

Isolation and molecular characterization of a novel *pseudomonas putida* strain capable of degrading organophosphate and aromatic compounds

Rupa Iyer*, Victor G. Stepanov, Brian Iken

Department of Biology and Biochemistry, University of Houston, Houston, USA

Corresponding Author: rriyer@uh.edu

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ABSTRACT

A bacterial strain designated in this study as POXN01 was found to be capable of degrading the synthetic organophosphorus pesticides paraoxon and methyl parathion. The strain was initially isolated through enrichment technique from rice field soil near Harlingen, Texas. Phylogenetic analysis based on 16S rRNA, *gyrB* and *rpoD* gene alignments identified the POXN01 isolate as a new strain of *Pseudomonas putida*, which is closely related to the recently discovered nicotine-degrading strain *Pseudomonas putida* S16. While being unable to metabolize nicotine, the POXN01 isolate was observed to actively proliferate using monocyclic aromatic hydrocarbons, in particular toluene, as nutrients. Search for the genetic determinants of paraoxon catabolism revealed the presence of organophosphorus-degrading gene, *opd*, identical to the one from *Sphingobium fuliginis* (former *Flavobacterium* sp. ATCC 27551). Assimilation of aromatic compounds likely relies on *phc ARKLMNOPQ* gene cluster for phenol, benzene and toluene catabolism, and on *benRABCDKGEF* cluster for benzoate catabolism. The observed versatility of POXN01 strain in degradation of xenobiotics makes it useful for the multi-purpose bioremediation of contaminated sites in both agricultural and industrial environmental settings.

Keywords: Organophosphates; Aromatic Hydrocarbons; Bioremediation; *Pseudomonas putida*

1. INTRODUCTION

Quality of life achieved by modern society would be im-

possible without tremendous development of chemical industry within the past century. As a side effect of this growth, large amounts of man-made chemicals have been released into the environment either intentionally (as fertilizers, pesticides, or waste deposits) or by accident. Consequently, a substantial number of terrestrial and marine habitats today are contaminated by various xenobiotic compounds, many of which are harmful to living organisms.

Among other xenobiotics, organophosphorus (OP) compounds are of great concern considering their environmental impact and threat to human health. These synthetic chemicals are potent cholinesterase inhibitors once intended for military use, but at the present time, widely employed as insecticides. Occupational exposure to the OP pesticides in agricultural industries and self-poisoning with OP compounds causes significant health problems [1-6]. In addition, there are risks associated with major international efforts to destroy the stockpiled chemical warfare agents [7].

Despite their high toxicity, OP pesticides are extensively used in developing countries [8]. Exposure to organophosphate pesticides both purposely and accidentally results in millions of poisonings worldwide and 200,000 to 500,000 deaths annually throughout Asia and the Western Pacific [9-12]. While being removed from large-scale use in developed countries, organophosphates still may threaten metropolitan water supplies through unintentional contamination [13,14].

Removing environmentally hazardous OP compounds through detoxification by microbial enzymes has become the focus of biodegradation research over the last few decades. Previous reports have identified several organophosphate-degrading bacterial species, among them are *Sphingobium fuliginis* (former *Flavobacterium* sp. ATCC 27551) [15,16] and *Brevundimonas diminuta* GM (former *Pseudomonas diminuta* GM) [17,18], both express-

*Corresponding author.

ing identical organophosphate hydrolase enzymes. Organophosphate hydrolase is a zinc-containing homodimeric membrane protein that has been found to hydrolyze paraoxon at a rate approaching the diffusion limit [19]. The enzyme is encoded by an *opd* (organophosphate degradation) gene and is capable of hydrolyzing a wide range of oxon and thion organophosphates [20,21]. The *opd* gene has been detected in soil microorganisms belonging to different taxonomic groups, which points to its horizontal spread using mobile genetic elements (transposons, plasmids, phages) as transfer vehicles [22-28].

Currently, the isolation of a microorganism capable of degrading a selected xenobiotic compound is a routine task. However, modern DNA sequencing technologies allow us to quickly assess biotransformation potentials of the newly discovered strains well beyond the originally targeted activity. A strain that can simultaneously degrade multiple types of xenobiotics will be a preferable tool for decontamination of heavily polluted sites like landfills and sewage collectors. It can also be included in a variety of environmental clean-up scenarios as a multi-purpose remedy for pollution.

We have isolated a bacterial strain capable of degradation of the OP pesticides paraoxon and methyl parathion as well as several commonly used industrial aromatic hydrocarbons. In the present paper, we describe characterization of this microorganism along with identification of molecular constituents responsible for its ability to detoxify xenobiotics.

2. MATERIALS AND METHODS

2.1. Ethics Statement

The soil sample collected for this study was taken from a rice field on private land by a student as part of a training program in biotechnology funded by the Texas Workforce Commission from 2011-2012. The student was given consent and access to the field by the landowner prior to collecting the sample. Sample collection included only a small portion of the topsoil and did not endanger any protected species.

2.2. Chemicals, Enzymes and Oligonucleotides

Paraoxon (*O,O*-diethyl *O-p*-nitrophenyl phosphate), methyl parathion (*O,O*-dimethyl *O-p*-nitrophenyl phosphorothioate), L-(-)nicotine, toluene, benzene, and phenol were obtained from Sigma-Aldrich (St. Louis, MO). Other chemicals were either from Sigma-Aldrich (St. Louis, MO) or from Fisher Scientific (Pittsburgh, PA). Egg white lysozyme and DNase-free ribonuclease A were obtained from Sigma-Aldrich (St. Louis, MO). Proteinase K was from Promega (Madison, WI). Synthetic

deoxyoligonucleotides (**Table 1**) were purchased from Eurofins MWG (Huntsville, AL).

2.3. Media for Bacterial Growth

A carbon-deficient minimal medium (CSM) had the following composition: 0.2 g L⁻¹ MgSO₄ * 7H₂O; 0.08 g L⁻¹ Ca(NO₃)₂ * 4H₂O; 0.005 g L⁻¹ FeSO₄ * 7H₂O; 4.8 g L⁻¹ K₂HPO₄; 1.2 g L⁻¹ KH₂PO₄. Immediately prior to inoculation, it was supplemented with an appropriate carbon source. Luria-Bertani medium (LB) was prepared from the following components: 10 g L⁻¹ BactoTryptone (Difco Laboratories, Detroit, MI); 5 g L⁻¹ yeast extract (Difco Laboratories, Detroit, MI); 10 g L⁻¹ NaCl. The pH of both media was adjusted to 7.5. For growth on a solid surface, the media were supplemented with 1.5 g L⁻¹ BactoAgar (Difco Laboratories, Detroit, MI).

2.4. Isolation of Paraoxon-Metabolizing Microbial Strains from a Soil Sample

Soil samples were collected from a rice field near Harlingen (Cameron County, TX) at 26°11'N, 97°35'W. Air-dried soil (1 g) was suspended in 50 mL of the LB medium. The suspension was kept for 2 days on a rotary shaker at 30°C and 200 rpm. Insoluble materials were allowed to settle out and an aliquot (100 µL) from the cleared supernatant was used to inoculate 3 mL of CSM supplemented with 0.1 mg mL⁻¹ paraoxon. The culture was incubated for 1 week on a rotary shaker at 30°C and 200 rpm. 100 µL of the bacterial suspension was transferred into 3 mL of fresh paraoxon-containing CSM, and the incubation step was repeated. After six consecutive subcultivations, the bacteria were plated on a CSM agar containing variable concentrations of paraoxon (0.1 - 2.0 mg mL⁻¹). After overnight incubation at 30°C, one distinct isolate that included large white or creamy-white circular colonies with irregular margins was found on the plates. This isolate, designated as POXN01, was determined to be a Gram-negative rod-shaped bacterium, which exhibited noticeable paraoxon- and methyl parathion-degrading activity and was capable of growing on minimal medium with paraoxon as a carbon source.

Table 1. Primers used for PCR and sequencing. M represents an equimolar mixture of A and C.

Primer ID	Target	Sequence (5' to 3')	Ref.
27F	16S rDNA	AGAGTTTGATCTGGCTCAG	[30]
U1510R	16S rDNA	GGTTACCTTGTACGACTT	[31]
F196	<i>opd</i> gene	CGCGGTCTTACAACTCTC	[32]
F450	<i>opd</i> gene	CGCCACTTCGATGCGAT	[32]
R757	<i>opd</i> gene	TCAGTATCATCGCTGTGACC	[32]
R840	<i>opd</i> gene	CTTCTAGACCAATGCCACTG	[32]
R977	<i>opd</i> gene	TCACTCTCAGTGGAAATGAAGG	this study

2.5. Effect of OP and Aromatic Compounds on Bacterial Growth

To assess the effect of xenobiotics on the growth of POXN01 strain, the bacterial suspension was spread on CSM agar plates supplemented with the increasing concentrations of organophosphate or monocyclic aromatic compounds. Paraoxon and methyl parathion concentration in agar was 100, 200 and 400 $\mu\text{g mL}^{-1}$, nicotine concentration was 8, 16 and 33 mg mL^{-1} , and toluene and benzene concentration was 33, 50 and 66 mg mL^{-1} . A 200 μl of 10,000-fold diluted (paraoxon, methyl parathion) or 5000-fold diluted (nicotine, toluene, benzene) stationary culture of POXN01 strain was evenly spread over the surface of an appropriate agar medium (plate diameter 10 cm, volume of solid medium 30 mL). The plates were incubated at 30°C for 20 hours, and photographed for further analysis.

2.6. Degradation of OP and Aromatic Compounds by Resting Cells

Bacterial cultures were grown in LB medium (150 mL) on a rotary shaker at 37°C and 200 rpm until cell density reached OD₆₀₀ equal to 1.0. Cells were harvested by centrifugation for 10 min at 600 g and 4°C, washed three times with 50 mM potassium phosphate (pH 7.0), and finally resuspended in 25 mL of the same buffer. The degradation experiment was initiated by mixing the cell suspension with an appropriate amount of a target compound. Concentration of paraoxon and methyl parathion in the degradation mixtures was 100 $\mu\text{g mL}^{-1}$, while concentration of nicotine, toluene, benzene and phenol was 3 mg mL^{-1} . The mixtures were incubated at 30°C under constant shaking (120 rpm). Aliquots were taken from the mixtures at regular intervals, centrifuged to remove cells and insoluble materials, and analyzed either for the removal of target compound or for the accumulation of its degradation products in aqueous phase. Decomposition of OP pesticides was monitored by measuring absorbance of the released metabolic product *p*-nitrophenol at 405 nm. Degradation of nicotine, toluene, benzene and phenol was tracked by UV spectrometry within the range of 220 - 340 nm. TLC analysis of the selected samples was done on Silica Alu Foil plates (Sigma-Aldrich, St. Louis, MO) and Whatman KC18F plates (Fisher Scientific, Pittsburgh, PA) in solvent systems chloroform : ethanol : methanol: 0.5 M NaOH 30 : 15 : 2 : 1.5 (v/v) and methanol: water 2:1 (v/v), respectively.

2.7. Isolation of Cellular DNA

Total cellular DNA was extracted using the CTAB method [29] with the following modifications: cells were resuspended in TE buffer containing lysozyme (50,000

units mL^{-1}) and ribonuclease A (300 Kunitz units mL^{-1}), and incubated for 1 hour at 37°C. Immediately prior to lysis, proteinase K ($>30,000$ units g^{-1}) was added to a final concentration 0.25 mg mL^{-1} . Cells were lysed with 0.5% (w/v) SDS for one hour at 37°C. The subsequent removal of polysaccharides and residual proteins was performed as described in the original protocol.

2.8. 16S rDNA Sequencing

Fragments of the 16S rRNA gene were amplified by PCR from cellular DNA using 16S rDNA-specific primers (Table 1) [30-32] and PCR Master Mix (Promega, Madison, WI). The PCR conditions were as follows: initial denaturation at 94°C for 4.5 min, 32 cycles consisting of denaturation at 94°C for 0.5 min, annealing at 52°C for 0.5 min, and extension at 72°C for 1 min, and final elongation at 72°C for 4 min. PCR products were column-purified [33] and sequenced bi-directionally by the dye-terminator method with the same primers used for amplification. Sequencing was performed by SeqWright, Inc. (Houston, TX).

2.9. Random Sequencing of Genomic DNA

Purified cellular DNA (5 μg) of the POXN01 isolate was converted into a shotgun DNA library using a Paired End DNA Sample Prep Kit (Illumina, Inc., San Diego, CA) according to the manufacturer's instructions. Sequencing was performed on an Illumina Genome Analyzer II with a paired-end module at the Solexa Sequencing Core Facility of M. D. Anderson Cancer Center (University of Texas, Houston, TX). After removal of duplicates and low quality sequences from the raw dataset with the FASTX toolkit [34], a total of 3.46 million usable pairs of 36 nucleotide-long reads was obtained for a total of 249.14 million nucleotides. For a 6 Mb bacterial genome, this would correspond to 41× coverage at the even distribution of reads. The reads were assembled into contigs using Velvet 1.2.07 [35] or Edena 3 dev120626 [36] *de novo* assemblers after several preliminary runs were undertaken for parameter optimization. Eventually the Velvet assembly was performed with a hash length of 19, coverage cut-off of 2 and an expected coverage of 12.5×. Other parameters were found to lesser affect length and number of the assembled contigs. The Edena assembly was performed with a minimum overlap size of 18 and a coverage depth limit of 3. Both sets of contigs were evaluated batchwise against an NCBI-maintained nucleotide sequence database, *nt* (as of July 27, 2012), using NCBI-BLAST-2.2.25 + software [37,38]. Sequences of the genes of interest from the POXN01 isolate were reconstructed from the contigs exhibiting high similarity to the homologues from closely related bacterial species. The reconstruction from contigs was supplemented with

alignments of the short reads to the homologous genes in order to validate the reconstructed sequences. The alignments were performed using either Mosaik 1.0.1388 [39] or BWA 0.5.9 [40] software packages.

2.10. Phylogenetic Analysis of Gene Sequences

Multiple DNA sequence alignments were performed using ClustalX 2.0.12 and corrected manually when necessary [41]. Phylogenetic affiliations were evaluated using a Phylip 3.69 software package [42]. Evolutionary distances for the neighbor-joining method of sequence clustering were calculated using the F84 model of nucleotide substitutions [43]. Maximum likelihood modeling of DNA sequence evolution was done with transition-transversion ratio set at 2. The topology of generated dendograms was validated by bootstrapping with 1000 replicates for neighbor-joining trees and 200 replicates for maximum likelihood trees. Trees were visualized using TreeGraph 2.0.45 [44].

2.11. Detection and Identification of Organophosphorus Hydrolase gene

The presence of an organophosphorus hydrolase gene in the bacterial genome was assessed by PCR with forward and reverse primers derived from the sequence of the parathion hydrolase gene of *Sphingobium fuliginis* (former *Flavobacterium* sp. strain ATCC 27551), accession number M29593 (**Table 1**) [30-32]. PCR was performed under the following conditions: initial denaturation at 94°C for 4.5 min, 32 cycles consisting of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min, and final chain elongation at 72°C for 4 min. PCR products were analyzed by electrophoresis in a 2% agarose gel with 1 × TBE as the running buffer. When necessary, the obtained DNA fragments were excised from the gel, purified as described in [33], and sequenced by the dideoxy-terminator method at SeqWright, Inc. (Houston, TX).

2.12. Nucleotide Sequence Accession Numbers

POXN01 nucleotide sequences were deposited in GenBank under the following accession numbers: KC189953 (*benABCDKGEF* operon), KC189954 (*benR* gene), KC 189955 (*catBCA* operon), KC189956 (*gyrB* gene), KC 189957 (*opd* gene fragment), KC189958 (*pcaIIFTBDC* gene cluster), KC189959 (*pcaRK* gene cluster), KC152 907 (*phcARKLMNOPQ* gene cluster), KC189960 (*rpoD* gene), KC189961 (16S rRNA gene fragment), KC189 962 (*todX* gene), KC189963 (*ttg2ABCDEFG* gene cluster), KC189964 (*ttg8* gene), KC189965 (*ttgRABC* gene cluster).

3. RESULTS AND DISCUSSION

The bacterial strain designated as POXN01 was isolated

from rice field soil by enriching for bacteria capable of using synthetic OP pesticide paraoxon as the sole source of carbon. When grown in pure culture in paraoxon-supplemented CSM medium, the isolate promoted a color change of the culture medium to yellow, which pointed to its ability to hydrolyze paraoxon to *p*-nitrophenol and diethylphosphate. The isolate was observed to form colonies on CSM agar supplemented with 100 - 400 µg mL⁻¹ paraoxon or methyl parathion as the only available carbon sources (**Figure 1**). Thus, the strain is capable of both degrading the OP pesticides and utilizing products of their degradation as nutrients. Paraoxon is apparently a better substrate for the POXN01 isolate than methyl parathion since paraoxon decomposition by POXN01 resting cells proceeds noticeably faster (**Figure 2**).

Initial attempt to identify the isolate relied on sequence analysis of 16S rRNA genes probed by PCR with standard 16S rDNA-specific primers. A fragment of 16S rDNA from strain POXN01 was obtained by PCR with primer pair 27F-U1510R. BLAST analysis revealed the amplicon to be identical to a number of sequences from GenBank database, and all of them belonged to environmental isolates related to the genus *Pseudomonas*. However, these isolates did not apparently form a coherent phylogenetic group, and the majority of them were not characterized beyond 16S rDNA sequencing. Thus, the exact taxonomic position of the POXN01 strain remained obscure.

Further insight into the phylogenetic affiliation of the POXN01 isolate was obtained from random sequencing of its genomic DNA using the Illumina short read sequencing system. The collected short reads were as

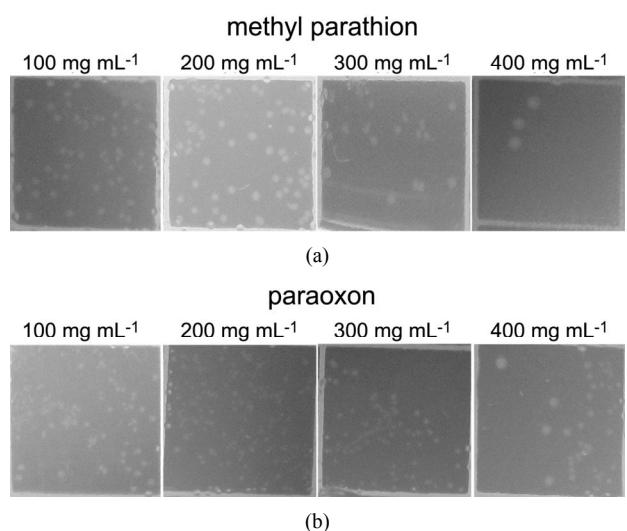


Figure 1. Growth of POXN01 isolate on CSM agar plates supplemented with methyl parathion (a) or paraoxon (b). A culture dilution factor of 10 - 4 was used for plate inoculation. Images depict colony growth within representative 1 cm² frames.

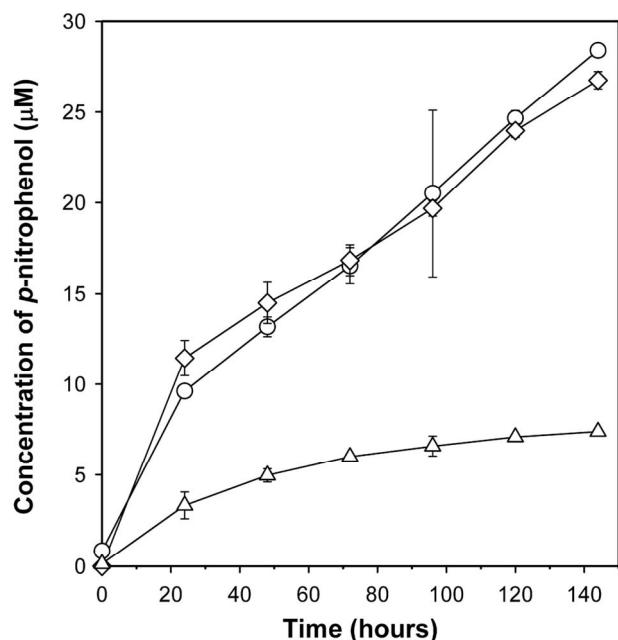


Figure 2. Degradation of paraoxon and methyl parathion by POXN01 isolate. Starting concentration of paraoxon and methyl parathion for degradative analysis was $100 \mu\text{g mL}^{-1}$. Concentration of *p*-nitrophenol, the metabolic byproduct of paraoxon and methyl parathion, in the media was derived from the absorbance at 405 nm. *P. putida* strain CBF10-2 harboring the *opd* gene (67) was used as a positive control. All values have been corrected for the spontaneous organophosphate hydrolysis by subtracting the amount of *p*-nitrophenol formed in the presence of *E. coli* JM109/pUC19 cells (non-OP-degrading strain). Shown are paraoxon degradation by POXN01 isolate (diamonds), methyl parathion degradation by POXN01 isolate (triangles), and paraoxon degradation by *P. putida* CBF10-2 (circles).

sembled using Velvet or Edena assemblers into two respective sets of contigs. Each set was used to perform a BLAST similarity search through the NCBI's *nt* nucleotide sequence database. In both cases the vast majority of top scoring hits were against *Pseudomonas putida* nucleotide sequences. Among bacteria with sequenced complete genomes, a nicotine-degrading strain *P. putida* S16 exhibited the highest degree of similarity to the POXN01 isolate (**Table 2**).

It is known that 16S rDNA similarity analysis alone does not provide satisfactory intrageneric resolutions for pseudomonads, and therefore, it is often supplemented by sequence comparison of other conserved genes [46-50]. Two housekeeping genes, *gyrB* and *rpoD*, are frequently utilized as molecular markers to elaborate phylogenetic relationships within the genus *Pseudomonas* [48,51-53]. Sequences of the POXN01 *gyrB* and *rpoD* genes were recovered from the obtained contigs of POXN01 genomic DNA and individually aligned with the homologous genes from type strains of 105 species belonging to genus *Pseudomonas*. This was combined with analogous

Table 2. Summary of POXN01 genome sequence assembly. Only contigs longer than 100 bp were considered. BLAST search was performed with E-value cut-off 10^{-6} . For each mentioned *P. putida* strain, the whole genome sequence is available.

	Edena	Velvet
Number of assembled contigs	8,230	9,725
Total number of base pairs in assembled contigs	4,843,570	4,885,502
Total number of base pairs in contigs with a single top BLAST hit in <i>nt</i> database	4,686,122	4,719,402
of those,		
total number of base pairs aligned to BLAST targets (percent identity among the aligned base pairs)	4,607,345 (97.67)	4,601,117 (97.64)
of those,		
with top hit to a sequence originating from a <i>Pseudomonas putida</i> strain	4,559,596 (97.82)	4,559,426 (97.80)
of those,		
from <i>P. putida</i> S16	4,323,490 (98.39)	4,317,142 (98.37)
from <i>P. putida</i> GB-1	86,772 (88.19)	69,629 (88.13)
from <i>P. putida</i> BIRD-1	44,467 (88.77)	49,508 (88.42)
from <i>P. putida</i> ND6	25,052 (86.17)	21,272 (85.74)
from <i>P. putida</i> KT2440	23,227 (86.32)	31,081 (86.92)
from <i>P. putida</i> F1	13,595 (87.10)	13,379 (86.95)
from <i>P. putida</i> DOT-T1E	14,812 (84.94)	20,855 (86.87)
from <i>P. putida</i> W619	15,230 (81.43)	19,994 (83.86)
from other <i>P. putida</i> strains	12,951 (90.38)	16,566 (92.83)
with top hit to a sequence originating from another species of genus <i>Pseudomonas</i>	30,686 (82.60)	28,832 (81.28)
with top hit to a sequence originating from an organism unrelated to genus <i>Pseudomonas</i>	17,063 (83.26)	12,859 (77.24)
Total number of base pairs in contigs with multiple equally good top BLAST hits in <i>nt</i> database	79,843	79,170
Total number of base pairs in contigs with no BLAST hits in <i>nt</i> database	77,605	86,930

alignment of 16S rDNA genes. An unrooted neighbor-joining tree was computed from concatenated individual alignments joined in the following order: 16S rDNA - *gyrB* - *rpoD*. The POXN01 isolate was found grouped with *P. monteili* within *P. putida* group of species (**Figure S1**). The refined analysis of POXN01 taxonomic affiliation was performed as above and included 20 strains of *Pseudomonas putida*, 8 other species belonging

to the *P. putida* intrageneric group (*P. fulva*, *P. parafulva*, *P. entomophila*, *P. monteili*, *P. mosselli*, *P. plecoglossicida*, *P. cremoricolorata*, and *P. oryzihabitans*), and *P. aeruginosa* taken as an outgroup. In both maximum likelihood and neighbor-joining trees, the POXN01 isolate was clustered together with *P. putida* S16 known for its ability to efficiently metabolize nicotine [54,55], *P. putida* BH, which can use phenol as a nutrient [56,57], and *P. putida* A10L, which can feed on 2-hydroxy-2-phenylacetate [58] (Figure 3). This whole branch is located somewhat closer to *P. monteili* than to *P. putida* type strain, ATCC12633, which explains the observed association of POXN01 isolates with *P. monteili* rather than with *P. putida* on the genus-wide tree.

Since the decomposition of paraoxon by the POXN01 strain apparently proceeds via the hydrolysis of a phosphoester bond yielding diethylphosphate and *p*-nitrophenol, the obtained fragments of its genomic DNA sequence were searched for the presence of an organophosphorus hydrolase gene. However, the sequenced part of the POXN01 genome did not contain any segment with significant similarity to the genes of known OP-degrading enzymes. Therefore, we attempted to identify organophosphorus hydrolase gene by PCR. Among OP-hydrolyzing enzymes, the most studied so far is a product of *opd* gene, which was found in *Sphingobium fuliginis* [27,59], *Brevundimonas diminuta* GM [17,27], and in several representatives of genera *Pseudomonas* and *Agrobacterium* (in the latter case the gene is designated *opdA*) [24-26,60]. Detection of the gene in the POXN01 strain was performed according to an established protocol that relies on analytical PCR with *opd*-specific primers [32]. PCR on the whole POXN01 genomic DNA yielded a single amplicon of the expected size for each of the primer pairs F196-R757, F196-R840, F450-R757, and F450-R840 (Figure 4). The largest amplicon obtained with primer pair F196-R977 was sequenced, and its sequence was found to be identical to that of the *opd* gene from *S. fuliginis* (Table 3).

Availability of sequenced fragments of POXN01 genome made it possible to search for the genes involved in degradation of other xenobiotics. In spite of high overall similarity to *P. putida* S16, no nicotine catabolism genes were identified in the POXN01 contigs. Furthermore, the POXN01 strain was unable to grow on nicotine-supplemented CSM and its resting cells did not degrade nicotine (data not shown). At the same time, POXN01 genome was found to contain several gene clusters related to the catabolism of monocyclic aromatic hydrocarbons (Table 3). In agreement with this finding, the POXN01 strain formed colonies on benzene- and toluene-supplemented CSM agar (Figure 5). Incubation of POXN01 resting cells with benzene, phenol, or toluene in phosphate buffer resulted in significant alteration of the UV absorption spectra of the solutions, which implies forma-

tion of water-soluble products of decomposition of these aromatic compounds (Figure 6). This was accompanied by accumulation of fluorescent metabolite(s) detected by TLC analysis (Figure S2). Overall, these observations revealed the ability of POXN01 strain to tolerate presence of various aromatic hydrocarbons, and to degrade

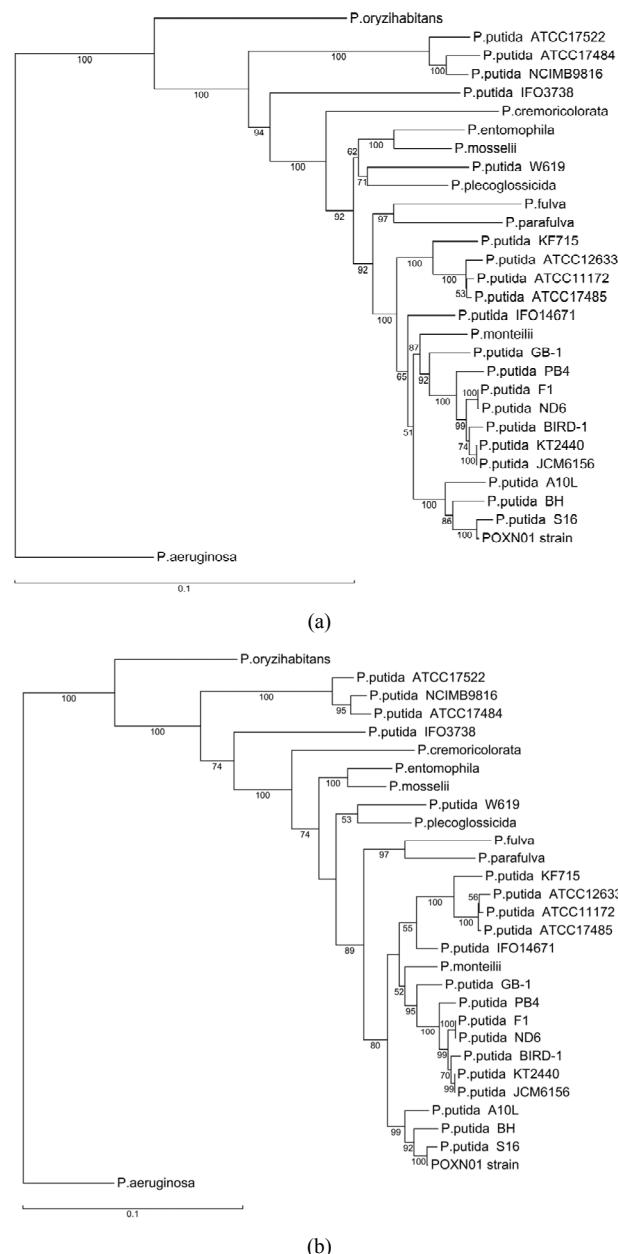


Figure 3. Phylogenetic trees derived from concatenated alignments of 16S rRNA - *gyrB* - *rpoD* genes of the POXN01 isolate and species belonging to the *Pseudomonas putida* intrageneric group. The trees were rooted using *Pseudomonas aeruginosa* as an outgroup. Bootstrap values expressed as percentages of the total number of replicates are shown next to each node (values below 50% are not shown). Scale of the total number of replicates are shown next to each node (values below 50% are not shown). Scale.

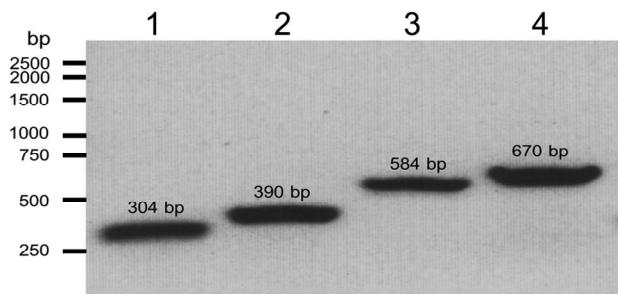


Figure 4. Detection of the *opd* gene in the genomic DNA of the POXN01 isolate. Electrophoretic analysis of PCR products obtained by amplification of the genomic DNA of the POXN01 strain with *opd*-specific primer pairs F450-R757 (lane 1), F450-R840 (lane 2), F196-R757 (lane 3) and F196-R840 (lane 4). The expected length of PCR fragments is 304 bp, 390 bp, 584 bp and 670 bp, respectively.

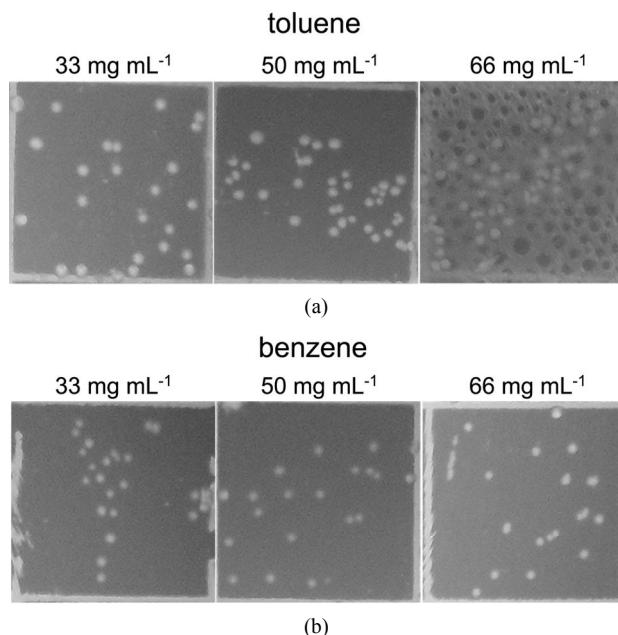


Figure 5. Growth of POXN01 isolate on CSM agar plates supplemented with toluene (a) and benzene (b). A culture dilution factor of $2 \times 10 - 4$ was used for plate inoculation. Images depict colony growth within representative 1 cm^2 frames.

them in the course of assimilation.

The initial steps of aromatic hydrocarbon degradation by the POXN01 strain likely involve *phc* and *ben* gene clusters. *phc* (phenol catabolism) cluster includes genes for catechol 1,2-dioxygenase (*phcA*), sigma54-dependent transcriptional activator (*phcR*), six subunits of multi-component phenol/benzene hydroxylase (*phcKLMNOP*), and conserved exported protein of unknown function (designated here as *phcQ*). Phenol/benzene hydroxylase can oxidize both phenol and benzene to catechol, which is then converted to *cis,cis*-muconate through intradiol cleavage catalyzed by catechol 1,2-dioxygenase [61]. *ben* cluster consists of nine genes involved in benzoate

uptake and degradation: transcriptional regulator (*benR*), three subunits of benzoate 1,2-dioxygenase (*benABC*), 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase (*benD*), two putative benzoate transporters (*benK*, *benE*), catechol 1,2-dioxygenase (designated here as *benG*), and benzoate-specific outer membrane porin (*benF*). The enzymes encoded in *ben* cluster convert benzoate to *cis,cis*-muconate via 1,2-*cis*-dihydroxybenzoate and catechol [62]. Thus, phenol, benzene, and benzoate upper degradation pathways converge at *cis,cis*-muconate, which is further processed by the enzymes of *catBCA* operon and *pca* regulon to tricarboxylic acid cycle intermediate, succinyl-CoA (Figure 7). At the same time, no close homologs of the known genes for the specialized toluene degradation enzymes have been found. While this may certainly result from incompleteness of the available POXN01 genome sequence, there is a possibility that the toluene ring fission is initiated by phenol/benzene hydroxylase. Indeed, this enzyme exhibits quite a relaxed specificity towards their aromatic hydrocarbon substrates, and can oxidize toluene albeit at a lower rate than phenol and benzene [63]. Similarly, it might hydroxylate *p*-nitrophenol released during paraoxon hydrolysis, thus making it susceptible to dioxygenase-mediated ring cleavage.

An ability of POXN01 strain to proliferate in the environment containing toxic substances while using them as nutrients implies tight control over their uptake and intracellular concentration. The strain maintains homeostasis using TtgABC efflux pump to expel aromatic hydrocarbons and some antibiotics from the cytoplasm [64,65]. It is supplemented by another putative transporter complex, Ttg2, which has been shown to significantly increase host tolerance of toluene [66]. Hydrocarbon uptake is mediated by a TodX-like outer membrane channel protein with a hatch domain regulating a passage of hydrophobic compounds to the periplasm [67]. In addition, the strain carries *ttg8* gene encoding a conserved protein of unknown function, which plays an important role in resistance to toluene [66]. The protein exhibits a pronounced similarity to O-antigen polymerases, and as such might control permeability of cell envelope to xenobiotics.

The discovered biotransformation capabilities of POXN01 strain may find application in a variety of tasks. The strain can be used to speed up mineralization of OP pesticides in agricultural systems and sewage water, to degrade chemical warfare agents, and to contain spills of petroleum hydrocarbons and related industrial chemicals. Further studies on POXN01 metabolism might reveal other useful properties of this microorganism.

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Table 3. Genes identified in POXN01 strain.

Gene	CDS length, bp	G + C content, %	Putative function of gene product	Closest homologous gene				
				Gene ID	Organism	Accession number	Percent nucleotide identity in CDS	Percent amino acid identity in gene product
<i>Rrs</i> (fragment)	1498	53.81	16S ribosomal RNA	16S rRNA	<i>Pseudomonas</i> sp. clone Filt.89	HM152676	100	-
<i>opd</i> (fragment)	823	56.38	organophosphate hydrolase	<i>opd</i>	<i>Sphingobium fuliginis</i>	M29593	100	100
<i>gyrB</i>	2418	57.28	DNA gyrase subunit beta	PPS_0012	<i>Pseudomonas putida</i> S16	NC_015733	98.96	99.75
<i>rpoD</i>	1848	60.34	RNA polymerase sigma factor RpoD (sigma 70)	PPS_0383	<i>Pseudomonas putida</i> S16	NC_015733	99.89	100
<i>phcA</i>	897	65.89	catechol 1,2-dioxygenase	PputGB1_33_13	<i>Pseudomonas putida</i> GB-1	NC_010322	85.71	89.36
<i>phcR</i>	1701	65.96	sigma 54-specific transcriptional regulator	PputGB1_33_12	<i>Pseudomonas putida</i> GB-1	NC_010322	90.65	96.82
<i>phcK</i>	255	64.31	phenol/benzene hydroxylase subunit	PputGB1_33_11	<i>Pseudomonas putida</i> GB-1	NC_010322	91.76	92.94
<i>phcL</i>	993	66.67	phenol/benzene hydroxylase subunit	PputGB1_33_10	<i>Pseudomonas putida</i> GB-1	NC_010322	91.74	95.77
<i>phcM</i>	267	60.67	phenol/benzene hydroxylase subunit	PputGB1_33_09	<i>Pseudomonas putida</i> GB-1	NC_010322	94.38	95.50
<i>phcN</i>	1512	62.76	phenol/benzene hydroxylase subunit	PputGB1_33_08	<i>Pseudomonas putida</i> GB-1	NC_010322	93.84	97.22
<i>phcO</i>	357	66.95	phenol/benzene hydroxylase subunit	PputGB1_33_07	<i>Pseudomonas putida</i> GB-1	NC_010322	89.07	90.75
<i>phcP</i>	1059	66.48	phenol/benzene hydroxylase subunit	PputGB1_33_06	<i>Pseudomonas putida</i> GB-1	NC_010322	90.17	95.46
<i>phcQ</i>	897	62.21	meta-pathway phenol degradation-like protein	PputGB1_33_05	<i>Pseudomonas putida</i> GB-1	NC_010322	91.97	96.65
<i>benR</i>	954	61.01	transcriptional regulator	PPS_2766	<i>Pseudomonas putida</i> S16	NC_015733	97.06	100
<i>benA</i>	1356	62.91	benzoate 1,2-dioxygenase subunit alpha	PPS_2765	<i>Pseudomonas putida</i> S16	NC_015733	98.89	99.33
<i>benB</i>	483	59.83	benzoate 1,2-dioxygenase subunit beta	<i>benB</i>	<i>P. putida</i> GJ31, plasmid pKW1	AY831458	99.58	100
<i>benC</i>	1008	65.77	benzoate 1,2-dioxygenase oxidoreductase subunit	PPS_2763	<i>Pseudomonas putida</i> S16	NC_015733	97.51	99.40
<i>benD</i>	759	64.82	1,6-dihydroxyhexa-2,4-diene-1-carboxylate dehydrogenase	PPS_2762	<i>Pseudomonas putida</i> S16	NC_015733	98.28	99.60
<i>benK</i>	1326	64.86	benzoate transporter	PPS_2761	<i>Pseudomonas putida</i> S16	NC_015733	98.49	99.54
<i>benG</i>	912	65.68	catechol 1,2-dioxygenase	PPS_2760	<i>Pseudomonas putida</i> S16	NC_015733	98.35	100

Continued

<i>benE</i>	1197	69.01	benzoate transporter	PPS_2759	<i>Pseudomonas putida</i> S16	NC_015733	98.74	99.74	99.74
<i>benF</i>	1248	62.82	outer membrane porin	PPS_2758	<i>Pseudomonas putida</i> S16	NC_015733	98.63	99.27	99.51
<i>catB</i>	1110	64.86	muconate cycloisomerase	PPS_3181	<i>Pseudomonas putida</i> S16	NC_015733	99.18	99.45	99.72
<i>catC</i>	288	64.58	muconolactone delta-isomerase	PPS_3180	<i>Pseudomonas putida</i> S16	NC_015733	98.95	100	100
<i>catA</i>	933	66.35	catechol 1,2-dioxygenase	PPS_3179	<i>Pseudomonas putida</i> S16	NC_015733	98.71	100	100
<i>pcaK</i>	1380	63.41	4-hydroxybenzoate transporter	PPS_4279	<i>Pseudomonas putida</i> S16	NC_015733	98.91	99.78	100
<i>pcaI</i>	828	64.25	beta-ketoadipate:succinyl-CoA transferase subunit alpha	PPS_4278	<i>Pseudomonas putida</i> S16	NC_015733	98.79	100	100
<i>pcaJ</i>	777	65.77	beta-ketoadipate:succinyl-CoA transferase subunit beta	PPS_4277	<i>Pseudomonas putida</i> S16	NC_015733	98.97	99.61	100
<i>pcaF</i>	1200	65.75	beta-ketoadipyl-CoA thiolase	PPS_4276	<i>Pseudomonas putida</i> S16	NC_015733	99.16	100	100
<i>pcaT</i>	1287	61.46	major facilitator superfamily metabolite/H(+) symporter	PPS_4275	<i>Pseudomonas putida</i> S16	NC_015733	98.91	99.76	99.76
<i>pcaB</i>	1350	69.11	3-carboxy-cis,cis-muconate cycloisomerase	PPS_4274	<i>Pseudomonas putida</i> S16	NC_015733	98.59	99.55	99.77
<i>pcaD</i>	789	66.41	beta-ketoadipate enol-lactone hydrolase	PPS_4273	<i>Pseudomonas putida</i> S16	NC_015733	98.60	98.85	99.23
<i>pcaC</i>	390	62.82	4-carboxymuconolactone decarboxylase	PPS_4272	<i>Pseudomonas putida</i> S16	NC_015733	99.23	100	100
<i>ttgR</i>	630	60.48	transcriptional regulator	PPS_4266	<i>Pseudomonas putida</i> S16	NC_015733	99.04	99.52	100
<i>ttgA</i>	1152	64.32	RND family efflux transporter, MFP subunit	PPS_4267	<i>Pseudomonas putida</i> S16	NC_015733	99.47	100	100
<i>ttgB</i>	3150	62.57	hydrophobe/amphiphile efflux-1 family protein	PPS_4268	<i>Pseudomonas putida</i> S16	NC_015733	99.39	100	100
<i>ttgC</i>	1455	65.15	RND efflux system outer membrane lipoprotein	PPS_4269	<i>Pseudomonas putida</i> S16	NC_015733	99.10	100	100
<i>ttg2A</i>	807	63.82	toluene tolerance protein similar to ABC transporter	<i>ttg2A</i>	<i>Pseudomonas putida</i> GM73	AF106002	99.50	100	100
<i>ttg2B</i>	795	62.52	toluene tolerance transmembrane protein	<i>ttg2B</i>	<i>Pseudomonas putida</i> GM73	AF106002	99.87	100	100
<i>ttg2C</i>	483	60.25	toluene tolerance protein	<i>ttg2C</i>	<i>Pseudomonas putida</i> GM73	AF106002	100	100	100
<i>ttg2D</i>	645	60.47	toluene tolerance protein	<i>ttg2D</i>	<i>Pseudomonas putida</i> GM73	AF106002	99.53	100	100
<i>ttg2E</i>	300	68.00	toluene tolerance protein	<i>ttg2E</i>	<i>Pseudomonas putida</i> GM73	AF106002	97.33	100	100
<i>ttg2F</i>	237	55.70	toluene tolerance protein	<i>ttg2F</i>	<i>Pseudomonas putida</i> GM73	AF106002	100	100	100
<i>ttg2G</i>	1263	63.18	UDP-N-acetylglucosamine 1-carboxyvinyltransferase transferase	PPS_0992	<i>Pseudomonas putida</i> S16	NC_015733	98.81	99.52	99.76
<i>ttg8</i>	603	62.19	toluene tolerance protein	PPS_4781	<i>Pseudomonas putida</i> S16	NC_015733	97.18	99.50	100
<i>todX</i>	1278	57.90	aromatic hydrocarbon degradation membrane protein	PPS_1340	<i>Pseudomonas putida</i> S16	NC_015733	99.29	99.76	99.76

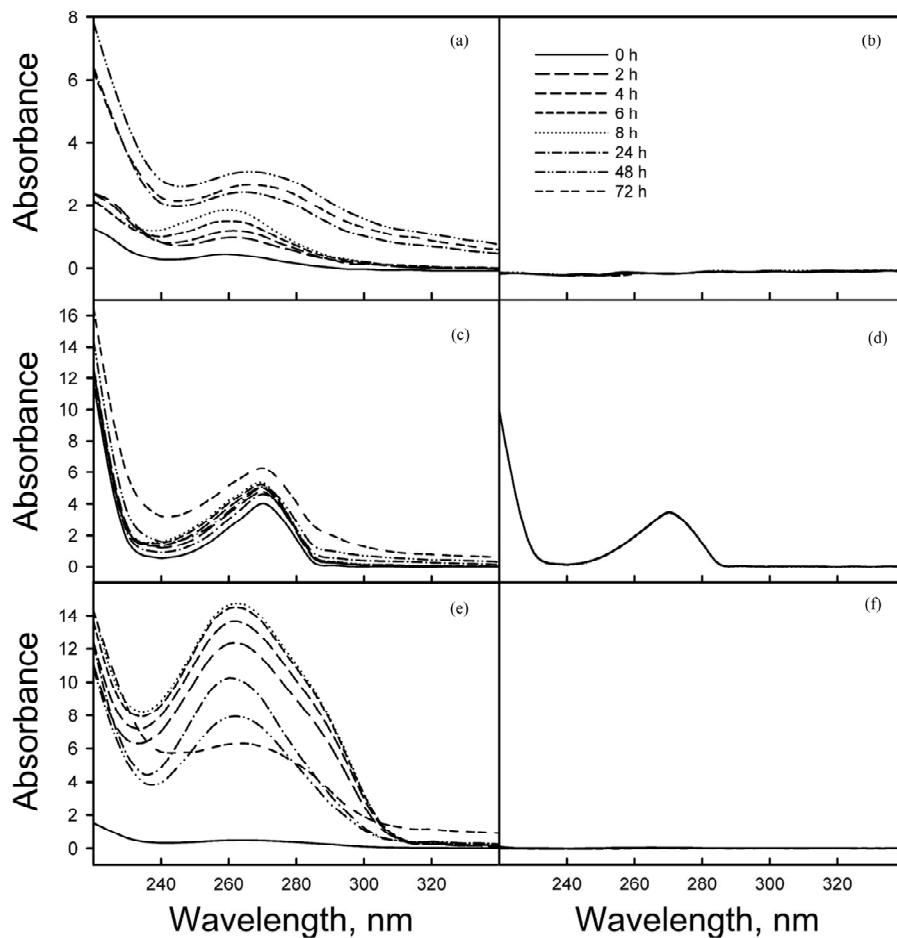


Figure 6. Spectral analysis of degradation of aromatic compounds by POXN01 resting cells. Benzene (a), (b), phenol (c), (d) or toluene (e), (f) were resuspended in 0.05 M potassium phosphate (pH 7.0) and incubated either with (a), (c), (e) or without (b), (d), (f) POXN01 cells as described in Materials in Methods. At indicated times, samples were withdrawn and centrifuged to remove cells and to separate aqueous and organic phases. UV spectra of clarified aqueous phases were measured against 0.05 M potassium phosphate (pH 7.0).

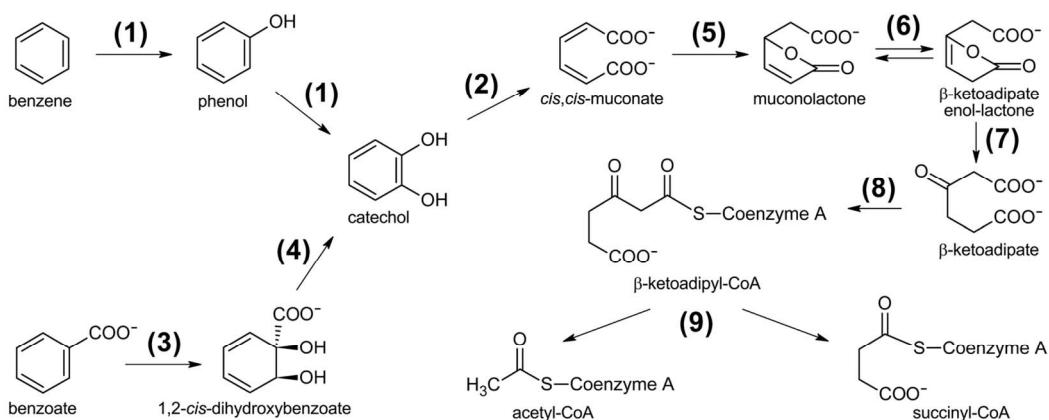


Figure 7. Putative pathway for the degradation of aromatic compounds by the POXN01 isolate. Gene products involved are as follows: (1) phenol/benzene hydroxylase (*phcKLMNOP*), (2) catechol 1,2-dioxygenase (*phcA*, *benG*, *catA*), (3) benzoate 1,2-dioxygenase (*benABC*), (4) 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase (*bend*), (5) muconate cycloisomerase (*catB*), (6) muconolactone delta-isomerase (*catC*), (7) beta-ketoadipate enol-lactone hydrolase (*pcaD*), (8) beta-ketoadipate:succinyl-CoA transferase (*pcaIJ*), (9) beta-ketoadipyl-CoA thiolase (*pcaF*).

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Abbreviations

OP: Organophosphorus;
CSM: Carbon-Deficient media;

TLC: Thin Layer Chromatography;
UV: Ultraviolet),
PCR: Polymerase Chain Reaction.



Figure S1. Neighboring-joining tree derived from concatenated alignments of 16S rRNA - *gyrB* - *rpoD* genes of the POXN01 isolate and type species of the genus *Pseudomonas*. Bootstrap values expressed as percentages of the total number of replicates are shown next to each node (values below 50% are not shown). Scale bar represents 10% sequence difference.

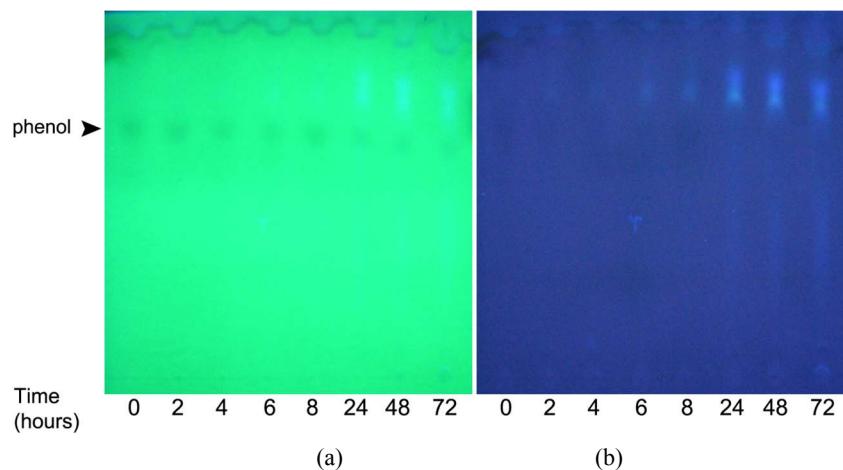


Figure S2. TLC analysis of products formed in the course of phenol degradation by POXN01 resting cells. Phenol degradation experiment was performed as described in Materials and Methods. Samples were collected at indicated times, cleared by centrifugation and kept frozen at (-80) °C prior analysis. Ascending TLC was carried out on Whatman KC18F plates using methanol: water 2:1 (v/v) as an eluent. Spots were visualized either under UVC light (200 - 280 nm) (a) or UVA light (340 - 400 nm) (b).