ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 3, pp. 299–305. © Pleiades Publishing, Ltd., 2012.
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EXPERIMENTAL ARTICLES

Metabolism of 3-Hydroxybenzoate and Gentisate by Strain *Rhodococcus opacus* 1CP

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Received September 28, 2011

Abstract—The ability of strain *Rhodococcus opacus* 1CP to utilize 3-hydroxybenzoate (3-HBA) and gentisate in concentrations up to 600 and 700 mg/L, respectively, as sole carbon and energy sources in liquid mineral media was demonstrated. Using high-performance liquid chromatography (HPLC) and thin-layer chromatography, 2,5-dihydroxybenzoate (gentisate) was identified as the key intermediate of 3-hydroxybenzoate transformation. In the cell-free extracts of the strain grown on 3-HBA or gentisate, the activities of 3-hydroxybenzoate 6-hydroxylase, gentisate 1,2-dioxygenase, and maleylpyruvate isomerase were detected. During growth on 3-HBA, low activity of catechol 1,2-dioxygenase was detected. Based on the data obtained, the pathway of 3-HBA metabolism by strain *R. opacus* 1CP was proposed.

Keywords: 3-hydroxybenzoate, gentisate, *Rhodococcus.*

DOI: 10.1134/S0026261712030137

Microbial degradation of mono- and dihydroxy derivatives of benzoic acid, intermediates of the biodegradation of a number of toxic xenobiotic, has been studied for decades. It is well known that mono- and dihydroxybenzoates are produced as a result of degradation of xylenols, cresols, phenol, benzene, benzoate, and their substitutive derivatives [1–3]. During recent years, much attention was paid to metabolism of 3-hydroxybenzoate (3-HBA) [4–6], since the microbiological, biochemical, and genetic aspects of its degradation have been poorly studied.

According to the published data, there are three key pathways of the aerobic metabolism of 3-hydroxybenzoate via the formation of the following intermediates: protocatechuate [7], gentisate [7], and 2,3-dihydroxybenzoate [8]. Two pathways of 3-HBA degradation are most common among gram-positive and gram-negative bacteria: via protocatechuate and gentisate.

Microorganisms degrade gentisate via its transformation into maleylpyruvate by gentisate 1,2-dioxygenase [9, 10]. The subsequent conversion of the generated maleylpyruvate occurs via two pathways, namely, isomerization catalyzed by maleylpyruvate isomerase [10] and hydrolysis catalyzed by maleylpyruvate hydrolase [11] with formation of fumaryl pyruvate and pyruvate with maleic acid, respectively, which are then incorporated into the main metabolic pathways.

The gram-positive bacterium *Rhodococcus opacus* 1CP is able to utilize a wide range of toxic organic compounds, including 4-chloro-, 2-chloro-, and 2,4-dichlorophenols [12–14], as well as *p*-cresol [15] and benzoate and its methyl derivatives [16–18], as the sole source of carbon and energy.

The goal of this work was to study the pathways of 3-hydroxybenzoate and gentisate metabolism by strain *R. opacus* 1CP.

MATERIALS AND METHODS

In our study, we used the strain *R. opacus* 1CP from the culture collection of the Laboratory of Enzymatic Degradation of Organic Compounds,G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences.

Cultivation Conditions

The strain was grown in Gorlatov liquid mineral medium (G) [14]. To obtain the inoculum, cells of a 2-day culture were washed off from the surface of a Luria Bertani agar slant (peptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 8 g/L).

To study the consumption of 3-HBA and gentisate, as well as the dynamics of formation of the key intermediates, the cultures were grown in 750-mL medical flasks with 100 mL of G medium at 29°C on a shaker (160 rpm).

The biomass for detection of the enzymatic activity in the cell-free extract was obtained by batch cultivation of strain *R. opacus* 1CP in a 5-L flask with 2 L of mineral medium. Gentisate or 3-hydroxybenzoate,

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which were used as the substrates, were added in portions (200 mg/L) upon their exhaustion. Consumption of the substrates was confirmed by the disappearance of UV absorption peaks; growth was detected by measuring the optical density (OD_{545}) on a Shimadzu UV-160 spectrophotometer (Japan). The biomass yield was 2.0–2.5 g/L. Bacterial cells were harvested by centrifugation at 5000*g* for 15–20 min, washed with 0.05 M of Tris–HCl buffer (pH 7.2), and stored at –20°C.

Study of 3-HBA exhaustion and the Dynamics of the Key Intermediate Formation

To study the decrease in the 3-HBA content, as well as the dynamics of formation of the key intermediates, batch cultivation of strain *R. opacus* 1CP was carried out in 100 mL of G medium with 3-hydroxybenzoate (400 mg/L) as a substrate. After inoculation and during the first day of incubation, 1-mL samples of thoroughly resuspended culture were taken at 2-h intervals and centrifuged at 5000 *g* for 10 min in order to remove the cells. Then, the supernatant was analyzed by high-performance liquid chromatography (HPLC). HPLC of the culture liquid was carried out using a Waters 996 chromatograph (United States), equipped with a Spherisorb ODS-2 column (5 μm, 250 mm ± 4/6 mm, Supelco, United States) and a UV detector at 254 nm. The analysis was performed in a methanol/water mixture supplemented with 1% acetic acid in a methanol gradient from 0 to 100%. The flow rate was 0.9 mL/min at 50°C. The elution time was 20 min. For identification of the tested compounds, comparison of their retention time (R_t) in the column and the retention time of the standard compounds was carried out.

To obtain preparations of the intermediates of 3-HBA transformation and for their identification, extracts of the culture liquid were obtained as described in [19]. Qualitative analysis of the extracts and isolation of the intermediates were performed as described in [15] by thin-layer chromatography (TLC) on 60 F_{254} silica gel plates (Merck, Germany). Identification of the tested compounds was based on determination of R_f values.

Obtaining Cell-Free Extracts and Determination of Protein Concentrations

To obtain cell-free extracts, the biomass was disrupted with a Hughes-type press. The disrupted biomass was supplemented with 0.01 mg/mL of DNase (Sigma, United States), incubated at 4°C for 15 min, and centrifuged at 15000–18000*g* for 30–40 min. The sediment was washed with 0.01 M Tris–HCl buffer (pH 7.2) containing 10% ethanol (vol/vol) and 0.25 mM of β-mercaptoethanol and recentrifuged under the same conditions. The obtained supernatants were combined and centrifuged at 18000*g* for 30 min. The cell-free extract was used for determination of the enzymatic activity.

The protein concentration was determined by the modified Bradford method using a series of serum albumin solutions as the standards [20].

Determination of the Enzymatic Activity in the Cell-Free Extracts

The enzymatic activity was determined on a Shimadzu UV-160 spectrophotometer (Japan) in a quartz cuvette with an optical length of 10 mm at 25°C. The activity of 3-hydroxybenzoate 6-hydroxylase was determined using the modified method by Wang et al. [21] at 340 nm; the gentisate 1,2-dioxygenase activity was determined by the rate of maleylpyruvate production at 334 nm [11]; the activity of maleylpyruvate isomerase was assessed by the rate of product formation at 330 nm [22]; the catechol 1,2-dioxygenase activity was determined using the modified method by Hayaishi et al. [23] at 260 nm; the activity of protocatechuate 3,4-dioxygenase was assessed by the rate of decrease in the protocatechuate content at 290 nm [24]; the activity of protocatechuate 4,5-dioxygenase was assessed by the rate of γ-carboxy-α-hydroxymuconate production at 410 nm [25], the activity of catechol 2,3-dioxygenase was determined by the rate of 2-hydroxymuconic semialdehyde production at 375 nm [26]; and the muconate cycloisomerase activity was assessed by the rate of muconate consumption at 260 nm [27].

An enzymatic activity unit corresponds to the enzyme quantity that, within 1 min, catalyzes transformation of 1 μmol of a substrate or production of 1 μmol of a product.

RESULTS AND DISCUSSION

Strain *R. opacus* 1CP is able to grow on 3-HBA and gentisate as sole carbon and energy sources.

Growth Characteristics of Strain R. opacus 1CP Exposed to Different 3-HBA and Gentisate Concentrations

The study of the growth dynamics of strain *R. opacus* 1CP in the presence of different 3-HBA and gentisate concentrations demonstrated that it was able to grow at substrate concentrations of up to 600 mg/L (Fig. 1). An increase in the 3-HBA concentration up to 700 mg/L resulted in complete inhibition of growth.

The kinetic parameters of growth (Table 1) demonstrated that optimal growth was observed at a 3-HBA concentration of 600 mg/L. The highest specific growth rate (0.192 h^{-1}) and the lowest biomass doubling time (3.61 h) were observed under these conditions. However, the duration of the lag phase during growth at 600 mg/L of 3-HBA was 6 hours longer (13 h) than at 400 mg/L (7 h). On the whole, an increase in the 3-HBA concentrations in the culture

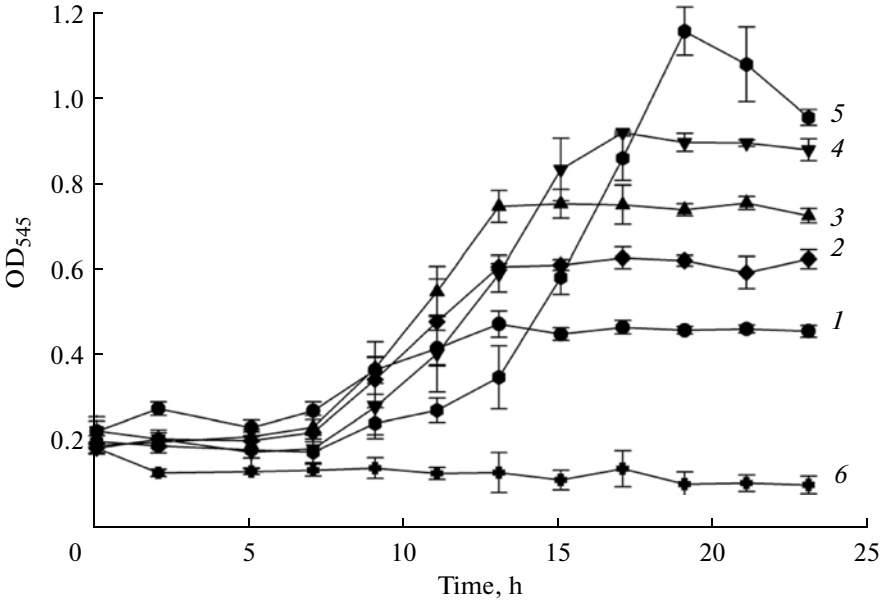

Fig. 1. Growth curves of strain *R. opacus* 1CP grown at different 3-HBA concentrations (mg/L): 200 (*1*), 300 (*2*), 400 (*3*), 500 (*4*), 600 (*5*), and 700 (*6*).

liquid resulted in the successive increase in the lag phase duration to the point of growth cessation.

The study of the *R. opacus* 1CP growth at different gentisate concentrations (200–800 mg/L) allowed us to determine the optimal gentisate concentration (700 mg/L), with the highest specific growth rate (0.213 h^{-1}) and the shortest biomass doubling time (3.25 h) demonstrating the greater availability of gentisate (as compared to 3-HBA) as a substrate. Moreover, the duration of the lag phase during growth on gentisate was shorter than during growth on 3-HBA (Fig. 2). At gentisate concentrations in the culture liquid of ≥800 mg/L, a drastic inhibition of growth was observed (Fig. 2).

The highest tolerable gentisate and 3-HBA concentrations for microbial growth are poorly investigated. It is well-known that *Salmonella typhimurium* and *Klebsiella pneumoniae* M5a are able to grow at the 3-HBA concentrations of 500 and 690 mg/L, respectively [1, 2] in liquid mineral media, whereas strain *Bacillus krulwichiae* grows on nutrient-rich media in the presence of 3 g/L 3-HBA [28]. However, it does not mean that these are the highest tolerable concentrations for these microorganisms; therefore, the capacity of the strains for 3-HBA degradation is difficult to assess. Growth on gentisate remains poorly studied. Usually, the enzymes involved in gentisate degradation were investigated using the microbial bio-

Table 1. Kinetic parameters of the growth of strain *R. opacus* 1CP on 3-HBA and gentisate as sole carbon and energy sources

Concentration, mg/L	Specific growth rate, μ, h^{-1}		Biomass doubling time, h	
	3-HBA	gentisate	3-HBA	gentisate
200	0.147	0.141	4.71	4.91
300	0.162	0.146	4.28	4.75
400	0.181	0.160	3.83	4.33
500	0.187	0.190	3.71	3.65
600	0.192	0.193	3.61	3.59
700	0	0.213	0	3.25
800	0	0	0	0

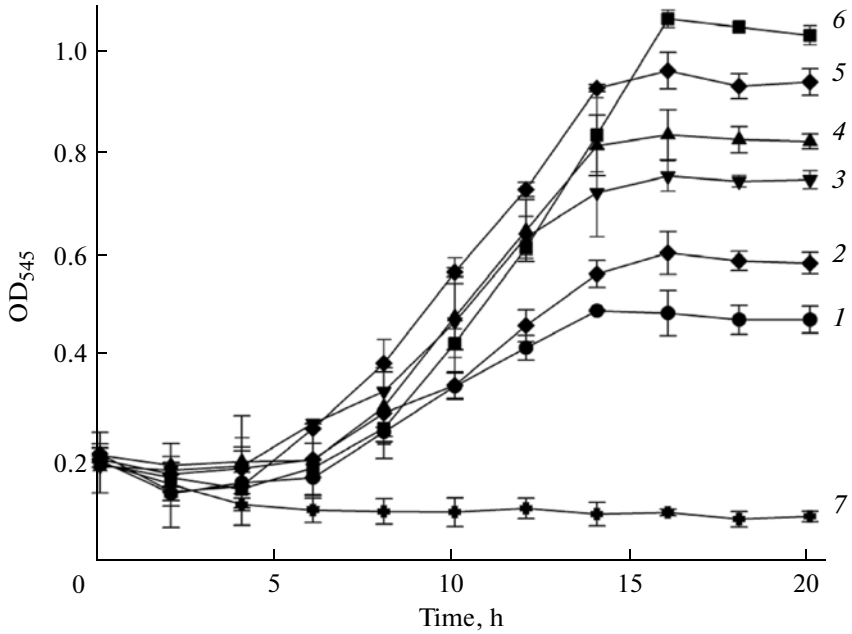

Fig. 2. Growth curves of strain *R. opacus* 1CP grown at different gentisate concentrations (mg/L): 200 (*1*), 300 (*2*), 400 (*3*), 500 (*4*), 600 (*5*), 700 (*6*), and 800 (*7*).

mass obtained during growth on 3-HBA or other compounds whose degradation pathways involve gentisate as the key intermediate [10, 29, 30].

Dynamics of 3-HBA Exhaustion and Accumulation of the Intermediates of 3-HBA Transformation by Strain R. opacus 1CP

Figure 3 demonstrates the exhaustion dynamics of 400 mg/L of 3-HBA in the culture liquid of strain *R. opacus* 1CP, as well as the dynamics of gentisate accumulation. Six hours after inoculation, gentisate ($R_t = 7.816$; $R_f = 0.072$) was detected in the culture liquid by HPLC and thin-layer chromatography. Subsequent accumulation of gentisate was observed, and, after 10-h cultivation, the gentisate concentration reached its peak value (1 mg/L), while the residual 3-HBA decreased to 50% of the initial concentration (Fig. 3). Then, the gentisate concentration decreased, and, after 14-h cultivation, neither gentisate nor 3-HBA were detected in the culture liquid. Throughout the cultivation, no 3-HBA metabolites other than gentisate (protocatechuate, 2,3-dihydroxybenzoate, catechol, and hydroxyquinol) were detected, as well as no intermediates of the subsequent gentisate degradation. After 24-h incubation in the presence of 3-HBA, the products of its metabolism were not detected in the culture liquid, which indicates complete degradation of the tested compound.

Enzymes Involved in the 3-HBA and Gentisate Metabolism by Strain R. opacus 1CP

In the cell-free extract of strain *R. opacus* 1CP grown in the presence of 3-HBA as a sole carbon and energy source, the activities of 3-hydroxybenzoate 6-hydroxylase, gentisate 1,2-dioxygenase, catechol 1,2-dioxygenase, and maleylpyruvate isomerase were detected (Table 2). The activities of the enzymes involved in other possible pathways of 3-HBA degradation (protocatechuate 3,4-dioxygenase, protocatechuate 4,5-dioxygenase, protocatechuate 2,3-dioxygenase, catechol 2,3-dioxygenase, and hydroxyquinol 1,2-dioxygenase) were not detected, which confirms that the strain degrades 3-HBA only via gentisate with its subsequent decomposition to maleylpyruvate. The low rates of synthesis of catechol 1,2-dioxygenase by the strain, the absence of catechol in the culture liquid, as well as the absence of activity of muconate cycloisomerase (the enzyme responsible for isomerization of muconate, the product of the intradiol degradation of catechol), indicate that the concomitant induction of catechol 1,2-dioxygenase by the strain was insignificant. This assumption was confirmed by a successive decrease in the synthesis of catechol 1,2-dioxygenase to the point of its cessation during fermentation.

During growth of some microorganisms (*Rhodococcus erythropolis* SI [9], *Klebsiella pneumoniae* M5a [2], and *Salmonella typhimurium* [1]) in the presence of gentisate, induction of 3-hydroxybenzoate 6-hydroxylase, the first enzyme of the 3-HBA degradation pathway, was detected. In the cell-free extract of *R. opacus* 1CP grown on gentisate, the activ-

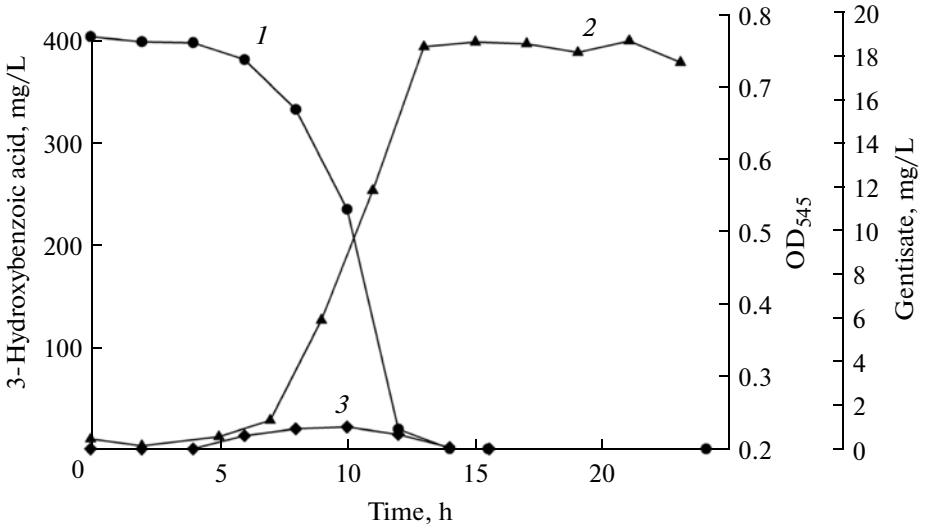

Fig. 3. Transformation of 400 mg/L of 3-HBA and accumulation of gentisate in the culture liquid of strain *R. opacus* 1CP (results of HPLC measurements): exhaustion of 3-HBA (*1*), biomass yield (*2*), and gentisate production (*3*).

ity of 3-hydroxybenzoate 6-hydroxylase was also detected. Moreover, the strain exhibited the activities of gentisate 1,2-dioxygenase and maleylpyruvate isomerase (Table 2). It should be noted that, during growth on gentisate, the strain exhibited no catechol 1,2-dioxygenase activity.

Possible Pathway of 3-HBA Degradation by Strain R. opacus 1CP

Taking into account the obtained intermediates of 3-HBA degradation, analyzed by HPLC and TLC, and considering the ability of the studied strain to grow on gentisate as a sole carbon and energy source, as well as the activities of 3-hydroxybenzoate 6-hydroxylase, gentisate 1,2-dioxygenase, and maleylpyruvate isomerase exhibited by bacterial cells grown in the presence of 3-HBA and gentisate, we proposed a pathway of 3-HBA degradation by strain *R. opacus* 1CP via 3-HBA hydroxylation with formation of gentisate as the key intermediate of 3-HBA degradation, with its subsequent decomposition to maleylpyruvate and maleylpyruvate transformation with formation of fumaryl pyruvate to the TCA cycle (Fig. 4).

Two pathways of 3-HBA degradation are most commonly used by gram-positive and gram-negative bacteria: via gentisate [1–3, 7, 9, 10] and protocatechuate [7, 31]. Members of the same bacterial genus may use different pathways of 3-HBA degradation. For instance, the first stage of 3-HBA transformation may serve as a taxonomic criterion for differentiation between *Pseudomonas testosterone* and *P. acidovorans*, utilizing 3-HBA via protocatechuate and gentisate, respectively [7]. Interestingly, some differences in 3-HBA degradation were observed within a single pathway. For example, Crawford demonstrated that the species of the genus *Bacillus* (*B. brevis* and *B. sphaericus*) degrading 3-HBA via gentisate transform maleylpyruvate synthesized by gentisate 1,2-dioxygenase via hydrolysis by maleylpyruvate hydrolase with formation of pyruvate and maleic acid [10]. Strains *R. erythropolis* S1 and *Corynebacterium glutamicum* transform maleylpyruvate via isomerization catalyzed by maleylpyruvate isomerase and resulting in formation of fumaryl pyruvate, which is then hydrolyzed to pyruvate and fumarate by fumaryl pyruvate hydrolase [3, 9]. The pathway of 3-HBA degradation by strain *R. opacus* 1CP is similar to that of *R. erythropolis* S1 and *C. glutamicum*. The characteris-

Table 2. Activities of the enzymes detected in cell-free extracts of *R. opacus* 1CP grown in the presence of 3-HBA and gentisate as sole carbon and energy sources

Enzyme	Specific activity, U/mg protein	
	3-HBA	gentisate
3-Hydroxybenzoate 6-hydroxylase	0.154	0.106
Gentisate 1,2-dioxygenase	0.651	0.654
Protocatechuate 2,3-dioxygenase	0	0
Protocatechuate 4,5-dioxygenase	0	0
Protocatechuate 3,4-dioxygenase	0	0
Catechol 1,2-dioxygenase	0.121	0
Muconate cycloisomerase	0	0
Catechol 2,3-dioxygenase	0	0
Hydroxyquinol 1,2-dioxygenase	0	0
2,3-Dihydroxybenzoate dioxygenase	0	0
Maleylpyruvate isomerase	1.543	1.840

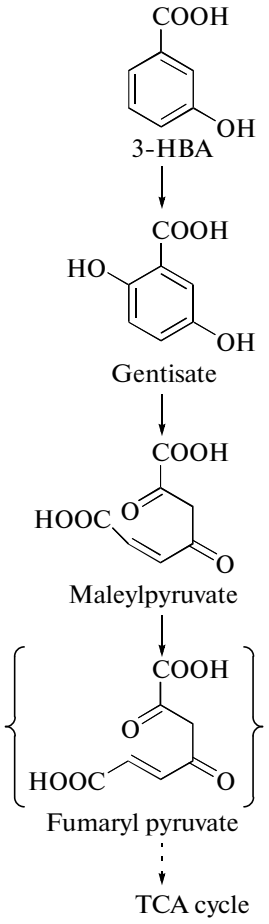

Fig. 4. Possible pathway of 3-HBA degradation by strain *R. opacus* 1CP.

tic trait of strain *R. opacus* 1CP is induction of catechol 1,2-dioxygenase during growth on 3-HBA.

Thus, we demonstrated the ability of strain *R. opacus* 1CP to degrade hydroxylated derivatives of benzoic acid, which expands the spectrum of known substrates utilized by these microorganisms [12–18]. The results of our study may be used for obtaining the active biomass of this strain for subsequent isolation and investigation of the enzymes involved in the degradation of 3-HBA and gentisate.

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