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Methylcatechol 1,2-Dioxygenase of *Rhodococcus opacus* 6a Is a New Type of the Catechol-Cleaving Enzyme

I. P. Solyanikova[1], E. I. Konovalova[1,2], and L. A. Golovleva[1,2]*

[1]*Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, 142290 Pushchino, Moscow Region, Russia; E-mail: golovleva@ibpm.pushchino.ru*
[2]*Pushchino State University, pr. Nauki 3, 142290 Pushchino, Moscow Region, Russia*

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Abstract—The strains *Rhodococcus* sp. 400, *R. rhodochrous* 172, and *R. opacus* 6a utilize 4-methylbenzoate as the only carbon and energy source. 4-Methylcatechol is a key intermediate of biodegradation. Its further conversion by all the strains proceeds via *ortho*-cleavage. The specific activity of catechol 1,2-dioxygenase assayed in crude extracts of *Rhodococcus* sp. 400 and *R. rhodochrous* 172 with 3- and 4-methylcatechols does not exceed the enzyme activity assayed with catechol. Two catechol 1,2-dioxygenases have been purified from the biomass of *R. opacus* strain 6a grown with 4-methylbenzoate. These enzymes differed in molecular mass and physicochemical and catalytic properties. One of these enzymes belongs to the type of enzymes cleaving the catechol ring and known as methylcatechol 1,2-dioxygenases. In bacteria of the *Rhodococcus* genus, such an enzyme is described here for the first time.

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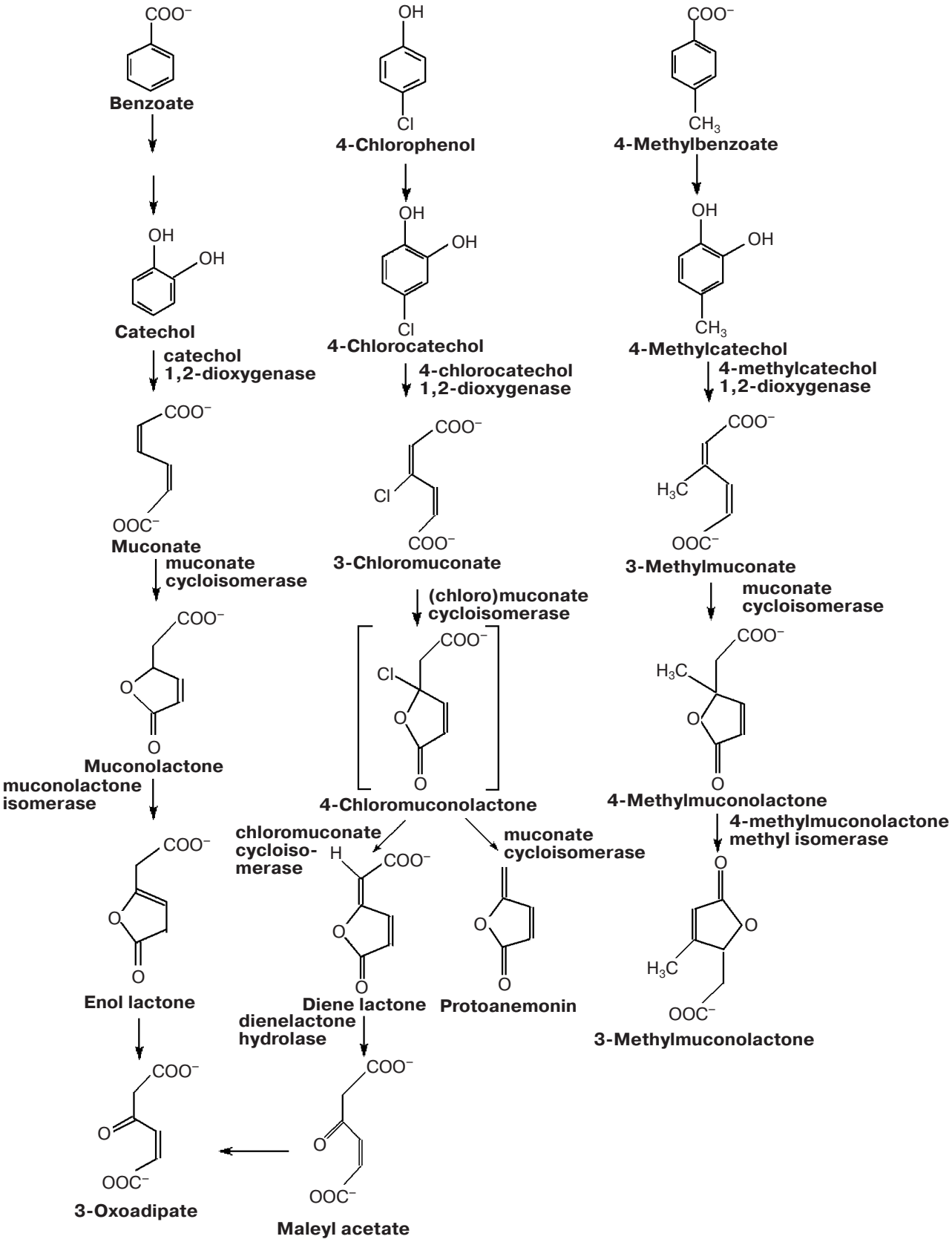
Key words: *para*-toluate, degradation, methylcatechol 1,2-dioxygenase, *Rhodococcus opacus* 6a

All diversity of initial stages of degradation of aromatic compounds by microorganisms results in formation of a limited set of intermediates; their subsequent conversion into Krebs cycle metabolites involves several pathways that are widespread in microorganisms. Catechol and its substituted analogs are widespread intermediates formed during microbial transformation. It is formed during primary microbial attack of such aromatic substances as phenol and benzoate [1, 2]. Subsequent *ortho*-cleavage of catechol in the so-called classical *ortho*-cleavage pathway results in 3-ketoadipate formation (Scheme). The enzyme of catechol cleavage, catechol 1,2-dioxygenase (C-1,2-DO), can oxidize chlorocatechol; usually the rate of its oxidation does not exceed 10% of the rate of catechol cleavage (Table 1), and the specificity constant (k_{cat}/K_m) is much higher for the unsubstituted substrate [1-7, 9]. C-1,2-DOs operating the ordinary *ortho*-cleavage pathway oxidize 3- and 4-methylcatechols at a rate that does not exceed the rate of catechol oxidation.

Degradation of chlorinated benzoates, benzenes, and phenols results in formation of chlorocatechol; its ring at the *ortho*-position is then cleaved by chlorocatechol 1,2-dioxygenase (CC-1,2-DO), and subsequent conversion involves the so-called modified *ortho*-cleavage pathway, because the enzymes and the reactions catalyzed by them differ from those in the classical *ortho*-cleavage pathway. Relative activity of CC-1,2-DO from various strains degrading chloroaromatic compounds with formation of chlorocatechols varies over a wide range from 14 to 130% versus the activity assayed with unsubstituted catechol (Table 1) [2-4, 13-18]. CC-1,2-DOs of the modified *ortho*-cleavage pathway oxidize 3-methylcatechol (3-MC) and 4-methylcatechol (4-MC), and the rates of their oxidation are 200-300% of the corresponding rate of catechol oxidation. However, the values of specificity constants of these enzymes are higher for chlorocatechols that are intermediates of initial substrate degradation.

Degradation of such compounds as *para*-cresol, *para*-toluate (methylbenzoate), and methyl salicylates results in formation of 4-MC. The *ortho*-cleavage pathway of methylcatechols found in several bacteria as well as enzymes catalyzing its particular reactions remains poorly studied [8, 10, 11]. The major emphasis is usually made on characterization of 4-methylmuconolactone methyl

Abbreviations: (C)C, (chloro)catechol; (C)C-1,2-DO, (chloro)catechol 1,2-dioxygenase; 4-MBZ, 4-methylbenzoate (*para*-toluate); MC-1,2-DO, methylcatechol 1,2-dioxygenase.
* To whom correspondence should be addressed.



Scheme of various pathways for degradation of substituted aromatic compounds by bacteria of the *Rhodococcus* genus. Left, classical *ortho*-cleavage pathway; center, modified *ortho*-cleavage pathway of chlorocatechol degradation; right, modified *ortho*-cleavage pathway of methylcatechol degradation

Table 1. Rates of oxidation of catechol, 3-methylcatechol (3-MC), 4-methylcatechol (4-MC), and 4-chlorocatechol (4-CC) by catechol dioxygenases (C-1,2-DO) and chlorocatechol 1,2-dioxygenases (CC-1,2-DO) from various bacteria

Bacterium	Growth substrate	Enzyme	Relative activity, %*			References
			+ 4-CC	+ 3-MC	+ 4-MC	
Pseudomonas arvilla C1	benzoate	C-1,2-DO	3-4	5-8	71-72	[1]
Pseudomonas sp. B13	–"–	–"–	11	11	92	[2]
Alcaligenes eutrophus CH34	–"–	–"–	5		20	[3]
Pseudomonas sp. PS12	benzene	–"–	40	68	82	[4]
Ralstonia eutropha JMP 134	2,4-D	–"–	7	7	41	[2]
Acinetobacter lwoffii	aniline	C-1,2-DO I	5.5	3	39	[5]
		C-1,2-DO II	7.8	22	51	
A. radioresistens	phenol	C-1,2-DO	n.d.	14.4	16.5	[6]
R. rhodochrous B259	benzoate	–"–	n.d.	79	68	[7]
R. rhodochrous N75	*p*-toluate	–"–	n.d.	64	76	[8]
R. opacus 1cp	benzoate	–"–	3.1	99	88	[9]
	p-toluate	–"–	n.d.	73	89	[10]
		CC-1,2-DO	113	191	253	
R. ruber P25	–"–	C-1,2-DO	n.d.	195	117	[11]
R. opacus 6a	–"–	–"–	n.d.	138	129	
		MC-1,2-DO	112.6	150	282	
Pseudomonas sp. MT1	methyl salicylate	–"–	22.7	31	291	[12]
Pseudomonas sp. B13	3-CBZ	CC-1,2-DO	96	337	316	[2]
Pseudomonas sp. pAC 27	–"–	–"–	80	n.d.	304	[3]
R. eutropha JMP 134	2,4-D	–"–	122	167	n.d.	[2]
Pseudomonas sp. PS12	1,2,4-trichlorobenzene	–"–	192	319	230	[4]
P. putida 87	3-CBZ	–"–	50.4	235	187	[13]
Pseudomonas sp. P51	chlorobenzene	–"–	n.d.	202	189	[14]
Xantobacter phlavus	dichlorobenzene	–"–	64	239	168	[15]
P. acidovorans	2-chloroaniline	–"–	128	307	205	[16]
R. opacus 1cp	2-chlorophenol	–"–	50	283	270	[17]
	4-chlorophenol	–"–	96	208	242	[18]

Note: n.d., not determined; 3-CBZ, 3-chlorobenzoate; 2,4-D, 2,4-dichlorophenoxyacetate.
* Enzyme activity with catechol was defined as 100%.

isomerase catalyzing conversion of 4-methylmuconolactone into 3-methylmuconolactone because it is considered as the key reaction in the *ortho*-cleavage of methylcatechols [19, 20]. Little attention has been paid to the enzymes catalyzing cleavage of the 4-MC ring. Study of these enzymes has shown that they do not represent a particular group with clearly defined properties. For example, in the *R. rhodochrous* N75 strain with well described modified *ortho*-cleavage pathway of methylcatechol, the C-1,2-DO differs from known C-1,2-DOs and CC-1,2-DOs in some characteristics, but catechol is its best substrate [8]. The rate of methylcatechol oxidation by another enzyme, C-1,2-DO from *R. ruber* P25, was higher than the rate of catechol oxidation; this enzyme exhibited low activity with chlorinated substrates [11]. This was basically the first evidence for the existence of enzymes adapted to cleavage of the aromatic ring of methylcatechols. However, the values of specificity constant for 4-MC of C-1,2-DOs from *R. ruber* P25 and from the *R. rhodochrous* N75 strain [8] grown on 4-methylbenzoate (4-MBZ) was lower than for catechol, and these enzyme can be arbitrarily referred to the type of methylcatechol 1,2-dioxygenases (MC-1,2-DO). C-1,2-DOsalD isolated from the *Pseudomonas* sp. MT1 grown

on substituted salicylates can be considered as the only representative of MC-1,2-DO. This enzyme is characterized by the highest specificity constant for 4-MC [12] (Table 1).

The goal of this study was to investigate catalytic properties of bacterial dioxygenases from the genus *Rhodococcus* that are involved in degradation of *para*-toluate.

MATERIALS AND METHODS

Microorganisms and methods of their cultivation. The strains *R. opacus* 6a, *R. rhodochrous* 172, and *Rhodococcus* sp. 400, capable of utilizing a wide range of substrates, were isolated earlier and maintained for a long time in a nutrient-rich agarose medium.

Strain cultivation and calculation of growth parameters were carried out as described earlier [10]. Strains were adapted to 4-MBZ in Erlenmeyer flasks with 0.25 g/liter substrate as the only source of carbon and energy for one month. For biomass preparation on *p*-toluate, the strains were cultivated in a 10-liter bioreactor to final optical density of 1.8-2.1 at 545 nm. Cells were sedimented by centrifugation (17,700*g*, 10 min), washed twice in 50 mM Tris-HCl buffer, and stored at −20°C.

Preparation of cell-free extract and assay of enzyme activities were described earlier [10]. One unit of activity was defined as the amount of enzyme catalyzing conversion of 1 μmol of substrate or formation of 1 μmol of product per minute.

Purification of enzymes from biomass of *R. opacus* 6a grown on *p*-toluate. The enzymes were purified using an FPLC system (Pharmacia, Sweden). Columns, sorbents, volumes of eluting solutions, and salt concentrations were described earlier [10]. The purified proteins were desalted and used in subsequent studies. Enzyme activity was monitored over purification stages using catechol as the substrate.

Determination of physicochemical properties of enzymes. Purity of enzyme preparations and molecular mass of subunits were determined by SDS-PAGE using the modified method of Laemmli [21]. The electrophoresis was performed using a Mini VE Complete system (Hoefer Pharmacia Biotech, USA), slabs (10 × 10.5 × 0.1 cm), and 4% stacking and 12% separating gels. Gels were stained with Coomassie G-250 [22]. Molecular mass of native enzymes was determined by gel filtration on a Superdex 200 column (16 × 70 cm, volume of 120 ml), calibrated using standards and (C)C-1,2-DO with known masses [10, 17]. Molecular masses of protein subunits were determined using the SDS-LMW standard kits (Sigma, USA). N-Terminal amino acid sequence of proteins was determined after SDS-PAGE and electrotransfer onto an Immobilon P membrane (Millipore, USA), which was carried out at 400 mA for 3.5 h.

Determination of kinetic characteristics. Apparent Michaelis constants (K_m) and V_{max} were determined using the method of double reciprocal plots and the ranges of substrate concentrations of 0.5-100 μM (catechol), 0.5-400 μM (3-MC and 4-MC), or 1-100 μM (CC). The effect of pH on the rate of enzymatic reaction was determined using catechol as substrate and the following buffer systems: Mes (pH 5.5-6.5), Mops (pH 6.5-7.5), and Tris-HCl (pH 7.2-8.7). The temperature dependence of the rate of the catechol-oxidation reaction was investigated in the range of temperatures 5-60°C using a Shimadzu temperature controller (Japan). Enzyme thermostability was investigated by incubating enzymes at various temperatures in thermostats.

Protein concentration was determined by the modified Bradford method [23] using bovine serum albumin as standard.

Reagents. The reagents used for preparation of mineral media were of analytical grades (chemical reagent factories, Russia). Biochemical reagents were purchased from Sigma and Serva (Germany); 3-MC was from Koch-Light (England), 4-MC from Fluka (Switzerland). The sorbents for column chromatography, the Resource Q and Resource Iso columns, were from Pharmacia, and reagents for polyacrylamide gel electrophoresis were from Bio-Rad (USA).

RESULTS AND DISCUSSION

Features of bacterial growth of genus *Rhodococcus* on *p*-toluate. Although cultivation of the bacteria on 4-MBZ in a liquid mineral medium did not require long term adaptation, most of the initially tested strains did not give significant increase in optical density. Subsequently, only three strains were characterized by high rate of growth on 4-MBZ and absorbance of cell culture suspensions reached one unit (Fig. 1). Cultivation of these strains (*Rhodococcus* sp. 400, *R. rhodochrous* 172, *R. opacus* 6a) in the bioreactor was accompanied by active substrate utilization and biomass yield up to 140 g of wet weight per 250-300 g of 4-MBZ for 72-96 h.

Using a semilogarithmic scale of the dependence of absorbance of a cultural liquid on cultivation time, the doubling rate of the *R. opacus* 6a strain was 0.14 h^{-1}. The doubling time and the economic coefficient of growth were 6.2 h and 0.01, respectively. These parameters were close to those obtained for the *R. opacus* strain 1 cp grown on *p*-toluate; this indicates similar growth characteristics of these strains [10].

Enzymes of *p*-toluate degradation in bacteria of *Rhodococcus* genus. Determination of enzyme activities in cell-free extracts of the three strains demonstrated the presence of C-1,2-DO and the absence of catechol 2,3-dioxygenase. Thus, forming 4-MC underwent *ortho*-cleavage (Table 2) also typical for the other representative

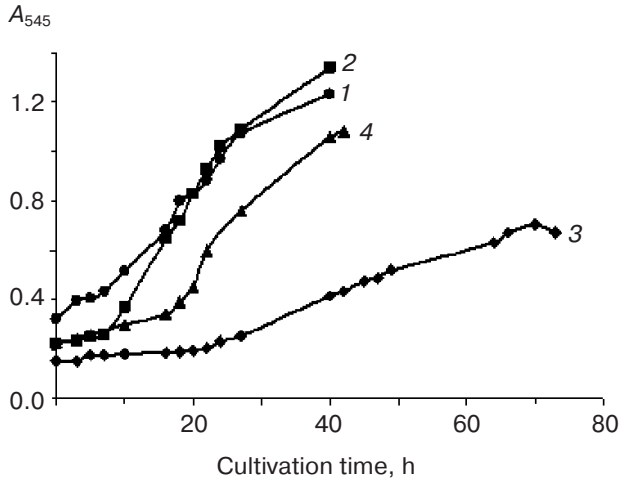


Fig. 1. Curves of growth on *p*-toluate for *Rhodococcus* sp. 400 (*1*), *R. rhodochrous* 172 (*2*), and *R. opacus* 6a, unadapted culture (*3*) and adapted culture (*4*).

of the *Rhodococcus* genus [10, 11]. There was significant difference in specific activities of C-1,2-DO of cell-free extracts of these strains. The rates of substrate conversion by extracts from the strain *R. opacus* 6a were one to two orders of magnitude higher than those determined in other strains.

In two strains, 400 and 172, relative activity of C-1,2-DO with 3-MC and 4-MC was lower than with unsubstituted substrate. C-1,2-DO from the strain *R. opacus* 6a differed from them in this respect. This included high values of relative rates of cleavage of 3-MC and 4-MC (139 and 235% versus catechol, respectively). The rate of 4-CC cleavage was also high (65.5%). Similar data were obtained for the strain *R. opacus* 1cp; during growth of this microorganism on *p*-toluate, C-1,2-DO activity in the cell-free extract was higher with 3-MC, 4-MC, and 4-CC than with the unsubstituted substrate [10].

Based on these results we chose the strain *R. opacus* 6a for subsequent studies. Determination of C-1,2-DO activity revealed the presence of only one peak of catalytic activity after ion-exchange chromatography; however, during hydrophobic chromatography the catechol oxidation activity separated into two peaks. The first peak exhibited low relative C-1,2-DO activity with 4-CC; however, its activity with 3-MC and 4-MC was higher than that assayed with catechol (Table 3). This enzyme was defined as C-1,2-DO. Relative activity of the enzyme from the second peak assayed with 3-MC and 4-MC was 1.5-2-fold higher than with catechol, and the enzyme activity with 4-CC was comparable with the activity assayed with catechol as substrate. Based on these catalytic properties, we defined this dioxygenase as MC-1,2-DO. Growth of the strain *R. opacus* 1cp on 4-MBZ was also characterized by separation of the catechol oxidation activity into two peaks, and the enzyme of one peak exhibited low activity with 4-MC and 4-CC, whereas the enzyme from the second peak actively involved into 4-MC cleavage shared identity in N-terminal sequence and close similarity in catalytic characteristics with CC-1,2-DO of the 4-CC branch of the same strain [10].

Purification of enzymes from strain *R. opacus* 6a. Methylcatechol 1,2-dioxygenase, MC-1,2-DO, was separated from the fraction with total catechol-oxidation activity in the stage of first hydrophobic chromatography, and then it was purified in five stages. Ion-exchange chromatography on Q-Sepharose (volume 150 ml, total protein 14.1 mg, specific activity 11.1 U/mg) was followed by hydrophobic chromatography on Phenyl-Sepharose with elution by $(NH_4)_2SO_4$ (the elution volume of 188 ml, total protein 7.5 mg, specific activity 11.4 U/mg), gel-filtration on Superdex 200 in the presence of 0.1 M NaCl (volume 33 ml, total protein 2.25 mg, specific activity 17.1 U/mg), ion-exchange chromatography on Resource Q (volume 3.3 ml, total protein 1.24 mg, specific activity 26.3 U/mg), hydrophobic chromatography on Resource Iso (volume 4 ml, total protein 2.94 mg, specific activity 9.12 U/mg). The resultant homogeneous enzyme preparation had specific activity of 9.12 U/mg of protein with yield of 10.2%.

Table 2. Specific activity of C-1,2-DO in cell-free extract of the strains cultivated on 4-MBZ

Substrate	Relative (specific, U/mg) activity of C-1,2-DO, %		
	R. opacus 6a	*Rhodococcus* sp. 400	*R. rhodochrous* 172
Catechol	100 (6.02)	100 (0.094)	100 (0.155)
3-MC	139 (8.39)	70 (0.066)	42.5 (0.066)
4-MC	235 (14.5)	100 (0.094)	55 (0.086)

Table 3. Activity of C-1,2-DOs from the *R. opacus* 6a strain that were separated at the stage of hydrophobic chromatography

Substrate	Specific activity, U/mg (relative activity, %)	
	C-1,2-DO	MC-1,2-DO
Catechol	1.15 (100)	11.4 (100)
3-MC	1.59 (139)	18.7 (164)
4-MC	1.29 (113)	28.7 (253)
4-CC	0	12.1 (106)

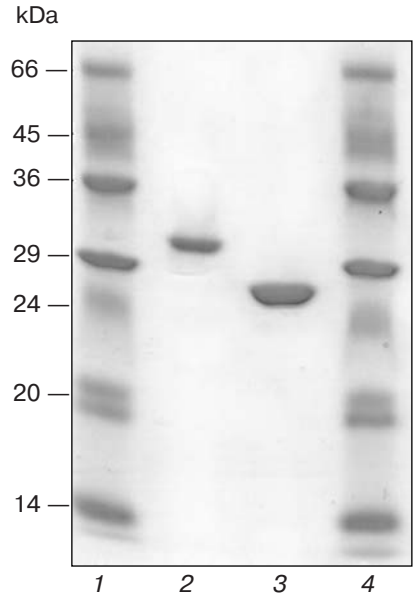


Fig. 2. SDS-PAGE of C-1,2-DO (*2*) and MC-1,2-DO (*3*) from *R. opacus* 6a. Lanes *1* and *4* contain molecular mass markers.

C-1,2-DO was also purified in five stages. The enzyme was eluted from a Q-Sepharose column at 0.2 M NaCl, from a Phenyl-Sepharose column at 0.3 M $(NH_4)_2SO_4$ (volume 141 ml, total protein 2.18 mg, specific activity 1.15 U/mg), from a Superdex 200 column (volume 20 ml, total protein of 0.7 mg, specific activity 15.8 U/mg), from a Resource Q column at 0.22 M NaCl (volume 7 ml, total protein 0.42 mg, specific activity 23.2 U/mg), and from a Resource Iso column at 0.27 M $(NH_4)_2SO_4$ (volume 4.3 ml, total protein of 0.64 mg, specific activity of 6.41 U/mg). Thus, although hydrophobic chromatography resulted in separation of the protein peaks, this purification stage was accompanied by significant enzyme inactivation. The resultant preparation of C-1,2-DO had specific activity of 6.41 U/mg of protein and the yield was 1.55%.

Physicochemical properties of dioxygenases. Molecular masses of subunits of MC-1,2-DO and C-1,2-DO were 27 and 33 kDa, respectively (Fig. 2), whereas molecular masses of these proteins were 63-65 and 68-70 kDa, respectively. This means that both enzymes are homodimers, and this is typical for most C-1,2-DO [2-8].

The sequence of 10 N-terminal amino acids of MC-1,2-DO was determined to evaluate similarity degree of this enzyme with C-1,2-DO and CC-1,2-DO from other bacteria, first of all with enzymes from the strain *R. opacus* 1cp (because there were minimal differences with 4-CC-1,2-DO both in molecular mass and subunit composition of the enzymes). The sequence of 10 N-terminal amino acids of MC-1,2-DO (ANT(X)VIYKIG) shared 50% identity with 4-CC-1,2-DO from *R. opacus* 1cp; however, in comparison with CC-1,2-DO from Gram-negative bacteria the identity parameter was significantly lower [24].

The temperature dependence of MC-1,2-DO activity was characterized by irregular bell shape with maximum activity observed at 45-50°C. Subsequent increase of temperature to 60°C caused enzyme inactivation (Fig. 3).

The temperature dependence of C-1,2-DO activity was less pronounced than that of MC-1,2-DO with rather small (less than 3-fold) increase of its activity during the increase of temperature from 10 to 40°C. The activity was maximal at 35-55°C. Increase of temperature to 65°C resulted in a sharp enzyme inactivation.

Determination of enzyme thermostability has shown that MC-1,2-DO is resistant to thermal treatment (Fig. 4). For example, at 50°C total inactivation of this enzyme was observed only after 90 min. C-1,2-DO was more

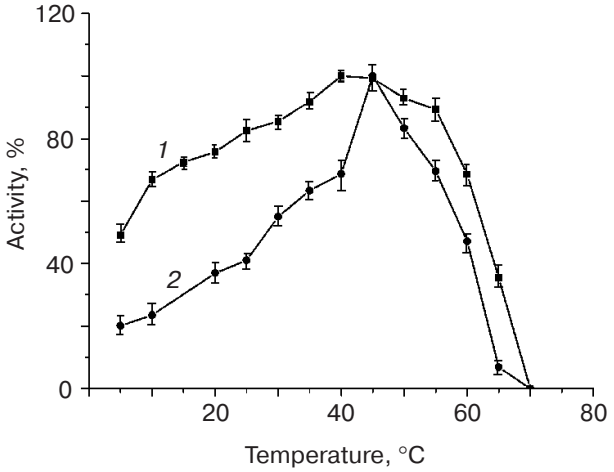


Fig. 3. Temperature optimum of enzyme activity: *1*) C-1,2-DO; *2*) MC-1,2-DO.

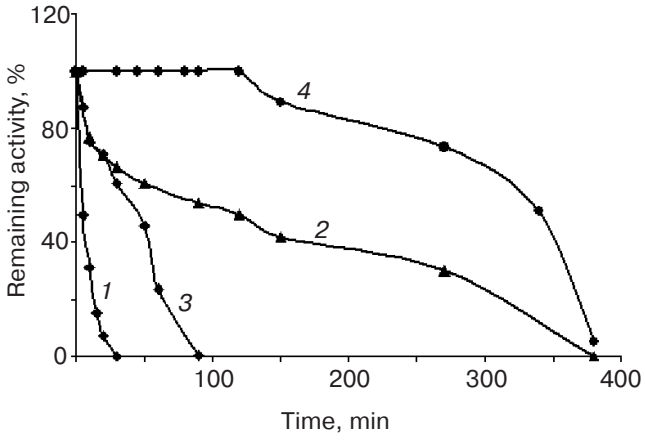


Fig. 4. Thermostability of C-1,2-DO at 50 (*1*) and 40°C (*2*) and MC-1,2-DO at 50 (*3*) and 40°C (*4*).

thermolabile: at 50°C its inactivation was observed within 30 min (Fig. 4).

The two enzymes differed in their stability during storage at various temperatures. At 25°C, MC-1,2-DO preserved its activity during 35 days, and at −10°C there was uniform decrease in its activity by 10% during 30 days and by 20% during 60 days. At 4°C, the enzyme retained 100% of its activity within 50 days and 83% of its activity after 90 days. At 25°C, C-1,2-DO demonstrated total loss of its activity within 28 days, and at −10°C the enzyme activity decreased by 25% during 30 days and by 50% during 60 days. As in the case of MC-1,2-DO, optimal temperature for enzyme storage was 4°C: the enzyme retained 100% of its activity within 60 days and 60% of its activity during 105 days. The pH optimum for MC-1,2-DO and C-1,2-DO was 7.4-7.6 and 7.3-7.5, respectively.

Catalytic properties of dioxygenases. MC-1,2-DO catalyzed oxidation of a wide range of substituted catechols. The enzyme exhibited maximal activity with methyl-substituted catechols. Some chlorinated catechols were also substrates for this enzyme. These included monochlorocatechols and 3,5-dichlorocatechol (Table 4). These results are similar to those obtained for CC-1,2-DO involved in degradation of various chloro-substituted aromatic compounds [2, 4, 16-18]. Nevertheless, calculation of the specificity constant values of MC-1,2-DO for the tested substrates has shown that the higher value of this parameter is for 4-MC. Now, only one MC-1,2-DO characterized by specificity constant value for 4-MC higher than for other substrates is known in the literature [12]. The type of one C-1,2-DO isolated from the biomass of the strain *R. opacus* 1 cp grown on *p*-cresol (its degradation involves 4-MC formation followed *ortho*-cleavage) remains unclear [25]. It was shown that this dioxygenase is also characterized by a high value of specificity constant with methylcatechols. However, taking into consideration that growth of the strain *R. opacus* 1 cp on *p*-toluate causes induction of dioxygenase, which is characterized by identity of the N-terminal sequence and similar catalytic properties with the enzyme of the 4-chlorocatechol branch (4-CC-1,2-DO), possible difference of the described enzyme (induced in bacteria during growth on *p*-cresol) from the strain 1 cp 4-CC-1,2-DO requires further investigation, especially due to insignificant differences in catalytic activities of these enzymes. Determination of an N-terminal amino acid sequence might solve this problem.

Table 4. Kinetic characteristics of MC-1,2-DO

Substrate	K_m, μM	V_{max}, U/mg	k_{cat}/K_m, %
Catechol	2.8	55.5	59.6
3-Methylcatechol	33.3	83.3	7.5
4-Methylcatechol	4.7	156.5	100
3-Chlorocatechol	4.2	15.2	10.8
4-Chlorocatechol	4.9	62.5	38.3
3,5-Dichlorocatechol	3.5	10.0	8.4
3-Methoxycatechol	133.3	22.2	0.6
3-Fluorocatechol	5.9	2.1	1.2

Table 5. Kinetic characteristics of C-1,2-DO

Substrate	K_m, μM	V_{max}, U/mg	k_{cat}/K_m, %
Catechol	2.4	50	100
3-MC	11.2	68.9	29.8
4-MC	8.9	64.5	34.6

Among substrate analogs 2-chlorophenol, 4-chlorophenol, and 4,5-dichlorocatechol were competitive inhibitors of MC-1,2-DO from *R. opacus* 6a with inhibition constant values of 115, 170, and 0.25 μM, respectively. 2-Chloro- and 3-chlorobenzoic acids did not inhibit the enzyme.

In contract to MC-1,2-DO, C-1,2-DO catalyzed transformation of the limited number of compounds. Among the tested analogs only catechol, 3-MC, and 4-MC were cleaved by this dioxygenase at a significant rate (Table 5). For these substrates, we have calculated Michaelis constant (K_m) and specificity constant (k_{cat}/K_m) (Table 5).

The minimal K_m value was with catechol as substrate, whereas the highest reaction rate was detected with 3-MC as substrate. There were similar values of specificity constant for the studied substrates. Thus, C-1,2-DO involved in degradation of 4-MBZ in the strains *R. opacus* 6a and *R. ruber* P25, and C-1,2-DO isolated from the strain *R. opacus* 1cp grown on *p*-cresol differ from C-1,2-DO of the classic *ortho*-cleavage pathway by their higher activity with 4-MC as substrate, although specificity constant of these enzymes is higher for catechol [10, 11, 25].

Activity of C-1,2-DO from *R. opacus* 6a was low with chlorinated substrates. Several structural analogs were tested for their ability to bind at the active site of this enzyme. 2-Chlorobenzoate and 3-chlorobenzoate at concentration 1 mM did not inhibit C-1,2-DO activity; this suggests lack of binding of these compounds at the active site of this enzyme. 2-Chlorophenol (35), 3-chlorophenol (25), 4-chlorophenol (139.6), 3-fluorocatechol (1.8), and *p*-hydroxybenzoic acid (35) acted as competitive inhibitors of this C-1,2-DO (the K_i values in μM are shown in parentheses).

Thus, the study of enzymes involved in degradation of *p*-toluate in *Rhodococcus* revealed diversity in culture responses to a new substrate. The study of bacterial strains of the *Rhodococcus* genus that utilize 4-MBZ as the only

source of carbon and energy has shown that they are characterized by *ortho*-cleavage of 4-MC formed from the initial compound. Nevertheless, a detailed study of dioxygenases catalyzing cleavage of the 4-MC ring indicates significant differences in their properties. There are three main variants on the *Rhodococcus* responses to 4-MBZ. The first is a nonspecific response, which is characterized by induction of dioxygenases with wide substrate specificity; employing these enzymes (C-1,2-DO of the classical *ortho*-cleavage pathway and CC-1,2-DO) the strains cleave the aromatic ring. The second response includes induction of C-1,2-DO, in which activity with MC is comparable or higher than the activity with unsubstituted catechol; however, due to high K_m values these enzymes are characterized by low specificity to substituted substrates. The third response consists in induction of highly specific enzymes characterized by the highest values of specificity constant for 4-MC, and consequently these enzymes provide highly effective response of bacterial cultures to the growth substrate. The presence of such enzyme, methylcatechol 1,2-dioxygenase in representatives of the genus *Rhodococcus* has been demonstrated for the first time.

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