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EXPERIMENTAL ARTICLES

Diversity of the *bphA1* Genes in a Microbial Community from Anthropogenically Contaminated Soil and Isolation of New Pseudomonads Degrading Biphenyl/Chlorinated Biphenyls

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Abstract—Molecular biological and cultivation-based approaches were used to investigate the microbial community of tehnogenic soil contaminated with poorly degradable toxic (chlorinated) aromatic compounds. Diversity of the *bphA1* genes, the key genes for the degradation of biphenyl/polychlorinated biphenyls (PCB) was assessed, and new bacterial degraders of biphemyl/PCB were isolated. Cloning of the PCR product obtained using the DNA isolated from soil as a template and the primers to the biphenyl 2,3-dioxygenase α-subunit gene (*bphA1*) revealed two types of the genes of aromatic dioxygenases (DO) with the highest similarity (97.8–99.5%) to the genes encoding the Rieske cluster of DO α-subunits (*bphA1*) from uncultured bacteria. Two biphenyl-degrading isolates obtained from an enrichment culture of a soil sample incubated with biphenyl were identified as *Pseudomonas* (VRP2-6 and VRP2-2). According to their 16S rRNA gene sequences, they exhibited the highest similarity to the type strain of *P. taiwanensis* (99%) and *P. alcaligenes* (100%), respectively. Analysis of the *bphA1* sequences of strains VRP2-6 and VRP2-2 revealed the similarity to those of the known biphenyl-degrading pseudomonads not exceeding 97.3%. The isolate VRP2-6 efficiently utilized *ortho*- and *para*-monochlorinated biphenyls and degraded dichlorinated biphenyl oxidizing both the *ortho*- and *para*-chlorinated rings of the biphenyl molecule. New pseudomonad strains may be of interest for development of biotechnologies aimed at monitoring and remediation of biphenyl/PCB-contaminated soils.

Keywords: bacterial degraders, *Pseudomonas*, biphenyl, polychlorinated biphenyls, molecular cloning, *bphA1* genes

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Environmental pollution with poorly degradable toxic organic compounds entering the environment as a result of the operation of industrial enterprises continues to be a topic of current importance and to attract ever-increasing attention of researchers dealing with environmental problems. Such compounds include biphenyl and its chlorinated derivatives (polychlorinated biphenyls (PCB)), which are classified into the group of persistent organic pollutants (POP) (http://chm.pops.int) by the United Nations Environment Programme (UNEP). Biphenyl and PCB adversely affect the human immune, nervous, reproductive, and endocrine systems; prolonged action of these toxicants on the human body can cause serious diseases, including cancer (Sharma et al., 2018). Biphenyl, being a component of oil, coal, and natural gas, causes large-scale environmental pollution; it is widely used in chemical industry for the synthesis of numerous organic compounds (Nam et al., 2014). Due to their exceptional resistance to physical and chemical factors, PCB were widely used in various industries in the 20th century. They were most widely used as the components of dielectric fluids (in transformers and capacitors), coolants, varnish-and-paint production, and insulating materials (Pieper and Seeger, 2008; Vasil'ev, 2017). Despite the ban on industrial production and use of PCB (Stockholm Convention, 2001), PCB utilization, as well as remediation of soils and water reservoirs contaminated with PCB, remains a relevant problem (Vasilyeva and Strizhakova, 2007; Sharma et al., 2018). Due to low toxicity and higher bioavailability of biphenyl (in comparison with its chlorinated derivatives), it is used as a model compound in the works on PCB biological degradation (Shumkova et al., 2015).

Over recent years, new technologies for detoxification and remediation of contaminated areas, using the biological potential of microorganisms, have been developed and introduced (Sharma et al., 2018). Gram-positive and gram-negative bacteria which are able to utilize or transform biphenyl and PCB partially are known and well-characterized, including members of the genus *Pseudomonas* (Master and Mohn, 2001;

Furukawa et al., 2004; Adebusoye et al., 2007; Nam et al., 2014, 2014; Chakraborty and Das, 2016; Suenaga et al., 2017). However, the search for and study of active degrading bacteria, which are promising for application in the biotechnologies aimed at remediation of the areas contaminated with biphenyl/PCB, are of current importance.

Biphenyl/PCB is degraded by bacteria to pentadienoic and chlorobenzoic acids in a four-step process. The first stage is inclusion of two hydroxyl groups into the aromatic ring of biphenyl, which occurs under the action of biphenyl 2,3-dioxygenase (BDO) with the formation of biphenyl 2,3-dihydrodiol. The latter is then oxidized by biphenyl-2,3-dihydrodiol-2,3-β-dehydrogenase (BphB) to form 2,3-dihydroxybiphenyl at the second stage. At the third stage, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) catalyzes *meta*-cleavage of the aromatic ring, which results in formation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA). At the final stage, conversion of HOPDA to pentadienoic and benzoic acids is catalyzed by 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD) (Pieper and Seeger, 2008). Biphenyl 2,3-dioxygenase (BDO) is the key enzyme for the decomposition of biphenyl/PCB. It is a multicomponent enzyme composed of the proteins of the electron transport system and the terminal oxygenase, which is a hexamer consisting of three α- and three β-subunits (BphA1 and BphA2, respectively), ferredoxin (BphA3), and ferredoxin reductase (BphA3). The α-subunit is believed to play a key role in the recognition and binding of the substrate (Furukawa et al., 2004). Thus, the *bphA1* gene (encoding the biphenyl 2,3-dioxygenase α-subunit) is an important marker for the study of the biodegradation potential of bacteria (Shumkova et al., 2015).

As part of this study, the microbial community of soil from the Perm Lubricant-Producing Plant territory, which was exposed to long-term contamination with chlorinated aromatic compounds (including PCB), was studied. The enterprise has been producing lubricants, coolants, greases, and technical fluids for over 60 years (http://www.nge.ru/passport-description-161.htm).

The goal of the present work was to identify the key biphenyl degradation genes (the *bphA* genes) and to assess their diversity in the microbial community of the soil collected in the territory of Perm Lubricant-Producing Plant, as well as to isolate active biphenyl/PCB-degrading bacteria from the studied contaminated soil.

MATERIALS AND METHODS

Studied samples. The subjects of this research were four soil samples collected at the Perm Lubricant-Producing Plant in 2016. The samples were collected from depths of 5–10 cm at various sites within the territory of the plant. Total soil obtained by thorough mixing of collected samples was used for microbiological and molecular genetic studies.

DNA isolation, PCR amplification, and cloning of the *bphA1* genes. Extraction of the total DNA from the soil sample was carried out using the MP Biomedicals commercial reagent kit (United States). The DNA concentration was determined using a Qubit™ Fluorometer (Invitrogen, United States) using the manufacturer's reagents.

The *bphA1* genes were amplified using the BPHD-f3 and BPHD-r1 primers (Table 1), specific for the biphenyl 2,3-dioxygenase α-subunit gene, the template of the total DNA, and a MyCycler device (Bio-Rad Laboratories, United States), according to the protocol (Iwai et al., 2010). The reaction products were separated by electrophoresis in 1% agarose gel at a voltage of 10 V/cm, stained with a solution of ethidium bromide (5 μg/mL), and photographed under UV illumination using a Gel Doc™ XR gel documentation system (Bio-Rad Laboratories, United States).

PCR fragments of the *bphA1* genes were cloned in *E. coli* JM109 cells as part of the pTZ57R/T vector (Thermo Scientific, United States). Subsequent selection of recombinant clones was carried out according to the blue–white screening by plating the transformants on Luria-Bertani (LB) medium (*Short Protocols in Molecular Biology*, 1995), supplemented with ampicillin (50 μg/mL), 100 mM IPTG, and X-gal (20 μg/mL) (Fermentas, Lithuania). The *bphA1* gene fragments were amplified using the BPHD-f3 and BPHD-r1 primers (Iwai et al., 2010) and the DNA template of the selected recombinant clones. Restriction fragment length polymorphism (RFLP) analysis of the *bphA1* genes was performed using the *Hha*I restriction enzyme (Fermentas, Lithuania). In order to prepare cloned DNA fragments (as part of the pTZ57R/T vector) for sequencing, the insertion was amplified using the standard M13 primers (M13F (5'-GTTTTCCCAGTCACGAC-3') and M13R (5'-CAGGAAACAGCTATGAC-3')), for which the pTZ57R vector has binding sites at both sides of the polylinker. Description of the sequencing and analysis of the cloned DNA sequences are presented below.

RT-PCR analysis. The real-time polymerase chain reaction method (RT-PCR) was used to characterize the microbial community (MC) of the studied soil. The 16S rRNA genes were amplified using the Eub338 primers (Table 1) (Fierer et al., 2005) and the template of the total DNA of the soil in order to assess the taxonomic composition of the MC. To detect the genes encoding the α-subunit of hydroxylating dioxygenases (PAH-RHD$_\alpha$) involved in the oxidation of various polycyclic aromatic hydrocarbons (PAH) and biphenyl/PCB, the primer pairs (Table 1) designed for the hydroxylating dioxygenase genes of gram-positive and gram-negative PAH- and biphenyl/PCB-degrading bacteria were used (Cébron et al., 2008). RT-PCR was

Table 1. Oligonucleotide primers

Amplified DNA fragments	Primers	Nucleotide sequence, 5'–3'	Reference
The 16S rRNA gene	Eub338 Eub518	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Fierer et al., 2005
The 16S rRNA gene	27F 1492R	AGAGTTTGATC(A/C)TGGCTCAG ACGG(C/T)TACCTTGTTACGACTT	Tiirola et al., 2002
The genes encoding the HDO α-subunit (G+)*	PAH-RHD$_\alpha$GPF PAH-RHD$_\alpha$GPR	CGGCGCCGACAAYTTYGTNGG GGGGAACACGGTGCCRTGDATRAA	Cébron et al., 2008
The genes encoding the HDO α-subunit (G–)**	PAH-RHD$_\alpha$GNF PAH-RHD$_\alpha$GNR	GAGATGCATACCACGTKGGTTGGA AGCTGTTGTTCGGGAAGAYWGTGCMGTT	Cébron et al., 2008
The *bphA1* gene encoding the BDO α-subunit***	BPHD-f3 BPHD-r1	AACTGGAARTTYGCIGCVGA ACCCAGTTYTCICCRTCGTC	Iwai et al., 2010

* HDO (G+), hydroxylating dioxygenases of gram-positive bacteria.
** HDO (G–), hydroxylating dioxygenases of gram-negative bacteria.
*** BDO, biphenyl dioxygenase.

performed in the presence of the Sybr Green I dye in the Sintol (Russia) and 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, United States) reagent kits using a CFX96 Touch™ Real-Time PCR Detection Systems device (Bio-Rad Laboratories, United States) according to the standard protocol (Jurelevicius et al., 2012).

Isolation of biphenyl/PCB-degrading strains was carried out by enrichment cultivation. The K1 mineral medium (50 mL) (Maltseva et al., 1999), a soil sample weighing 0.5 g, and biphenyl (1 g/L) (as the sole source of carbon and energy) were added to each 250-mL Erlenmeyer flask. Cultivation was carried out under conditions of aeration on a thermoshaker (120 rpm) for 1 month at 28°C. The suspension obtained was plated onto the K1 agar medium, and biphenyl was added to the lid of an inverted petri dish. Incubation was carried out at 28°C until colonies appeared. Purity of the cultures was tested by cultivation on the rich LB medium.

The morphological and physiological features of the isolated microorganisms were studied by the standard methods (*Manual of Methods for General Bacteriology*, 1981). Bacterial growth under conditions of the change in ambient osmolarity was studied both on Raymond's agar mineral medium (Rozanov and Nazina, 1982) supplemented with biphenyl as the sole carbon and energy source and on Raymond's enriched medium (containing 2.5 g/L yeast extract and 5 g/L trypton) at the following NaCl concentrations (g/L): 0, 30, 50, and 70. The cultivation was carried out for 7 days in a thermostat at 28°C. Assessment of the growth of bacterial strains under different temperature conditions was carried out on solid media (K1 mineral medium supplemented with biphenyl and enriched LB medium) at the following temperatures: 4, 10, 28, 37, and 45°C. The growth of the colonies was assessed on the seventh day of cultivation.

Ability of bacteria to degrade aromatic compounds was assessed by cultivation in the liquid K1 medium on a thermoshaker (120 rpm) at 28°C and/or on the agar K1 medium on petri dishes in a thermostat at 28°C. Naphthalene and phenanthrene (in the form of fine powders; 0.5 g/L), as well as *ortho*-phthalic, salicylic, *para*-hydroxybenzoic, benzoic, and protocatechuic acids (in the form of aqueous solutions of sodium salts; 0.5 g/L in acid equivalent), were used as sources of carbon and energy. Toluene and phenol were added to the lid of an inverted petri dish, and bacteria were cultivated in toluene and phenol vapors. During growth experiments, concentrations of biphenyl were 0.5 and 1.0 g/L. Biphenyl was added to flasks as a fine powder; the volume of the cultivation medium was taken into account. The growth of strains in liquid media containing the substrates listed above was assessed by measurements of the optical density (OD) of the culture using an UV-Visible BioSpec-mini spectrophotometer (Shimadzu, Japan) at $\lambda_{max} = 600$ nm. Specific growth rates of the strains were calculated according to the following formula: $\mu = (\ln N_t - \ln N_0)/t - t_0$, where μ is the specific growth rate, h^{-1}; $\ln N_t$ is the natural logarithm of the value of OD of the culture when the stationary phase of growth was reached; $\ln N_0$ is the natural logarithm of the value of OD of the culture at the beginning of the log phase; t is time of cultivation when the stationary phase of growth was reached; and t_0 is the cultivation time at the beginning of the log phase.

Genetic typing and identification of degrading bacteria. DNA from pure bacterial cultures was isolated by the standard method (*Short Protocols in Molecular Biology*, 1995). Genetic similarity/difference of the isolated strains was determined by the BOX-PCR

method according to the standard protocol (Versalovic et al., 1994).

Bacteria were identified by amplification of the 16S rRNA gene using the 27F and 1492R standard bacterial primers (Table 1) with subsequent determination and analysis of nucleotide sequences of the amplified 16S rRNA genes (as described below). The search for homologous sequences was performed using the ezTaxon database (http://www.ezbiocloud.net/eztaxon).

Investigation of the key genes for biphenyl/PCB degradation (the *bphA1* genes) in the isolated strains was carried out by amplification with the BPHD-f3 and BPHD-r1 primers (Table 1) (Iwai et al., 2010), followed by sequencing and analysis of the nucleotide sequences of the *bphA1* genes. The *bphA1* gene fragments of the strains VRP2-2 and VRP2-6 were deposited in the GenBank database under the accession numbers KY978889.1 and KY978890.1, respectively.

Sequencing and analysis of the 16S rRNA and *bphA1* genes. Nucleotide sequences were determined using the Big Dye Terminator Cycle Sequencing Kit v. 3.1 reagent kit (Applied Biosystems, United States) and a 3500XL Genetic Analyzer automated sequencer (Applied Biosystems, United States) according to the manufacturer's recommendations. Analysis of nucleotide sequences was carried out using the Sequence Scanner v. 2.0 and MEGA 6.0 software packages (http://www.megasoftware.net). The search for homologous sequences was performed using the international GenBank database (http:/www.ncbi.nlm.nih.gov/Blast.cgi). The UPGMA algorithm (MEGA 6.0) was used to construct the tree of similarity. The statistical significance of branching (bootstrap analysis) was evaluated on the basis of 1000 alternative trees.

Plasmid DNA. The presence of plasmid DNA was detected by pulsed-field gel electrophoresis using a CHEF DR II device (Bio-Rad Laboratories, United States). Bacteria were grown in the K1 mineral medium (10 mL) supplemented with biphenyl (1 g/L) to $OD_{600} = 1.0$. Subsequent cell preparation, electrophoresis, and data processing were carried out as previously described (Egorova et al., 2013).

Degradation of mono- and dichlorinated biphenyls. During the study of the degradation of 2-CB, 4-CB, and 2,4'-diCB, 1 mL of the cells ($OD_{600} = 2.0$) grown on biphenyl (1 g/L) and washed twice with the K1 medium were transferred to 5-mL vials with Teflon caps. Mono-CB and 2,4'-diCB substrates were added to final concentrations of 250 and 44.6 mg/L, respectively. Incubation was carried out on a shaker (200 rpm) at 28°C. Accumulation of chlorobenzoic acids in the culture medium was registered by HPLC using an LC-20AD Prominance chromatograph (Shimadzu, Japan) with a C-18 column (150 × 4.6 mm; Sigma-Aldrich, United States) and a SPD-20A UV detector (at 205 nm) in the acetonitrile–0.1% H_3PO_4 (70 : 30) system. Identification of metabolic products was performed by comparing the retention time of the formed and standard compounds (2-CBA and 4-CBA) on the column (Maltseva et al., 1999). The amount of obtained products was determined by the size of the area and the height of the peaks in the chromatogram, relative to these values of the standard compounds. The formation of other products of CB transformation, 2-hydroxy-6-oxo-6-(chlorophenyl)hexa-2,4-dienoic acids (HOPDA), was determined using a UV-Visible BioSpec-mini spectrophotometer (Shimadzu, Japan) with λ_{max} from 390 to 440 nm (Maltseva et al., 1999). To determine the amounts of 2-CB, 4-CB, and 2,4'-CB, the total reaction volume was extracted with the concentrated H_2SO_4–12.5% sodium dodecyl sulfate–hexane mixture (1 : 10 : 25) for 60 min at 30°C at a stirring speed of 200 rpm. The extracts obtained were dehydrated with Na_2SO_4 and analyzed by the GC/MS method on a GC6890N gas chromatograph (Agilent Technology, United States) with a MSD5973N mass selective detector (Agilent Technology, United States), according to the RD 52.18.578-97. Methodological Instructive Regulations (http://docs.cntd.ru/document/1200036915). The concentration of chlorinated biphenyls was calculated according to the calibration graphs.

Statistics. Experiments were carried out in three replicates. The data obtained were processed using the standard Microsoft Excel software packages. All numerical data were checked for reliability during statistical processing.

RESULTS AND DISCUSSION

Molecular genetic characteristics of the microbial community of the contaminated soil. Application of the method of quantitative analysis (RT-PCR) of the total DNA isolated from the soil collected in the territory of the Perm Lubricant-Producing Plant revealed the presence of the bacterial 16S rRNA genes in the amount of 1.05×10^{11} ($\pm 1.59 \times 10^8$) gene copies per 1 g of soil. High numbers of copies of the PAH-RHD_α genes (2.41×10^8 ($\pm 3.57 \times 10^7$) per 1 g of soil) encoding dioxygenases involved in the oxidation of PAH and biphenyl by gram-negative bacteria were detected by RT-PCR. At the same time, when using the PAH-RHD_αGPF and PAH-RHD_αGPR primers (Table 1), the presence of the hydroxylating dioxygenase genes of gram-positive bacteria was not detected. The proportion of bacteria capable of degradation of PAH and biphenyl/PCB (represented by the ratio of the number of copies of the PAH-RHD_α gene to the number of copies of the 16S rRNA gene (per 1 g of soil)) was rather high in the studied microbial community, approximately 10^{-3}. Thus, the bacterial genes that control the initial stages of degradation of polyaromatic compounds (PAH and biphenyl/PCB) were found in the microbial community of the studied contaminated soil.

We used oligonucleotide primers specific to the biphenyl 2,3-dioxygenase α-subunit gene (Table 1) (Iwai et al., 2010) for precise determination of the presence of biphenyl/PCB-degrading bacteria, as well as for the investigation of the diversity of genetic systems for the degradation of these compounds in the studied MC. The PCR product of the desired size (approximately 500 bp) was obtained using the template of the total soil DNA and was cloned in the *E. coli* cells. As a result of cloning, a library consisting of 72 recombinant clones containing the *bphA1* genes was constructed. To identify the similarity and distinction between the cloned *bphA1* gene fragments, RFLP analysis using the *Hha*I restriction endonuclease was carried out. The results of this analysis indicated that the restriction profile of the cloned DNA regions differed in size and number of restriction fragments (data not shown). In total, four genomogroups of the cloned *bphA1* genes were identified on the basis of RFLP analysis. Nucleotide sequences were determined and comparative analysis with homologous sequences from the GenBank database was carried out for the members of these groups (Table 2).

Most of the cloned DNA fragments, assigned to three different genomogroups on the basis of RFLP analysis and constituting 80% of the total number of recombinant clones obtained, had high similarity (97.8–99.5%) with the genes encoding the Rieske cluster of the dioxygenase α-subunit of uncultured bacteria from the soil exposed to long-term PCB contamination (Aguirre de Cárcer et al., 2007). The sequenced DNA fragment of the clone Pp105 (genomogroup VI) had a high level of similarity (98.4–99.3%) to the genes of the large α-subunit of the biphenyl 2,3-dioxygenase (the *bphA1* gene) of uncultured bacteria (Vezina et al., 2008) and not more than 90.5% similarity with the similar genes of bacteria of the genus *Pseudomonas* (*P. putida* plasmid pKF715A, *Pseudomonas* sp. Cam-1, *P. alcaliphila* JAB1, and *P. pseudoalcaligenes* KF707) (Table 2). These results indicate the presence of heterogeneous genetic systems in bacteria involved in the degradation of polyaromatic compounds by the microbial community of the soil collected in the territory of the Perm Lubricant-Producing Plant. The results of the studies carried out using molecular biological methods, including high-throughput sequencing methods, indicated a huge variety of aromatic dioxygenases, including biphenyl dioxygenases (BDO), in the soil MC and in other environments. The biphenyl dioxygenase genes of cultivated bacteria were shown to be only a small part compared to the diversity of the BDO genes in the metagenome of the microbial community of soil (Aguirre de Cárcer et al., 2007; Iwai et al., 2010; Standfuss-Gabisch et al., 2012; Jurelevicius et al., 2012).

Identification, morphological, physiological, and genetic characteristics of the isolated bacteria. Two aerobic bacterial strains (designated VRP2-2 and VRP2-6), which were able to use unsubstituted biphenyl as a substrate of growth, were isolated from the technologically contaminated soil by the method of enrichment cultivation. The cells of the strains were gram-negative non-spore-forming motile rods. When growing on LB medium, the isolate VRP2-2 was represented by yellow shiny round smooth convex colonies, 1–3 mm in size. Colonies of strain VRP2-6 were 2–4 mm in size; they were creamy yellow dull round convex colonies with a wavy edge. The cells of the isolates were motile regular-shaped rods with rounded ends; they were oxidase- and catalase-positive. Both strains grew actively in the temperature range of 4–45°C on the rich medium (LB) and in the range of 4–37°C on the K1 mineral medium supplemented with biphenyl as the sole source of carbon. Growth experiments under conditions of the changes in the ambient osmolarity indicated that strains VRP2-2 and VRP2-6 were able to grow (on both LB medium and on K1 medium supplemented with biphenyl) at a NaCl concentration up to 50 g/L.

The results of genotyping showed that the strains differed from each other at the molecular genetic level (Fig. 1). The strains also differed by the presence of plasmids in their cells. Thus, a plasmid of ~280 kb was detected by pulsed-field gel electrophoresis in the cells of strain VRP2-6 grown on the K1 medium with biphenyl. No plasmid DNA was detected in the cells of strain VRP2-2 (Fig. 2).

Based on analysis of the 16S rRNA gene, strains VRP2-2 and VRP2-6 were assigned to the genus *Pseudomonas* and had the highest similarity to the type strain *Pseudomonas alcaligenes* NBRC 14159^T (100% similarity) and *Pseudomonas taiwanensis* BCRC 17751^T (99% similarity), respectively. Some pseudomonad strains were described in the literature as active degraders of biphenyl and PCB (Adebusoye et al., 2007; Hatamian-Zarmi et al., 2009; Li et al., 2009; Nam et al., 2014; Chakraborty and Das, 2016; Ridl et al., 2018), among which the strains (designated KF) isolated from technologically polluted soil from the territory of a biphenyl producing plant in Japan were the best-studied ones (Furukawa et al., 1989). The KF strains were shown to belong to different *Pseudomonas* species, including *P. furukawaii* (strain KF707), *P. putida* (strains KF703 and KF715), *P. toyotomiensis* (strain KF710), and *P. aeruginosa* (strain KF702). Complete sequencing of the genomes of these strains was carried out, which made it possible to determine and analyze the genetic structures underlying the unique metabolic potential of these bacteria, including their ability to degrade biphenyl and PCB to non-hazardous compounds (Watanabe et al., 2015; Fujihara et al., 2015; Suenaga et al., 2015, 2017; Kimura et al., 2018).

Biodegradation characteristics of the isolated bacteria. Strains VRP2-2 and VRP2-6 were shown to use not only biphenyl, but also monoaromatic hydrocar-

Table 2. Comparison of cloned nucleotide sequences of the hydroxylating dioxygenase gene fragments (the *bphA1* genes) with homologous sequences from the GenBank database

Clone (genomogroup)/ strain, size of the analyzed DNA fragment	Homologous genes in GenBank	GenBank accession nos. of compared nucleotide sequences	Similarity, %	Reference
Pp64 (I) clone, 451 bp	Genes encoding the Rieske cluster of the DO α-subunit; LhIspA24/ LhIspA20/LhIspA21 clones, uncultured bacteria	EF565833.1/ EF565845.1/ EF565830.1	97.8–99.5	Aguirre de Cárcer et al., 2007
Pp116 (II) clone, 427 bp				
Pp123 (III) clone, 448 bp				
Pp105 (VI) clone, 466 bp	The phenylpropionate DO gene (*hca*); AN-11 clone, uncultured bacterium	DQ521968.1	99.3	No data
	The biphenyl DO gene (*bph*); AN-70 clone, uncultivated bacterium	DQ521951.1	99.0	No data
	bphA1; G7-12 clone, uncultured bacterium	EF596918.1	98.4	Vezina et al., 2008
	bphA1; Pseudomonas putida, pKF715A plasmid	AP015030.1	90.7	Suenaga et al., 2017
	The *bph* operon, *Pseudomonas* sp. Cam-1	AY027651.1	90.5	Master and Mohn, 2001
	bphA1; Pseudomonas alcaliphila JAB1	CP016162.1	90.7	Ridl et al., 2018
	bphA1; Pseudomonas furukawaii KF707	AP014862.1	90.7	Kimura et al., 2018
Strains VRP2-2 and VRP2-6, 453 bp	*bphA1; Pseudomonas putida* B6-2	CP015202.1	97.3	Li et al., 2009
	bphA1; Pseudomonas sp. B3B	AJ544517.1	97.1	Kahl and Hofer, 2003
	bphA1; Pseudomonas sp. B6K	AJ544520.2	93.0	Kahl and Hofer, 2003
	bphA1; Pseudomonas alcaligenes B-357	EF596934.1	90.7	Vézina et al., 2008
	The *bph* operon, *Pseudomonas* sp. Cam-1	AY027651.1	90.0	Master and Mohn, 2001
	bphA1; Pseudomonas putida, pKF715A plasmid	AP015030.1	89.8	Suenaga et al., 2017
	bphA1; Pseudomonas alcaliphila JAB1	CP016162.1	89.8	Ridl et al., 2018
	bphA1; Pseudomonas furukawaii KF707	AP014862.1	89.8	Kimura et al., 2018
	bphA1; Pseudomonas sp. B4	AJ544519.1	89.8	Kahl and Hofer, 2003

bons (*ortho*-phthalic and benzoic acids) as the sole source of carbon and energy. Strain VRP2-6 grew actively on *para*-hydroxybenzoic, protocatechuic, and salicylic acids, while strain VRP2-2 did not utilize these compounds (Table 3). These strains were unable to grow on naphthalene, phenanthrene, phenol, or toluene.

Our study suggests that the strains VRP2-2 and VRP2-6 degrade biphenyl by the classical pathway via formation of pentadienoic and benzoic acids, followed by decomposition of benzoic acid (Pieper and Seeger, 2008). Both strains were shown to grow on benzoic acid actively (Table 3). The results of PCR analysis indicated that the *benA* gene encoding the small subunit of the benzoate 1,2-dioxygenase enzyme (the key enzyme for benzoate decomposition in bacteria) was present in the genomes of both strains (data not shown); this indicates the metabolic pathway of benzoate degradation via formation of pyrocatechol (the key intermediate) (Ridi et al., 2018).

When grown in liquid medium with biphenyl, strain VRP2-6 exhibited a higher growth rate (0.098 h^{-1}) than

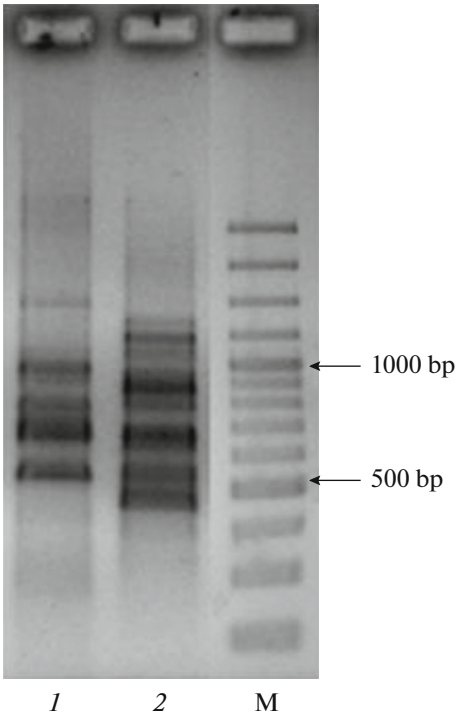

Fig. 1. BOX-PCR profiles of the strains VRP2-2 (*1*) and VRP2-6 (*2*). M is O'GeneRuler™ 100 bp Plus DNA Ladder molecular weight marker (Fermentas, Lithuania).

Fig. 2. Electropherogram of plasmid DNA from *Pseudomonas* strains: *1*, DNA Size Markers–Yeast Chromosoma molecular weight marker (Bio-Rad Laboratories, United States); *2*, strain VRP2-2; *3*, strain VRP2-6.

strain VRP2-2 (0.042 h^{-1}) (Fig. 3); the former strain was tested for ability to decompose chlorinated biphenyls. Strain VRP2-6 was shown to utilize high concentrations of *ortho*- and *para*-monoCB (250 mg/L), oxidizing the nonchlorinated ring of the CB molecule (Table 4). When cultivated on 2-CB and 4-CB, the amount of chlorinated biphenyls in the medium decreased, respectively, 4.9 and 4.3 times, by the third hour of growth. After 24 h of incubation, 2-CB was almost completely utilized (97.1% of the theoretically possible), and 4-CB was decomposed by 82.3%. Intermediate metabolic products (2-hydroxy-6-oxo-(chlorophenyl)hexa-2,4-dienoic acids (HOPDA)) were not detected in the medium, which may indicate high activity of the enzyme systems for the destruction of monoCB in strain VRP2-6 (Maltseva et al., 1999). During the periods under study, chlorobenzoic acids were detected in the amount of 2.0–3.5 mg/L (Table 4). The presence of such small amounts of CBA (no more than 2% of the theoretically possible) may indicate subsequent degradation of these chlorine-containing metabolites by the cells of the strain. Few strains of the genus *Pseudomonas* are known to be able to completely utilize the relevant CBA formed during the degradation of monochlorinated biphenyls (Chae et al., 2000; Kim and Picardal, 2001; Hatamian-Zarmi et al., 2009).

Transformation of dichlorinated biphenyl (2,4'-diCB) by strain VRP2-6 was less active than that of monochlorinated biphenyls. Thus, by 24 h of growth, the amount of the substrate in the culture medium remained at the level of 80% of the initial concentration. Accumulation of the product of *meta*-cleavage of dichlorinated biphenyl (3,8-Cl HOPDA with λ_{max} = 395/396 nm) was recorded, indicating 2,3-dioxygenation of the *para*-chlorinated ring of 2,4'-diCB (Maltseva et al., 1999). Moreover, a small amount of 4-CBA was accumulated in the medium, which may indicate 2,4'-diCB transformation preferably via the pathway of 2,3-dioxygenation of the *ortho*-chlorinated ring (Maltseva et al., 1999). Thus, the BDO of the strain VRP2-6 can oxidize both the *ortho*- and *para*-substituted rings of the 2,4'-diCB molecule. The strain *Pseudomonas* sp. SA-6 was previously reported to use 2,4'-diCB as the sole source of carbon and energy, oxidizing the *ortho*-chlorinated ring of the biphenyl molecule, with subsequent degradation of the formed 4-CBA (Adebusoye et al., 2007). Further research should be carried out in order to determine the metabolic patterns of the degradation of chlorinated biphenyls differing in the number and position (*ortho*-,

Table 3. Growth of strains VRP2-2 and VRP2-6 on aromatic compounds

Substrate	Strains	
	VRP2-2	VRP2-6
Biphenyl	++	+++
Naphthalene	–	–
Phenanthrene	–	–
Phenol	–	–
Toluene	–	–
ortho-Phthalic acid	+	++
Salicylic acid	–	+++
para-Hydroxybenzoic acid	–	+++
Protocatechuic acid	– (d.s.m.)	+++
Benzoic acid	+++	+++

Symbol "–" indicates not detected; d.s.m. indicates dark staining of the culture medium; symbol "+" indicates OD_{600} in the range of 0.1–0.3 units; symbol "++" indicates OD_{600} in the range of 0.4–0.7 units; symbol "+++" indicates OD_{600} over 0.7 units.

para-, and *meta*-) of chlorine atoms in the molecule, as well as of the products of their degradation (chlorinated benzoates) by the strain *Pseudomonas* sp. VRP2-6.

The *bphA1* genes of *Pseudomonas* spp. VRP2-2 and VRP2-6. PCR with specific primers (Iwai et al., 2010) indicated the presence of the *bphA1* gene encoding the α-subunit of biphenyl 2,3-dioxygenase (the key enzyme for the degradation of biphenyl) in the isolated strains. This fact confirms that degradation of biphenyl/PCB involves the enzymes encoded by the *bph* gene cluster of the classical upper biphenyl degradation pathway; these enzymes convert biphenyl to benzoic acid (Pieper and Seeger, 2008). Analysis of nucleotide sequences of the *bphA1* gene fragments of strains VRP2-2 and VRP2-6 showed their identity and the highest similarity (89.8–97.3%) to the biphenyl 2,3-dioxygenase α-subunit genes of the biphenyl/PCB-degrading bacteria of the genus *Pseudomonas* (Table 2). Phylogenetic analysis of the translated amino acid sequences of the *bphA1* genes of the strains VRP2-2 and VRP2-6 showed that they formed a separate branch in the BDO tree (Fig. 4). This branch was most closely located to the branch of biphenyl 2,3-dioxygenases of biphenyl-degrading strains *P. putida* B6-2 (Li et al., 2009) and *Pseudomonas* sp. B3B (Kahl and Hofer, 2003). Levels of similarity of the *bphA1* genes of the strains isolated in the present work with those of the pseudomonad strains mentioned above were 97.3 and 97.1%, respectively (Table 2). Analysis of the genome of *P. putida* B6-2 (a degrader of aromatic compounds) showed that the genome contained the classical cluster of the *bph* genes (*bphABCKHJID*) and the genes (operons) responsible for decomposition of benzoate, catechol, *para*-hydroxybenzoate, and salicylate (Li et al., 2009). The *bphA1* genes of the studied strains had low percentage of similarity (approximately 90%) to those of well-characterized strains *P. furukawaii* KF707 and *P. putida* KF715 (Table 2, Fig. 4). The latter strains degrade actively chlorinated biphenyls containing various numbers of chlorine atoms (in the *ortho*-, *para*- and *meta*-positions) in the biphenyl molecule, as well as PCB mixtures (Suenaga et al., 2017; Kimura et al., 2018). The *bph* gene cluster was previously shown to be located on one of the four plasmids of *P. putida* KF715 (a 483-kb pKF715A plasmid), whereas the identical *bph* genes of the plasmid-free strain KF707 were located on the chromosome (Kimura et al., 2018). It was shown that the mobile element containing the *bph* genes (plasmid pKF715A) could be integrated into the chromosome (Suenaga et al., 2017). The study of our strains revealed a plasmid of approximately 280 kb in strain VRP2-6 (Fig. 2). Hence, localization of the *bph* genes on the plasmid should not be excluded. The presence of identical *bphA1* genes in strain VRP2-2 strain (100% similarity) is an interesting fact. However, no plasmids were detected in the cells of this strain, which indicated the chromosomal localization of these genetic elements. The study of the composition and functioning of the genetic structures (including mobile elements: plasmids and transposons), which are responsible for the biphenyl/PCB degradation in the strains isolated from the same biotope, will be continued.

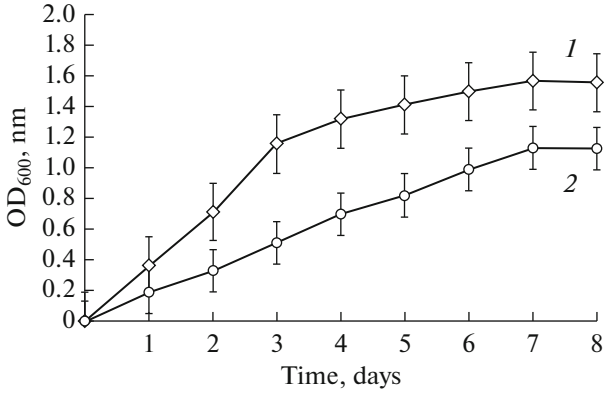

Fig. 3. Growth of *Pseudomonas* sp. VRP2-6 (*1*) and *Pseudomonas* sp. VRP2-2 (*2*) on biphenyl (1 g/L).

Thus, the results of the study of contaminated soil (the territory of the Perm Lubricant-Producing Plant) revealed the presence of a community of bacteria, which was able to decompose aromatic compounds, including biphenyl and its toxic chlorinated derivatives (polychlorinated biphenyls). Two active biphenyl-degrading members of the genus *Pseudomonas* were isolated from the soil by the method of enrichment cultivation. One of the isolates (VRP2-6) utilized *ortho*- and *para*-monochlorinated biphenyls and degraded dichlorinated biphenyl (2,4'-diCB) effi-

Table 4. Degradation of chlorinated biphenyls by *Pseudomonas* sp. strain VRP2-6

Substrate	Incubation time, h	Substrate concentration		Product of degradation				
		mg/L	%*	HOPDA		CBA	mg/L	%*
				$\lambda_{max,}$ nm	OD, units			
2-CB	0	250.00 ± 0.01	100.00	N.d.	N.d.		0.36 ± 0.02	0.17
	3	51.45 ± 0.04	20.58	N.d.	N.d.	2-CBA	2.45 ± 0.05	1.18
	24	7.43 ± 0.02	2.90	N.d.	N.d.		3.50 ± 0.03	1.68
4-CB	0	250.00 ± 0.01	100.00	N.d.	N.d.		0.30 ± 0.03	0.16
	3	57.80 ± 0.03	23.12	N.d.	N.d.	4-CBA	2.00 ± 0.04	0.96
	24	44.30 ± 0.05	17.70	N.d.	N.d.		2.35 ± 0.03	1.13
2,4'-CB	0	44.60 ± 0.01	100.00	N.d.	N.d.		N.d.	N.d.
	3	40.80 ± 0.02	91.48	396	0.696	4-CBA	0.085 ± 0.002	0.27
	24	35.70 ± 0.03	80.00	395	0.804		0.090 ± 0.005	0.28

2-CB, 2-monochlorinated biphenyl; 4-CB, 4-monochlorinated biphenyl; 2,4'-CB, 2,4'-dichlorinated biphenyl; CBA, chlorobenzoic acid; HOPDA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; N.d., not detected; * % of theoretically possible.

ciently. Phylogenetic analysis of the genes (*bphA1*) encoding the α-subunit of biphenyl DO in isolated pseudomonads (belonging to different species) showed their identity and high similarity (although not exceeding 97.3%) to the *bphA1* genes of the known biphenyl/PCB-degrading bacteria of the genus *Pseudomonas*. This type of the *bphA1* genes was not detected during molecular study of the total DNA pool isolated from contaminated soil, which may indicate the presence of a small number of cultured bacteria containing similar nucleotide sequences *(bphA1*) in the soil. At the same time, two other types of the *bphA1*-like genes, which were phylogenetically close to the genes of aromatic dioxygenase genes (including phenylpropionate DO and biphenyl DO) of uncultured bacteria, were identified in the soil DNA (using molecular methods: amplification, cloning, and sequencing). These results agree with the data of other

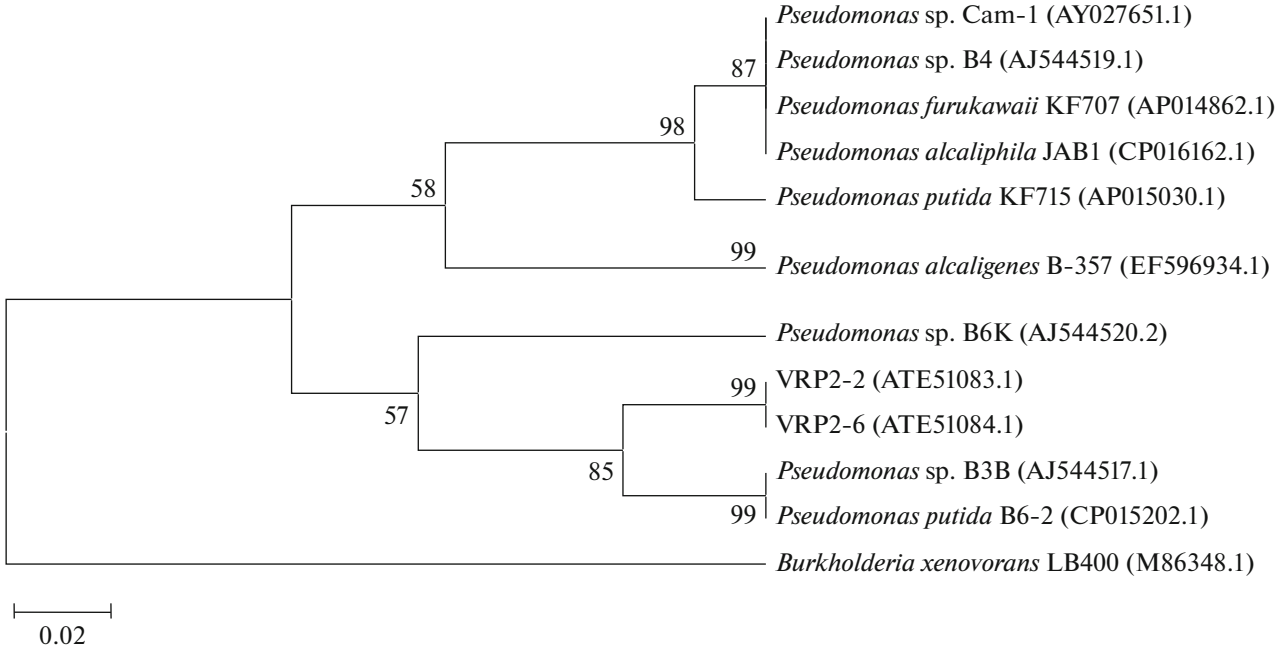

Fig. 4. Position of the *bphA1* genes of *Pseudomonas* spp. VRP2-2 and VRP2-6 on the phylogenetic tree constructed on the basis of comparative analysis of translated amino acid sequences using the UPGMA method. The numerals indicate the accuracy of the branching determined using bootstrap analysis. The amino acid sequence of the biphenyl 2,3-dioxygenase large subunit of *Burkholderia xenovorans* LB400 (M86348.1) was used as an outgroup.

researchers who reported that biphenyl dioxygenases (the BDO genes) of cultured bacteria are only a small part of the BDO diversity in the metagenomes of soil microbial communities (Iwai et al., 2010; Standfuss-Gabisch et al., 2012; Jurelevicius et al., 2012). Subsequent comprehensive study of new *Pseudomonas* spp. strains, VRP2-2 and VRP2-6, aimed at the possible application of their genetic and metabolic potential in biotechnologies for monitoring and remediation of the territories contaminated with biphenyl and PCB, will be carried out.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals performed by any of the authors.

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