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## SHORT COMMUNICATIONS

# Degradation of 4-Chlorophenol by the Strain *Rhodococcus opacus* 6a

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4-Chlorophenol (4-CP), a highly toxic pollutant, is degraded by bacteria under aerobic conditions via hydroxy hydroquinone or 4-chlorocatechol (4-ClC) [1, 2]. Degradation of 4-CP through 4-ClC includes the stage of aromatic ring opening in the *ortho*- or *meta*-position preceding dechlorination [2, 3]. In the literature, a limited number of pure cultures of gram-positive and gram-negative bacteria were reported to degrade high concentrations of 4-CP [1, 4, 5]. The major cause of insignificant biological degradation of chlorophenols is, first, that their degradation via chlorocatechols requires the enzymes of the so-called modified *ortho* pathway, which are specialized for degradation of chlorinated substrates, and, second, that chlorophenols are strong competitive inhibitors for practically all chlorocatechol 1,2-dioxygenases, the key enzymes of this modified *ortho* pathway [2].

The strain *Rhodococcus opacus* 6a was isolated on 4-CP by the enrichment culture method from the soil sampled on the territory of a petrol station (Pushchino). Apart from 4-CP, *R. opacus* 6a utilized 3-chlorobenzoate, ferulic acid, *p*-cresol, and *p*-toluate (4-methylbenzoate, 4-MBA) as the sole carbon and energy sources. The strain was subcultured on meat infusion agar for 10 years and then adapted de novo to 4-MBA. It was shown that growth on 4-MBA resulted in induction of catechol 1,2-dioxygenase (1,2-CTD) and methylcatechol 1,2-dioxygenase (MCat-1,2-DO). MCat-1,2-DO was characterized by the high activity with 3-methylcatechol (3-MCat), 4-methylcatechol (4-MCat), and 4-chlorocatechol (3-ClC). The data on the catalytic activity of MCat-1,2-DO suggest that even if the strain loses its ability for complete degradation of 4-CP after prolonged storage, it should be able at least to transform this toxicant, taking into consideration that the enzymes of initial attack are usually characterized by broad substrate specificity and the formed 4-ClC can be decomposed by MCat-1,2-DO. Besides, the induction of specific enzymes involved in 4-CP degradation is possible in the case when their coding genes are stably inherited during growth on a rich medium and are not lost in the absence of selective pressure of 4-CP.

The goal of this work was adaptation of the strain *R. opacus* 6a to 4-CP after long-term growth on a rich medium, study of the effect of this toxicant on colony morphology, and characterization of the enzymes involved in 4-CP degradation.

**Adaptation of *R. opacus* 6a to 4-CP.** Originally, the growth of *R. opacus* 6a in a liquid medium with 4-CP (50 mg/l) as the sole carbon and energy source was accompanied by a long (up to 40 h) lag phase and an increase in optical density ($D_{545}$) from 0.07–0.1 to 0.15–0.2 U. After cultivation of this strain for 3 months on solid and liquid media with 4-CP in the concentrations of 100 and 50 mg/l, respectively, under repeated subculturing, the character of its growth changed: the lag phase shortened to 5 h and the culture density reached 0.73 U. At cultivation of *R. opacus* 6a in a 10-l bioreactor, the lag phase was 3 h, the additives (4-CP, 150–250 mg/l) were introduced at intervals of 20–60 min depending on respiration, the whole process lasted 48 h, and the total 4-CP uptake was 35 g. The culture reached the optical density of 1.9 U. These values make it possible to name the strain *R. opacus* 6a among the best 4-CP degrading cultures.

**Phenotypic variation of *R. opacus* colonies during growth on 4-CP.** Cultivation of the strain *R. opacus* 6a revealed morphological heterogeneity of the population typical of many cultures, which is expressed in the splitting of a homogeneous bacterial population into variants with different morphological, physiological, biochemical, and genotypic properties [6]. The process of dissociation was studied at cultivation of *R. opacus* 6a on agar slants with 100 mg/l 4-CP; then, the strain was inoculated into a flask and the quantity and appearance of colonies were determined as follows: for five days, samples were taken from the inoculated flask every 24 h, and 100 μl of its serial dilutions (from $10^{-1}$ to $10^{-15}$) were plated on a rich medium. The counting of

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**Table 1.** Appearance of the phenotypic variants in the culture of *R. opacus* 6a

| Day of growth | $D_{545}$ | CFU | Quantity of *R*-forms, % | Quantity of *S*-forms, % |
|---|---|---|---|---|
| 0 | 0.163 | $1.0 \times 10^7$ | 100 | 0 |
| 1 | 0.244 | $1.5 \times 10^8$ | 100 | 0 |
| 2 | 0.284 | $6.0 \times 10^9$ | 95 | 5 |
| 3 | 0.327 | $2.5 \times 10^{10}$ | 75 | 25 |
| 4 | 0.376 | $3.0 \times 10^{13}$ | 50 | 50 |
| 5 | 0.380 | $5.0 \times 10^{16}$ | 30 | 70 |

CFU is the number of colony-forming units.

colonies on day 7 showed that in the beginning of cell cultivation in the flask with 4-CP, the strain *R. opacus* 6a formed rough colonies (*R*). On day 2 of cultivation, the initial rough form was split into rough and smooth (*S*-form). The forming colonies had smooth and somewhat glossy surfaces. Table 1 shows dissociation of the strain depending on the time of cultivation in a 4-CP-containing liquid mineral medium. It should be noted that *S*-form colonies growing on a rich medium were 2–2.5 larger than *R*-form colonies.

Similar processes of phenotypic variation were revealed for strain *R. opacus* 1cp [7]. However, in strain *R. opacus* 1cp smooth colonies constituted only 1–5%, and rough colonies quantitatively predominated throughout the experiment, whereas in strain *R. opacus* 6a the quantity of smooth colonies reached 70% after 5 days of cultivation.

**The enzymes of 4-CP degradation in R. opacus 6a.** The cell-free extract of *R. opacus* 6a grown on 4-CP exhibited activities of all the enzymes of the modified *ortho* pathway: ClC-1,2-DO, chloromuconate cycloisomerase (CMCI), and dienelactone hydrolase (DLH). The activities of ClC-1,2-DO with catechol, 3-chlorocatechol, 4-ClC, 3-MCat, and 4-MCat were as follows (U/mg of protein): 0.2367 (100%), 0.0266 (11.2%), 0.240 (101.4%), 0.4515 (190.7%), and 0.536 (226.4%), respectively. The activity of CMCI with 3-chloromuconate was 0.15 U/mg of protein and that of DLH with *cis*-dienelactone was 0.94 U/mg of protein. As for the activity of ClC-1,2-DO in the cell-free extract, it was prima facie similar to the activity in the cell-free extract of the same strain grown on 4-MBA. As a whole, it confirmed our assumption that the strain growth on 4-CP may be provided by the enzymes participating in 4-MBA degradation; however, the high rate of growth on 4-CP and high biomass yield suggest that this process is catalyzed by highly effective enzymes, which allow the strain not to transform 4-CP but to degrade it completely.

**Chlorocatechol 1,2-dioxygenase** was purified 81.8-fold in 5 steps, and the activity yield was 15.8%. The main physicochemical and catalytic properties of ClC-1,2-DO were determined: the enzyme was a homodimer with the subunit molecular mass of 27 kDa. Unlike many ClC-1,2-DO, it was stable during storage at 4°C and preserved 100% activity for a year. The enzyme lost 25% of its activity after 3 months at –10°C, 15% after 40 days, and 80% after 3 months at 25°C; the temperature optimum was 50–55°C and the pH optimum was 7.2. ClC-1,2-DO catalyzed degradation of a wide range of substrates: catechol, methyl- and chlorocatechols. The highest value of the specificity constant $V_{cat}/K_M$ was calculated for 4-ClC (Table 2). The results demonstrate that ClC-1,2-DO described in the present work differs from MCat-1,2-DO isolated from the biomass of *R. opacus* 6a grown on 4-MBA. Although 4-ClC is degraded by both enzymes at a rate comparable with the rate of oxidation of unsubstituted catechol, 4-MCat and 4-ClC are the best substrates for MCat-1,2-DO and ClC-1,2-DO, respectively (Table 2).

Some compounds served as competitive inhibitors for ClC-1,2-DO. The values of inhibition constant for 3,5-diClC, 2-CP, and 4-CP were 0.5, 71.6, and 21.5 μM, respectively. The enzyme was not inhibited by phenol.

**DLH** was purified in 4 steps; the degree of purification was 132-fold; and the activity yield was 5.97%. Like the homologous enzymes, DLH is a monomer with the mass of about 26 kDa. DLH is active with a number of substrates with the relative transformation rate of 100% (*cis*-dienelactone), 229% (2-chloro-*cis*-dienelactone), and 28% (2-methyl-*cis*-dienelactone). The Michaelis constant with *cis*-dienelactone was

**Table 2.** Substrate specificity of ClC-1,2-DO of the modified *ortho* pathway in strain *R. opacus* 6a grown on 4-chlorophenol

| Substrate | $K_M$, μM | $V_{max}$, U/mg | $k_{cat}$, $min^{-1}$ | $k_{cat}/KM$, $min^{-1}$ $\mu M^{-1}$, % | |
|---|---|---|---|---|---|
| | | | | ClC-1,2-DO | MCat-1,2-DO[a] |
| Catechol | 62.5 | 20.0 | 540 | 0.32 (3.4) | 535.2 (59.5) |
| 3-Chlorocatechol | N.d. | N.d. | N.d. | N.d. | 97.7 (10.9) |
| 4-Chlorocatechol | 1.4 | 13.3 | 359 | 9.5 (100) | 344.4 (38.3) |
| 3-Methylcatechol | 33.3 | 11.5 | 311 | 0.35 (3.7) | 67.5 (7.5) |
| 4-Methylcatechol | 31.2 | 27.8 | 751 | 0.89 (9.4) | 899.0 (100) |

[a] The data are given for methylcatechol 1,2-dioxygenase from *R. opacus* 6a grown on 4-MBA; N.d., not determined.

19.2 μM; the maximum rate was 20 U/mg of protein; and the pH optimum was 8.2–8.5. DLH was extremely unstable and quickly inactivated in the course of purification. Addition of ammonium sulfate stabilized the enzyme.

**Immobilization of *R. opacus* 6a cells** on polycaproamide fiber was carried out using cell suspension with $D_{545}$ of 0.62 relative units. Nonimmobilized cells were washed off with the medium. 4-ClC was added in the concentration of 50–300 mg/l. The carrier with 200 ml of the medium containing 300 mg/l 4-CP was used as a control. Nonimmobilized culture degraded 50 and 100 mg/l 4-CP in 23 and 35 h, respectively; 150 mg/l 4-CP was degraded in 148 h and 200 mg/l inhibited growth. At cell immobilization on fiber, the culture completely utilized 150 mg/l of the toxicant in 24 h. Immobilized cells of the strain consumed the toxicant in the concentrations of 200 and 250 mg/l, but the process of utilization was slower.

Thus, the present work shows the stability of 4-CP$^+$ phenotype of strain *R. opacus* 6a and, accordingly, of the genes encoding 4-CP degradation enzymes at long-term (for more than 10 years) cultivation on a rich medium in the absence of selective pressure. Due to the capacity for effective degradation of large 4-CP amounts (up to 35 g in 48 h) and the presence of several dioxygenases (1,2-CTD, MCat-1,2-DO, and ClC-1,2-DO), which degrade the substrates carrying different substitutes, the strain *R. opacus* 6a is a unique research object and a promising tool for creation of systems of bioremediation of chlorophenol contamination.

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