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SHORT COMMUNICATIONS

# Degradation of *para*-Toluate by the Bacterium *Rhodococcus ruber* P25

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The pathways of bacterial degradation of methyl aromatic compounds are presently less studied than the pathways for their unsubstituted or halogenated analogues. Previously, it has been considered that in the presence of an intact methyl group the only pathway of their degradation includes formation of methylcatechols followed by cleavage of the ring in the *meta* position, because *ortho*-degradation of methylcatechols leads to formation of methylmuconolactones, and the presence of an alkyl substitute at the C-4 atom prevents further decomposition of 4-methylmuconolactone via the classical 3-oxoadipate pathway [1]. However, several strains (*Ralstonia eutropha* JMP134, *Rhodococcus rhodochrous* N75, and *Rhodococcus* sp.) were later shown to possess a modified *ortho*-pathway of methylcatechol degradation [2–4]. The main emphasis in these works was placed on the study of the properties of 4-methylmuconolactone methyl isomerase, which catalyzes the reaction of transformation of 4-methylmuconolactone into 3-methylmuconolactone and thus enables further transformation of this compound to the intermediates in central metabolism. At the same time, insignificant attention was paid to characterization of the enzymes that cleaved the aromatic ring of the formed 4-methylcatechol (4-MCat).

The strain *R. ruber* P25 was isolated by the enrichment culture method from the soil contaminated with industrial wastes of halogenated compounds (Perm'). The strain can utilize biphenyl, phenol, naphthalene, salicylate, gentisate, *ortho*-phthalate, benzoate, and their chlorinated derivatives (mono- and dichlorinated biphenyls, 2- and 4-chlorobenzoic acids) as the only carbon and energy source [5]. Later on, the variant growing on chlorophenols and 2,4-dichlorophenoxyacetate was obtained. However, nothing was known about the ability of this strain to degrade methyl-substituted aromatic compounds. As a model compound, by analogy with the substrates chlorinated in the *para* position, we used *para*-toluate (4-methylbenzoate, 4-MBA).

The aim of this work was to study the process of degradation of a methyl-substituted aromatic compound, 4-MBA, by *R. ruber* P25.

Cultivation, determination of growth parameters, and biomass production were carried out as described [6]. The apparent Michaelis constants ($K_M$) and $V_{max}$ values were determined by the double reciprocal method in the coordinates $1/V_0$ from $1/S$, where $S$ was the substrate concentration of 2–50 μM (catechol, Cat) and 1–400 μM (3-methylcatechol, 3-MCat and 4-MCat). Inhibition constants ($K_i$) were determined graphically by the Dixon's method, by plotting the dependence of $1/V$ on the inhibitor concentration at three fixed substrate concentrations [7].

It was established that the strain *R. ruber* P25 could grow on *para*-toluate after a short period of adaptation. The culture reached the maximal optical density ($D_{545}$) of 0.668 after 145 h (i.e., by day 7). The specific growth rate (μ) was 0.015, and the cell doubling time ($t_d$) was 46.2 h. The lag phase was 72.5 h. Adaptation of the strain to the given substrate for several months resulted in a considerable decrease of the duration of the lag phase (to 5 h), and the culture reached the maximal $D_{545}$ of 0.8–0.9 after 70 h of incubation.

The 4-MBA degradation pathway included the formation of 4-MCat followed by the aromatic ring opening in the *ortho* position. The cell-free extract of *R. ruber* P25 obtained from the biomass grown on 4-MBA exhibited no catechol 2,3-dioxygenase activity but the activities of catechol 1,2-dioxygenase (Cat 1,2-DO) and muconate cycloisomerase (MCI) were detected. Specific activities were 1.8 U/mg of protein (Cat 1,2-DO with Cat) and 0.062 U/mg of protein (MCI with muconate). The relative activity of Cat 1,2-DO in the cell-free extract with Cat, 3-MCat, 4-MCat, 3-chlorocatechol (3-CCat), and 4-chlorocatechol (4-CCat) was 100, 186, 102, 2.1, and 2.4%, respectively. These data demonstrate an unusual substrate specificity of

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The pathways of degradation of aromatic compounds and the rates of oxidation of Cat, 3-MCat, and 4-MCat by the key Cat 1,2-DO enzymes of bacteria from the genus *Rhodococcus*

| Bacterium | Growth substrate | Pathway* | Key enzyme | Relative activity (%) of Cat 1,2-DO with | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | Substrate | | | |
| | | | | Cat | 3-MCat | 4-MCat | |
| *R. rhodochrous* NCIMB13259 | Benzyl alcohol | A | Cat 1,2-DO | 100 | 79 | 68 | [9] |
| *R. rhodochrous* N75 | *p*-Toluate | A | Cat 1,2-DO | 100 | 64 | 83 | [8] |
| *R. ruber* P25 | *p*-Toluate | A | MCat 1,2-DO | 100 | 195 | 117 | This publication |
| *R. opacus* lcp | Benzoate | B | Cat 1,2-DO | 100 | 99 | 88 | |
| | *p*-Toluate | C | Cat 1,2-DO | 100 | 73 | 89 | [6] |
| | | | CCat 1,2-DO | 100 | 208 | 242 | [6] |
| R. *opacus* lcp | 2-CP | D | CCat 1,2-DO | 100 | 283 | 270 | [10] |
| | 4-CP | E | CCat 1,2-DO | 100 | 208 | 242 | [11] |

Note: Pathway: A, the modified *ortho*-pathway of methylcatechol degradation; B, the ordinary *ortho*-pathway of catechol degradation; C, the presence of enzymes from the pathways of Cat and 4-CCat degradation has been shown; D, the new modified *ortho*-pathway of degradation of chlorocatechols; E, the modified *ortho*-pathway of degradation of chlorocatechols.

dioxygenase, because the Cat 1,2-DO enzymes of the ordinary *ortho*-pathway of catechol degradation are usually characterized by low catalytic activity with methyl-substituted substrates.

The enzymes were purified to an electrophoretically homogenous state on an FPLC device (Pharmacia, Sweden). Cat 1,2-DO was purified in 4 steps with a 20% yield of activity: ion exchange chromatography on Q-Sepharose (total protein 165 mg, specific activity 2.9 U/mg of protein), hydrophobic chromatography on Phenyl-Sepharose (total protein 37 mg, specific activity 5.8 U/mg of protein), gel filtration on Superdex 75 (total protein 55 mg, specific activity 3.9 U/mg of protein), and repeated ion exchange chromatography on Resourse Q (total protein 33 mg, specific activity 5.4 U/mg of protein). Cat 1,2-DO, like most of the enzymes of this group, was a homodimer with the subunit molecular mass of 35 kDa as determined by electrophoresis in polyacrylamide gel under denaturing conditions. The temperature optimum of the enzyme activity with Cat was 50°C; this was comparable with the data of described Cat 1,2-DO. The pH optimum in Tris–HCl buffer was within 7.2–7.4, i.e., a little lower than in other Cat 1,2-DO enzymes [8].

The relative activity of the purified enzyme with substrates Cat : 3-MCat : 4-MCat was 100 : 195 : 117% (5.7, 11.1, and 6.7 U/mg of protein). Thus, the rate of 3-MCat oxidation was higher than the rates of oxidation of unsubstituted and *para*-substituted catechol. The activity with 3- and 4-CCat was no more than 5% of the activity with Cat. The Michaelis constants for Cat, 3-MCat, and 4-MCat were 7, 40, and 25 μM, respectively, i.e., the affinity to Cat was higher than to its methyl-substituted analogs. The inhibitory analysis showed that the competitive inhibitors for Cat 1,2-DO of *R. ruber* P25 were tetrachlorocatechol, 3,5-, 3,6-, 4,5-dichlorocatechols, and 2-chlorophenol (2-CP) (inhibition constants were 1, 0.3, 0.4, 0.5, and 25 μM, respectively). In concentrations of up to 200 μM, 4-chlorophenol (4-CP), 4-MBA, and 4-chlorobenzoate did not inhibit Cat 1,2-DO of *R. ruber* P25. It means that the *para*-substituted phenol and benzoates have the least affinity to the active center of Cat 1,2-DO of *R. ruber* P25. The isolated Cat 1,2-DO differed from all analogous enzymes of gram-positive bacteria, because, in contrast to Cat 1,2-DO of the usual *ortho*-pathway, it catalyzed the oxidation of methylcatechols at a higher rate than the rate of catechol oxidation. In this respect, it was similar to the CCat 1,2-DO of modified *ortho*-pathways but, unlike the latter, it could not catalyze the oxidation of chlorosubstituted substrates at a high rate (table).

MCI was purified in three steps: Q-Sepharose (protein 165 mg, specific activity 0.14 U/mg of protein), hydrophobic chromatography on Phenyl-Sepharose (protein 32 mg, specific activity 1.8 U/mg of protein), and heating at 60°C for 15 min followed by centrifugation (protein 5.9 mg, specific activity 2.2 U/mg of protein). MCI was purified 36-fold with the 42% yield of activity. Determination of the catalytic properties of MCI showed that it was stable at 60°C. $K_M$ for muconate was 6.67 μM.

Thus, *R. ruber* P25 can degrade not only chlorosubstituted but also methyl-substituted aromatic compounds. Adaptation to the new substrate took several months and was accompanied by the emergence of $4\text{-MBA}^+$ phenotype, which was provided by induction of the enzymes absent from the studied pathways of degradation of chlorinated substrates by this strain (unpublished data). The enzyme of *R. ruber* P25 differs in substrate specificity from dioxygenases of the intradiol cleavage of (substituted) catechols from other

*Rhodococcus* bacteria. The described enzyme is most similar to methylcatechol 1,2-dioxygenase, the new type of dioxygenases that open the ring of methylcatechols, the existence of which has been shown previously only in *Pseudomonas* sp. MT1 [12].

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