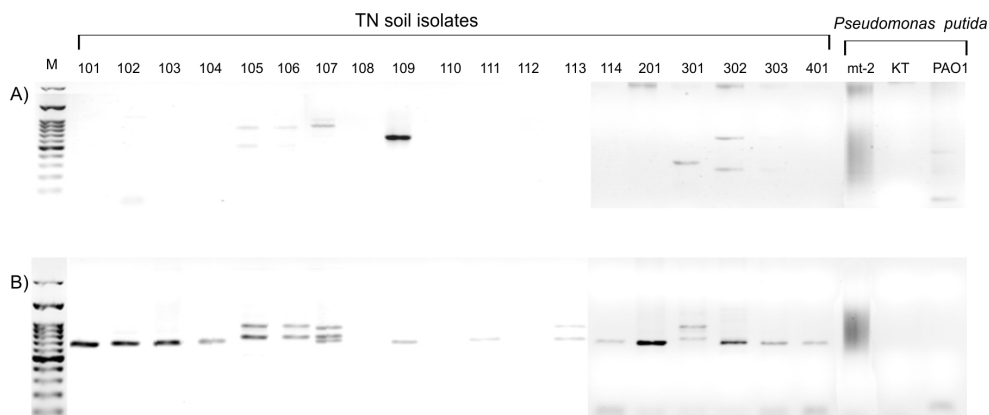


Figure 5. PCR screen for A) naphthalene dioxygenase (NAPH; 377 bp) and B) toluene dioxygenase (TOD; 757 bp) in 19 environmental isolates and three *Pseudomonas putida* strains. M; 100 bp Nippon Genetics H3 RTU DNA ladder with the heaviest band of 500 bp.

In this study, we have tried to isolate the novel genes from the aromatic degradation pathway from the microbial strains originating from the contaminated environment with a view of diversifying biocatalysis toolbox. However, we were able to confirm limited diversity of the targeted genes such as TAUT and TDO, suggesting association of these genes to catabolic plasmids and the sharing of this information amongst the strains. Considering that the large number of aromatic compounds catabolic genes is plasmid associated, the level of their diversity and evolution are under strong influence of the selective pressure such as the presence of certain pollutants in the environment (ANDREONI and GIANFREDA, 2007). Exposure of natural microbial populations to aromatic hydrocarbons may impose a selective advantage to strains possessing plasmids encoding enzymes for hydrocarbon catabolism, resulting in an overall increase in the plasmid frequency in the community (LEAHY and COLWELL, 1990). On the other hand, inability to detect NAPH genes using degenerate primers may indicate that there is still potential

variability in genes responsible for naphthalene biodegradation amongst versatile aromatic degraders isolated from the contaminated environment and employed in this study. Indeed, as with other areas of microbial ecology, the emergent technology of high-throughput pyrosequencing and metagenomic approach have the potential to dramatically impact the study of aromatic biodegradation and to reveal gene clusters with low similarity to homologous genes from already described organisms and thus to result in novel biocatalysts.

Selection of obtained sequences from this study is supplied as on-line Supplemental Information.

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**OGRANIČEN DIVERZITET GENA IZ PUTEVA RAZGRADNJE AROMATIČNIH
JEDINJENJA MEĐU ZEMLJIŠNIM BAKTERIJSKIM IZOLATIMA SA
SPOSOBNOŠĆU RAZGRADNJE AROMATIČNIH JEDINJENJA**

Gordana MINOVSKA, Tanja NARANČIĆ, Mina MANDIĆ, Lidija ŠENEROVIĆ, Branka
VASILJEVIĆ and Jasmina NIKODINOVIĆ-RUNIĆ

Institut za molekularnu genetiku i genetičko inženjerstvo, Univerzitet u Beogradu, Beograd,
Srbija

Izvod

Identifikacija i karakterizacija novih gena koji pripadaju putevima mikrobiološke razgradnje aromatičnih jedinjenja je od velikog značaja, jer su se pokazali kao izuzetno dobri biokatalizatori. U ovoj studiji, korišćenjem PCR metodologije, analizirano je prisustvo pet različitih gena iz biodegradativnog puta aromatičnih jedinjenja među 19 sredinskih izolata sa sposobnošću razgradnje širokog spektra aromatičnih jedinjenja. U slučaju 4-oksalokrotonat tautomeraze i toluen dioksidogeneze, koji su detektovani kod većine sredinskih izolata, sekvence fragmenata su ukazivale na veoma ograničen diverzitet ova dva gena i visoku homologiju sa već poznatim sekvencama opisanim kod vrsta roda *Pseudomonas*. Korišćenjem degenerisanih prajmera konstruisanih na osnovu poznatih katehol- i naftalendioksidogenaznih gena vrlo mali broj fragmenata je amplifikovan kod sredinskih izolata. Samo dve katehol 2,3-dioksidogeneze iz dva izolata roda *Bacillus* su sekvenciranjem ukazale na različitost u odnosu na poznate sekvence, a pokazale međusobnu sličnost od 80-90%. Potencijalno tri nove katehol 1,2-dioksidogeneze su identifikovane kod *Bacillus* sp. TN102, *Gordonia* sp. TN103 i *Rhodococcus* sp. TN112. Visok stepen homologije tautomeraza i toluen dioksidogeneza među sredinskim izolatima izolovanim iz zagađene sredine ukazuje na horizontalni transfer gena, dok je ograničen uspeh u detektovanju preostala tri gena ukazao na potencijal da se među ovim izolatima mogu naći nove varijante gena iz puteva razgradnje aromatičnih jedinjenja.

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