2017, Vol. 66, No 2, 189–199

ORIGINAL PAPER

Gene Expression during BTEX Biodegradation by a Microbial Consortium Acclimatized to Unleaded Gasoline and a *Pseudomonas putida* Strain (HM346961) Isolated from It

JESÚS A. MORLETT CHÁVEZ[1, 2], JORGE Á. ASCACIO MARTÍNEZ[2], WILLIAM E. HASKINS[3–5], KARIM ACUÑA ASKAR[6] and HUGO A. BARRERA SALDAÑA[1]*

[1] Laboratory of Genomics and Bioinformatics, Autonomous University of Nuevo Leon, Monterrey Nuevo León, Mexico
[2] Laboratory of Biotechnology, Department of Biochemistry and Molecular Medicine, Faculty of Medicine, Autonomous University of Nuevo Leon, Monterrey Nuevo León, Mexico
[3] Departments of Biology and Chemistry, University of Texas at San Antonio, San Antonio, TX, USA
[4] RCMI Proteomics, University of Texas at San Antonio, San Antonio, TX, USA
[5] Protein Biomarkers Cores, University of Texas at San Antonio, San Antonio, TX, USA
[6] Laboratory of Environmental Bioremediation, Department of Microbiology, Faculty of Medicine, Autonomous University of Nuevo Leon, Monterrey Nuevo León, Mexico

Submitted 21 September 2016, revised and accepted 6 December 2016

Abstract

Pseudomonas putida strain (HM346961) was isolated from a consortium of bacteria acclimatized to unleaded gasoline-contaminated water. The consortium can efficiently remove benzene, toluene, ethylbenzene and xylene (BTEX) isomers, and a similar capability was observed with the *P. putida* strain. Proteome of this strain showed certain similarities with that of other strains exposed to the hydrocarbon compounds. Furthermore, the toluene di-oxygenase (*tod*) gene was up-regulated in *P. putida* strain when exposed to toluene, ethylbenzene, xylene, and BTEX. In contrast, the *tod* gene of *P. putida* F1 (ATCC 700007) was up-regulated only in the presence of toluene and BTEX. Several differences in the nucleotide and protein sequences of these two *tod* genes were observed. This suggests that *tod* up-regulation in *P. putida* strain may partially explain their great capacity to remove aromatic compounds, relative to *P. putida* F1. Therefore, new *tod* and *P. putida* strain are promising for various environmental applications.

Key words: *Pseudomonas* spp. BTEX, dioxygenases, LC/MS/MS, bioremediation, biodegradation

Introduction

Benzene, toluene, ethylbenzene and xylene (BTEX) isomers are aromatic hydrocarbons that constitute the major components of gasoline (Potter, 1992). The irresponsible use of these compounds and their release into soil and water causes considerable damage to the environment (Díaz *et al*., 2001; Lawniczak *et al*., 2011). In comparison to other gasoline hydrocarbons, these compounds are soluble in water and have genotoxic properties (Dean, 1985; Tsao *et al*., 1998), although under favorable conditions, BTEX is biodegradable by a wide variety of microorganisms (Gibson and Subramanian, 1984; Lawniczak *et al*., 2011; Lisiecki *et al*., 2014). The scientific community is increasingly interested in these microorganisms and their genomes for developing biotechnological processes that remove aromatic compounds (Pieper and Reineke, 2000; Díaz *et al*., 2001; Owsianiak *et al*., 2009).

Numerous bacterial species have been discovered that can metabolize chemical components found in gasoline (Cyplik *et al*., 2011; Dalvi *et al*., 2014). Recently, we reported that a consortium acclimatized to unleaded gasoline degraded 95% of total BTEX and *Pseudomonas*, *Shewanella*, *Burkholderia*, *Alcanivorax*, *Rhodococcus* and *Bacillus*, were identified by 16S rDNA. While, *Pseudomonas putida* strain, isolated from that consortium, was capable of removing 90% of total BTEX (Morlett-Chávez *et al*., 2010). Moreover, bacteria from the genus *Rhodococcus* have the ability to grow under a wide variety of xenobiotic compounds including aliphatic and aromatic hydrocarbons (Kim *et al*., 2004). Other microorganisms that can remove aromatics are *P. putida*-DOT T1E and *Pseudomonas mendocina*-KR1

* Corresponding author: H.A. Barrera Saldaña, Laboratory of Genomics and Bioinformatics, Autonomous University of Nuevo Leon, Monterrey Nuevo León, Mexico; **e-mail: habarrera@gmail.com**

that utilize toluene (Ramos-Gonzales *et al*., 2003); *Pseudomonas fluorescens*-CA-4 utilizes ethylbenzene (Díaz *et al*., 2001); and *P. putida* F1 utilizes benzene, toluene and ethylbenzene (Zylstra and Gibson, 1989). The ability to remove BTEX by microorganisms is attributed to the expression of proteins capable of oxidizing and cleaving aromatic-rings; these enzymes are known as dioxygenases (Tarasev *et al*., 2007).

Several studies based on proteins or mRNAs have been employed to elucidate the pathway followed by microorganisms during aromatic compounds biodegradation. The knowledge of genomic and biochemical basis of proteins related with biodegradation could improve degradation efficiency and use them as substrate hydrocarbons broad range (Sabirova *et al*., 2006). The principal main of this study was use semi-quantitative proteomic analysis to investigate the catabolic potential of the new bacterial *P. putida* strain, isolated from an enriched consortium of microorganisms acclimatized to unleaded gasoline and capable of removing BTEX, in the presence and absence of these aromatic compounds. In order to confirm our results, the *bed* and *tod* genes from the new bacterium were amplified by PCR, their mRNAs were quantified by RT-PCR, and their nucleotide sequences were obtained from the amplified products.

Experimental

Material and Methods

Reagents. BTEX isomers (o-,m-,p-) were purchased from Sigma-Aldrich Química (Monterrey, NL, México). Bacteriological agar (BIOXON, Becton-Dickinson, Monterrey, NL, Mexico), mineral medium reagents and other chemicals used in this study were reagent grade or better and purchased from either Sigma-Aldrich Química (Monterrey, NL, México) or CTR Scientific (Monterrey, NL, México).

Culture enrichment. The consortium used in this study was obtained from an acclimatized biomass and kept according to Morlett-Chávez *et al.* (2010). The *P. putida* strain was isolated from this consortium, was fed weekly with 50 mg l^{-1} of BTEX, and maintained in the conditions mentioned above.

Bioassays with BTEX. Bioassays with BTEX at 50 mg l^{-1} were prepared as suggested Acuna-Askar *et al.* (2006) and Morlett-Chávez *et al.* (2010). As final concentration of cell suspension 3×10^{10} cell/ml. were used All bioassays were incubated at 250 rpm at 36 ± 2°C and substrate concentrations were monitored at 0, 8, 16, 24 and 32 h. Three replicates were run for each set of bioassays to evaluate substrate biodegradation kinetics.

Bioassays with separate BTEX substrates. Bioassays to test the degradability of individual BTEX chemicals were run as mention above (Acuna-Askar *et al*., 2006; Morlett-Chávez *et al*., 2010).

Chemical analysis. BTEX concentrations were analyzed by a Varian 3400 GC/FID chromatograph. A Petrocol™ (Supelco, Bellefonte, PA) 100 m × 0.25 mm ID × 0.5 µm film DH fused silica GC capillary column was used. Column, injector and detector conditions were held as reported in a prior study (Acuna-Askar *et al*., 2006). Results were analyzed and statistically evaluated using the computer software SigmaPlot® 10.0 U (Cincinatti, OH, USA)(Standard Methods 1998).

Differential proteomic analysis

Sample preparation and SDS-PAGE. Consortium and *P. putida* strain biomass were recovered from culture by centrifugation (5000 rpm 10 min^{-1}) at 0, 8, 16, 24, 32 h, during BTEX biodegradation. A quantity of 100 mg (wet weight) of this biomass and 1 ml of buffer were added for protein extraction (B-PER- Bacterial Protein Extraction Reagent) per the manufacturer's instructions (Pierce, Rockford IL, USA). The samples were kept at 4°C and a protease inhibitor cocktail was added. Afterwards, cell lysis was performed by sonication (Sonicor-ultrasonic processor Up 400 a, Copiague N.Y. USA) with six cycles (75 W × 30 seg) and intervals of 30 seconds each. The sonicated residue was centrifuged at 14,000 rpm × 30 min at 4°C to remove cellular debris, the rest of protocol was run as described by Sabirova *et al.* (2010).

Protein identification. Protein identification was run as previously described by Morlett-Chávez *et al.* (2010) and Dalvi *et al.* (2014).

Genetic studies. To confirm our proteomic results and unambiguously identify the enzymes associated with biodegradation, the following genetic studies were performed for the *bed* and *tod* genes analyzed.

Amplification of the catabolic *bed* and *tod* genes. Three vials that contained 1 ml of the consortium, *P. putida* strain, and *P. putida* F1, respectively, were centrifuged at 3,000 rpm × 5 min to obtain a pellet, and the supernatant was discarded. Genomic DNA was extracted from each pellet with the DNAeasy kit (Qiagen, GMBH, Hilden, Germany). Also, *E. coli* DNA was extracted to use it as a negative control for the amplification reaction. Using the extracted DNA, *bed* and *tod* genes were amplified by PCR, employing primers described in Table I, and PCR was carried out with an initial denaturing step for 5 min at 94°C followed by 35 cycles at 94°C for 45 s, *bed* 54°C and *tod* 60°C both for 45 s and 72°C elongation for 45 s, followed by a final elongation at 72°C for 5 min. The amplicons were revolved in 1% agarose gels, stained with ethidium bromide (0.5 g ml^{-1}) and detected in

Table I
Primers for amplifying bed and tod genes.

	Name	Code	Sequence
Primers	Bed	Fwdbzm	5' GAAGGGGACGTAGAATCATG
		Rvbzm	3' GCTAACGATTGCGTCTTGA
	Tod	Fwdtolm	5' TGAAAAGTGAGAAGACAATG
		Rvtolm	3' GATTCAGAGTGTCGCCTTCA

a photo-documenter (UVP Inc, Upland, CA, USA). The amplicons were purified using the Wizard SV Genomic DNA Purification System kit (Promega, Madison WI, USA). Sequences and bioinformatics analysis were ran in accordance with Morlett-Chávez *et al*. (2010).

Reverse Transcriptase/Polymerase Chain Reaction. Total RNA was extracted from the consortium, *P. putida* strain and *P. putida* F1, grown in the presence of BTEX as a mixture and from its individual components. The RNA from microorganisms was isolated during the exponential phase ($DO_{600nm} = 1.5$) using trizol and following the manufacturer's protocol (Invitrogen, Carlsbard California, USA). Finally, this was treated with DNAse for 30 min at 37°C (Invitrogen) to eliminate traces of genomic DNA. In addition, total RNA was extracted from *E. coli* (grown in the presence of glucose) to use it as negative control in gene expression analyses. One microgram of total RNA was used as a template for reverse-transcription into complementary DNA (cDNA) using the Reverse AidH Minus First Strand cDNA synthesis kit (Fermentas, ST. Leon-Rot, Germany), and *bed*-reverse and *tod*-reverse oligonucleotides as primers. The program was of 94°C × 5 min; 94°C × 45 sec, 60°C × 45 sec and 72°C × 45 sec; the final extension was 72°C × 10 min. The cDNAs from the consortium, *P. putida* strain and *P. putida* F1 were used as templates for studying *bed* and *tod* gene expression. The genomic and cDNA from *P. putida* F1 were amplified as a positive control, while total RNA was used as a negative control. Also, 16S RNA was retro-transcribed and used as an internal control for verification of cDNA synthesis and amplification.

Results

BTEX biodegradation kinetics. The enriched consortium showed better kinetics to remove these compounds, followed by *P. putida* strain and the reference strain (*P. putida* F1). Our results showed that *P. putida* strain had removed 50% of the compounds in the first 16 h and 85% at 32 h; however, the consortium had already removed more than 95% in 32 h and ~75% at 16 h, while the reference strain removed ~80% in 32 h and less than 25% at 16 h. An important detail to consider is that *P. putida* strain had a slow lag phase (8 h) in comparison to the consortium (5 h) (Fig. 1).

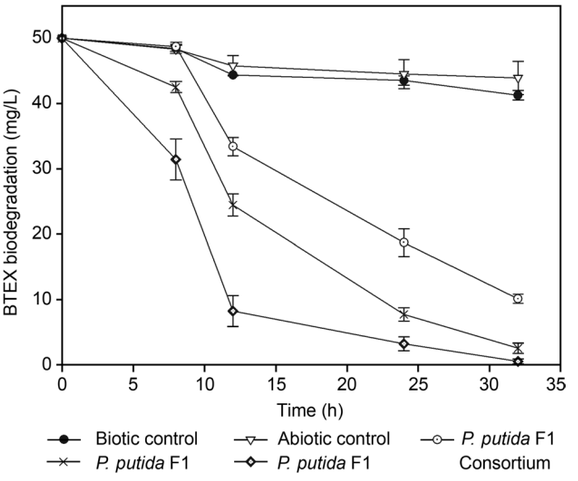

Fig. 1. Removal kinetics of BTEX-mixture (50 mg l^{-1}) by consortium and *P. putida* strain.

Bioassays were set with a BTEX mixture having 50 mg l^{-1} of each chemical. All bioassays were shaken at 45 × g, at a temperature of 36 ± 2°C and monitored for substrate concentrations at 0, 8, 16, 24 and 32 h. Results indicate the *P. putida* strain exposed to ethylbenzene show mayor degradation (97.7%), followed by benzene (94.8%), toluene (94.8%) and xylene (87.8%).

Kinetic results obtained from biodegradation experiments with *P. putida* strain and the consortium exposed to the BTEX or to its individual chemical components are shown in Fig. 2. These results indicated that the *P. putida* strain primarily biodegraded ethylbenzene (97.7%), followed by benzene (94.8%), toluene (90.8%) and xylenes (87.8%).

Differential proteomic analysis

SDS-PAGE. Comparison of SDS-PAGE semi-quantitative results allowed us to identify putative peptide bands that may possibly be related to the aromatic compounds biodegradation. The proteins were extracted from *P. putida* strain and the consortium exposed to BTEX at multiple time points. As expected, the protein banding pattern and the protein abundance within particular bands changed during the time course of BTEX biodegradation. In Fig. 3a, four conspicuous bands with different molecular weights were observed: band 1 (~ 60 kDa), band 2 (~ 48 kDa), band 3 (~ 22 kDa), and band 4 (~ 19 kDa). These bands were activated at the 8th hour. At the 16th and 24th hours, several proteins showed differences in the molecular weight with respect to the proteins mentioned above: band 5 (~ 45 kDa), band 6 (~ 35 kDa), and band 7 (~ 23 kDa). SDS-PAGDE also showed that the consortium and *P. putida* strain expressed different proteins when exposed to glucose.

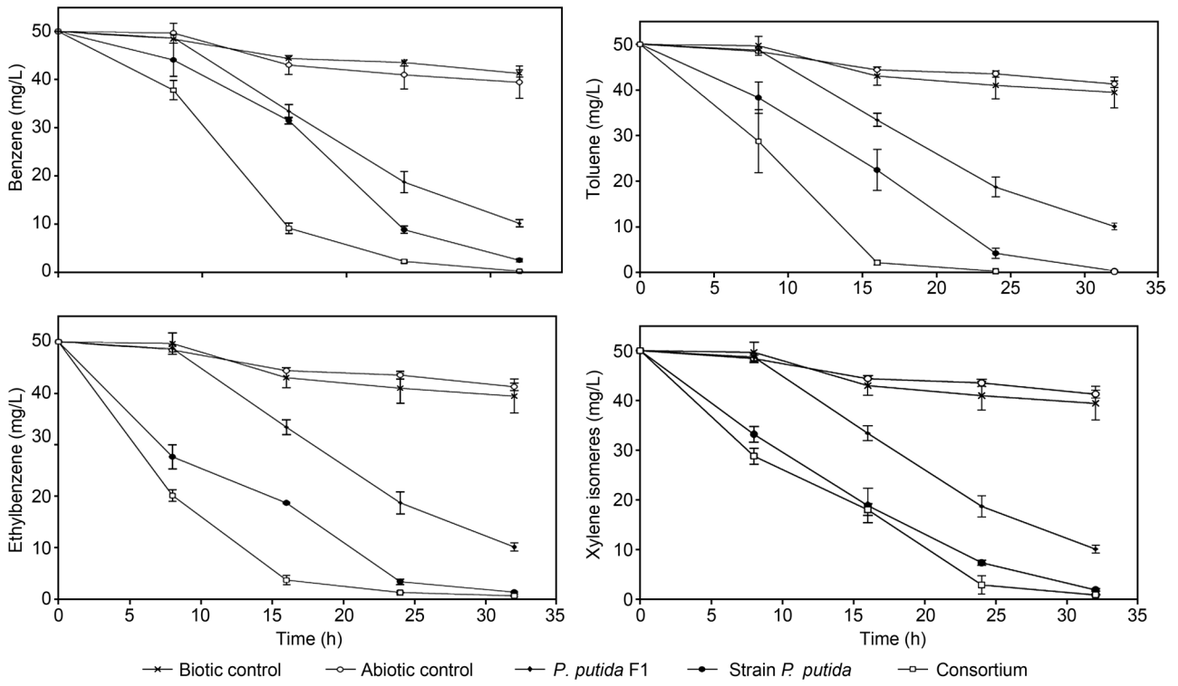

Fig. 2. Kinetics of BTEX-separate biodegradation by consortium and *P. putida* strain.

One set for each individual BTEX chemical at the initial concentration of 50 mg l^{-1} was run with each of the following three cultures: a) consortium, b) *P. putida* strain and c) *P. putida* F1. Similarly, all bioassays were shaken at 45 × g, at a temperature of 36 ± 2°C and monitored for substrate concentrations at 0, 8, 16, 24 and 32 h. Three replicates were also run for each set of samples.

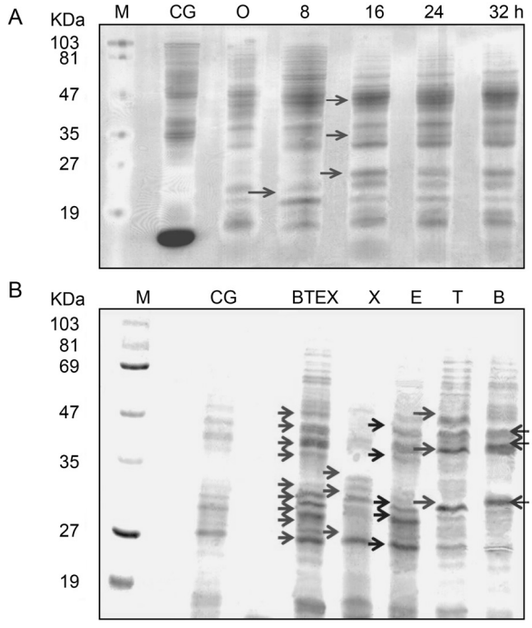

Fig. 3. Protein profile of *P. putida* strain exposed to the BTEX-mixture.

Protein bands for *P. putida* strain bioassays with and without BTEX (using only glucose as source of carbon) were resolved by SDS-PAGE and stained with Coomassie bright blue. A) Cells were collected at indicated different times of cultivation and proteins were recovered and resolved by SDS-PAGE. B) Protein expression profile of *P. putida* strain exposed to BTEX-mixture and -separate. Samples were collected at 24 h. As a control, a *P. putida* strain fed with glucose (CG).

In another experiment, the protein profile of *P. putida* strain and the consortium when exposed to the BTEX components individually were compared. Figure 3b shows proteins differentially expressed in the presence of the individual BTEX chemical components: the more abundant bands were observed in the cells exposed to benzene: 1) ~ 43 kDa, 2) ~ 40 kDa, and 3) ~ 30 kDa; in toluene: 1) ~ 48 kDa, 2) ~ 38 kDa, and 3) ~ 29 kDa; in ethylbenzene: 1) ~ 43 kDa, 2) ~ 29 kDa, 3) ~ 28 kDa, and 4) ~ 27 kDa; and in xylene: 1) ~ 33 kDa, ~ 31 kDa, and ~ 28 kDa. Similar results were observed in the analyses of proteins from the consortium (Fig. 4a and 4b).

Capillary LC/MS/MS with protein database searching. Various peptides revealed by time and carbon source *via* SDS-PAGE (Fig. 3a and 3b), were identified by capillary LC/MS/MS and protein database searching. These include: cytochrome C (35,526 Da); transcriptional regulator Lys R (33,602 Da); hydrogenase Fe-S (19,306 kDa); ferrodoxine, union dominion Fe-S (12,458 Da); NADH dehydrogenase (18,308 Da); 2,3 catecol-dioxygenase (32,189 Da); 4- oxalocrotonate decarboxylase; polihydroxyalconato depolymerase; tioredoxina-disulphure reductase (38,518 Da); formate dehydrogenase (21,699 Da); and *tod*F hydratase (23,902 Da) (Table II). All of these proteins are involved in the metabolism of aromatic/aliphatic compounds.

Genetic studies. The dioxygenases are enzymes involved in oxidative hydroxylation, the initial step of

Table II
PCR conditions to amplify bed and tod genes and PCR cycles.

PCR conditions		PCR Cycles			
DNA (100 ng)	1 µl		Time	Temperature	Cycle
Buffer 10 × PCR	2 µl	Initial denatured	5 min	94°C	1 X
MgCl2	2 µl	Denatured	45 sec	94°C	35 X
dNTPs (10 mM)	0.5 µl	Annealing	45 sec	*Bed* 54°C	
Primers 5' (10 mM)	0.4 µl	Extension	45 sec	72°C	
Primers 3' (10 mM)	0.4 µl	Extension	10 min	72°C	1 X
Taq DNA polymerase	0.2 µl				
Sterile Water	14.5 µl				
Total Volume	20 µl				

aromatic compound biodegradation. Proteomic analysis revealed novel proteins, in addition to dioxygenases, that are also involved in BTEX removal. To confirm that the dioxygenase genes are strongly expressed in response to BTEX, we amplified the α-subunit of the *bed* and *tod* genes involved in the catabolism of benzene and toluene, respectively, and evaluated their expression by RT-PCR.

Amplification of *bed* and *tod* genes. Genomic DNA was extracted from: a) consortium, b) *P. putida* strain, c) *P. putida* F1, and d) *E. coli* DH5α, and used them as templates for PCR, *bed* and *tod* genes were amplified. Results show an amplification of 1400 pb corresponding to the *bed* gene, and 1353 pb for *tod*. In both cases the genes were amplified from the cultures mention above, except for the one corresponding to *E. coli*. The amplicons were directly extracted from the agarose gel, purified and sequenced. Nucleotide sequences obtained were compared with the sequences reported in GenBank (genes *tod* and *bed* from *P. putida* F1). The *bed* gen of *P. putida* strain resulted similar in 98% while the *bed* gen of *P. putida* F1 was similar in 99%, with regard to the *bed* gen from *P. putida* F1-GenBank. Interestingly, the *tod* gene of *P. putida* strain was similar in 90%, while the *tod* gene of *P. putida* F1 showed 99% similarity, with respect to the *tod* gen from *P. putida* F1-GenBank (Fig. 5a). The virtual translation of the *tod* gen of the *P. putida* strain suggests that it encodes a protein with several amino acids changes including amino acids in the enzyme's catalytic site. Also, by comparing the amino acid sequence of Tod, the *P. putida* strain protein is 95% similar to the one of *P. putida* F1 Tod in the GenBank (Fig. 5b).

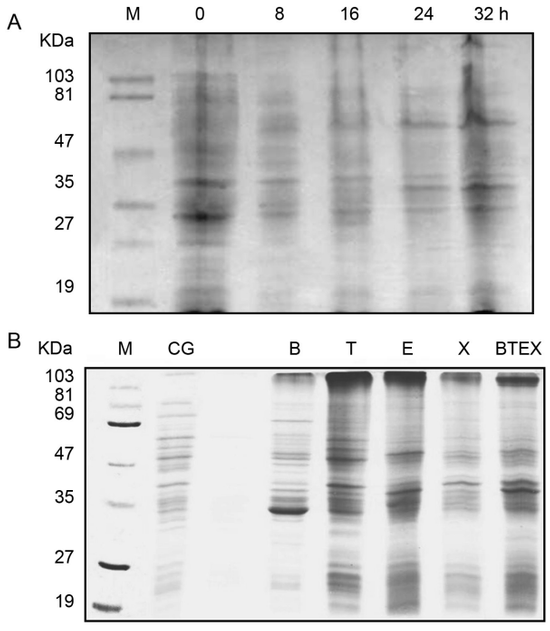

Fig. 4. Consortium proteins expressed in the presence of BTEX. Protein profile for consortium bioassays with and without BTEX (using only glucose as source of carbon) were resolved by SDS-PAGE and stained with Coomassie bright blue. A) Protein expression profile of the consortium exposed to BTEX and the cells were collected at different points of cultivation. B) Protein expression profile of the consortium using BTEX-separate and -mixture as the only source of carbon. Samples were collected at 24 h and 1.5 D.O. ($D.O._{600\ nm}$). The control was a consortium growth in glucose (CG).

RT-PCR of *tod* gene. The dioxygenase enzymes catalyze the first reaction during BTEX degradation; these proteins were strongly observed by proteomic analysis. In this context, the expression of the genes *bed* and *tod* was analyzed in the consortium and the *P. putida* strain grown in the presence of BTEX and with its individual components. To achieve this, cells were collected during the exponential phase and their total RNA was isolated. By RT-PCR *bedα* and *todα* cDNAs were amplified. Fig. 6 shows the results for *todα*, which indicates that its gene was expressed in *P. putida* strain exposed to BTEX, toluene, ethylbenzene and xylene, but not in the presence of benzene. Similar results were observed for the consortium. Table III summarizes these results. Interestingly, in *P. putida* F1, *todα* gene expression was induced only in the presence of BTEX

```
Query  1        TCAGCGTGTCGCCTTCAGCGCGTCCCAGTCGGGGGATGTCATCATCCGCAGCCAATGGGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271737  TCAGCGTGTCGCCTTCAGCGCGTCCCAGTCGGGGGATGTCATCATCCGCAGCCAATGGGC

Query  61       ATAGAGCCCGCGGGCAGCTTCCTCGCTGTAGACGTTGTTGCTGATCCGCCCGGGGTAAAC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271797  ATAGAGCCCGCGGGCAGCTTCCTCGCTGTAGACGTTGTTGCTGATCCGCCCGGGGTAAAC

Query  121      CGGGTCGTTGTCGACGGTCTGGTCCATGCTCATCTCGGCATTGAAAGGGCGGCTCCGCGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271857  CGGGTCGTTGTCGACGGTCTGGTCCATGCTCATCTCGGCATTGAAAGGGCGGCTCCGCGC

Query  181      CTTGTGGCCTCGCAGGATGTGCTGGATCTCGACCCAGTTCTCCCCGTCGTCCTGCTCGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271917  CTTGTGGCCTCGCAGGATGTGCTGGATCTCGACCCAGTTCTCCCCGTCGTCCTGCTCGAA

Query  241      CACGCCACCGGCAGAGAAGGTGCGCAGCGTCTGGCGCCGGAACTCTTCCTTGATATCGTC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271977  CACGCCACCGGCAGAGAAGGTGCGCAGCGTCTGGCGCCGGAACTCTTCCTTGATATCGTC

Query  301      AGGAGCATCAGCATCGACCACCGTAAACGCCCATACCTCGACCTCGTTCGGCCCGCGCGG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272037  AGGAGCATCAGCATCGACCACCGTAAACGCCCATACCTCGACCTCGTTCGGCCCGCGCGG

Query  361      ATGCCATGTCCGGACCGTATTGATACCTGGGAGGAAGGAACACGTGGGGAAGACGGTCAT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272097  ATGCCATGTCCGGACCGTATTGATACCTGGGAGGAAGGAACACGTGGGGAAGACGGTCAT

Query  421      GTGCTCGACCATGAGTTTCGAGCCGCGCTCCACGCTACCCAGACGTTCGGCCGCCTTTTC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272157  GTGCTCGACCATGAGTTTCGAGCCGCGCTCCACGCTACCCAGACGTTCGGCCGCCTTTTC

Query  481      CGACGCGGGGCCTTCGGTCCAGTAGCTGGTGACCTTTGGCCCCATGATGGCAAGCATCAG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272217  CGACGCGGGGCCTTCGGTCCAGTAGCTGGTGACCTTTGGCCCCATGATGGCAAGCATCAG

Query  541      ATTGGGGTCGCCGACATAGAAGCCACTTCCATGTCCGCCCCATGACGCACGGTACTGCTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272277  ATTGGGGTCGCCGACATAGAAGCCACTTCCATGTCCGCCCCATGACGCACGGTACTGCTT

Query  601      GCCAACTGTCGGCGGAGCAAGGTCGGCCATTTCAAGGTCTTCTGGCAGGCCTGCCAGGAT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272337  GCCAACTGTCGGCGGAGCAAGGTCGGCCATTTCAAGGTCTTCTGGCAGGCCTGCCAGGAT
```

Fig. 5A. Nucleotide sequence and identity search.

The sequence obtained from the *tod* gen of *P. putida* Strain was used to search for matches in the GenBank.

```
tod          MNQTDTSPIRLRRSWNTSEIEALFDEHAGRIDPRIYTDEDLYQLELERVFARSWLLLGHE 60
FMB08        MNQTDTSPIRLRRSWNTSEIEPLFDEHAGRIDPRIYTDEDLYQLELERVFARSWLLLGHE 60
             ********************.**************************************

tod          TQIRKPGDYITTYMGEDPVVVVRQKDASIAVFLNQCRHRGMRICRADAGNAKAFTCSYHG 120
FMB08        TQIRKPGDYITTYMGEDPVVVVRQKDASIAVFLNQCRHRGMRICRADAGNAKAFTCSYHG 120
             ************************************************************

tod          WAYDTAGNLVNVPYEAESFACLNKKEWSPLKARVETYKGLIFANWDENAVDLDTYLGEAK 180
FMB08        WAYDTAGNLVNVPYEAESFACLNKKEWSPLKARVETYKGLIFANWDENAVDLDTYLGEAK 180
             ************************************************************

tod          FYMDHMLDRTEAGTEAIPGVQKWVIPCNWKFAAEQFCSDMYHAGTTSHLSGILAGLPEDL 240
FMB08        IYMDHMLDRTEPRTEAIPGVQKWVIPCNWKFAAEQFCSDIYHAGTTSHLSGILAGLPEDL 240
             :**********. ************************:********************

tod          EMADLAPPTVGKQYRASWGGHGSGFYVGDPNLMLAIMGPKVTSYWTEGPASEKAAERLGS 300
FMB08        EMADLAPPTVGKQYRASWGGHGSGFYVGDPNLRPAIMGPKVTSYWTEGPASEKAAERLGS 300
             ******************************  *************************

tod          VERGSKLMVEHMTVFPTCSFLPGINTVRTWHPRGPNEVEVWAFTVVDADAPDDIKEEFRR 360
FMB08        VERGSKLMVEHMTVFPTCSFLPGINTVRTWHPRGPNEIEVWAFTEVDADAPDDIKEELRS 360
             ************************************:****** ************:**

tod          QTLRTFSAGGVFEQDDGENWVEIQHILRGHKARSRPFNAEMSMDQTVDNDPVYPGRISNN 420
FMB08        QTLSTFSAGGVFEQDDGENWVEIQHILRGHKTRSRPFNAEMSMDQTVDNDPVYPGRISNN 420
             *** ***************************:****************************

tod          VYSEEAARGLYAHWLRMMTSPDWDALKATR 450
FMB08        VYSEEAARGLYAHWLRMMTSPDWDALKATR 450
             ******************************
```

Fig. 5B. Aminoacidic sequences of Tod-*P. putida* strain and Tod-*P. putida* F1.

The aminoacidic sequence deduced for Tod-*P. putida* strain was used to search for matches in the Swiss protein bank. Tod-*P. putida* F1 strain was found to differ only in eleven residues.

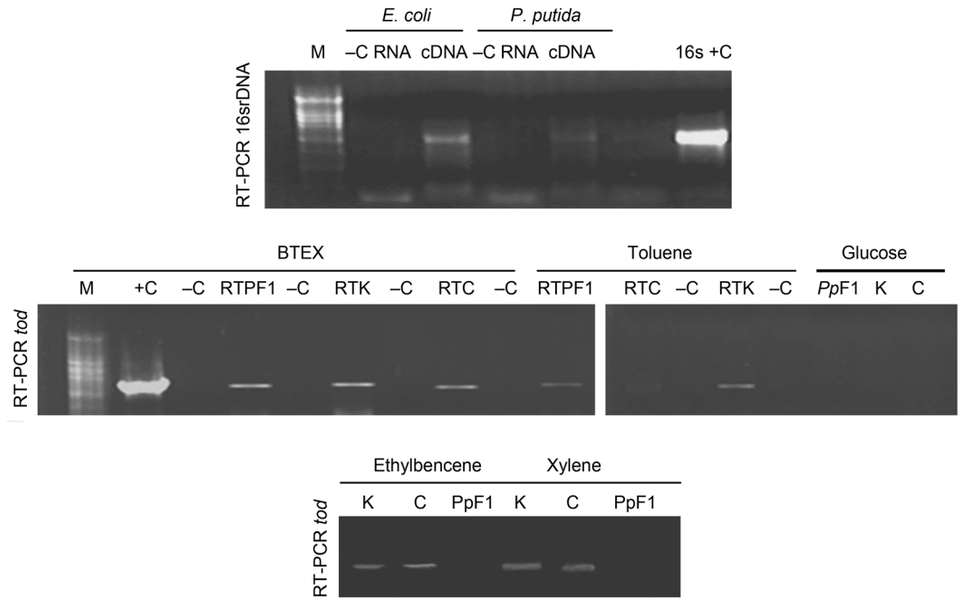

Fig. 6. Gene expression analyses of *P. putida* strain and consortium exposed to BTEX and their individual components. Total RNA was extracted from the consortium, the *P. putida* strain and *P. putida* F1 grown in the presence of BTEX as a mixture and of its individual components. Its retro-transcription yield cDNA was used as template for studying *bed* and *tod* genes expression. +C = positive control, –C = negative control (total RNA, K = consortium, C = *P. putida* strain and PpF1 = *P. putida* F1 (ATCC 700007).

or toluene. In the case of *bedα*, the gene was expressed in *P. putida* strain and the consortium exposed to BTEX and benzene (data not shown).

Table III
Conditions for cDNA formatted from total RNA of the strain FMB08, consortia, reference strain and *E. coli* DH5.

Conditions RT-PCR		
RNA (400 ng)	4 µl	65°C × 5 min
Primer 3' (200 ng µl-1)	1 µl	
dNTPs (10 mM)	1 µl	
Sterile Water	8 µl	
5X first Strand	4 µl	65°C × 2 min
DTT 0.1 M	2 µl	
M.MVLRT (200 U)	1 µl	37°C × 50 min

Inactivate 1 µl RNAase 37°C × 30 min

Discussion

Biodegradation kinetics of BTEX. In this study, we have established that the enriched consortium acclimatized to unleaded gasoline, and *P. putida* strain, are bio-degraders of aromatic compounds (50 mg l^{-1} BTEX). Results show that most of the oxidation occurs in the first 8 and 24 hours (Fig. 1). Our findings are in agreement with previous studies including our prior report (Morlett-Chávez *et al.*, 2010). *Pseudomonas* genus, have been found in BTEX contaminated sites and reported to use a different BTEX metabolic pathway (Lima-Morales *et al.*, 2016). Lee and Lee (2001) reported that *Ralstonia* sp. was capable of completely removing 3 mg l^{-1} of BTEX under aerobic conditions. These results can be explained because a specialized consortium is required to removal different aromatic compounds such as BTEX (Bell *et al.*, 2013). Furthermore, some organisms are capable of growth on the majority of the hydrocarbons, whereas others may be specialized to only a few of the substrates (Ciric *et al.*, 2010). In contrast, bacterium can rarely use multiple aromatic compounds (Gescher *et al.*, 2006). Bell *et al.* (2013); Gescher *et al.* (2006) indicated due to the high toxicity of organic contaminants, these are not completely mineralized by a single bacterium. Moreover, intermediates produced during biotransformation results more toxic than the initial compound (Lawniczak *et al.*, 2011). By last, similar results were reported by Morlett-Chávez *et al.* (2010), where the F distribution and the Tukey's statistical tests provide evidence that the consortium exhibited higher biodegradation efficiency than the FMB08 isolate.

Differential proteomic analysis

SDS-PAGE. Our strategy allowed us to distinguish several proteins up-regulated by BTEX. This strategy had also been used by other authors to identify proteins involved in aromatic compound metabolism (Demanèche *et al.*, 2004; Sabirova *et al.*, 2006; Peters

et al., 2007; Patrauchan *et al.*, 2008). Interestingly, Peters *et al.* (2007) found two protein bands of 57 and 60 kDa when *Geobacter metallireducen* was exposed to to *p*-cresol. In other work, Kim *et al.* (2004) identified α dioxygenase (~ 50 kDa) y β dioxygenase (~ 25 kDa) related to *o*-xylene degradation. Also, Maeda *et al.* (2001) reported a molecular weight of ~ 50 kDa for the enzyme biphenyl-dioxygenase α-subunit and ~ 23 kDa for subunit β and toluene dioxygenase α and β subunits at 52.5 and 21.5 kDa, respectively.

Table IV
Identified proteins of consortium and FMB08 strain exposed to mixture-BTEX.

Protein	Hydrocarbons	Found to be similar to:	Identification No.	MW (Da)	pI	Coverage %
Aromatic ring oxidation						
P1	BTEX	ABC-type oligopeptide transport system	giI84359023	59,033	8.7	40
P2	BTEX	Porine E	giI26986977	48,297	5.4	31
P3	BTEX, B	Outer membrane Protein F	giI85058985	40,061	4.5	55
P4	T	Thioredoxin-disulfide reductase	giI84516681	38,518	5.5	25
P5	T	Outer membrane protein II	giI148368	25,538	4.8	34
P6	BTEX	NADH dehydrogenase	giI113871846	18,308	8.7	97
P7	BTEX, T	OmpF porin	giI15131544	38,442	4.6	100
P8	BTEX	LrgA family protein	giI148653125	18,800	10	93
P9	B	TRAP-T family transporter	giI84385307	21,269		7
Aromatic ring cleavage						
P10	BTEX	Glyoxilase/dioxygenase	giI148548089	32,189	5.8	100
P11	BTEX	Ferredoxin, Fe-S union domain	giI149118333	12,458	4.4	99
P12	BTEX	Cytochrome C family protein	giI117921118	35, 62	8.6	100
P13	BTEX	Catechol dioxygenase	giI78063176	35,000		27
P14	BTEX, T	Formate dehydrogenase subunit B	giI34733215	21,699	6.4	99
P15	BTEX, T	Mot/TolQ/ExbB	giI119774393	19,762	8.9	95
P16	BTEX, T	Fe-S-cluster containing hydrogenase	giI77973841	19,306	8.4	100
P17	BTEX, T	TodF Hydratase	giI135977	23,902	4.7	15
P18	BTEX, X	Transcriptional regulator LysR	giI126990316	33,780	6.9	16
P19	BTEX, E	hypothetical protein ebD82	giI56477892	7,688	4.7	100
P20	BTEX	4Fe-4S cluster binding	giI26250216	17,594	8.1	96
Protein in the fatty acids catabolism						
P21	BTEX	Polyhydroxyalconate depolymerase	giI73538528	46, 97	7.9	100
P22	B	C4-dicarboxylate transport system	giI149187948	21,274	9.0	7
P23	BTEX	4 oxalocronate decarboxylase	giI148548088	28,222	4.9	5
Other proteins identified						
P24	BTEX	BB2842 hypothetical protein	giI33601818	11,724	5.6	100
P25	BTEX	Hypothetic protein OB2597	giI84503169	10,824	6.3	100
P26	BTEX	Hypothetic protein ED21	giI149186419	8,154	4.4	100
P27	BTEX	Hypothetic protein c2B002	giI56476484	9,822		100
P28	BTEX	Conserved hypothetic protein	giI121531209	8,857	6.7	100
P29	BTEX	Hypothetic protein azo 1735	giI119898026	12,147	4.5	100
P30	BTEX	Hypothetical protein Aave1976 protein	giI120610656	12, 662	5.7	100
P31	BTEX, E	Enlongation factor Tu	giI2886756	43,337		8
P32	B	Translation elongation factor of translation	giI96718	43,324	5.3	10
P33	BTEX	Hypothetic protein BammMC	giI118700962	28,692	11.5	3
P34	X	Tryptophan synthetase	giI464911	28,422	5.1	3
P35	BTEX, B, E	50s Ribosomal protein (L1)	giI26987185	24,236	9.5	12
P36	BTEX, B	Hypothetic protein RF_034	giI67458826	21,986	5.0	6
P37	BTEX	Electron transport complex protein Rnfb	giI59711541	20,523	4.5	100
P38	B	putative sulfate transport protein	giI145627930	10,698	6.5	100

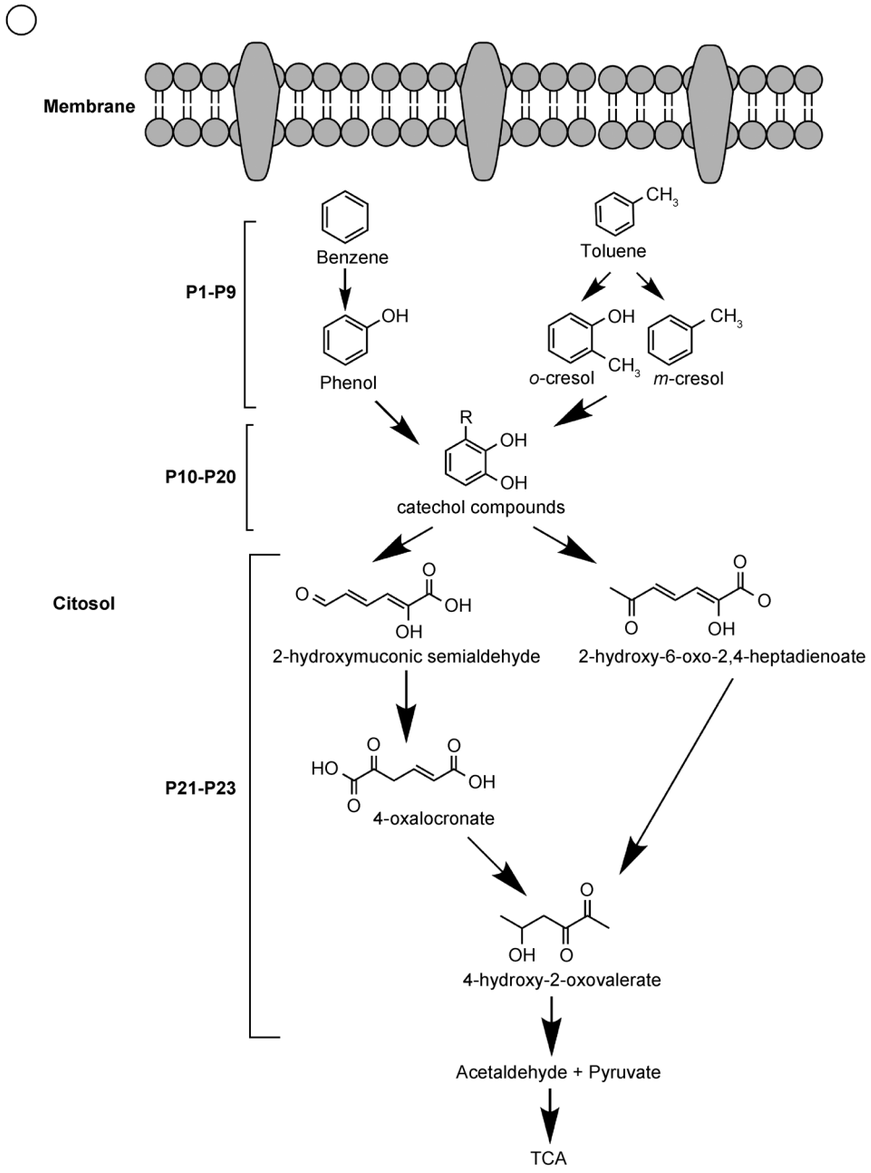

Fig. 7. BTEX catabolism in *P. putida* strain.

Capillary LC/MS/MS and protein database searching. All of the proteins identified by capillary LC/MS/MS and protein database searching are involved in the metabolism of hydrocarbon aromatic/aliphatic compounds. These can be classified by function and subcellular localization as membrane proteins, proteins involved in aromatic ring oxidation and cleavage, and proteins involved in fatty acid catabolism (Fig. 7).

Membrane proteins. The identified membrane proteins are thioredoxin-disulfide reductase (38,518 Da), and formate dehydrogenase (21.6 kDa). In *E. coli* this protein is a membrane protein that uses formate as an electron donor in reduction of nitrate to nitrites (Kane *et al*., 2007). Other membrane proteins identified in the study are: Lrga (18.8 kDa), transport systems ABC (59 kDa), membrane protein F (40 kDa), porin (36 kDa), permease (21 kDa) and membrane proteins II (25 kDa), all of these had been reported previously by Sabirova *et al.* (2006) and Peters *et al.* (2007).

Aromatic ring oxidation. Is well known that BTEX compounds, after penetrating the cell membrane of microorganisms, receive an initial hydroxylation by mono- or dioxygenases. These proteins are formed by a) flavoprotein reductase, b) ferredoxine, and c) the ISP presents α- and β-subunits with the former acting as the catalytic site (Bagnéris *et al*., 2005; Witzig *et al*., 2006, Szczepaniak *et al*., 2016). During initial hydroxylation catechol or protocatechuate is produced and

those products are identified as central intermediate products. On that subject, we identified a) hydrogenase Fe-S (19.3 kDa), b) Ferredoxin-dominion of union Fe-S (12.4 kDa), and c) NADH dehydrogenase (18.3 kDa) and monooxygenases (cytochrome P450). Similar results were reported by Dalvi *et al.* (2014); Patrauchan *et al.* (2008) and Sabirova *et al.* (2006). Prior, Fong *et al.* (1996) reported the presence of a protein (39 kDa) identified as a dehydrogenase NAD^+ dependent, necessary to convert benzene into catechol.

Aromatic ring cleavage. Following with the metabolic pathway of aromatic compounds, central intermediate products are the substrate for ring cleavage mediated by dioxygenases. We identified catechol 2,3-dioxygenase (32.1 kDa); and its transcriptional regulator known as Lys R (33.2 kDa). Lima-Morales *et al.* (2016) identified catechol 2,3 dioxygenases gene in *Pseudomonas* strains. Patrauchan *et al.* (2008) identified ethylbenzene dioxygenases, benzene dioxygenases and catechol dioxygenases enzymes. In addition, from the consortium we identified a protein similar to 4-oxalocrotonate decarboxylase (28.2 kDa) which degrades benzoate, toluene, and xylene. However, intermediates central can follow different routes where cleavage is ran by non oxygenolitic enzymes (Gescher *et al*., 2006).

Protein in the catabolism of fatty acids. The catechol dioxygenase enzyme cleaves the catechol-aromatic ring, allowing formation of ketoadipate enollactone, which is degraded by beta-oxidation pathway (Patrauchan *et al*., 2008; Dalvi *et al*., 2014). Herein, we identified a protein similar to polyhydroxyalkonate depolymerase (46.9 kDa) and 4-oxalocronate decarboxylase. Shöber *et al.* (2000) and Dalvi *et al.* (2014), respectively, indicate that these enzymes are responsible for fatty acid degradation to acetate, butyrate, and succinate. Finally, bacteria must produce precursors of metabolites like acetyl CoA to produce energy and biomass. We also identified an enzyme similar to glyoxylase (32.1 kDa) and linked to tricarboxylic acid cycle.

Genetic analyses. To confirm the key enzymes in BTEX catabolism that were revealed by differential proteomic analysis, we amplified the catabolic genes *bed* and *tod*. The analysis of the *bed* gene sequence indicates that is similar in a 98% to the reference *bed*-gene (GenBank gi|151068). When comparing the nucleotide sequence of *tod*, it showed a 90% similarity to the *tod* gene of *P. putida* F1 (GenBank gi|148512152). The alignment of both amino acid sequences showed 95% similarity. The 5% difference corresponds to amino acid substitutions (A22P, F181I, A192P, M219I, V337I, F357L, and A391T) in the catalytic site. This difference may be linked to the increase of catabolic potential of the *P. putida* strain in comparison with *P. putida* F1. Szczepaniak *et al*. (2016) indicated the alfa subunit is found in dioxygenase enzymes with similar catabolic activity but with different substrate specificity. Bagnéris *et al*. (2005) indicates that amino acid substitution (I301V, T305S, I307L, and L309V) of the *tod* catalytic region increases the preference for ethylbenzene. In addition, genetic studies indicate that the *tod* gene expressed in consortium and *P. putida* strain exposed to BTEX, T, E, and Xylene. In contrast, the *tod* gene is only expressed in the reference strain exposed to BTEX and toluene. Previous studies have reported similar results, Patrauchan *et al*. (2008) amplified the genes *Etb* and *Bph*, being the products of these genes ethylbenzene-benzene and biphenyl enzymes, respectively.

In conclusion, the enriched consortium acclimatized to unleaded gasoline is capable of removing 95% of the BTEX in this experiment, while *P. putida* strain alone removes up to 90%. Differential proteomic analyses allowed us to identify proteins that are up-regulated in the presence of BTEX. These proteins are homologous to other proteins related to BTEX biodegradation that were reported in previous studies. *bed* and *tod* genes were identified, which are up-regulated when exposed to BTEX. The *tod* gene of *P. putida* strain is 90% similar to the counterpart from the reference strain. The protein tod of *P. putida* strain is a 95% to the enzyme Tod in the reference strain. The *bed* gene only gets expressed in consortium and strain exposed to BTEX and benzene. The nucleotide sequence from *bed* gene has a 98% similarity with the one from reference strain. Analyzing the amino acid sequence of Bed, it was possible to prove that it is 100% identical to the Bed enzyme in the reference strain. These results are expected to provide directions for future studies on BTEX removal and other environmental applications.

Acknowledgments

JM thanks the Mexican Council of Science and Technology (CONACyT) for a doctoral fellowship. KAA, HBS and JAM thank the CONACyT for its support (grant SEP-2004-CO1-47370) and PAICyT CN072-09. The authors also thank the RCMI Proteomics Core at the University of Texas at San Antonio (UTSA) (NIH G12 RR013646) for their assistance with experiment design, sample preparation, data collection, results interpretation, and manuscript preparation. We thank the Computational Biology Initiative (UTSA/UTHSCSA) for providing access and training and analysis software use, and the NCRR/NIH for their support of the RCMI Proteomics Core at UTSA (NIH G12 RR013646). The authors also thank Sergio Lozano-Rodriguez, M.D. for his critical reading of the manuscript.

Literature

Acuna-Askar K., M.A. de la Torre-Torres, M.J. Guerrero-Munoz, M.T. Garza-Gonzalez, B. Chavez-Gomez, I.P. Rodriguez-Sanchez and H.A. Barrera-Saldana. 2006. Biodegradation kinetics of BTE-OX and MTBE by a diesel-grown biomass. *Water Sci. Technol.* 53(11): 197–204.

Bagnéris C., R. Cammack and J.R. Mason. 2005. Subtle difference between benzene and toluene dioxygenases of *Pseudomonas putida*. *Appl. Environ. Microb*. 71: 1570–1580.

Bell T.H., E. Yergeau, D.F. Juck, L.G. Whyte and C.W. Greer. 2013. Alteration of microbial community structure affects diesel biodegradation in an Arctic soil. *FEMS Microbiol. Lett.* 85: 51–61.

Ciric L., J.C. Philp and A.S. Whiteley. 2010. Hydrocarbon utilization within a diesel-degrading bacterial consortium. *FEMS Microbiol. Lett.* 303: 116–122.

Cyplik P., M. Schmidt, A. Szulc, R. Marecik, P. Lisiecki, H.J. Heipieper, M. Owsianiak, M. Vaishtein and L. Chrzanowski. 2011. Relative quantitative PCR to assess bacterial community dynamics during biodegradation of diesel and biodiesel fuels under various aeration conditions. *Bioresource Technol.* 102: 4347–43562.

Dalvi S., C. Nicholson, F. Najar, B.A. Roe, P. Canaan, S.D. Hartson and B.Z. Fathepure. 2014. *Arhodomonas* sp. Strain *Seminole* and its genetic potential to degrade aromatic compounds under high-salinity conditions. *Appl. Environ. Microb*. 80(21): 6664–6676.

Díaz E., A. Fernández, M.A. Prieto and J.L. García. 2001. Biodegradation of aromatic compounds by *Escherichia coli*. *Microbiol. Mol. Biol. R.* 65: 523–569.

Dean B.J. 1985. Recent findings on the genetic toxicity of benzene, toluene, xylenes, and phenol. *Mutat Res.* 145: 153–181.

Demanèche S., C. Meyer, J. Micoud, M. Louwagie, J.C. Willison and Y. Jouanneau. 2004. Identification and functional analysis of two aromatic ring-hydroxylating dioxygenases from a *Sphingomonas* strain degrading various polycyclic aromatic hydrocarbons. *Appl. Environ. Mic*rob. 70: 6714–6725.

Fong K.P., C.B. Goh and H.M. Tan. 1996. Characterization and expression of the plasmid-borne *bed*D gene from *Pseudomonas putida* ML2, which codes for a NAD_1-dependent cis-benzene dihydrodiol dehydogenase. *J. Bacteriol.* 178: 5592–5601.

Gescher J., O. Ismail, E. Ölgeschläger, W. Eisenreich, J. Wörth and G. Fuchs. 2006. Aerobic benzoyl-coenzyme A (CoA) catabolic pathway in *Azoarcus evansii*: conversion of ring cleavage product by 3,4-dehydroadipyl-CoA semialdehyde dehydrogenase. *J. Bacteriol*. 18(8): 2919–2927.

Gibson D.T. and V. Subramanian. 1984. Microbial degradation of aromatic hydrocarbons, pp. 181–252. In: Gibson D.T. (ed.) *Microbial degradation of organic compounds.* Marcel Dekker, New York, NY.

Kane R.S., A.Y. Chakicherla, P.S. Chain, R. Schmidt, M.W. Shin, T.C. Legler, K.M. Scow, F.W. Larimer, S.M. Lucas, P.M. Richardson and others. 2007. Whole-genome analysis of the methyl tert-butyl ether-degrading beta-proteobacterium *Methylibium petroleiphilum* PM1. *J. Bacteriol.* 189: 1931–1945.

Kim D., J.C. Chae, G.J. Zylstra, Y.S. Kim, S.K. Kim, N.H. Nam, Y.M. Kim and E. Kim. 2004. Identification of a novel dioxygenase involved in metabolism of o-xylene, toluene, and ethylbenzene by *Rhodococcus* sp. strain DK17. *Appl. Environ. Microb.* 70: 7086–7092.

Lawniczak L., E. Kaczorek and A. Olszanowski. 2011. The influence of cell immobilization by biofilm forming on the biodegradation capabilities of bacterial consortia. *World J. Microb. Biot.* 27: 1183–1188.

Lima-Morales D., R. Jáuregui, A. Camarinha-Silva, R. Geffers, D.H. Pieper and R. Vilchez-Vargas. 2016. Linking microbial community and catabolic gene structures during the adaptation of three contaminated soils under continuous long term pollutant stress. *Appl Environ Microb.* 82(7): 2227–37.

Lisiecki P., L. Chrzanowski, A. Szulc, L. Lawniczak, W. Bialas, M. Dziadas, M. Owsianiak, J. Staniewski, P. Cyplik, R. Marecik and others. 2014. Biodegradation of diesel/biodiesel blends in saturated sand microcosms. *Fuel* 116:321–327.

Lee S.K., and S.B. Lee. 2001. Isolation and characterization of a thermotolerant bacterium *Ralstonia* sp. strain PHS1 that degrades benzene, toluene, ethylbenzene, and o-xylene. *Appl. Environ. Microb.* 56: 270–275.

Maeda T., Y. Takahaxhi, H. Suenaga, A. Suyama, M. Goto and K. Furukawa. 2001. Functional analyses of bph-tod hybrid dioxygenases, which exhibits high degradation activity toward trichoroethylene. *J. Biol. Chem.* 276: 29833–29838.

Morlett-Chávez J.A., J.A. Ascacio-Martínez, A.M. Rivas-Estilla, J.F. Velázquez-Vadillo, W.E. Haskins, H.A. Barrera-Saldaña and K. Acuña-Askar. 2010. Kinetics of BTEX biodegradation by a microbial consortium acclimatized to unleaded gasoline and bacterial strains isolated from it. *Int. Biodeter. Biodegr.* 64(7): 581–587.

Owsianiak M., L. Chrzanowski, A. Szulc, J. Staniewski, A. Olszanowski, A.K. Olejnik-Schmidt and H.J. Heipieper. 2009. Biodegradation of diesel/biodiesel blends by a consortium of hydrocarbon degraders: effect of the type of blend and the addition of biosurfactants. *Biosource Technol.* 100: 1497–1500.

Peters F., D. Heintz, J. Johannes, A. van Dorsselaer and M. Boll. 2007. Genes, enzymes, and regulation of para-cresol metabolism in *Geobacter metallireducens*. *J. Bacteriol.* 189: 4729–4738.

Patrauchan M.A., C. Florizone, S. Eapen, L. Gómez-Gil, B. Sethuraman, M. Fukuda, J. Davies, W.W. Mohn and L.D. Eltis. 2008. roles of ring-hydroxylating dioxygenases in styrene and benzene catabolism in *Rhodococcus jostii* RHA. *J. Bacteriol*. 190: 37–47.

Pieper D.H. and W. Reineke. 2000. Engineering bacteria for bioremediation. *Curr. Opin. Biotech.* 11: 262–270.

Potter T.L. 1992. Fingerprinting petroleum products: unleaded gasolines, pp. 83–92. In: Kostecki P.T. and E.J. Calabrese (ed.). *Petroleum contaminated soils, vol 2.* Lewis Publishers, Chelsea, Mich.

Ramos-González M.I., A. Ben-Bassat, M.J. Camposm and J.L. Ramos. 2003. Genetic engineering of a highly solvent-tolerant *Pseudomonas putida* strain for biotransformation of toluene to p-hydroxybenzoate. *Appl. Environ. Microb.* 69: 5120–5127.

Sabirova J.S., M. Ferrer, D. Regenhardt, K.N. Timmis and P.N. Golyshin. 2006. Proteomic insights into metabolic adaptations in *Alcanivorax borkumensis* induced by alkane utilization. *J. Bacteriol.* 188: 3763–3773.

Shöber U., C. Thiel and D. Jendrossek. 2000. Poly (3-hydroxyvalerato) depolimerase of *Pseudomonas lemoignei*. *Appl. Environ. Microb.* 66(4): 1385–1392.

Smith R.K. 1990. The biodegradation of aromatic hydrocarbons by bacteria. *Biodegradation* 1: 191–206.

Szczepaniak Z., J. Czarny, J. Staninska-Pieta, P. Lisiecki, A. Zgoła-Grześkowiak, P. Cyplik, L. Chrzanowski, L. Wolko, R. Marecik, W. Juzwa and others. 2016. Influence of soil contamination with PAH on microbial community dynamics and expression level of genes responsible for biodegradation of PAH and production of rhamnolipids. *Environ. Sci. Pollut. Res.* 23(22): 23043–23056.

Tarasev M., C.S. Kaddis, S. Yin, J.A. Loo, J. Burgner and D.P. Ballou. 2007. Similar enzymes, different strctures: phtalato dyoxigenases is an α3α3 hexamer, not an α3β3 trimer like "normal" rieske oxygenases. *Arch. Biochem. Biophys.* 466: 31–39.

Tsao C.W., H.G. Song and R. Bartha. 1998. Metabolism of benzene, toluene and xylene hydrocarbons in soil. *Appl. Environ. Microb*. 64: 4924–4929.

Witzig R., H. Junca, H.J. Hecht and D.H. Pieper. 2006. Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: Links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Appl. Environ. Microb.* 72: 3504–3514.

Zylstra, G.J. and D.T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1. *J. Biol. Chem.* 264: 1940–1946.