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DIVERSITY OF THE GENE OF BENZOATE DIOXYGENASE IN BACTERIAL ASSOCIATIONS ISOLATED FROM LONG TERM ORGANOCHLORINE-CONTAMINATED SOILS

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❀ **Background.** Communities of bacteria with specific enzymes are formed in the soil with long-term organochlorine contamination. **The aim** of this study was to analyze the diversity of the *benA* gene encoding the α-subunit of the benzoate 1,2-dioxygenase in aerobic bacterial associations isolated from the soils of the Chapayevsk-city (Samara region, Russia). **Materials and methods.** The soil samples were taken on the territory, contaminated with organochlorine compounds for a long time. As a selection factor in the enrichment cultures were used 4-chlorobenzoic acid and chlorobenzene, in the pure cultures – benzoic acid. The isolation of total DNA from bacterial associations was performed using a commercial FastDNA Spin Kit for Soil kit (USA). Amplification was performed on a MyCycler instrument (USA). Determination of the nucleotide sequence was performed on an automatic sequencer Genetic Analyzer 3500XL (USA). The search and analysis for *benA* gene homologs was carried out using international GenBank databases and BLAST system (http://www.ncbi.nlm.nih.gov). **Results.** As a result of selection, 12 associations of aerobic bacteria were obtained. Fragments of the *benA* gene (α-subunit of benzoate dioxygenase) were obtained with the total DNA of six bacterial associations selected on chlorobenzene and with the total DNA of three bacterial associations selected on 4-chlorobenzoate. Pure cultures of aerobic bacterial strains using benzoic acid as a carbon source were isolated from *benA*-positive associations. It was established that the amplified fragments with the DNA of the A1, A4, A5, B1, B2, B3, B4 and B6 association strains form a single phylogenetic cluster with the α-subunit gene of the benzoate dioxygenase of the *Pseudomonas putida* strain KT2440 (level of similarity is 96–98%). The amplified fragment with the DNA of strain B5-170 (association B5) forms a cluster with the gene of the α-subunit of the benzoate dioxygenase of the strain *Pseudomonas* sp. VLB120 (93% similarity).

❀ **Keywords:** benzoate dioxygenase; gene; strain; bacterial associations; chloroaromatic compounds.

РАЗНООБРАЗИЕ ГЕНА БЕНЗОАТ ДИОКСИГЕНАЗЫ В БАКТЕРИАЛЬНЫХ АССОЦИАЦИЯХ, СФОРМИРОВАВШИХСЯ ПОД ДАВЛЕНИЕМ ХЛОРОРГАНИЧЕСКОГО ЗАГРЯЗНЕНИЯ

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❀ Из образцов грунта, отобранных на территории, длительное время загрязненной хлорорганическими соединениями, в результате селекции получены 12 ассоциаций аэробных бактерий. В качестве фактора отбора при накопительном культивировании использовали 4-хлорбензойную кислоту и хлорбензол. В результате скрининга установлено, что в ДНК шести бактериальных ассоциаций, селектированных на хлорбензоле, и в ДНК трех бактериальных ассоциаций, селектированных на 4-хлорбензоате, присутствует ген *benA* (α-субъединица бензоат 1,2-диоксигеназы). Из *benA*-положительных ассоциаций выделены чистые культуры аэробных бактериальных штаммов, использующих бензойную кислоту в качестве источника углерода. Установлено, что амплифицированные фрагменты с ДНК штаммов ассоциаций A1, A4, A5, B1, B2, B3, B4 и B6 формируют единый филогенетический кластер с геном α-субъединицы бензоат диоксигеназы штамма *Pseudomonas putida* KT2440 (уровень сходства 96–98 %), тогда как амплифицированный фрагмент с ДНК штамма B5-170 ассоциация B5 формирует кластер с геном α-субъединицы бензоат диоксигеназы штамма *Pseudomonas* sp. VLB120 (уровень сходства 93 %).

❀ **Ключевые слова:** бензоат диоксигеназа; ген; штамм; бактериальные ассоциации; хлорароматические соединения.

BACKGROUND

As a result of mass production and use of the industrial artificially-synthesized substituted and unsubstituted aromatic compounds in the 20th century, a considerable amount of these pollutants have been emitted into the environment. The major storage sites are soils and bottom sediments in industrial regions [1], as seen in the city of Chapaevsk (Samara region, Russia), with the JSC "Middle-Volga Chemical Plant". From 1967 to 1987, organochlorine compounds included in the list of persistent organic pollutants (POPs; Stockholm Convention, 2001) were produced at this plant [2–4]. These compounds have high chemical and physical stability, which results in their long-term persistence in the soil [4].

The availability of POPs in the environment activates adaptation processes in biotopes; in particular, POPs initiate changes in the content of microbiocenosis of contaminated soils. The microorganisms capable of decomposing pollutants and using them as the source of carbon and energy get privilege. Such features are typical for aerobic bacteria having enzymes that can breakdown aromatic rings with broad substrate specificity [5–7]. Bacterial dioxygenases, such as benzoate 1,2-dioxygenase [5, 8], perform the first stage of transformation of POPs and other aromatic compounds by catalyzing penetration of hydroxy groups in the chemically stable aromatic ring of the molecule [8].

Benzoate dioxygenase (BDO; EC1.14.12.10) has been studied in a wide range of representatives of Gram-positive and Gram-negative aerobic bacteria and decomposes a wide range of aromatic compounds [6, 9–11]. Benzoate dioxygenase is a double-component system; one of the components is oxygenase, consisting of α- and β-subunits [5, 6], and the other component consists of. The α-subunit appears to be responsible for the BDO substrate specificity [8]. Analysis of the genes determining the α-subunit of BDO demonstrated that they form a separate cluster (subfamily) on the phylogenetic tree of bacterial dioxygenases capable of oxidizing the aromatic ring [8].

The goal of this work was to study the diversity of *benA* genes coding for the α-subunit of benzoate dioxygenase in aerobic bacterial associations isolated from the soils of Chapaevsk (Samara region, Russia) and in bacteria with further selective cultivation in the presence of the chlorine aromatic compounds. These bacterial associations are formed as a result of adaptation to long-term effects of high concentrations of compounds of the POP group, and can provide information about the evolutionary processes taking place in microbe communities under the effect of the negative environmental factors (chemical contamination).

MATERIALS AND METHODS

Soil characteristics and sampling. Soil samples were taken at the production site of SVJhZ JSC (Chapaevsk, Russia). Previous examination demonstrated that the soil was contaminated with the organochlorine compounds, including ones of the POP group. Concentration of pollutants exceeds maximum allowable concentrations by 3.5–17.8 times [2, 3].

Enrichment cultivation. Bacterial associations were obtained by means of the storage cultivation of 1g of soil of every sample in 100 ml of the mineral medium K1, with the following composition (g/l): $K_2HPO_4 \cdot 3H_2O$ – 3.2, $NaH_2PO_4 \cdot 2H_2O$ – 0.4, $(NH_4)_2SO_4$–0.5, $MgSO_4 \times 7H_2O$ – 0.15, $Ca(NO_3)_2$–0.01, with an added selective factor of 4chlorobenzoic acid (4CBA) (0.5 g/l) or chlorobenzene (0.5 g/l) [12]. Cultivation was done in 250 ml Erlenmeyer flasks for 30 days in an environmental shaker-incubator ES-20/60 (BioSan, Latvia) at 120 rpm and 28 °C. Twelve bacterial associations were obtained (Table 1).

Isolation of the pure bacterial strains. Bacterial associations obtained as a result of cultivation on chlorobenzene and 4-CBA were plated on agarized medium with vitamin K1 and 0.3 g/l sodium benzoate as the source of carbon. Cultivation was done in a hemostat TC-1/80 SPU (Russia) at 28 °C prior to occurrence of colonies. Culture purity was checked during plating on nutrient-rich LB agar of the following composition (g/l): yeast extract – 0.5, tryptone – 1.0, sodium chloride – 1.0, agar – 1.5.

DNA analysis. DNA from bacterial associations was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, USA). DNA from pure bacterial cultures was extracted by previously described methods [13]. DNA concentration was determined using the Qubit™ Fluorometer (Invitrogen, USA).

Identification of isolated strains. Morphological and physiological properties of isolated bacterial strains were studied using previously established methods [14]. Bacteria were identified during amplification of 16S rRNA using

Table 1

Bacterial associations obtained during storage cultivation

Cultivation substrate	Number of soil sample					
	Ch1	Ch2	Ch3	Ch4	Ch5	Ch6
4chlorobenzoic acid	A1	A2	A3	A4	A5	A6
Chrolobenzene	B1	B2	B3	B4	B5	B6

universal primers 27F 5'-AGAGTTTGATC(A/C)TGGCTCAG-3' and 1492R5'-ACGG(C/T)TACCTTGTTACGACTT-3' [15] with further sequencing (see below). The search for homologous sequences was conducted in the database EzTaxon (http://www.ezbiocloud.net/eztaxon).

Amplification of benzoate dioxygenase gene. Gene *benA* was amplified using primers benAF [5'-GCCCACGAGAGCCAGATTCCC-3'] and benAR [5'-GGTGGCGGCGTAGTTCCAGTG-3'] [16]. Primers were selected for the conserved area of gene *benA* from *Acinetobacter baylyi* ADP1 (amplified area: nucleotide 175 to nucleotide 712, fragment size – 521 bp) [16]. PCR was conducted using 25 μl of mixture containing of buffer for *Taq*-polymerase with $MgCl_2$ (Sintol, Russia), 0.25 mm of dNTP, 0.5 μm of every primer, two units of active *Taq*-polymerase (Sintol, Russia) and 2 μl of DNA-matrix. DNA of pure cultures and bacterial associations was used as the DNA-matrix. Amplification was done using MyCycler (BioRad Laboratories, USA) under the following conditions: initial denaturant step at 95 °C for 5 min, then 30 cycles (c), 40 c at 94 °C, 50 c at 60 °C with reduction at each step for 0.4 °C, 1 min at 72 °C, and for the final step, 7 min at 72 °C.

Restriction analysis and DNA visualization. RFLP analysis using restriction endonucleases *Hha*I and *Hae*III (Fermentas, Lithuania) was conducted on amplicons obtained from DNA strains isolated from associations A5, B3 and B5. Electrophoresis was conducted in a horizontal 0.8% agarose gel in TBE buffer (Thermo Scientific, Lithuania) at a voltage of 10 V/cm. DNA visualization was conducted using UV illumination after staining with ethidium bromide solution (0.5 μg/ml) and was documented using Gel Doc™ XR (BioRad, USA). In order to determine the size of amplified fragments, a marker of molecular weight 100 bp (Plus DNA Ladder, Fermentas, USA) was used. The anticipated fragment sizes amounts were 520 bp.

Sequencing and analysis of fragments of the benzoate dioxygenase gene. Nucleotide sequences of amplified DNA fragments was determined using a Big Dye Terminator Cycle Sequencing Kit and an automatic sequencer (Genetic Analyzer 3500XL, Applied Biosystems, USA). Searches for homologs of gene *benA* were conducted in GenBank and analyzed using BLAST (http://www.ncbi.nlm.nih.gov). Multiple alignments of nucleotide sequences were done using the ClustulX program (http://www.ebi.ac.uk). A tree of similarity was constructed with using a sequential clustering algorithm (UPGMA) implemented in the CLC Sequence Viewer 6 (http://www.clcbio.com/products/clcsequenceviewer). Phylogenetic and cluster analysis and visualization of the tree were conducted in the program MEGA7. Statistical accuracy of branching (bootstrap analysis) was based on 1000 alternative trees. Nucleotide sequences of fragments of genes *benA* obtained in the research were registered in the database GenBank (see Table 3).

RESULTS AND DISCUSSION

Microbial communities able to decompose the complex organochlorine compounds were formed as a result of the long-term selection in contaminated soils at the site of JSC "Middle-Volga Chemical Plant" (Chapaevsk, Russia) [2]. Chlorobenzene and chlorobenzoic acid were used as selective factors in subsequent artificial conditions, as potential metabolites of organochlorine substances present in the soil for a long time. Selection under the effect of high concentrations of these compounds resulted in obtaining associations of the aerobic bacteria designated as A1–A6 and B1–B6 (see Table 1).

Screening of the overall DNA of 12 bacterial associations was conducted for the presence of nucleotide sequences coding for the α-subunit of benzoate 1,2-dioxygenase. PCR products of expected size – 500 fp (Fig. 1) – were obtained from the DNA of nine associations. However, specific amplification of DNA from associations A2, A3 and A6 was not observed. Strains carrying the gene *benA* may have been eliminated from the microbial community during cultivation on 4-CBA. This phenomenon can be explained with the fact that strains performing hydrolytic dehalogenation of 4-CBA prior to decomposition of the aromatic ring of molecule obtained preference [6, 12]. For a number of strains of *Acinetobacter*, *Arthrobacter* and *Pseudomonas,* multiple genes and enzymes transform 4-BCS by means of hydroxylation, with the further formation of 4-hydroxybenzoic and 3,4-dihydroxybenzoic acid metabolites [6, 12, 17, 18].

Selection of the storage cultures with positive amplification of gene *benA* resulted in obtaining pure cultures of bacterial strains able to use benzoic acid (benzoic acid sodium salts) as the sole source of carbon and energy. Based on the morphophysiological features of the isolated strains in associations A1, A4, B1, B2, B4 and B6, each association had a single strain-destructors of benzoate: these were strain A1-69, A4-72, B1-169, B2-174, B1-72, B6-173, respectively. Three strains that could utilized benzoate (A5-67, A5-68, A5-70) were present in the A5

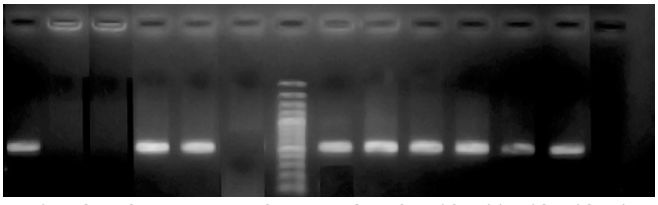

Fig. 1. Electrophoregram of the amplification products of gene benA coding for α-subunit of benzoate dioxygenase with DNA bacterial associations. *1* – A1, *2* – A2, *3* – A3, *4* – A4, *5* –A5, *6* –A6, *7* –marker of molecular weight of O'GeneRuler™ 100bp Plus DNA Ladder (Fermentas, Lithuania), *8* –B1, *9* –B2, *10* –B3, *11* –B4, *12* –B5, *13* –B6, *14* –negative control

association, five strains in association B3 could use sodium benzoate as the growth substance (B3-162, B3-163, B3-164, B3-165 and B3-166), and four strains-destructors of the benzoate (B5-167, B5-168, B5-170 and B5-171) were present in association B5.

Table 2 provides results of examinations that demonstrate the most probable taxonomic position of the isolated bacteria. Based on the analysis of gene 16S pPHK, the strains isolated from *benA*positive associations were identified as *Achromobacter*, *Ochrobactrum* and *Pseudomonas*.

Fragments of the gene coding for the α-subunit of benzoate 1,2-dioxygenase of the anticipated size (~500 bp) were obtained on DNA-matrix of the isolated strains by means of primers *benA-F* and *benA-R*. The RFLP analysis of amplified fragments of gene *benA* (~500 bp long) from the strains of associations A5, B3 and B5 was conducted using restriction endonucleases *Hha*I and *Hae*III. It was found, that the analyzed nucleotide sequences form three groups corresponding to the associations from which the strains were obtained (data not shown). Thus, within each group similar genes are available, which allows using one strain of benzoate from these associations for further analysis. Nucleotide sequences of amplified fragments from the DNA of bacterial associations were determined, as well as with DNA of the strain of benzoate isolated from the examined associations (Table 3).

Detected nucleotide sequences demonstrated the highest level of similarity with the gene for the α-subunit of benzoate 1,2-dioxygenase of *Pseudomonas*-strains. It should be noted that the level of similarity of fragments amplified with the overall DNA association coincided with the level of similarity of fragments of gene *benA* amplified with the DNA of pure cultures. We used the names of bac-

Table 2

Comparison of nucleotide sequences of 16S rRNA of the isolated degradative strains of benzoate with homologous sequences of typical strains

Association	Strain	Typical strain	Similarity, %
A1	A1-69	*Pseudomonas japonica* NRBC103040^T	99.9
A4	A4-72	*Pseudomonas alcaligenes* NBRC14159^T	100
A5	A5-67	*Ochrobactrum anthropi* ATCC49188^T	99.8
	A5-68	*Pseudomonas alcaligenes* NBRC14159^T	99.9
	A5-70	*Pseudomonas alcaligenes* NBRC14159^T	100
B1	B1-169	*Pseudomonas japonica* NRBC103040^T	99.9
B2	B2-174	*Ochrobactrum anthropi* ATCC49188^T	99.5
B3	B3-162	*Pseudomonas xanthomarina* KMM 1447^T	100
	B3-163	*Pseudomonas xanthomarina* KMM 1447^T	100
	B3-164	*Achromobacter spanius* LMG 5911^T	99.7
	B3-165	*Achromobacter spanius* LMG 5911^T	99.8
	B3-166	*Pseudomonas taiwanensis* BCRC17751^T	100
B4	B4-172	*Pseudomonas taiwanensis* BCRC17751^T	99.9
B5	B5-167	*Achromobacter spanius* LMG 5911^T	99.6
	B5-168	*Pseudomonas japonica* NRBC103040^T	100
	B5-170	*Pseudomonas alcaligenes* NBRC14159^T	100
	B5-172	*Pseudomonas xanthomarina* KMM 1447^T	99.9
B6	B6-173	*Ochrobactrum anthropi* ATCC49188^T	99.7

terial associations for designation of examined nucleotide sequences during visualization of the phylogenetic tree in the further analysis.

Phylogenetic analysis of the examined nucleotide sequences was conducted based on comparison with homologous sequences from the database GenBank (Fig. 2). Amplified areas of functional genes were similar to the genes of subfamily of BDO bacteria of different phyla performing breakdown of aromatic compounds (see Fig. 2). Past research demonstrates the possibility of horizontal gene transfer allowing decomposition of the chlorine aromatic substances among the Protista [18–21]. This phenomenon can explain the presence of genes with a high level of similarity in the genome of strains and bacterial associations spatially remote from each other.

On the contrary, analysis of the similarity of bacterial degradation genes from different phyla demonstrates that these genes can have considerable differences [5]. This study determined that nucleotide sequences of amplified fragments of gene *benA* from the overall DNA of associations A1, A4 and A5, and from DNA of appropriate strains, had a higher level of similarity (78–99% at 96–100% overlapping) to the analogous gene of Gram-negative bacteria and a lower level of similarity with genes of BDO α-subunits of Gram-positive bacteria (79–87% at 83–97% overlapping) among over a thousand sequences presented in the database (http://blast.ncbi.nlm.nih.gov/).

Similar regularity was detected during analysis of similarity of nucleotide sequence of gene fragments amplified on the DNA of associations and bacterial strains obtained during cultivation on chlorobenzene (see Table 2, Fig. 1, 2). The level of similarity with the analogous dioxygenases of Gram-positive bacteria of classes *Actinobacteria*

Table 3

Analysis of nucleotide sequences of the amplified fragments of gene *benA*

Association, fragment size, fp (number in GenBank)	Strain, size, fp (number in GenBank)	The closest homologous gene (number in GenBank)	Level of similarity, %	Overlapping, %
A1 419 (MK403888)	A1-69 420 (MK403897)	*benA* (α-subunit) *Pseudomonas putida* B6-2 (CP015202.1)	98	100
A4 415 (MK403889)	A4-72 416 (MK403898)	*benA* (α-subunit) *Pseudomonas putida* KT2440 (LT799039.1)	98	100
A5 420 (MK403890)	A5-67 423 (MK403899)	*bedA* (α-subunit) *Pseudomonas putida* JY-Q (CP011525.1) *benA* (α-subunit) *Pseudomonas putida* KT2440 (LT799039.1)	99 98	100 100
B1 408 (MK403891)	B1-169 408 (MK403900)	*bedA* (α-subunit) *Pseudomonas putida* JY-Q (CP011525.1) *benA* (α-subunit) *Pseudomonas putida* KT2440 (LT799039.1)	97 96	100 100
B2 398 (MK403892)	B2-174 395 (MK403901)	*benA* (α-subunit) *Pseudomonas putida* KT2440 (LT799039.1)	98	100
B3 399 (MK403893)	B3-164 399 (MK403902)	*benA* (α-subunit) *Pseudomonas putida* KT2440 (LT799039.1)	98	100
B4 401 (MK403894)	B4-172 403 (MK403903)	*benA* (α-subunit) *Pseudomonas putida* B6-2 (CP015202.1)	99	100
B5 400 (MK403895)	B5-170 401 (MK403904)	*benA* (α-subunit) *Pseudomonas* sp. VLB120 (CP003961.1) *benA* (α-subunit) *Pseudomonas putida* S16 (CP002870.1)	93 95	100 99
B6 411 (MK403896)	B6-173 418 (MK403887)	*benA* (α-subunit) *Pseudomonas putida* KT2440 (LT799039.1)	97	100

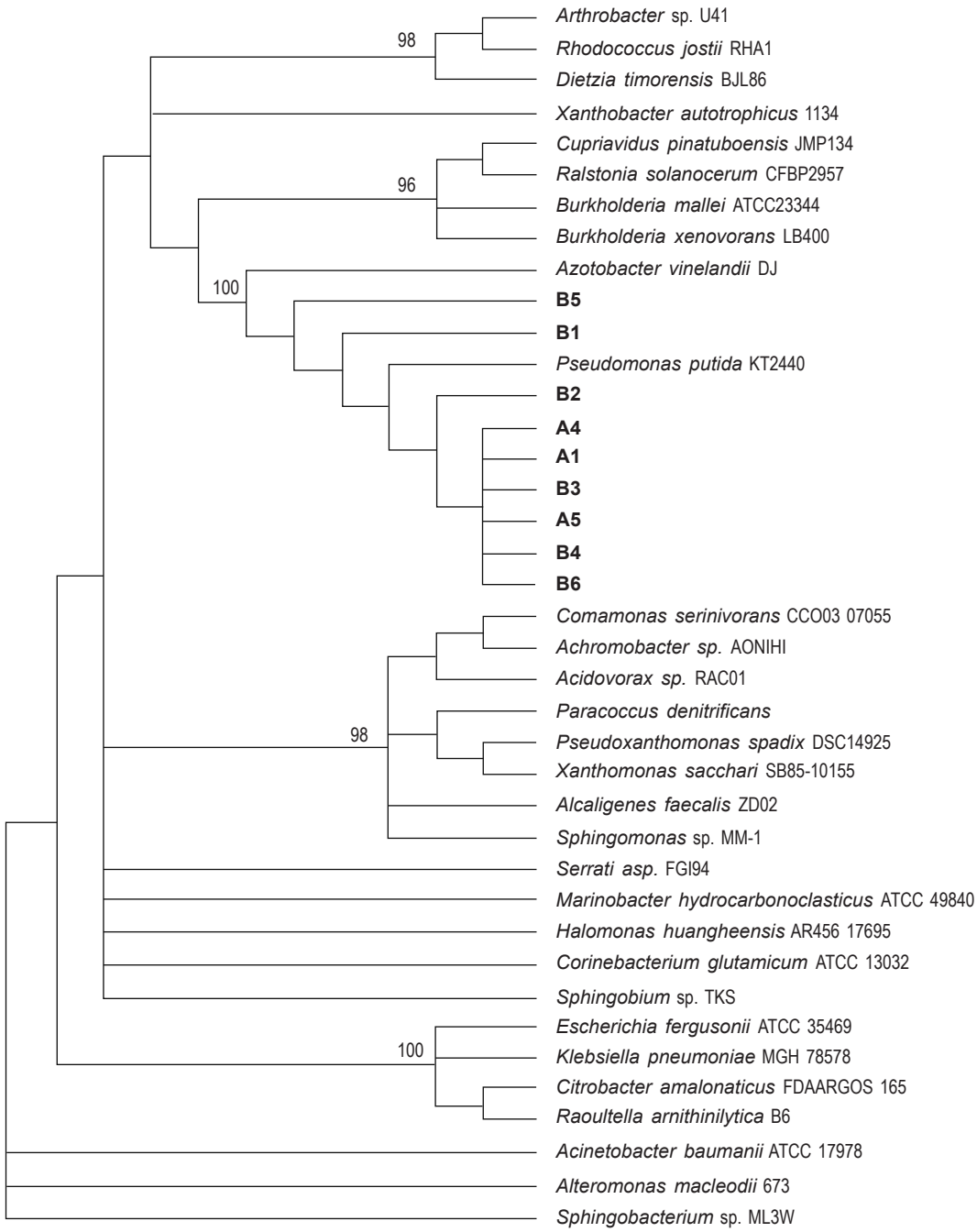

Fig. 2. Tree of similarity of the detected genes with the well-known genes of α-subunit of benzoate 1,2-dioxygenase constructed with method UPGMA. The scale corresponds to 10 nucleotide substitutions per every 100 nucleotides. Bootstrap-analysis was conducted using 1000 repeats. Values next to the branches demonstrate location of sequences in these groups. Bold fonts underline nucleotide sequences examined in the work. Designation of strains, as well as their generic and specific names are provided for the analyzed sequences

and *Bacilli* amounted to 83–88% at 73–100% overlapping; the level of similarity with the gene for the BDO α-subunit of Gram-negative bacteria of classes β- and γ-*Proteobacteria* ranged from 80–99% at 85–100% overlapping.

Cluster analysis demonstrated that fragments of genes *benA* amplified with DNA of strains and associations extracted from the soils of Chapaevsk, at the 100% credible level, form the uniform cluster with the genes of BDO strains of the genus *Pseudomonas* (see Fig. 2). Thus, the obtained results do not preclude horizontal transfer of BDO genes among bacteria of different phylums. However, more probable is the hypothesis of a convergent origin of genes *benA* due to adaptation to similar effects of

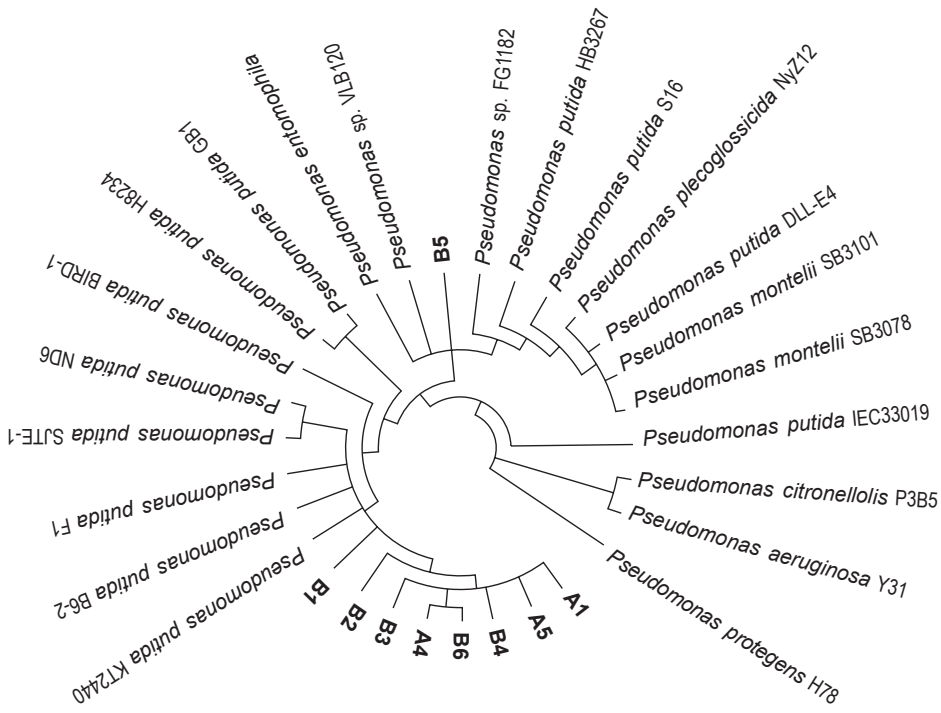

Fig. 3. Tree of similarity of nucleotide sequences homologous to the examined areas of genes of α-subunit of benzoate 1,2-dioxygenase of strains of genus *Pseudomonas* constructed with method UPGMA. The scale corresponds to 10 nucleotide substitutions per every 100 pairs of nucleotides. Bootstrap-analysis was conducted on 1000 repeats. Bold fonts underline nucleotide sequences examined in the work. Designation of strains, as well as their generic and specific names are provided for the analyzed sequences

organochlorine compounds present in the environment, as well as in the cultivation medium [19–22].

As cluster analysis of amplified sequences and known nucleotide sequences of gene *benA* demonstrated that genes presented in the examined bacterial associations most likely form one cluster with BDO genes of the strains of genus *Pseudomonas* (see Fig. 2), further analysis was conducted inside the cluster (Fig. 3). The closest nucleotide sequence for gene *benA* amplified with DNA of associations A1, A4, A5, B1, B2, B3, B4, and B6 and of appropriate individual strains are the genes coding α-subunit BDO of the strains *Pseudomonas putida* KT2440 (GenBank LT799039.1) and *Pseudomonas putida* B6-2 (CP015202.1) – 96–99% of similarity at 100% overlapping. Analyzed genes form a separate cluster on the phylogenetic tree (see Fig. 3) adjacent to the cluster of gene *benA* of strains *P. putida* KT2440, *P. putida* B6-2 (GenBank CP015202.1), *P. putida* F1 (GenBank CP000712.1), *P. putida* SJTE-1 (GenBank CP015876.1) and *P. putida* ND6 (GenBank CP003588.1). These results suggest that genes coding BDO α-subunit are spread in bacterial associations, which are well described for strains attributed to species *Pseudomonas putida* [9]. It is likely that genes of biodegradation can undergo horizontal transfer on plasmids between representatives of different phylums [19–21, 23].

The sequence of gene *benA* amplified with DNA of the strain B5-170 of association B5 was placed in one cluster with the genes of BDO of the strains *Pseudomonas* sp. VLB120 and *P. entomophila* L48 (see Fig. 3), with the level of similarity of 93% at 100% overlapping, but in different clusters with gene *benA* of strain of *Pseudomonas putida* S16, despite that fact that the level of similarity with this gene in homologous search amounted to 95%. The strain from *P. entomophila* L48 (GenBank CT573326), pathogenic bacterium attributed to saprophytic soil aerobic bacteria, was detected to have enzymatic systems ensuring metabolism of benzoic, 4-hydroxybenzoic, 3-hyndoxybenzoic and 3,4-dihydroxybenzoic acids; in this strain, BDO is involved only in transformation of unsubstituted benzoic acid and genes are located on the primary chromosome [24]. A strain of *Pseudomonas* sp. VLB120 (GenBank CP003961) with BDO genes on the plasmid that is able to decompose octanol, toluene and styrene has been extracted from the soils of Stuttgart, Germany [25]. However, detection of the high similarity genes *benA* in spatially remote bacterial strains supports the theory of transfer of genetic material between bacteria of genus *Pseudomonas* in the process of adaptation to high level of contamination of aromatic and chlorine aromatic compounds [19, 21, 22].

In this research, fragments of the gene α-subunit BDO were detected to have unique nucleotide sequences; this is confirmed by the absence of 100% similarity with the sequences of the known genes *benA* placed in international databases. It was detected that the examined fragment of gene *benA* spread among bacterial strains of associations A1, A5 and B4 contains one substitution (cytosine is substituted with thymine). Two substitutions in the structure of nucleotides were detected in genes of the BDO α-subunit in associations A4, B3 and B6 (cytosine is substituted with thymine in all associations); in addition, adenine is substituted with cytosine (association A4), guanine is substituted with thymine (association B3) and guanine is substituted with adenine (association B6). The substitutions in nucleotides were detected in the fragments of gene *benA* spread in association B2, and six substitutions in nucleotides were detected in the sequence of fragments of gene *benA* in association B1. The greatest number of differences was detected in the nucleotide sequence of the examined fragment of gene α-subunit of BDO typical for association B5 (14 substitutions).

Detected spot mutations are not observed during analysis of the amino acid sequence of the fragment of the α-subunit of BDO in the representatives of associations A1, A5, B2, B4 and B6. One amino acid substitution was detected in the BDO sequence of associations A4 (serine is substituted with arginine) and B3 (aspartic acid is seen in the protein content instead of glutamic acid). Two amino acid substitutions were detected in the sequence of the examined enzyme typical for the strains of association B1 (tyrosine and alanine are substituted with serine). The greatest number of differences were detected in the amino acid sequence of the BDO α-subunit of association B5 – four substitutions (alanine is twice substituted with serine, lysine is substituted with glutamine, and asparagine is substituted with lysine). The current study did not examine whether the detected differences in nucleotide and amino acid sequences of the BDO α-subunit allowed the examined bacterial communities to more effectively use benzoate as the source of carbon and energy, in comparison with the well-known strains and their decomposition of aromatic compounds.

One of the adaptation mechanisms that allows aerobic bacteria to adapt to survival under stress conditions is mutations, including the spot mutations [26]. Mutations can also occur as a result of chemical compounds present in the bacteria surroundings. Bacterial associations examined in this work were affected by organochlorine compounds of the POP group for a long time, which can cause an increase in mutation frequency once they are present in the organism [4]. Probably, a combination of these factors resulted in the formation of unique sequences of genes of α-subunit of BDO among different types of bacterial communities available in the soils at the industrial site of JSC "Middle-Volga Chemical Plant" (Chapaevsk, Russia).

CONCLUSION

Genes determining α-subunits of benzoate dioxygenase were identified in bacterial associations exposed to long-term effects of organoaromatic pollutants under natural and artificial conditions. Amplified nucleotide sequences showed phylogenetic similarity with genes *benA* from strains of genus *Pseudomonas*, which degraded of different aromatic compounds.

Additional information

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