Identification and characterization of dioxygenase genes in new polychlorinated biphenyls degraders

Hamdy A. Hassan[1], El-Sayed A.El-Absawy[2], Reham M. El.attar[1], Nashwa MH Rizk[1]

[1]Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Sadat city University, Sadat city, Egypt .
[2] Bioinformatics Department, Genetic Engineering and Biotechnology Research Institute, Sadat city University, Sadat city, Egypt
hamdyhassan1973@yahoo.com

Abstract: Polychlorinated biphenyls (PCBs) are one of the most dangerous xhenobiotics in the environment, because they are normally thermodynamically stable and not readily degraded. In addition, PCBs pose potential health risks to humans, as they can be toxic, mutagenic, and carcinogenic. Two bacterial isolates were isolated from the oxidation park of Sadat City, Egypt where this region is known to be highly contaminated by PCBs. The two isolates were characterized by sequencing 16S rRNA and were identified as *Pseudomonas sp* HA-OP21 and *Burkholderia sp.* HA-OP24. The PCBs degrading ability of the two strains was determined and it was found that the first strain can degrade monochlorinated biphenyls while the second can utilize polychlorinated biphenyls as sole carbon source and energy , these findings suggests that our strains contain genes that encode catabolic dioxygenases enzymes ,which are involved in PCBs degradation. Using previously designed primers, we managed to amplify Rieske non-heme iron oxygenase (ISPα) gene of toluene/biphenyl subfamily and catechol 2,3dioxygenase gene that considered the most important catabolic genes involved in PCBs biodegradation.
[Hamdy A. Hassan, El-Sayed A.El-Absawy, Reham M. El.attar, Nashwa MH Rizk. **Identification and characterization of dioxygenase genes in new polychlorinated biphenyls degraders.** *Life Sci J* 2013;10(3):889-900] (ISSN: 1097-8135). http://www.lifesciencesite.com. 133

Key words: PCBs, xhenobiotics, dioxygenases, Rieske non-heme iron oxygenase, catechol 2,3 dioxygenase

1.Introduction

Polychlorinated biphenyls (PCBs) are a class of 209 compounds with the molecular formula $C_{12}H_{10-n}Cl_n$ where $1 \leq n \leq 10$.They were commercially produced as complex mixtures for various uses, being employed mainly as dielectric fluids in capacitors and transformers (WHO, 1993). Their thermal and chemical stability, resistance to chemical corrosion and general inertness have contributed to their persistence in the environment (Haluska *et al.,* 1995).They are ubiquitous contaminants of public concern because of their persistence and bioaccumulation in the environment, as well as their potential toxicity to humans and ecotoxicology (Borlakoglu and Haegeles, 1991). The conventional methods for remediation of PCB-contaminated soil, that include incineration or relocation to specialized landfills, are often prohibitively expensive. The alternative biodegradation strategy for in situ PCB removal uses microorganisms capable of metabolizing PCBs. Microorganisms play an important role in the field of environmental science by degrading and transforming toxic compounds into non-toxic or less toxic forms. Nevertheless polychlorinated biphenyls (PCBs) are still very persistent, resulting from the inability of environmental microbial populations to degrade these compounds efficiently (Sylvestre, 2004).

Lunt and Evans (Lunt and Evans, 1970) were the first to isolate microorganisms capable of growing on biphenyl as sole source of carbon and energy and since then several biphenyl-degrading organisms have been isolated. These organisms belong to both Gram-negative and Gram-positive genera and comprise various *Pseudomonas, Burkholderia, Achromobacter, Comamonas, Ralstonia, Acinetobacter, Rhodococcus*and *Bacillus* isolates (Pieper, 2005). *Burkolderia xenovorans* LB400 is a model bacterium for PCBs degradation and is able to degrade a broad range of PCBs (Haddock *et al*., 1995; Seeger *et al*., 2001) also *Rhodococcus jostii* RHA1 is another potent PCB-degrading soil bacterium (Seto *et al*., 1995; Warren *et al*., 2004; McLeod *et al*., 2006). Anaerobic bacteria, such as the Clostridium members, which remove chlorine from PCB, have also been studied (Hou and Dutta, 2000).

The biodegradation of PCBs by aerobic bacteria had been well studied. However, it was observed that only lightly chlorinated PCB congeners, those with four or less chlorine atoms, were degraded (Ahmed and Focht, 1973; Ahmad *et al.*, 1991; Fava *et al.,* 1994). Highly chlorinated PCB congeners, those with five or more chlorine atoms, remain biorefractory to aerobic bacteria, although there had been few reports on the aerobic degradation of penta- and hexachlorobiphenyls (Bedard *et al.,*

1987). Thus, the degradation of PCB mixture will involve a complex microbial network characterized with a diversity of substrate specificities (Borja *et al.,* 2006).

Biphenyl 2,3-dioxygenase is the enzyme that catalyzes the first step in PCBs degradation. It belongs to the toluene/biphenyl family of Rieske non-heme iron oxygenases (Gibson and Parales 2000). Studies on various biphenyl 2,3-dioxygenases have revealed considerable differences in their congener selectivity patterns, as well as their preference for the ring attacked (Erickson and Mondello 1993; Kimura *et al.,* 1997; Seeger *et al.,* 1999; Zielinski *et al.,* 2002).They comprise ferredoxin, ferredoxin reductase and a terminal oxygenase. The ferredoxin and ferredoxin reductase act as an electron transport system to transfer electrons from NADH to the terminal oxygenase. The terminal oxygenase is the one that interacts directly with the substrate and which influences the range of PCB substrate oxygenated by the enzyme (Pieper, 2005). It is iron–sulfur protein (ISP) comprising a 2Fe–2S Rieske center and composed of a large α- and a small β-subunit. The ISPα-subunit gene was found to be crucially responsible for recognition and binding of the substrates, and also for substrate specificity (Furukawa *et al.,* 1993; Kimura *et al.,* 1997). Biphenyl is subject to 2,3-dioxygenase, giving, after dehydrogenation, 2,3-dihydroxybiphenyl which is then subjected to extradiol ring cleavage by 2,3-dihydroxybiphenyl 1,2-dioxygenases. (Pieper, 2005)

2,3- dihydroxybiphenyl 1,2-dioxygenases genes have a particular significance in the degradation of PCBs. They belong to the family of extradiol dioxygenases and use mononuclear Fe2+ to cleave dihydroxybiphenyl. A large collection of genes coding for such an activity have been cloned and sequenced in the last years, being classified as a diverse gene family (Eltisand Bolin, 1996). Usually, extradiol dioxygenases, which are involved in biphenyl degradation, belong to the I.3.A subfamily of extradiol dioxygenases (Eltis and Bolin, 1996) and are specialized for transformation of 2,3-dihydroxybiphenyls. These enzymes differ in their substrate specificity, but seem to be generally capable of transforming various chloro substituted derivatives (Dai *et al.,* 2002). The 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate thereby formed undergoes hydrolysis, yielding benzoate and 2-hydroxypenta-2,4-dienoate as reaction products. This sequence of reactions forms the so-called biphenyl upper pathway (Pieper, 2005).

PCR was used as a molecular tool for detection of functional/catabolic genes in environmental isolates or environmental DNA, and diversity is usually assessed by sequencing of genes from isolates or PCR clone libraries (Yeates *et al.,* 2000; Buchan *et al.,* 2001; Hamelin *et al.,* 2002).

In this work, new polychlorinated biphenyl (PCBs)-degrading bacteria were obtained from soils highly contaminated with PCBs and PCR was used with the DNA of our new isolates as a molecular tool for the detection of Rieske non-heme iron oxygenase (ISPα) gene of toluene/biphenyl subfamily and catechol 2,3 dioxygenase gene that considered the most important catabolic genes involved in PCBs biodegradation.

2. Material and methods

Site description

The study site was located in Sadat City. Sadat City is one of the largest industrial cities in Egypt. It is a desert area and includes more than 109 factories with several industrial activities. The final outputs of industrial as well as urban effluents are expelling out of the populated area of the city. The treatment of such emissions is carried out depending on the oxidation pond system (Elaxadaponds). However, the sweeping overflow of this discharge move downward to the lower land forming large pools (2.6-4 hectares, 2-4 m depth). The Oxidation Pond site had been polluted with PCBs compounds. The PCBs content in these ponds were detected with range from 212 to 320 mg PCBs kg-l (Hamdy, 2013).

Isolation, purification and preservation of bacterial isolates

1 g of soil was incubated in 1 L Erlenmeyer flasks containing 100 ml of mineral medium (Buffer:Na_2HPO_4 x12H_2O 14 g, KH_2PO_4 2 g, H2O to 1L, pH 7.4;100x Salt-solution :$Ca(NO_3)_2$ x 4H_2O 5 g,$MgSO_4$ x 7H2O 20 g ,FeIII-ammonium-citrate 1 g (28 % Fe),Trace elements solution 100 ml , H_2O to 1L; Trace elements (sterilized by filtration) : HCL(25%) 1.3 ml, $ZnCl_2$ 70 mg, $MnCl_2$ x 4H_2O 100 mg, H_3BO_4 62 mg, $CoCl_2$ x 6H_20 190 mg, CuCl x 2H_2O 17 mg, $NiCl_2$ x 6H_2O 24 mg, $NaMoO_4$ x 2H_2O 36 mg, H_2O to 1L. Liquid medium was assembled by supplementing the buffer with 1% (vol/vol) of salt solution. Biphenyl was added as the sole source of carbon and energy in appropriate concentrations, usually 2 mM. After one month of cultivation at 30°C with shaking, 10 % of the culture was transferred to fresh medium and cultured for one more month. The culture was monitored for the presence of microorganisms until turbidity of the medium was observed by measuring at 600 nm. Each isolated colony was recultured on solid minimal media supplemented with crystals of biphenyl in the lid of the plate as sole carbon source. All isolates were purified and stored in minimal media and glycerol

added to a final concentration of 50% and stored at -80°C.

Screening of 2,3-dihydroxybiphenyl 1,2-dioxygenase and catechol 2,3-dioxygenase activity

For screening 2,3-dihydroxybiphenyl 1,2-dioxygenase activity, a sterile solution 10 mM of 2,3-dihydroxybiphenyl (dissolved in 1 % of the final volume of ethanol, to which water was added to give the final molarity) was sprayed on the bacterial colonies. Also catechol 2,3-dioxygenase activity was screened by spraying with catechol. Positive clones could be easily identified due to yellow coloration around the colonies. Colonies turning yellow were purified by repeated subculturing and streaking on minimal medium agar plates with Biphenyl as sole carbon source.

Growth on different PCBs compounds

Pure cultures were tested for their ability to grow on a variety of defined carbon sources. The tests were performed in minimal media supplemented with the tested compounds as a sole carbon source. Substrates were added to 10 ml MM in Balch tubes at a concentration of 100 ppm and inoculated with 105–106 cells/ml of phosphate buffer (pH 7.4), tubes were crimp sealed with Teflon-coated stoppers and incubated on shaker at 100 rpm. Tested substrates included BETX, dibenzofuran (DBF), monochlorobiphenyls (2-CB and 4-CB), dichlorobiphenyls (2,3CB and 2,4CB) and polychlorinated biphenyls (2,4,5,2′,4′,5′-Chlorobiphenyl). Stock solutions of all substrates were autoclaved prior to use. Growth was evaluated by microscopy and visual monitoring of turbidity . In these substrate-screening studies, growth was scored as positive if turbidity was notably greater than in controls lacking the test compound, microscopic examination revealed an increase in cell numbers.

DNA Extraction and amplification of 16S rRNA gene fragment from the new isolates

Genomic DNA of *Pseudomonas sp* HA-OP21 and *Burkholderia sp*. HA-OP24 were isolated from cells pregrown on biphenyl according to the protocol for bacterial DNA extraction with the GeneJET Genomic DNA Purification Kit (#K0721) provided by Fermentas .Yield of genomic DNA was visualized on 1% agarose gels and quantified spectrophotometrically by using (Gene Quant, Pharmacia) in 1cm covet and diluted to 2 µg/µl. Purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm (a pure DNA has an A260/A280 ratio of 1.7-1.9).

The new PCBs degraders were identified partially using two primer sets targeting the 16S rRNA, the first primer set (16F8 5`AGAGTTTGATCCTGGCTCAG 3`, 16R518 5`CGTATTACCGCGGCTGCTGG3`) and the second primer set (16F945 5`GGGCCCGCACAAGCGGTGG 3`, 16R1492 5`TACGGYTACCTTGTTACGACTT 3`) corresponding to position 1-518 and 945-1492 respectively according to the *E. coli* numbering system. The 16S rRNA were directly determined from PCR fragments after purification with Gene JET™(#K0831) PCR Purification Kit (Fermentas).

Primer design and PCR conditions

Three genes for degradation of aromatics (ISPa gene segments of the toluene/biphenyl subfamily, C23O type 1.2.A, C23O type 1.3.A) were screened by PCR. Primers details including sequence, annealing temperature and product size are given in Table 1. PCR amplification was carried out in a 50µl reaction mixture containing 100 ng of pure strain DNA as templates. the PCR mixture contained 1.25 U Taq polymerase, 10 pmol of the forward primer, 10 pmol of the reverse primer, 200 µM of each dNTP and 5 µl of 10x Taq reaction buffer (20mM MgCl2). The Taq polymerase, dNTPs and PCR buffer were purchased from fermentas. The thermal cycling program was as follows: hot start at 94°C for 5 min followed by 35 cycles of 94°C for 40 sec, annealing for 40 sec, 72°C for 1 min and a final step of 72 °C for 8 min. The PCR products (4 µl of the PCR reaction) were checked for integrity by agarose gel electrophoresis. 1% agarose gel was dissolved in 1x TBE buffer, melted and poured into an electrophoresis chamber, A volume of 10% (v/v) loading buffer (60% glycerin, 0.3 % xylolcyanol 0,3 % bromophenolblue) was added to the DNA samples. Samples were loaded onto the gel and the electrophoresis was performed at 50 Volt/cm in 1x TBE buffer. DNA was visualized by soaking in a dilute solution of ethidium bromide. DNA fragments were separated and their sizes examined and determined under UV light. The DNA extraction was processed by the GeneJET Gel Extraction Kit (#K0831).

Sequence analysis of the PCR amplified catabolic genes and 16S rRNA gene fragments

The purified PCR products of catabolic gene sequences and the 16S rRNA gene sequences were sequenced double stranded. Primers used for sequencing reactions were the same as those used in the original PCR. Nucleotide similarity searches were performed using BlastN program from the National Center for Biotechnology Information website. Phylogenetic trees of partial 16s rRNA gene sequence of strains *Pseudomonas sp* HA-OP21 and

Burkholderia sp. HA-OP24 and related bacteria were constructed using the clustalx1.83 program and N-J trees were visualized with tree view program. Vectorial representations were imported and edited on Microsoft Office Power point program. Phylogenetic analysis of the catabolic genes and the related genes also constructed with the same programs.

Data deposition

The catabolic gene sequences reported in this study are available from GenBank under accession numbers from JQ015315 to JQ015320, while the 16S rRNA gene sequences are available under accession numbers HQ918250, HQ918248, JF264747, and JF264749.

3. Result

Isolation and characterization of PCBs-degrading bacteria

Soil samples were collected from oxidation ponds in Sadat city. Enrichment cultures were set up in minimal media with biphenyl crystals as sole source of carbon source and energy. After two transfers, aliquots were plated on minimal media agar plates with biphenyl as sole carbon source. Purified single colonies exhibiting yellow coloration after spraying with 2,3- dihydroxybiphenyl were purified by streaking on agar plates with biphenyl as sole carbon source and the DNA of the two strains was isolated. The two isolates were characterized by sequencing 16S rRNA partially with two primer sets. Analysis using the RDPII database revealed that the isolate HA-OP21 belongs to the genus *Pseudomonas* whereas isolate HA-OP24 belongs to the genus *Burkholderia.* The 16S rRNA genes of strains *Pseudomonas* sp. strain HA-OP21 and *Burkholderia* sp. strain HA-OP24 were sequenced, analyzed and compared with sequences deposited in publicly accessible database using NCBI BlastN search tool.

Phylogenetic relationships between isolated strains with related aromatic compounds degraders

Two phylogenetic relationships between isolated strains with related aromatic compounds degrader's taxa were determined by a neighbor-joining distance analysis of their 16S rRNA gene sequences. For the first primer set (Figure 1) and for the second primer set (Figure 2). *Pseudomonas* sp. HA-OP1 has high similarity in case of the first primer set with *Pseudomonas* sp. BZ2, *Pseudomonas* sp. H1, *Pseudomonas veronii* UFZ-B547, *Pseudomonas* sp. Cam-1, *Pseudomonas aeruginosa* W3.1 and uncultured soil bacterium clone NAP7d37 which have previously been reported to be able to grow on aromatic hydrocarbons, polyaromatic hydrocarbons, chlorobenzene, polychlorinated biphenyls, anthracene and naphthalene respectively (Figure 1) (Master and Mohn, 1998; Singleton *et al*., 2005). In case of the second primer with *Pseudomonas* sp. C16w, *Pseudomonas* sp. CT-1, *Pseudomonas* sp. DK2009-3a *Pseudomonas* sp. BFXJ-8, *Pseudomonas* sp. Geo33 and uncultured *Pseudomonas* sp. clone G85 which have previously been reported to be able to grow on hydrocarbons, naphthalene, fenamiphos & oxamyl, phenol, polyaromatic hydrocarbons and acetate respectively (Figure 2) (Hoefel *et al* ., 2006; Adav *et al*., 2010). It also showed high similarity in case of the first and second primer set with *Pseudomonas* sp. ARDRA PS2, *Pseudomonas veronii* strain S1f-34 and *Pseudomonas* sp. A3YXyl2-4, which have been reported as BETX degrader (Junca and Pieper, 2004; Hendrickx *et al.,* 2006) and also with the polychlorinated biphenyl degraders *Pseudomonas frederiksbergensis* OUCZ24 and *Pseudomonas pseudoalcaligenes* KF707 (Figures1, 2) (Leigh *et al.,* 2006) .

Whereas *Burkholderia* sp. HA-OP24 showed high similarity in case of the first primer set with *Pandoraea* sp. HA-OP28 and uncultured *Eubacterium* clone WR112 which have been reported as aromatic hydrocarbons and polychlorinated biphenyls degraders (Figure 1) (Nogales *et al*., 1999). In case of the second primer it showed high similarity with uncultured *Eubacterium* WD285 clone and uncultured *Eubacterium* clone WD202 that they have been reported as polychlorinated biphenyl degraders (Figure 2) (Nogales *et al*., 2001). It also showed high similarity in case of the first and second primer set with *Burkholderia fungorum* DBT1, *Burkholderia* sp. isolate N2P5, *Burkholderia xenovorans* LB400 and *Pandoraea* sp. Y1 that they have been reported as dibenzothiophene, polyaromatic hydrocarbons, polychlorinated biphenyls and aromatic hydrocarbons degraders respectively (figures 1,2)(Mueller *et al*., 1997; Zocca *et al.,* 2004; Chain *et al.,* 2006).

Substrate diversity of bacterial strains

The two strains were tested for the use of a range of a wide range of chlorinated biphenyls as well as BETX, biphenyl and dibenzofuran (DBF) as sole carbon and energy sources (Table 2). The chlorinated biphenyl include 2CB; 4CB; 2,3CB; 2,4CB and 2,4,5,2′,4′,5′-Chlorobiphenyl, and it was found that both strains were able to utilize BETX, Biphenyl, dibenzofuran, 2CB; 4CB quite rapidly. Whereas di-CBs including 2,3-, 2,4-DCB and 2,4,5,2′,4′,5′-Chlorobiphenyl can be utilized by only *Burkholderia*sp.HA OP24.

Transformation of 2,3-Dihydroxybiphenyl and catechol by resting cells from PCBs degraders

To analyze the differences in extradiol dioxygenase activities (2,3 dihydroxybiphenyl dioxygenase or catechol 2,3 dioxygenase) in the two strains, the resting cells grown on biphenyl were incubated with 2,3DHB and Catechol for detection of the *meta*- cleavage activity in both the upper and lower pathway of PCBs degradation, the activities were detected colorimetric by the production of yellow metabolites. The yellow color observed could be considered as an indication of meta-cleavage product of hydroxyl 6-oxo-6-penta 2,4-dienoic acid (HOPDA) in the case of 2,3DHB and and 2-hydroxymuconic semialdehyde for catechol. A rapid coloration due to *meta*-cleavage of DHB and catechol were observed with high activities in both 2-CB and 4-CB cells.

Table 1. Primers used in this study

Gene	Primer name	Sequence 5'-3'	Anneling temperature	Product size (bp)	Refrence
ISP(α) of toluene/biphenyl subfamily	bphAF668-3a bphAR1153-2a	GTTCCGTGTAACTGGAARTWYGC CCAGTTCTCGCCRTCRTCYTGHTC	50°c	550	Witzig *et al.*, 2006
Catechol extradiol dioxygenases (I.2.A) subfamily	C23O-ORF-F C23O-ORF-R	AGGTGWCGTSATGAAMAAAGG TYAGGTSAKMACGGTCAKGAA	50°c	934	Junca and Pieper, 2003
Catechol extradiol dioxygenases (I.3.A) subfamily	EXDO-K2-F EXDO-K2-R	GAAAAAGTGGGTTTGATGGAGG CGCTTATGCCKCGTCATCACCC	55°c	810	Brennerova *et al.*,2009

PCR detection of biphenyl degradation genes

The primer set bphAF668-3 & bphAR1153-2 (Witzig *et al.,* 2006) was used in PCR for amplifying iron-sulfur protein (ISP- α) genes of the toluene/ biphenyl oxygenase subfamily of *Pseudomonas* sp. Strain HA-OP21 and *Burkholderia* sp. Strain HA-OP24. The expected amplification product of 550bp fragment was resulted from both strains (Figure 3a). Amplicons resulting from PCR were purified and sequenced double stranded. Nucleotide sequence similarity was performed using BlastN programs from the National Center for Biotechnology Information website and a phylogenetic tree of ISPα genes of the two strains and related ISPα genes from other isolates were constructed (Figure 4). The analysis of the phylogenetic tree showed that the majority are related to rieske non-heme iron oxygenase (ISPα) gene of toluene/biphenyl subfamily. They showed high similarity (99%) to the rieske non-heme iron oxygenase α subunit gene of the bacterium *Pseudomonas* sp. Strain 3YdBTEX2 and was closely related (similarity 94%) to putative rieske non-heme iron oxygenase α subunit (ipbAa) gene of the bacterium *Pseudomonas* sp. IpA-92.

The nucleotide sequence of ISP-α gene of *Pseudomonas* sp. Strain HA-OP21 and *Burkholderia* sp. Strain HA-OP24 were compared. The nucleotide sequence conservation is 99% and showed long stretches of identical sequence indicating that these genes are very closely related. *Pseudomonas* sp. Strain HA-OP21 ISP-α gene differs from *Burkholderia* sp. Strain HA-OP24 ISP-α gene at 6 positions. Erickson *et al.,* (Erickson *et al.,* 1993) proposed that any alterations in the sequence of the large subunit of the terminal biphenyl dioxygenase resulted in modifying the PCB congener specificity of the enzyme. So we suggest that the differences in the degrading abilities between *Pseudomonas* sp. Strain HA-OP21 and *Burkholderia* sp. Strain HA-OP24 was due to differences in the sequence of the large subunit of the terminal biphenyl dioxygenase.

Two previously designed primer sets that have been previously designed were used in PCR for amplifying diverse types of catechol 2,3 dioxygenase genes. The primer set C230-F & C230-F (Junca and Pieper, 2003) that was previously designed to amplify 934bp comprising the complete open reading frames of the subfamily I.2.A C23O genes was tested with our strains. A clear single bands with the expected size 934bp (figure 3b) was resulted from both strains and the nucleotide sequence similarity search showed that they are closely related to (similarity 92%) the catechol 2,3-dioxygenase (bphE) gene of biphenyl-utilizing bacterium *Pseudomonas* sp. IC (Figure 5). While primers EXDO-K2-F & EXDO-K2-R (Brennerova *et al.,*2009) were designed to target 3- isopropylcatechol 2,3-dioxygenase encoding gene involved in the isopropyl benzene degradation (a second subgroup of subfamily 1.3A). Also a clear single bands with the expected size 810bp (figure 3c) were resulted from both strains and it was found that they showed high similarity (97%) to isopropylbenzene dioxygenase gene of the bacterium *Pseudomonas putida* RE204 (Figure 6).

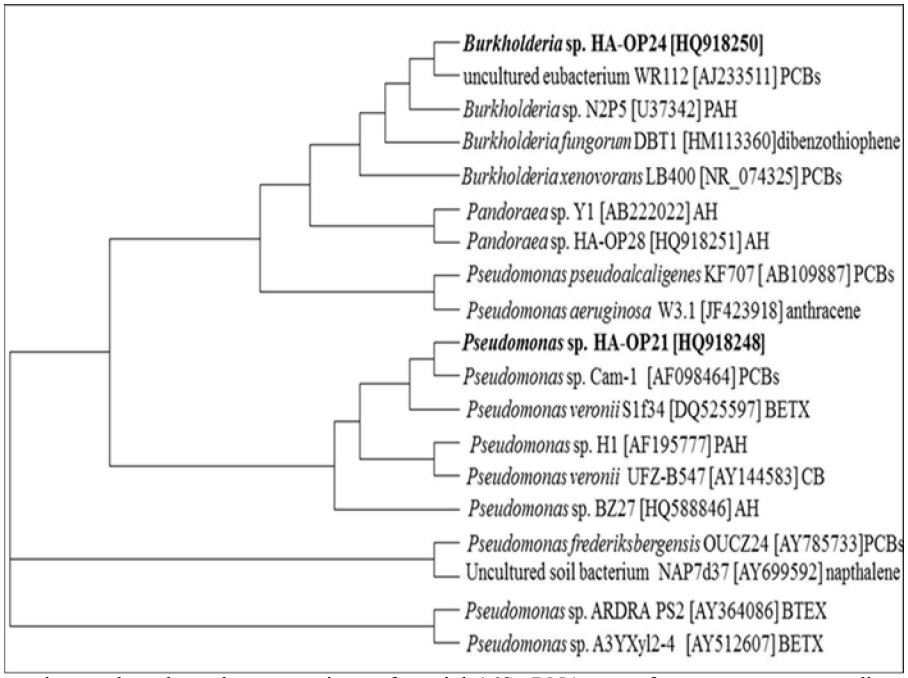

Figure 1. Phylogenetic tree based on the comparison of partial 16S rRNA gene fragments, corresponding to position 1-518 according to the *E.coli* numbering system, showing the relationship of the new PCBs degraders strains (with bold line and the accession numbers after the strain name) with the nearest strain for each, that have already reported before as aromatic degraders. polychlorinated biphenyls (PCBs), Polyaromatic hydrocarbon (PAH), aromatic hydrocarbon (AH), benzene - ethylbenzene - toluene - xylene (BETX), and chlorobenzene (CB).

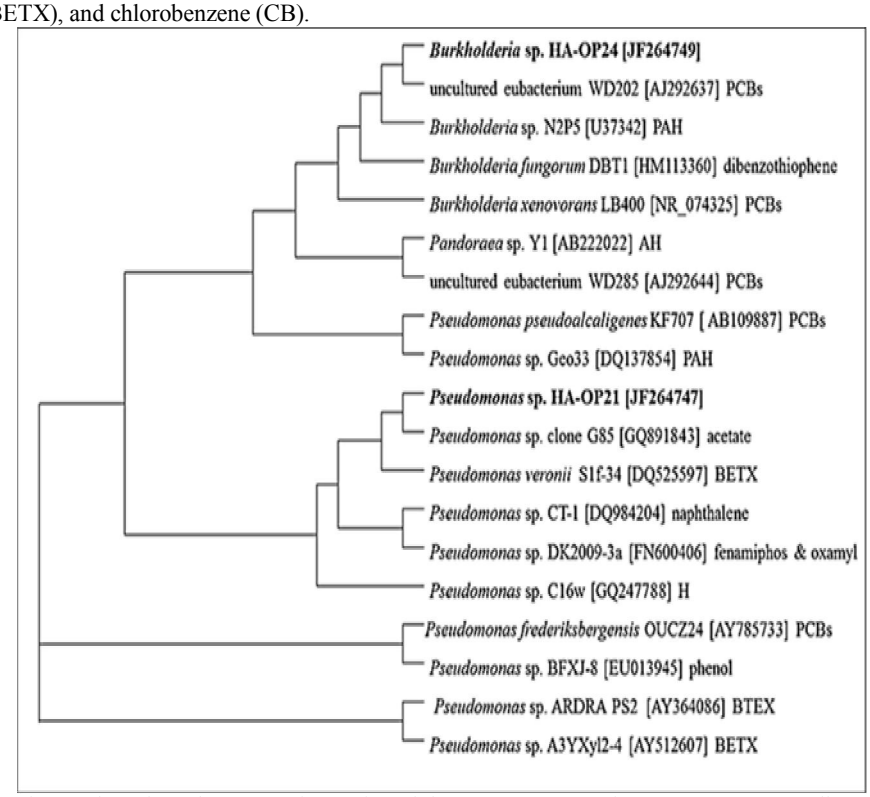

Figure 2. Phylogenetic tree based on the comparison of partial 16S rRNA gene fragments, corresponding to position 945-1492 according to the *E.coli* numbering system, showing the relationship of the new PCBs degraders strains (with bold line and the accession numbers after the strain name) with the nearest strain for each, that have already reported before as aromatic degraders. Polychlorinated biphenyls (PCBs), Polyaromatic hydrocarbon (PAH), aromatic hydrocarbon (AH), benzene - ethylbenzene - toluene - xylene (BETX), hydrocarbons (H).

Table 2. Substrate utilization by *Pseudomonas* sp. HA-OP21 and *Burkholderia* sp. HA-OP24

Carbon source / Strains	*Pseudomonas* sp. HA-OP21	*Burkholderia* sp. HA OP24	Control
BTEX	+	+	-
Biphenyl	+	+	-
Dibenzofuran	+	+	-
2-Chlorobiphenyl	+	+	-
4-Chlorobiphenyl	+	+	-
2,3-Chlorobiphenyl	-	+	-
2,4-Chlorobiphenyl	-	+	-
2,4,5,2',4',5'-Chlorobiphenyl	-	+	-

+ growth; - No growth detected; control (without microorganisms)

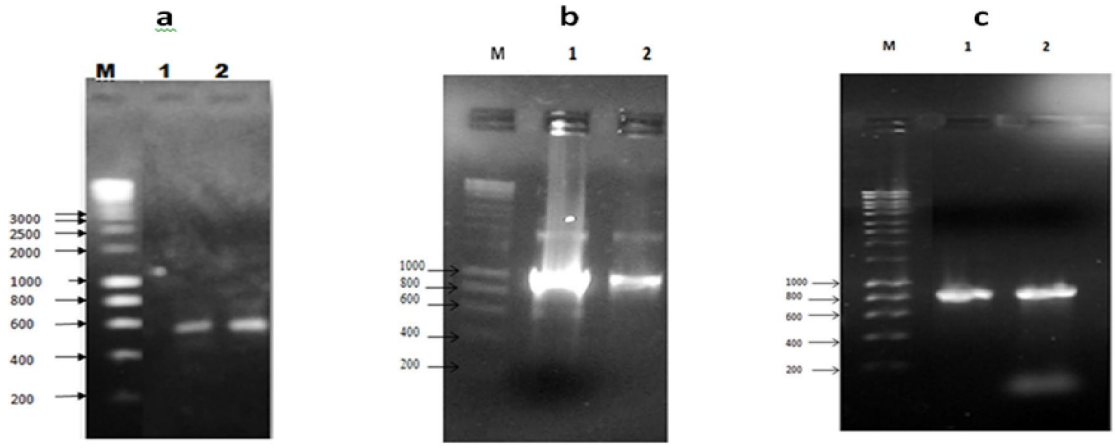

Figure 3. Agarose gel electrophoresis of the PCR product obtained (lane 1, 2) from genomic DNA of *Pseudomonas* sp. Strain HA-OP21 and *Burkholderia* sp. Strain HA-OP24 respectively using bphAF668-3 & bphAR1153-2 primers (a), C23OF & C23OR primers (b) and EXDO-K2-F & EXDO-K2-R primers (c). Molecular weight marker Hyperladder 1(M) (fermentas).

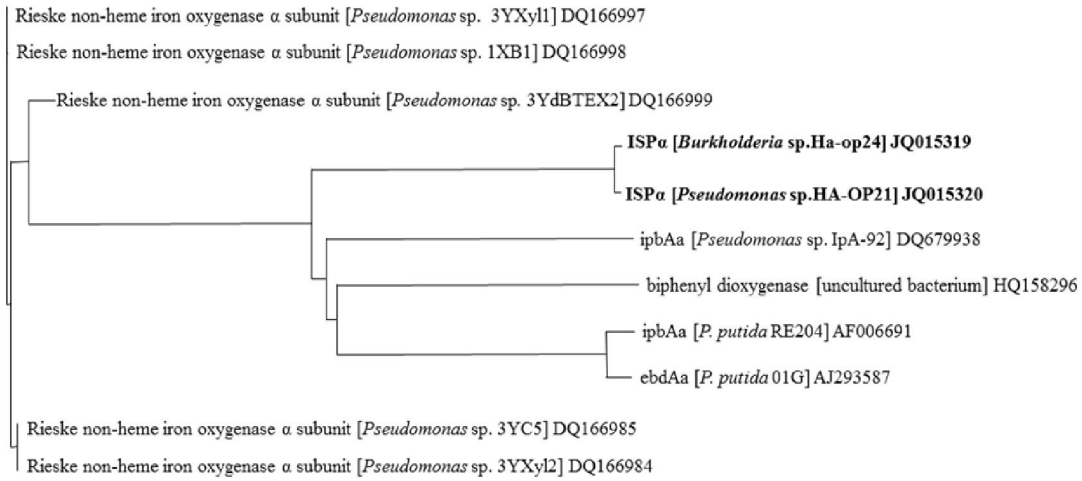

Figure 4. Phylogenetic tree of Rieske non-heme iron oxygenase α subunit from different bacteria including the Biphenyl dioxygenase large subunit from *Burkholderia* sp. HA-OP24 and *Pseudomonas* sp. HA-OP21 (referred to it with bold line and the accession numbers after the strain name). ClustalX 1.8 and Treeview 1.6.1 computer programs were used to construct the phylogenetic tree based on nucleotide sequence alignments.

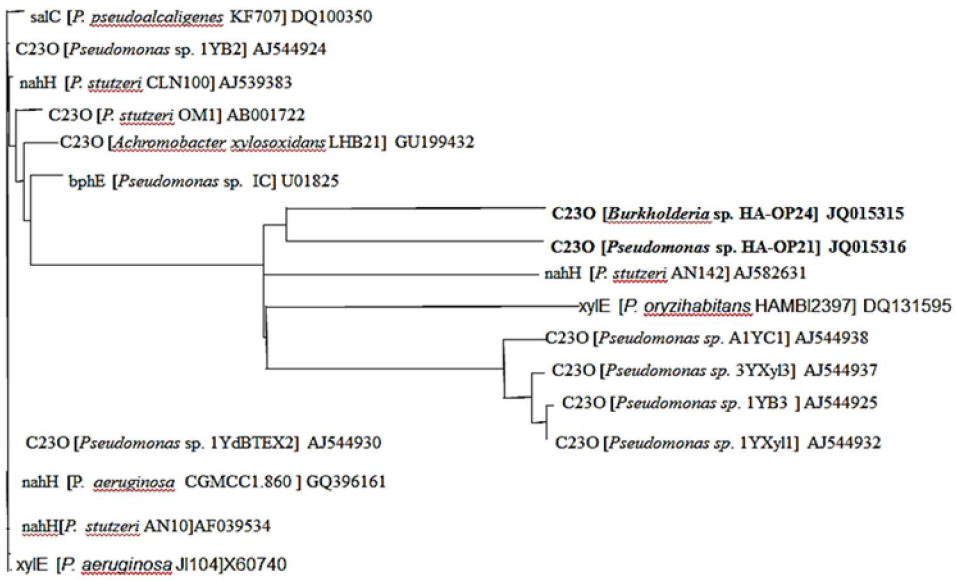

Figure 5. Phylogenetic tree of extradiol dioxygenase genes type I from different bacteria including the extradiol dioxygenase genes from *Burkholderia* sp.HA-OP24 and *Pseudomonas* sp.HA-OP21 referred to it with bold line and the accession numbers after the strain name. ClustalX 1.8 and Treeview 1.6.1 computer programs were used to construct the phylogenetic tree based on nucleotide sequence alignments.

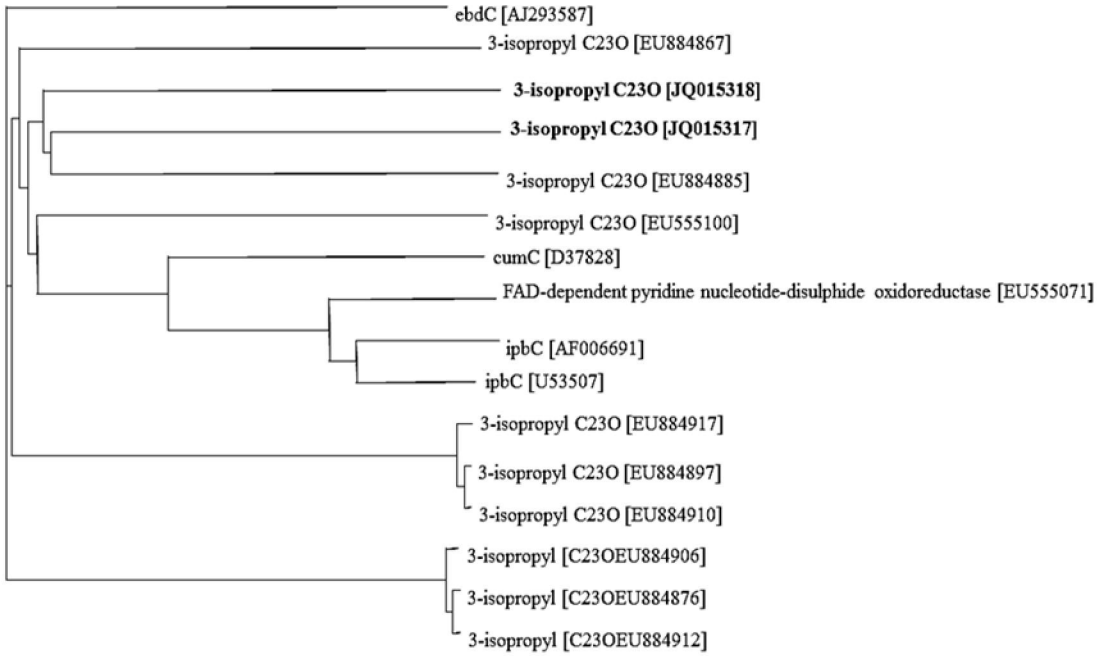

Figure 6. Phylogenetic tree of 3-isopropylcatechol dioxygenase from different bacteria including the 3-isopropylcatechol dioxygenase from Burkholderia sp.HA-OP24 and Pseudomonas sp.HA-OP21 referred to it with bold line and the accession numbers after the strain name. ClustalX 1.8 and Treeview 1.6.1 computer programs were used to construct the phylogenetic tree based on nucleotide sequence alignments. For strain names AJ293587[*P. putida*01G3], EU884867[Uncultured bacterium clone s8], JQ015317 [*Burkholderia* sp.HA-OP24], JQ015318 [*Pseudomonas* sp.HA-OP21], EU884885 [Uncultured bacterium, clone s102], EU555100 [Uncultured bacterium clone s221_K2], D37828 [*P. fluorescens*IP01], EU555071 [Uncultured bacterium clone s8_Tn-color], AF006691 [*P. putida* RE204], U53507 [*Pseudomonas* sp. JR1], EU884917 [Uncultured bacterium clone s223], EU884897 [Uncultured bacterium clone s157], EU884910 [Uncultured bacterium clone s199], EU884906 [Uncultured bacterium clone s188], EU884876 [Uncultured bacterium clone s73], EU884912 [Uncultured bacterium clones 209].

4. Discussion

One of the challenges for successful PCBs bioremediation is the development of effective methods to analysis bacterial community structures and encourages the growth of selected microorganisms that are capable of transforming PCBs. Many PCB-degrading bacteria have been isolated and characterized (Bopp, 1986; Bedard *et al.,* 1987; Ahmad *et al.,* 1990). In the present study, We described the isolation and characterization of two PCBs degraders, *Pseudomonas* sp. Strain HA-OP21, that can grow only on monochlorinated biphenyls and *Burkholderia* sp. Strain HA-OP24 that have the ability to transform the higher chlorinated biphenyls and behave like *Burkholderia* sp. strain LB400 and *Rhodococcus jostii* RHA1, this may be explained by the highest activities, which were observed against 2,3DHB and catechol due to the high induction of *meta*-cleavage enzymes 2,3 dihydroxybiphenyl dioxygenase or catechol 2,3 dioxygenase in both upper and lower pathways respectively, this indication may be a good marker for isolation of PCBs degraders with broad spectrum. We suggest that our new PCBs degraders can metabolize biphenyl by *meta*-cleavage based on a simple test for induction of 2,3-dihydroxybiphenyl 1,2-dioxygenase and catechol 2,3 dioxygenase activities. The reaction product is yellow in color as a result of the production of the yellow *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid in the upper pathway and 2-hydroxymuconic semialdehyde in the lower pathway, this indication may be a good marker for isolation of PCBs degraders with broad spectrum.

The genetic organization of biphenyl catabolic genes has been elucidated in various groups of microorganisms (Pieper, 2005). Most of these studies have been conducted in which PCR was used as a molecular tool for detection of these catabolic genes in environmental isolates or environmental DNA, and diversity is usually assessed by sequencing of genes from isolates or PCR clone libraries. Several primer sets suitable for amplification conserved regions of the C23O genes as well as Rieske non-heme iron oxygenase ISPα gene segments have been reported previously (Kahl and Hofer, 2003; Junca and Pieper, 2003; Junca and Pieper, 2004 ; Hendrickx *et al.,* 2006). The ISPα gene was chosen as an indicator gene, because it has been impacted in substrate specificity and its DNA sequence is conserved for oxygenases targeting the same substrate (Witzig *et al.,* 2006).

Witzig et al. (Witzig *et al.,* 2006) used the PCR–single-strand conformation polymorphism (SSCP) technique to assess the diversity and distribution of toluene/biphenyl ISPα gene in a subset (36 isolates) of bacterial strains recovered from sites contaminated with BTEX and have the ability to convert catechol by *meta*-cleavage to the yellow product 2-hydroxymuconic semialdehyde. Products of the expected size could be amplified with DNA extracted from 33 out of the 36 bacterial isolates. Degenerative primer set (Witzig *et al.,* 2006) was used with our new bacterial isolates and a single band with the expected size was obtained. A comparison of the nucleotide sequences obtained in this study with the reported nucleotide sequences (Witzig *et al.,* 2006) was made and it was high identity (94% - 99%) the nucleotide sequences were related to Rieske non-heme iron oxygenase (ISPα) gene of toluene/biphenyl subfamily.

Erickson et al (Erickson *et al.,* 1993) proposed that the alterations in the sequence of the large subunit of the terminal biphenyl dioxygenase resulted in modifying the PCB congener specificity of the enzyme. As an example, the biphenyl dioxygenase genes of strains LB400 and KF707 exhibit dramatic differences in PCB substrate range despite nearly identical amino acid sequences. Similar comparisons have been reported (Gibson *et al.,* 1993).The differences in biphenyl dioxygenase activity result from amino acid differences in the large subunit of the terminal dioxygenase. Also by making a comparison between the nucleotide sequence of ISPα gene of *Pseudomonas* sp. Strain HA-OP21 and *Burkholderia* sp. Strain HA-OP24, it was found that there is difference in 6 nucleotides, So we suggest that the differences in the degrading abilities between *Pseudomonas* sp. Strain HA-OP21 and *Burkholderia* sp. Strain HA-OP24 was due to differences in the sequence of the large subunit of the terminal biphenyl dioxygenase, and the possibility to modify the PCB congener specificity of the biphenyl dioxygenase by site directed mutagenesis within this divergent region of the BphA subunit. These results suggest that it may be possible to expand the range of biodegradable PCB congeners and therefore facilitate the PCBs bioremediation.

Overall, we managed to isolate and characterize two bacterial PCBs degraders that showed high activities against 2,3DHB and catechol due to the high induction of *meta*-cleavage enzymes. Also, we amplified (ISPα) gene of toluene/biphenyl subfamily and catechol 2,3 dioxygenase gene that considered the most important catabolic genes involved in PCBs biodegradation.

Acknowledgements

The authors are grateful to the Science and Technological Development Fund (STDF) Government of Egypt for financial support to carry out this work as a part of project 46.

References

1. **Adav, S.S.; Lee, D.J. and Lai, J.Y. (2010).** Microbial community of acetate utilizing denitrifiers in aerobic granules. Appl. Microbiol. Biotechnol. 85:753-762.
2. **Ahmad, D.; Masse, R.; Sylvestre, M. (1990).** Cloning and expression of genes involved in 4-chlorobiphenyl transformation by *Pseudomonas testosteroni*: homology to polychlorobiphenyl-degrading genes in other bacteria. Gene 86:53–61.
3. **Ahmad, D.; Masse, R.; Sylvestre, M. and Sandossi, M., (1991).** Bioconversion of 2-hydroxy-6-oxo-6-(40-chloro-biphenyl) hexa-2,4-dienoic acid: the meta-cleavage product 185 of 4-chloro biphenyl. J. Gen. Microbiol. 137:1375–1385.
4. **Ahmed, M.; Focht, D.; 1973**. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. 19:42–82.
5. **Bedard, D.; Wagner, R. and Brennan, M. (1987).** Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcalegenes eutrophus* H850. Appl. Environ. Microbiol. 53, 1094–1102.
6. **Brennerova, M.V.; Josefiova, J.; Brenner, V.; Pieper, D.H and Junca, H. (2009).** Metagenomics reveals diversity and abundance of *meta*-cleavage pathways in microbial communities from soil highly contaminated with jet fuel under air-sparging bioremediation. Environmental Microbiology. 9:2216–2227.
7. **Bopp, L.H. (1986).** Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. 1:23–29.
8. **Borja, J.Q.; Auresenia, J.L. and Gallardo S.M. (2006).** Biodegradation of polychlorinated biphenyls using biofilm grown with biphenyl as carbon source in fluidized bed reactor. Chemosphere. 64:555–559.
9. **Borlakoglu, J.T. and Haegeles K.D. (1991).**Comparative aspects in the bioaccumulation, metabolism and toxicity with PCBs. Comp Biochem Physiol C. 100:327–338.
10. **Buchan, A.; Neidle, E.L. and Moran, M.A. (2001).** Diversity of the ring cleaving dioxygenase gene pcaH in a salt marsh bacterial community.Appl. Environ. Microbiol. 67:5801–5809.
11. **Chain, P.S.; Denef, V.J.; Konstantinidis, K.T.; Vergez, L.M.; Agulló, L.; Reyes, V.L.; Hauser, L.; Córdova, M.; Gómez, L.; González, M.; Land, M.; Lao, V.; Larimer, F.; LiPuma, J.J.; Mahenthiralingam, E.; Malfatti, S.A.; Marx, C.J.; Parnell, J.J.; Ramette, A.; Richardson, P.; Seeger, M.; Smith, D.; Spilker, T.; Sul, W.J.; Tsoi, T.V.; Ulrich, L.E.; Zhulin, I.B. and Tiedje, J.M. (2006).***Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility.Proc. Natl. Acad. Sci. 103:15280-15287.
12. **Dai, S.; Vaillancourt, F.; Maaroufi, H.; Drouin, N.; Neau, D.; Snieckus, V.; Bolin, J. and Eltis, L. (2002).** Identification and analysis of a bottleneck in PCB biodegradation. Nat Struct Biol. 9:934–939.
13. **Eltis, L.D. and Bolin, J.T. (1996).**Evolutionary relationships among extradioldioxygenases.J Bacteriol. 178:5930–5937.
14. **Erickson, B.D. and Mondello, F.J. (1993).**Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene.Appl Environ Microbiol 59:3858–3862.
15. **Fava, F.; Di Gioia, D.; Cinti, S.; Marchetti, L. and Quatroni, G. (1994).**Degradation and dechlorination of low chlorinated biphenyls by a 3-membered bacterial coculture. Appl. Microbiol. Biotechnol. 41, 117–123.
16. **Furukawa, K.; Hirose, J.; Suyama, A.; Zaiki, T. and Hayashida, S. (1993).**Gene components responsible for discrete substrate specifity in the metabolism of biphenyl (bph operon) and toluene (tod operon). J Bacteriol. 175:5224–5232.
17. **Gibson, D.T.; Cruden, D. L.; Haddock, J.D.; Zylstra, G. J.and Brand, J. M. (1993).** Oxidation of polychlorinated biphenyls by *Pseudomonas sp.* strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. 175:4561-4564.
18. **Gibson, D.T. and Parales, R.E. (2000).**Aromatic hydrocarbon dioxygenases in environmental biotechnology.CurrOpinBiotechnol. 11:236–243.
19. **Haddock, J.D.; Horton, J.R. and Gibson, D.T. (1995).**Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2,3-dioxygenase from Pseudomonas sp. strain LB400. J Bacteriol. 177:20–26.
20. **Haluska, L.; Barancikova, G.; Balaz, S.; Dercova, K.; Vrana, B.; Paz-Weisshaar, M.; Furciova, E. and BIielek, P. (1995).** Degradation of PCB in different soils by inoculated *Alcaligenesxylosoxidans*. Sci. Total Environ. 175:275–285.
21. **Hamdy A.H. (2013).** Detection of New Polychlorinated Biphenyl (PCB) Degraders Express *Meta*-cleavage Enzymes in PCB

Contaminated Site. Life Science Journal.10:1123-1132.

22. **Hamelin, J.; Fromin, N.; Tarnawski, S.; Teyssier-Cuvelle, S. and Aragno, M. (2002).** nifH gene diversity in the bacterial community associated with the rhizosphere of Moliniacoerulea, an oligonitrophilic perennial grass. Environ. Microbiol. 4:477-481.
23. **Hendrickx, B.; Junca, H.; Vosahlova, J.; Lindner, A.; Ruegg, I.; Bucheli-Witschel, M.; Faber, F.; Egli, T.; Mau, M.; Schlomann, M.; Brennerova, M.; Brenner, V.; Pieper, D.H.; Top, E.M.; Dejonghe, W.; Bastiaens, L. and Springael, D.(2006).** Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. J. Microbiol. Methods. 64:250–265.
24. **Hoefel, D.; Ho, L.; Aunkofer, W.; Monis, P.T.; Keegan, A.; Newcombe, G. and Saint, C.P. (2006).**Cooperative biodegradation of geosmin by a consortium comprising three gram-negative bacteria isolated from the biofilm of a sand filter column. Lett. Appl. Microbiol. 43:417-423.
25. **Hou, L. and Dutta, S.K. (2000).** Phylogenetic characterization of several para- and meta-PCB dechlorinating Clostridium species: 16s rDNA sequence analyses. LettApplMicrobiol. 30:238–243.
26. **Junca, H. and D.H. Pieper. (2003).** Amplified functional DNA restriction analysis to determine catechol 2,3dioxygenase gene diversity in soil bacteria. Microbiological Methods.55:697– 708.
27. **Junca, H. and D.H. Pieper. (2004).** Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. Environ. Microbiol. 6:95–110.
28. **Kahl, S. and B. Hofer.(2003).** A genetic system for the rapid isolation of aromatic-ring-hydroxylatingdioxygenase activities.Microbiology. 149:1475–1481.
29. **Kimura, N.; Nishi, A.; Goto, M. and Furukawa, K. (1997).**Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. J Bacteriol. 179:3936–3943.
30. **Leigh, M.B.; Prouzová, P.; Macková, M.; Macek, T.; Nagle, D.P. and Fletcher, J.S. (2006).**Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. Appl Environ Microbiol. 72:2331-2342.
31. **Lunt, D. and Evans, W.C. (1970).**The microbial metabolism of biphenyl.Biochem J. 118:54–55.
32. **Master, E.R. and Mohn, W.W.(1998).**Psychrotolerant bacteria isolated from arctic soil that degrade polychlorinated biphenyls at low temperatures. Appl. Environ. Microbiol. 64:4823-4829.
33. **McLeod, M.P.; Warren, R.L.; Hsiao, W.W.; Araki, N.; Myhre, M.; Fernandes, C.; Miyazawa, D.; Wong, W.; Lillquist, A.L.; Wang, D.; Dosanjh, M.; Hara, H.; Petrescu, A.; Morin, R.D.; Yang, G.; Stott, J.M.; Schein, J.E.; Shin, H.; Smailus, D.; Siddiqui, A.S.; Marra, M.A.; Jones, S.J.; Holt, R.; Brinkman, F.S.; Miyauchi, K.; Fukuda, M.; Davies, J.E.; Mohn, W.W. and Eltis, L.D. (2006).** The complete genome of *Rhodococcus*sp. RHA1 provides insights into a catabolic powerhouse. Proc. Natl. Acad. Sci. 103:15582-15587.
34. **Mueller, J.G.; Devereux, R.; Santavy, D.L.; Lantz, S.E.; Willis, S.G. and Pritchard, P.H. (1997).**Phylogenetic and physiological comparisons of PAH-degrading bacteria from geographically diverse soils.Antonie Van Leeuwenhoek 71:329-343.
35. **Nogales, B.; Moore, E.R.; Abraham, W.R. and Timmis, K.N. (1999).** Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil.Environ.Microbiol. 1:199-212.
36. **Nogales, B.; Moore, E.R.; Llobet-Brossa, E.; Rossello-Mora, R.; Amann, R. and Timmis, K.N. (2001).**Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. 67:1874-1884.
37. **Pieper, D.H. (2005).** Aerobic degradation of polychlorinated biphenyls.ApplMicrobiolBiotechnol. 67:170–191.
38. **Seeger, M.; Camara, B. and Hofer, B. (2001).**Dehalogenation, denitration, dehydroxylation, and angular attack on substituted biphenyls and related compounds by a biphenyl dioxygenase.J Bacteriol. 183:3548–3555.
39. **Seeger, M.; Zielinski, M.; Timmis K.N. and Hofer, B. (1999).** Regiospecificity of dioxygenation of di- to pentachlorobiphenyls

and their degradation to chlorobenzoates by the bph-encoded catabolic pathway of Burkholderia sp. strain LB400. Appl Environ Microbiol. 65:3614–3621.

40. **Seto, M.; Kimbara, K.; Shimura, M.; Hatta, T.; Fukuda, M. and Yano, K. (1995).**A novel transformation of polychlorinated biphenyls by *Rhodococcus*sp. strain RHA1. Appl. Environ. Microbiol. 61:3353-3358.
41. **Singleton, D.R.; Powell, S.N.; Sangaiah, R.; Gold, A.; Ball, L.M. and Aitken, M.D. (2005).**Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. Appl. Environ. Microbiol. 71:1202-1209 .
42. **Sylvestre, M. (2004).**Genetically modified organisms to remediate polychlorinated biphenyls.Where do we stand?.International Biodeterioration& Biodegradation. (54):153 – 162.
43. **Warren, R.; Hsiao, W. L.; Kudo, H.;Myhre, M.; Dosanjh, M.; Petrescu, A.; Kobayashi, H.; Shimizu, S.; Miyauchi, K.; Masai, E.; Yang, G.; Stott, J.M.; Schein, J.E.; Shin, H.; Khattra, J.; Smailus, D.; Butterfield, Y.S.; Siddiqui, A.; Holt, R.; Marra, M.A.; Jones, S.J.; Mohn, W.W.; Brinkman, F.S.; Fukuda, M.; Davies, J. and Eltis, L.D. (2004).** Functional characterization of a catabolic plasmid from polychlorinated biphenyl-degrading *Rhodococcus* sp. strainRHA1. J. Bacteriol. 186:7783-7795.
44. **WHO (World Health Organization), (1993)**. Polychlorinated Biphenyls andTerphenyls.
45. **Witzig, R.; Junca,H.; Hecht, H.; and Pieper, D.H. (2006).** Assessment of Toluene/Biphenyl Dioxygenase Gene Diversity in Benzene-Polluted Soils: Links between Benzene Biodegradation and Genes Similar to Those Encoding IsopropylbenzeneDioxygenases. Appl. Environ. Microbiol.72:3504–3514.
46. **Yeates, C.; Holmes, A.J. and Gillings, M.R. (2000).** Novel forms of ring-hydroxylating dioxygenases are widespread in pristine and contaminated soils. Environ. Microbiol. 2:644–653.
47. **Zielinski, M.; Backhaus, S. and Hofer, B. (2002).** The principal determinants for the structure of the substrate-binding pocket are located within a central core of a biphenyl dioxygenase alpha subunit. Microbiology. 148:2439–2448.
48. **Zocca, C.; Di Gregorio, S.; Sidler, S.; Toffanin, A.; Lizzari, D.; and Vallini, G.(2004).** Identification of two new sets of genes for dibenzothiophene transformation in *Burkholderia* sp. DBT1. Biodegradation 15:111-123.

7/22/2013