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EXPERIMENTAL ARTICLES

Degradation of 4-Chlorobiphenyl and 4-Chlorobenzoic Acid by the Strain *Rhodococcus ruber* P25

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Abstract—The strain *Rhodococcus ruber* P25 utilizes 4-chlorobiphenyl (4CB) and 4-chlorobenzoic acid (4CBA) as sole carbon and energy sources. 4CB degradation by washed cells of strain P25 was accompanied by transient formation of 4CBA, followed by its utilization and release of equimolar amounts of chloride ions into the medium. The strain *R. ruber* P25 possessed active enzyme systems providing 4CBA degradation via the stages of formation of intermediates, *para*-hydroxybenzoate (PHBA) and protocatechuic acid (PCA), to compounds of the basic metabolism. The involvement of protocatechuate 4,5-dioxygenase in 4CBA degradation by rhodococci was revealed. It was established that the initial stage of 4CBA degradation (dehalogenation) in the strain *R. ruber* P25 was controlled by the *fcbA* and *fcbB* genes encoding 4-CBA-CoA ligase and 4-CBA-CoA dehalogenase, respectively. The genes encoding 4CBA dehalogenase components have not been previously detected and characterized in bacteria of the genus *Rhodococcus*.

Keywords: *Rhodococcus*, 4-chlorobiphenyl, 4-chlorobenzoic acid, destruction, *fcb* genes, protocatechuate 4,5-dioxygenase.

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Chlorinated aromatic hydrocarbons are widespread in the environment. The sources of these compounds are synthesized substances and chemical industry wastes. Most of the chlorinated aromatic compounds are toxic, resistant to degradation, and cause soil and water pollution. Among the most widespread pollutants are polychlorinated biphenyls (PCB) comprising a group of stable organic pollutants (http://chm.pops.int) and belonging to class 1 of hazard of chemical agents (http://www.atsdr.cdc.gov) [1]. PCB may contain 1 to 10 chlorine atoms in the two aromatic rings of the molecule. Anaerobic degradation of PCB in the environment (the initial stage of destruction of this class of compounds) most often results in the formation of low-chlorinated biphenyls containing up to 5 chlorine atoms in *ortho*- and *para*-positions [2]. Aerobic microorganisms involved in the further process of degradation of low-chlorinated chlorobiphenyls, in most cases, transform them to the stage of formation of chlorobenzoic and pentadienoic acids [3, 4].

In recent years, environmental bacteria have been reported to utilize mono- and dichlorinated biphenyls [3, 5–7]. Complete degradation of *para*-CB by the bacteria *Pseudomonas* sp. DJ-12, *P. cepacia* P166 and *Burkholderia* sp. SK-3 has been observed [5, 8, 9]. The strain *Rhodococcus* sp. B7a described in 2010 degrades *para*-CB via the formation of 4CBA followed by its utilization [10]. The strain *R. ruber* P25 investigated in the present work is an active biphenyl and PCB destructor capable of complete utilization of *ortho*- and *para*-(mono-di)chlorinated biphenyls, including 4-chlorobiphenyl (4CB) [11, 12].

The goal of the present work was to study the biochemical and genetic aspects of destruction of *para*-chlorinated biphenyl and *para*-chlorobenzoic acid by the strain *R. ruber* P25 which can use these compounds as the sole carbon and energy sources.

MATERIALS AND METHODS

The strain *Rhodococcus ruber* P25 (=IEGM896) was isolated by the method of enrichment cultivation from soil polluted with the wastes of chemical plants producing halogen-containing compounds (Perm, Russia). The strain can utilize a broad range of aromatic compounds (biphenyl, phenol, naphthalene, salicylate, gentisate, *ortho*-phthalate, and benzoate), as well as their substituted derivatives (*para*-, *ortho*-chlorinated biphenyls and chlorobenzoates, *para*-methylbenzoate, and 2,4-dichlorophenoxyacetate) as a sole carbon and energy source [11, 12].

Growth characteristics of the strain *R. ruber* P25 were studied in a K1 liquid mineral medium [13]. The

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strain was grown in 750-mL Erlenmeyer flasks in 200 mL of the mineral medium at 28°C under aeration on a shaker at 220 rpm. The substrates were biphenyl, 4-chlorobiphenyl (4CB), and 4-chlorobenzoic acid (4CBA) at concentrations of 1.0, 0.5, and 0.7 g/L, respectively. The number of colony-forming units (CFU/mL) was determined by the method of serial dilutions followed by plating and counting of the colonies on petri dishes with agarized Luria–Bertrani (LB) medium [14]. Specific growth rate and doubling time of the culture were calculated by the standard formulas.

4CB and 4CBA degradation was studied in the experiments with washed cells. Strain P25 was grown in liquid K1 medium with biphenyl (1 g/L) at 28°C to $OD_{600} = 1.0$. The cells twice washed with K1 medium (1 mL, $OD_{600} = 2.0$) were transferred into vials with Teflon-lined cups. The substrates (4CB and 4CBA) were added to the final concentrations of 94.25 mg/L (500 μM) and 0.7 g/L, respectively. The vials were shaken on a shaker at 28°C. The 4CB-containing vials were sampled after 1, 5, and 24 h of incubation; 4CBA-containing ones, after 5, 24, 48, 72, and 96 h. Each variant of the experiment was repeated three times.

4CB and 4CBA extraction, qualitative and quantitative analysis were carried out as described in the work of Egorova et al. [10].

Identification of 4CB and 4CBA biodegradation products was performed in the culture liquid purified from bacterial cells by centrifugation (3 min, 9660 *g*) in a miniSpin centrifuge (Eppendorf, Germany).

The products of *meta*-cleavage of the chlorobiphenyl aromatic ring, 2-hydroxy-6-oxo-phenylhexa-2,4-dienoic acids (HOPDA), were detected in an UV-Visible BioSpec-mini spectrophotometer (Shimadzu, Japan) at λ of 390 to 440 nm [4].

In the supernatant, CBA, hydroxybenzoic (PHBA) and protocatechuic (PCA) acid were detected by HPLC analysis in a LC-10ADvp chromatograph (Shimadzu, Japan) with a Lichrosorb RP-18 10U column (250 × 4.6 mm) (Alltech, United States) and an UV detector at 205 nm. The analysis was performed in the acetonitrile–0.1% H_3PO_4 solution (70 : 30) system. Retention times of the tested and standard compounds in the column were compared for identification. The quantity of the products was assessed by the peak area and peak height on the chromatogram relative to the respective values for the standard compounds.

The dynamics of 4CB and 4CBA dehalogenation by strain *R. ruber* P25 was controlled by measuring the optical density of silver chloride at λ_{460}. This compound was formed after the reaction between chloride ions and silver nitrate in the cell-free culture liquid [13].

Purification and determination of enzyme activities. The strain biomass for enzyme isolation and purification was grown in batch culture as described above, using 4CBA as the sole carbon and energy source (1 g/L). The cells were precipitated by centrifugation and washed twice in 50 mM Tris–HCl buffer, pH 7.4. The biomass was stored at –20°C.

The cell-free extract was obtained by the method of extrusive disintegration in a Hughes press as described [15].

Enzyme activities were determined by spectrophotometry in an UV-160 spectrophotometer (Shimadzu, Japan) in quartz cuvettes with an optical path of 1 cm at –25°C.

The activity of *para*-hydroxybenzoate hydroxylase (PHBH, EC 1.14.13.33) was determined by the NADH- or NADPH-dependent decrease in absorption at 340 nm. The reaction mixture contained 100 mM Tris–HCl, pH 7.8, 1 mM *para*-hydroxybenzoate, 0.2 mM NADH or NADPH, 0.5 mM EDTA, and 10 μM FAD [16]. The enzyme was preincubated with FAD and NADPH for 5 min; the reaction was started by adding PHBA. PHBH activity was calculated by NADH (the molar extinction coefficient for NADH or NADPH is 6220 $M^{-1}\,cm^{-1}\,L^{-1}$).

The activity of protocatechuate 4,5-dioxygenase (PC 4,5-DO, EC 1.13.11.8) was determined by the increase in absorption at 410 nm corresponding to the formation of 2-hydroxy-4-carboxymuconic semialdehyde, using the molar extinction coefficient of 11220 $M^{-1}\,cm^{-1}\,L^{-1}$ for calculation [17].

The activity of protocatechuate 3,4-dioxygenase (PC 3,4-DO, EC 1.13.11.3) was determined by the decrease in absorption at 290 nm corresponding to the loss of protocatechuate. The reaction mixture contained 50 mM Tris–HCl buffer, pH 7.5, and the substrate; the reaction was started by adding the enzyme. The activity was calculated using the molar extinction coefficient of 6770 $M^{-1}\,cm^{-1}\,L^{-1}$ [18].

The activities of catechol 1,2-dioxygenase (Cat1,2-DO, EC 1.13.11.1) and catechol 2,3-dioxygenase (Cat 2,3-DO, EC 1.13.11.2) were determined as described [19].

The quantity of the enzyme catalyzing transformation of 1 μmol of the substrate or formation of 1 μmol of the product per minute was taken as a unit of activity.

PHBH was purified with the 50 mM Tris–HCl buffer, pH 7.2, containing 10 μM FAD, 1 mM EDTA, and 0.5 mM DTT (buffer A). The cell-free extract was applied to the Q-Sepharose column (2.6 × 20, carrier volume 60 mL) pre-equilibrated with the starting buffer. Proteins were eluted by the increasing 0–0.5 M NaCl linear gradient in 1200 mL of the starting buffer at a rate of 2 mL/min. The fractions with PHBH activity were combined; ammonium sulfate was added to a final concentration of 1.6 M; after centrifugation (20000 *g*, 20 min), the fractions were applied to a Buthyl-Sepharose column (1.6 × 20, carrier volume 30 mL) pre-equilibrated in buffer A with 1.6 M ammonium sulfate. Proteins were eluted by the

Table 1. Products of 4-chlorobiphenyl degradation by washed cells of *R. ruber* P25

Time, h	Cl^-, mg/L	HOPDA		4CBA	
		λ_{max}	OD, U	mg/L	%
0*	n.d.	434	0.047 ± 0.001	1.09 ± 0.02	1.4
1	2.6 ± 0.1	"	0.268 ± 0.005	11.82 ± 0.09	15.1
5	7.5 ± 0.4	"	0.526 ± 0.005	51.65 ± 0.10	66.0
24	12.6 ± 0.1	"	0.577 ± 0.004	32.08 ± 0.08	41.0

Note: * samples for the analysis were taken 3–5 min after 4-chlorobiphenyl introduction; "n.d." stands for "not determined."

decreasing 1.6–0 M ammonium sulfate linear gradient in 300 mL of the starting buffer. The fractions with PHBH activity were combined, concentrated in a cell with the PM-10 membrane, and applied to a Superdex 75 column (1.6 × 60) equilibrated in buffer A with 0.1 M NaCl. The most active fractions were combined and applied to a Resource Q column (6 mL) equilibrated with buffer A. Elution was performed with an increasing 0–0.5 M NaCl linear gradient at a rate of 2 mL/min. The most active fractions were combined, concentrated in the cell with the PM-10 membrane, and stored in the presence of 10 μM FAD, 1 mM EDTA, and 0.5 mM DTT. PHBH activity during purification was determined using PHBA and NADH.

During the purification of protocatechuate dioxygenase, the cell-free extract was applied to the Q-Sepharose column (2.6 × 20, carrier volume 60 mL) equilibrated with 50 mM Tris–HCl buffer, pH 7.5 (buffer B), and eluted with the increasing 0–0.5 M NaCl linear gradient in 1200 mL of the starting buffer at a rate of 2 mL/min. The fractions containing PCDO activity were combined and applied to a Resource Phe column (1-mL volume) equilibrated with buffer B containing 1.6 M ammonium sulfate. Elution was performed with the decreasing 1.6–0 M ammonium sulfate gradient in 25 mL of the starting buffer at a rate of 1 mL/min. After gel filtration in the Superdex 75 column (16 × 60) equilibrated in buffer B with 0.1 M NaCl, up to 1.6 M ammonium sulfate was added to the active fractions, which, upon centrifugation, were applied to a Resource Iso column (1 mL); the proteins were eluted with the decreasing 1.6–0 M ammonium sulfate linear gradient in 25 mL of the buffer at the rate of 2 mL/min. It was followed by ion exchange chromatography in the Resource Q column (6 mL) with elution by the increasing 0–0.5 NaCl gradient in 90 mL of the starting buffer at a rate of 2.5 mL/min. Active fractions were combined and used for the characterization of the enzymes. PCA was used as a substrate during PCDO purification.

All procedures for assessment of protein purity and quantity, determination of protein composition, and weight were performed as described [15].

Apparent Michaelis constants (K_m) and V_{max} values were obtained by the double reciprocal method in the coordinates $1/V_0$ from $1/S$, where S was the substrate concentration.

Total DNA from the strain P25 was isolated by the standard procedure [14].

PCR was performed using the primers constructed on the basis of the known *fcb* gene sequences of the strains *Arthrobacter* sp. SU and *A. globiformis* KZT1 (GenBank M93187, AF304300) [20]. PCR was carried out in a MyCycler (BioRad Laboratories, United States) with one cycle at 95°C for 5 min followed by 30 cycles: 30 s at 94°C, 30 s at 60°C, and 2 min 10 s at 72°C. PCR products were analyzed by electrophoresis in 1% agarose gel at 60–90 V in Tris–acetate buffer (89 mM Tris, 12.5 mM Na_2EDTA, 89 mM Na acetate, pH 8.0). Agarose gels were stained with ethidium bromide solution (2 μg/mL) for 20 min and photographed in the UV light using a Gel Doc™ XR gel documentation system (BioRad Laboratories, United States). The λ/*Hin*dIII DNA (Sigma, Germany) and the molecular marker IX (Sigma, Germany) were used as markers.

Amplification products were sequenced by the Sanger method using a CEQ Cycle Sequencing kit in an automated MegaBACE 1000 sequencer (JSC GE Healthcare, United States) according to the manufacturer's instructions. Nucleotide sequences were analyzed using the CLUSTAL X 1.83 [21], TREECON version 1.3b [22], and BLAST (http://www.ncbi.nlm.nih.gov) software packages. Homologous sequences were searched in the GenBank databases (http://www.ncbi.nlm.nih.gov).

RESULTS

Degradation of 4-Chlorobiphenyl by the Strain Rhodococcus ruber P25

The experiments with washed cells demonstrated the ability of the strain *R. ruber* P25 to degrade 4CB; as a result, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) with $\lambda_{max} = 434$ nm, chloride ions, and 4CBA were found in the cultivation medium (Table 1). It was shown that while the content of HOPDA was

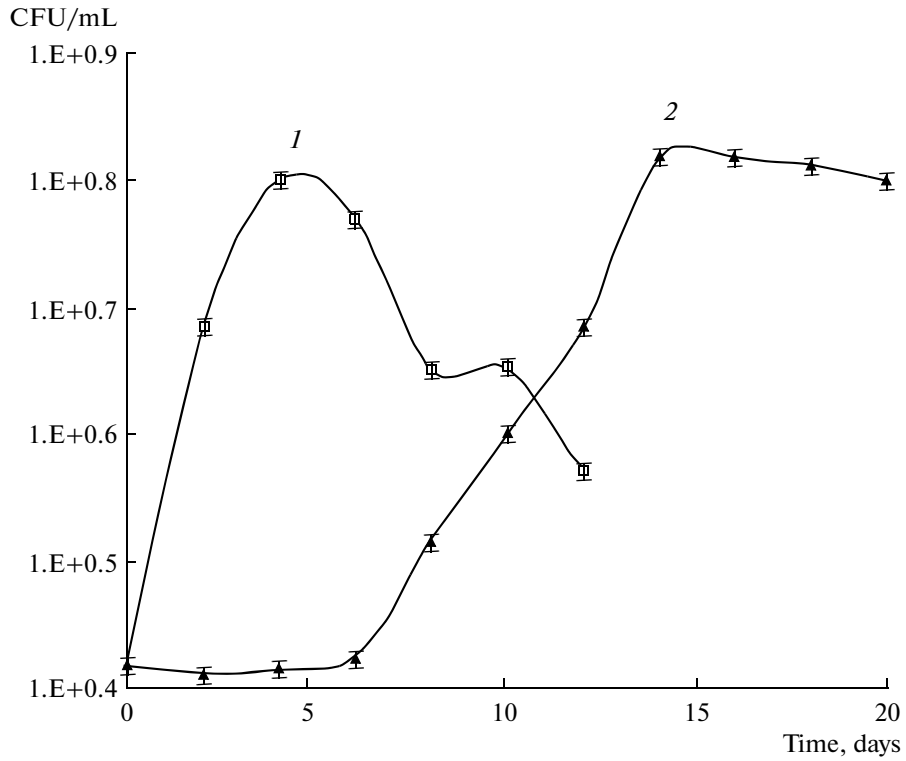

Fig. 1. Growth of the strain *Rhodococcus ruber* P25 on biphenyl (*1*) and 4-chlorobiphenyl (*2*).

practically unchanged by hour 24 of 4CB destruction, the content of 4CBA considerably decreased (Table 1).

Growth experiments confirmed our suggestion that strain *R. ruber* P25 could use not only biphenyl but also 4CB (0.5 g/L) as a sole carbon and energy source (Fig. 1). During the growth on 4CB, transition to the stationary growth phase occurred on day 15 of the cultivation, with culture density reaching 1.5×10^8 CFU/mL. The cell doubling time in the exponential growth phase was 14.3 h. Growth on 4CB was accompanied by slight coloration of the medium (λ_{max} = 434 nm), indicating formation and slow utilization of the 4-chlorobiphenyl *meta*-cleavage product: (10-CI)2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (10-CI HOPDA). It was shown that by the end of the stationary growth phase (day 20), the cultivation medium contained 35 mg/L 4CB (7% of the initial concentration), 12.97 mg/L 4CBA, and 84.63 mg/L chloride ion. The low 4CB and 4CBA concentrations and considerable content of chloride ions in the medium suggested active utilization of both the initial substrate and the 4-chlorobenzoic acid formed during its degradation.

Degradation of 4-Chlorobenzoic Acid by the Strain R. ruber P25

Figure 2 shows that in the course of 96-h incubation, washed cells of the strain *R. ruber* P25 degraded 0.6 g/L 4CBA, with accumulation of free chloride ions in the medium in stoichiometric ratio to the substrate loss. Trace amounts of the intermediates of substrate metabolism detected during 4-chlorobenzoate degradation (*para*-hydroxybenzoic and protocatechuic acids) were subsequently utilized, according to the data of liquid chromatography and enzyme activity analysis (see below).

The growth of strain *R. ruber* P25 on 4CBA as a sole carbon and energy source (Fig. 3) was accompanied by a considerable increase in cell number (by four orders of magnitude in 8 days) and correlated with a decrease in the substrate concentration and with accumulation of chloride ions in the medium. At the same time, growth was accompanied by formation of the products of 4CBA degradation: *para*-hydroxybenzoate (up to 8%) and protocatechuate (up to 13%). The cell doubling time in the exponential growth phase was 7.6 h and the specific growth rate was 0.09 h^{-1}.

Based on the above results, it may be supposed that the metabolic pathway for degradation of the tested substrates consists of the following stages (Fig. 4): (1) 4CB degradation to 4CBA via the stage of 10-Cl HOPDA formation; (2) 4CBA dechlorination with the formation of PHBA; and (3) oxidation of PHBA to compounds of the basic metabolism.

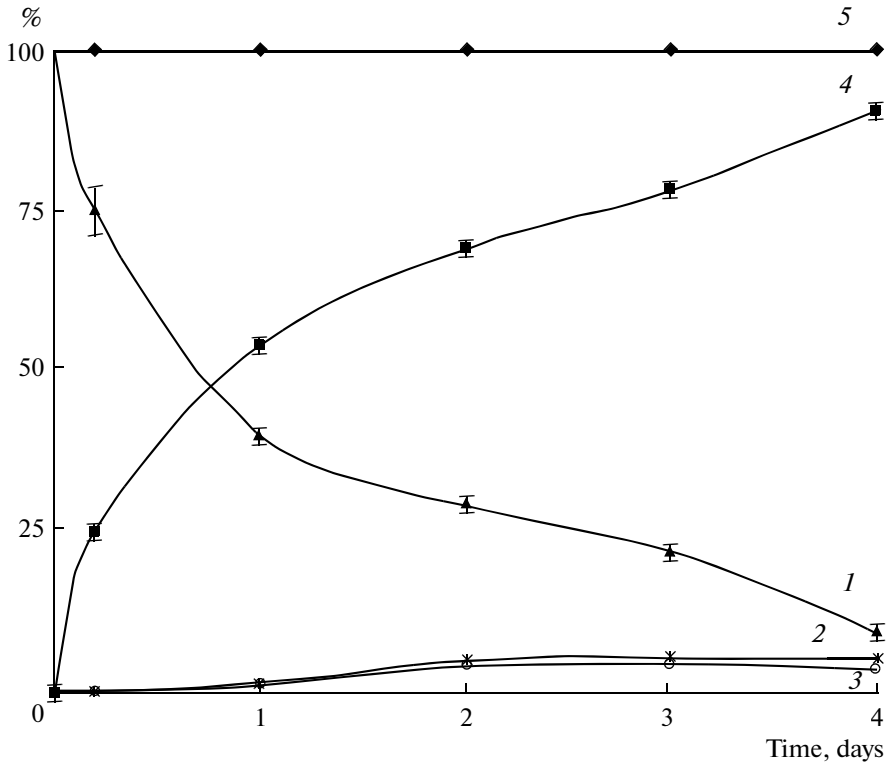

Fig. 2. Dynamics of 4CBA degradation by washed cells of *Rhodococcus ruber* P25: 4CBA (*1*); PCA (*2*); PHBA (*3*); Cl^- (*4*); and 4CBA (chemical control) (*5*).

Genes and Enzymes of Strain R. ruber P25 Involved in 4CBA Degradation

The *fcb* genes controlling 4CBA dehalogenation. The nucleotide sequences corresponding to the *fcb* gene regions (*fcbA* and *fcbB*) responsible for hydrolytic dehalogenation of 4CBA were amplified on the DNA template of strain *R. ruber* P25 [13]. The sizes of the amplified gene fragments *fcbA* and *fcbB* encoding 4-chlorobenzoate-CoA-ligase and 4-chlorobenzoate-CoA-dehalogenase, respectively, was 590 bp for the *fcbA* gene and 598 bp for the *fcbB* gene. Comparative analysis of the nucleotide sequences of the *fcb* genes of *R. ruber* P25 with the similar sequences from the GenBank international database showed high levels of homology between the *fcbA* gene region (corresponding to the region from nucleotide 402 to 927 of *Arthrobacter* sp. SU *fcbA*, GenBank M93187) and those from strains of the genus *Arthrobacter*: 98–99% similarity with the *fcbA* sequences of the strains *Arthrobacter* sp. KZT1, FG1, SU, and TM1, 95% similarity with the *fcbA* sequences of the strain *Arthrobacter* sp. FHP1 (Fig. 5a). The *fcbB* gene fragment of *R. ruber* P25 (corresponding to the region from nucleotide 262 to 774 of *Arthrobacter* sp. SU *fcbB*, GenBank M93187) was also shown to have high levels of similarity with the *fcbB* gene sequences of *Arthrobacter* sp. SU and FG1 (98%), *Arthrobacter* sp. TM1 and KZT1 (97%), and *Arthrobacter* sp. FHP1 (96%) (Fig. 5b). The level of homology between the nucleotide sequences of the *fcb* genes of *R. ruber* P25 and those of gram-negative 4CBA-degrading bacteria (*Pseudomonas* sp. DJ-12 and *Alcaligenes* sp. AL3007) was about 60% (Fig. 5).

It was also established that strain *R. ruber* P25 possessed an active enzyme system providing the oxidation of *para*-hydroxybenzoic acid to protocatechuic acid and extradiol cleavage of PCA to the compounds of the basic metabolism (Fig. 4). The cell-free extract of strain *R. ruber* P25 grown on 4CBA was shown to contain *para*-hydroxybenzoate hydroxylase (PHBH) and protocatechuate dioxygenase (PCDO) activities (Tables 2 and 3). Specific PHBH activity was 0.68 U/mg of protein; PCDO activity was not amenable to quantitative expression and was tested only by the appearance of yellow coloration of the reaction mixture. The presence of these enzyme activities corresponded to the formation of PHBH and PCA as intermediates during 4CBA degradation (Figs. 2, 3). The cell-free extract had no activity of the enzymes of catechol ring cleavage: catechol 1,2-dioxygenase (Cat 1,2-DO) or catechol 2,3-dioxygenase (Cat 2,3-DO), which was in agreement with the metabolic profile data.

***para*-Hydroxybenzoate hydroxylase** (PHBH) was purified to a homogenous state in four steps (Table 2). Preliminary studies showed that EDTA and DTT had a positive effect on its stability, while the presence of FAD was necessary for the maintenance of PHBH

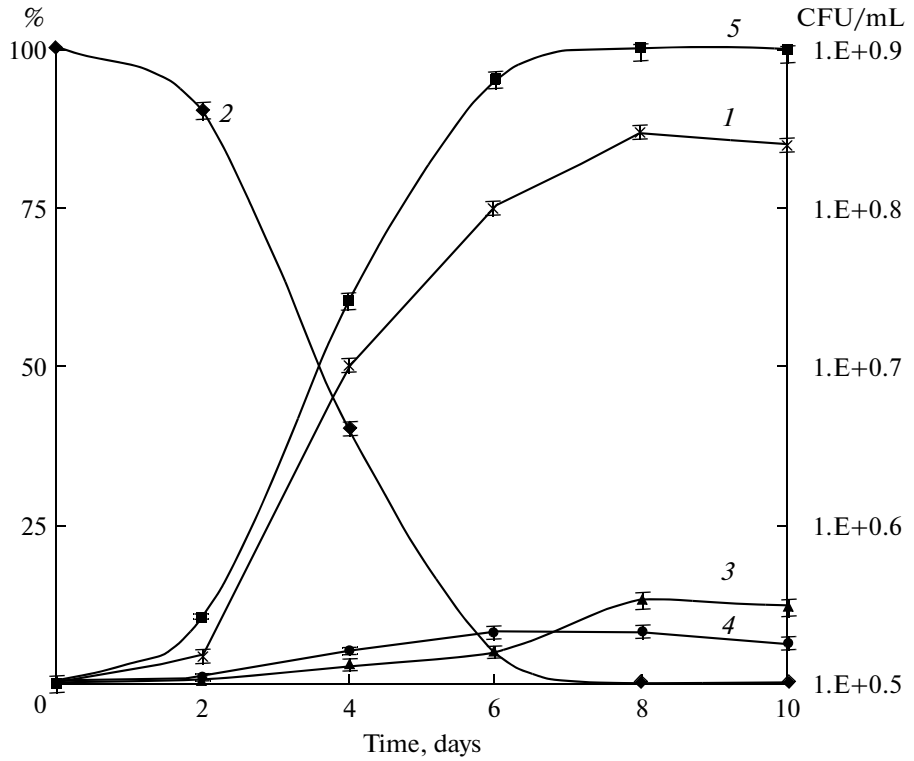

Fig. 3. Growth of the strain *Rhodococcus ruber* P25 (*1*) on 4CBA and content (%) in the cultivation medium of: 4CBA (*2*), PCA (*3*), PHBA (*4*), and Cl^- (*5*).

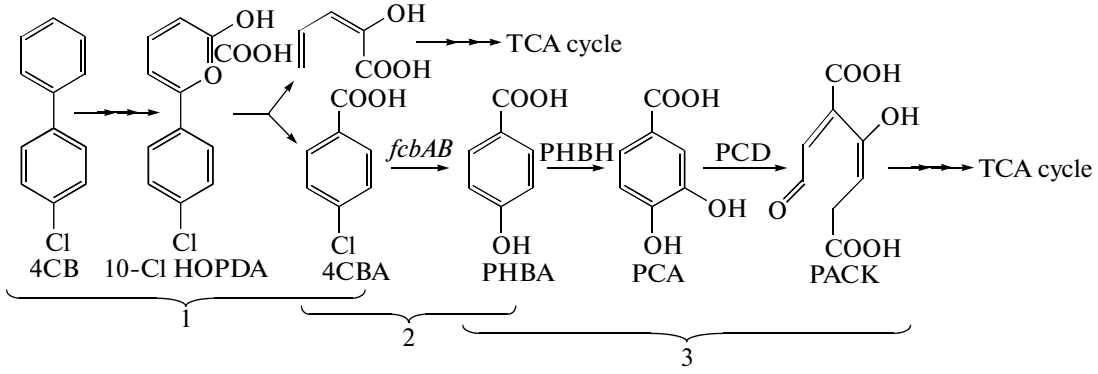

Fig. 4. The scheme of the metabolic pathway of 4-chlorobiphenyl degradation by the strain *Rhodococcus ruber* P25. The legends denote the compounds, genes, and enzymes used in the present work. 4-Chlorobiphenyl, 4CB; 10-Cl hydroxy-oxo-phenyl hexadienoic acid, 10-Cl HOPDA; 4-chlorobenzoic acid, 4CBA; *para*-hydroxybenzoic acid, PHBA; protocatechuic acid, PCA; 2-oxy-4-carboxy-*cis,cis*-muconic acid semialdehyde, PACK; tricarboxylic acid cycle, TCA cycle; the genes encoding 4-chlorobenzoate-CoA-ligase and 4-chlorobenzoate-CoA-dehalogenase, *fcbA* and *fcbB*, respectively; *para*-hydroxybenzoate hydroxylase, PHBH; protocatechuate dioxygenase, PCD.

activity of the tested strain. After the first three stages of purification, the enzyme was purified 70-fold with a 15% yield of activity. Application of ion-exchange chromatography at the last stage of purification resulted in an abrupt decrease of the specific enzyme activity.

According to the results of SDS-PAAG electrophoresis, PHBH had two subunits with molecular masses of 43 and 45 kDa (data not shown). The following catalytic characteristics were determined: K_m^{PHBA} = 9.85 μM; K_m^{NADH} = 14.2 μM. Specific $PHBH_{P25}$ activity upon NADPH application was 14.25% of the specific $PHBH_{P25}$ activity upon NADH application.

Protocatechuate dioxygenase (PCDO) of the strain *R. ruber* P25 was isolated in a homogenous state as a

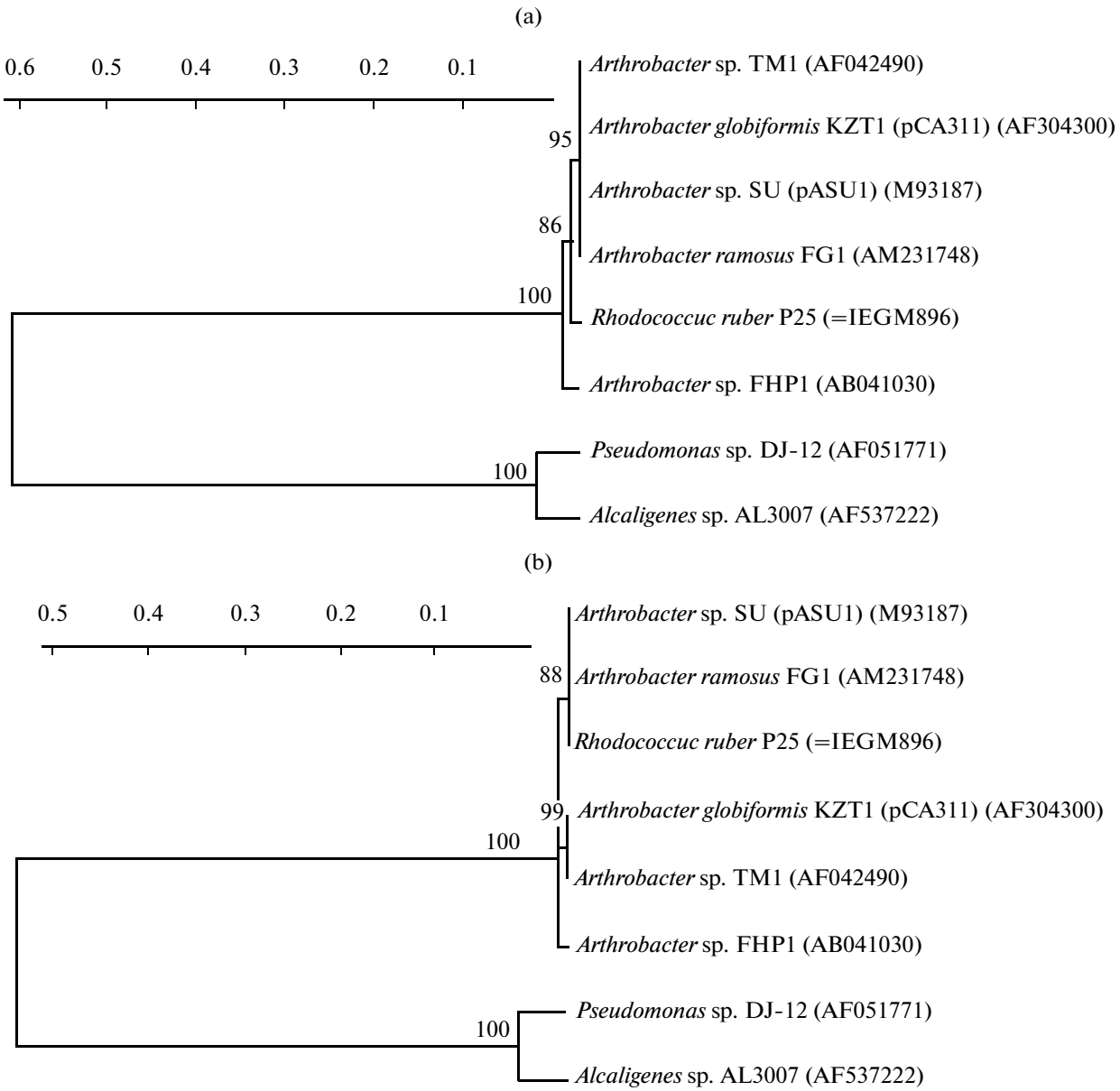

Fig. 5. Dendrograms of similarity of the nucleotide sequences homologous to the investigated regions of the *fcbA* (a) and *fcbB* (b) genes of the strain *R. ruber* P25. The scale corresponds to 10 nucleotide replacements per every 100 nucleotides. Bootstrap analysis was performed in 1000 repeats.

result of six-stage purification (Table 3). Since PCDO activity in the cell-free extract and after ion-exchange chromatography could be determined only qualitatively, the degree of purification and the yield of activity were assessed in comparison to the preparation after the first hydrophobic chromatography. The data show that the last stages of purification gave a sufficiently high yield of the enzyme.

The enzyme isolated as a result of combining the stages of hydrophobic and ion-exchange chromatography had a specific activity of 15 U/mg protein, $K_m^{PCA} = 5.26$ μM. The enzyme was found to be stable during a week at 4°C and completely lost its activity under heating at 60°C for 5 min.

The product of enzymatic cleavage of protocatechuate had the yellow color typical of extradiol cleavage. Spectroscopy data showed that the cleavage occurred by the C4–C5 bond with formation of 2-oxy-4-carboxy-*cis,cis*-muconic acid semialdehyde (Fig. 4).

Thus, we have shown that the strain *R. ruber* P25 degraded 4CB to 4CBA, performed hydrolytic dechlorination of 4-chlorobenzoate with PHBA formation, and possessed active enzyme systems providing PHBA degradation via PCA to the compounds of the basic metabolism.

Table 2. The scheme of purification of PHBH from the *R. ruber* P25 biomass grown on 4CBA

Purification stage	Volume, mL	Protein, mg	Total activity, U	Specific activity, U/mg protein	Purification, times	Yield, %
Cell-free extract	27.0	177.5	120.3	0.68	1	100
Q-Sepharose	68.0	23.8	56.8	2.4	3.5	47.2
Buthyl-Sepharose	44.0	18.3	127.4	7.0	10.3	106.0
Superdex-75	12.0	0.38	18.5	48.2	71.1	15.4
Resource Q	1.8	0.21	2.5	11.7	17.2	2.1

Table 3. The scheme of purification of PCA from the *R. ruber* P25 biomass grown on 4CBA

Purification stage	Volume, m	Protein, mg	Total activity, U	Specific activity, U/mg protein	Purification, times	Yield
Cell-free extract	27.0	177.5	–	–*	–	–
Q-Sepharose	31.0	–	–	–*	–	–
Resource Phe	15.0	0.7	0.6	0.8	1.0	100
Superdex-75	6.0	0.11	0.43	4.1	5.1	72.6
Resource Iso	1.4	0.05	0.13	6.4	8.0	53.3
Resource Q	0.25	0.005	0.28	15.0	18.7	47.2

Note: * PCD activity was monitored by formation of a yellow product at 410 nm.

DISCUSSION

At present, there are two ways of successful application of microorganisms for remediation of soils and water objects polluted with toxic agents: (1) the search of natural active strains degrading the toxicants for their further application in ecobiotechnologies and (2) creation of genetically modified bacteria using the biodegradative (genetic) potential of the same natural, well characterized degrader strains. Numerous bacteria have been isolated and studied at research laboratories which can degrade a broad range of toxic pollutants, including chloroaromatic hydrocarbons. Comprehensive investigation of bacteria by the modern methods of genomics, proteomics, and systematic biology makes it possible to ascertain the biodegradation potential of the organisms under study. Particular attention of researchers is focused on the strains degrading polychlorinated biphenyls, widespread and persistent toxic aromatic compounds. The emphasis is put on the search for bacteria that can degrade *ortho*- and *para*-chlorinated biphenyls, because these very compounds accumulate in the environment as a result of anaerobic destruction of PCB [3].

The results of this work show that the strain *R. ruber* P25 actively degraded *para*-chlorinated biphenyl. It is known that the strains utilizing monochlorobiphenyls as growth substrates in most cases oxidize the non-chlorinated ring of the biphenyl molecule [4]. The key enzymes of chlorobiphenyl catabolism, i.e., biphenyl 2,3-dioxygenases with a broad substrate specificity against CB that contain substitutes in different positions, have been studied best of all [3, 23]. The analysis of 4CB degradation products showed that strain P25 oxidized the unsubstituted ring of the chlorobiphenyl molecule with formation of 10-Cl HOPDA and 4-chlorobenzoic acid as intermediate products (Fig. 1, Table 1). However, the results [11, 24] on destruction of (di-tri)chlorobiphenyls containing chlorine in both rings of the molecule (4,4'-, 2,4'-, 2,4,4'-CB, and 2,4,2'-CB) showed that the strain *R. ruber* P25 also possessed the activities oxidizing both the *para*- and *ortho*-chlorinated biphenyl ring,

indicating that the strain possessed biphenyl dioxygenase(s) with broad substrate specificity. At the same time, the experiments on amplification of the *bph* genes with the total DNA of the strain *R. ruber* P25 using different pairs of primers constructed on the basis of the known sequences of biphenyl dioxygenase (*bph*) genes possessing different activities against chlorobiphenyls [25] showed no presence of these genes in the strain under study (the authors' data, not shown in the article). This fact indicates that the strain *R. ruber* P25 possibly contains unique functional genes/enzymes controlling the initial stages of destruction of chlorinated biphenyls, including 4-chlorobiphenyl. Previously the 4CB-degrading strain *Pseudomonas* sp. DJ-12 was shown to contain the *pcb* genes involved in chlorobiphenyl destruction, which substantially differed from the well-characterized *bph* genes [8].

4CBA is one of the intermediates during degradation of 4CB (as well as of diCB and higher chlorinated biphenyls) by the strain *R. ruber* P25 [11, 24]. In addition, all our studies confirmed that 4CBA was not a final product of chlorobiphenyl utilization, undergoing further degradation. The growth experiments with 4CBA as a sole carbon and energy source, as well as the experiments on destruction of this compound by washed cells, confirmed the presence of enzyme activities responsible for 4CBA utilization in the strain *R. ruber* P25.

The natural and genetically modified bacterial strains capable of utilizing *para*-chlorinated benzoates and different pathways of 4CBA catabolism have been described in the literature. The pathways of 4CBA degradation via 4-chlorocatechol (the modified *ortho*-pathway) are known for *Pseudomonas cepacia* P166 (performing 4CB and 4CBA degradation) [9, 26]. The present work showed that the strain *R. ruber* P25 degraded 4CBA by hydrolytic dechlorination with the formation of PHBA (Fig. 4). Such pathway of 4CBA destruction has been described for a number of gram-positive (e.g., *Arthrobacter globiformis* KZT1, *Arthrobacter* sp. SU, *Arthrobacter* sp. TM1) and gram-negative (*Pseudomonas* sp. CBS3, *Pseudomonas* sp. DJ-12) bacteria, and the enzyme systems and genes/operons controlling 4-chlorobenzoate dechlorination have been well characterized [8, 13, 27–29]. Our results obtained from the analysis of the nucleotide sequences of the *fcbA* and *fcbB* genes demonstrate the presence in strain P25 of the genes having a high level of homology with the *fcb* genes of bacteria of the genus *Arthrobacter* (Fig. 5). The presence of *fcb* genes in environmental bacteria of the genus *Rhodococcus* have never been previously described in the literature. It should be noted that, in addition to the strain *R. ruber* P25, we have isolated 4CBA-degrading strains of the genus *Arthrobacter* from the same soils polluted with the wastes of chemical plants producing halogenated compounds [12]. It is known that the genes controlling 4CBA degradation may be components of plasmids and transposons [28, 29]; in addition, the *fcb* genes of *Arthrobacter* have been successfully expressed in the cells of rhodococci [20]. Previously we have shown that the strain *R. ruber* P25 contains three plasmids of 110, 90, and 80 kb. The results of experiments on elimination of the plasmids suggest that the genes responsible for 4CBA degradation are located in a 110-kb plasmid (data not shown). Thus, there is a possibility of horizontal transfer of the *fcb* gene cluster among the bacteria of the soil ecosystem existing under a high selective pressure of halogen-containing pollutants.

The pathway of PHBA degradation via the stage of protocatechuic acid production to the compounds of the basic cellular metabolism has been described for aerobic bacteria (www.brenda-enzymes.org) [30]. PHBH catalyzes incorporation of an oxygen atom into PHBA in the presence of a NADH (or NADPH) cosubstrate with formation of protocatechuic acid (Fig. 4). The enzymes catalyzing this type of reaction have been isolated and characterized in many degraders of aromatic compounds: gram-negative bacteria of the genera *Pseudomonas*, *Acinetobacter, Klebsiella* (www.brenda-enzymes.org) and gram-positive bacteria, including *Rhodococcus* spp. [16]. These enzymes may be homo- and heterodimers and tetramers. PHBH of the strain *R. ruber* P25 is a heterodimer. In agreement with the previously published data on the significant similarity of PHBH from different bacteria [16, 31], the subunits differ insignificantly in molecular mass, which is comparable with those of the known PHBH. The enzyme isolated from *R. ruber* P25 is little different from other PHBH of gram-positive bacteria in the K_m values for PHBA and in the preference for NADH, but not NADPH, as a co-substrate [16]. The PHBH isolated from *Corynebacterium glutamicum* [31], the preferable substrate for which is NADPH, is an exception.

Protocatechuic acid may be degraded via one of the three metabolic pathways described for aerobic bacteria, with manifestation of different activities performing intradiol or extradiol cleavage of the aromatic ring. Protocatechuate 3,4-dioxygenase (PC 3,4-DO) belongs to the large group of intradiol oxygenases that cleave the aromatic ring with formation of 3-carboxy-*cis,cis*-muconate [30]. Extradiol cleavage of PCA under the influence of protocatechuate 2,3-dioxygenase with formation of 2-hydroxy-5-carboxy-muconic aldehyde has been described for bacteria of the genus *Bacillus* [32]. PCA catabolism may proceed by the *meta*-pathway with formation of 2-hydroxy-4-carboxy-muconic aldehyde; the reaction is performed by protochatechuate 4,5-dioxygenase. Such type of metabolism has been described for a number of gram-negative bacteria, including *Comamonas testosteroni* T-2 [33] and *Pseudomonas* sp. [17]. Numerous 4,5-DOs characterized up to now have been isolated from the strains degrading 2,3- and 3,4-dichlorobenzoates, 3CBA, benzoate, gallate, methyl gallate, and syringate (www.brenda-enzymes.org). We have

shown that during the growth of the strain *R. ruber* P25 on 4CBA, the PCA aromatic ring is cleaved via the *meta*-pathway, between the atoms C4 and C5. Concerning the catalytic characteristics, the isolated enzyme differs from the known PCA 4,5-DOs both in the substrate affinity and in the catalytic activity. The distinctive feature of the isolated enzyme is its relatively high thermostability compared to the enzymes described in literature [17, 33].

It is interesting that an extradiol PCDO and two intradiol PCDOs were induced in the strain *R. ruber* P25 during its growth on 4CBA and PHBA, respectively (data not shown). The literature shows the examples of induction of isoenzyme dioxygenases in the same strain. The presence of several isoforms of the same enzyme in a cell is supposed to enhance the survival of bacteria under changing environmental conditions [34]: some or other isoform can work with greater efficiency under the influence of various physical and chemical factors on different substrates.

Based on the above, it is proposed to further investigate the characteristics of the functioning of biodegradative systems in *Rhodococcus ruber* P25.

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