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Catechol 1,2-Dioxygenase from *Rhodococcus rhodochrous* N75 Capable of Metabolizing Alkyl-Substituted Catechols

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Abstract Catechol 1,2-dioxygenase was purified from cells of *R. rhodochrous* N75 grown at the expense of benzoate and *p*-toluate as the sole sources of carbon. A single catechol 1,2-dioxygenase was found to be induced with either growth substrate. The enzyme has an estimated M_r of 71,000 consisting of two identical subunits. Catechol 1,2-dioxygenase from *R. rhodochrous* N75 exhibits some unusual properties including: broad substrate specificity, extradiol cleavage activity with 4-methylcatechol and low K_m values for halocatechols, suggesting that this enzyme is distinct from other known catechol and chlorocatechol 1,2-dioxygenases.

Key words: Catechol 1,2-dioxygenase, *Rhodococcus*, methylcatechol

Catechol dioxygenases, which cleave the aromatic ring of catecholic substrates via the insertion of molecular oxygen, are key enzymes in the degradation of aromatic compounds by aerobic microorganisms. These enzymes are categorized into two large classes based on the nature of their products. Intradiolic or *ortho*-cleaving dioxygenases such as catechol 1,2-dioxygenases open the aromatic ring of catechol-like substrates between the vicinal ring hydroxyl groups to form a dicarboxylic acid [2, 16, 19, 21–24, 27, 30], whereas extradiol or *meta*-cleaving dioxygenases such as catechol 2,3-dioxygenases open the ring adjacent to one of the hydroxyl groups to form a semialdehyde [12–14, 18, 26]. Microorganisms have been shown to possess more than one type of catechol 1,2-dioxygenase. Several catechol 1,2-dioxygenase type enzymes found in the degradative pathway for chlorinated aromatic compounds have been shown to exhibit higher affinities and activities towards chlorinated catechols than catechol 1,2-dioxygenase of the classical *ortho*-cleavage pathway and have been termed chlorocatechol 1,2-dioxygenase, type II catechol 1,2-dioxygenase or pyrocatechase II [3, 8, 9, 17, 25, 29]. Powlowski and Dagley [27] discovered another type of isofunctional catechol 1,2-dioxygenase from the fungus *Trichosporon cutaneum* which displayed a higher activity towards 4-methylcatechol than catechol. The isozyme, referred to as 4-methylcatechol 1,2-dioxygenase, was readily separated from the classical catechol 1,2-dioxygenase by DEAE-Sephacel anion exchange chromatography. 4-Methylcatechol 1,2-dioxygenase from *T. cutaneum* was shown to be induced specifically for the degradation of methyl-substituted aromatic compounds such as *p*-toluate and *p*-cresol. Microorganisms have, therefore, evolved new types of isofunctional enzymes which possess different catalytic properties for the transformation of substituted aromatic substrates. Since *Rhodococcus rhodochrous* N75 was found to exhibit considerable catabolic activities towards methylaromatic compounds [4–6], the possibility was raised that this strain might induce isofunctional enzymes for the catabolism of such compounds. In addition, although a number of catechol 1,2-dioxygenases from various microorganisms have been studied, there are only a few reported cases where these enzymes have been characterized from Gram-positive bacteria [17, 19, 22, 23, 28, 30]. The present studies describe the purification and characterisation of catechol 1,2-dioxygenase from *R. rhodochrous* N75.

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MATERIALS AND METHODS

Chemicals

Catechol was obtained from Sigma Chemical Company Ltd, 3-methylcatechol and 4-methylcatechol from Aldrich Chemical Company Ltd., and 4-ethylcatechol from Lancaster Syn. Ltd. (Morecambe, U.K.). Other alkylcatechols were a

generous gift from Professor R. B. Cain, The University, Newcastle upon Tyne, and Professor G. W. Kirby, University of Glasgow. 3-Chlorocatechol, 4-chlorocatechol, 4-bromocatechol and 4-fluorocatechol were kindly provided by Dr. S. Schmidt, University of Hamburg, Germany.

Enzyme Assays

Activity of catechol 1,2-dioxygenase was assayed spectrophotometrically using a Shimadzu UV-160 spectrophotometer or Hewlett Packard 8452A diode array spectrophotometer at 25°C by monitoring the increase of absorbance at 260 nm for catechol and at 255 nm for 4-methylcatechol. The reaction mixture contained, in a total volume of 3 ml: 50 mM Tris-HCl buffer (pH 8.0), 0.2 mM substrate, 1.3 mM EDTA. The reaction was initiated by adding a substrate following 5 min incubation of the enzyme solution in the buffer mixture. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 mol of the product per min. Molar absorption coefficients (litre·mol^{-1}·cm^{-1}) were those reported by Dorn and Knackmuss (1978b); ε=16,800 at 260 nm for muconate, ε=12,400 at 260 nm for 3-chloromuconate, ε=14,900 at 260 nm for 3-fluoromuconate, ε=14,300 at 255 nm for 3-methylmuconate.

Alternatively, catechol 1,2-dioxygenase activity was assayed polarographically by measuring the oxygen consumption using a Clarke oxygen electrode (Rank Brothers, Cambridge) connected to a chart recorder. The reaction mixture contained enzyme solutions in 3 ml of 50 mM Tris-HCl buffer (pH 8.0) which had been aerated by stirring for 5 min. The reaction was then initiated by adding substrate solutions by means of microsyringe.

Enzyme Purification

Catechol 1,2-dioxygenase was purified from cells of *R. rhodochrous* N75 grown at the expense of benzoate and *p*-toluate as the sole sources of carbon. Each batch of cells was subjected to the same purification procedure. Cell-free crude extracts were prepared using 50 g (wet weight) of cells. All the procedures were performed at 4°C unless otherwise stated. The extract was brought to 45% ammonium sulphate saturation by the slow addition of fine solid ammonium sulphate with stirring. This solution was allowed to equilibrate for 40 min with stirring and then the protein precipitate was removed by centrifugation at 13,000 rpm for 20 min. The resulting supernatant was brought to 60% ammonium sulphate saturation, equilibrated and centrifuged. The resulting protein precipitate was then resuspended in a small volume of 50 mM MOPS buffer (pH 7.4) containing 1 mM DTT (buffer A) and the suspension dialyzed overnight against 2 l of buffer A with one buffer change. The dialyzed sample was loaded onto a column of DEAE-Toyopearl 650C (2.5×18 cm) which had been previously equilibrated with buffer A. The column was then washed with the same buffer until no protein was evident in the eluate. The enzyme was eluted from the column with 500 ml of buffer A that contained KCl in a gradient running from 0 to 0.5 M. Fractions of 5 ml were collected at a flow rate of 1 ml/min and each fraction was checked for activity with the substrates catechol and 4-methylcatechol. Active fractions were pooled and concentrated by ultrafiltration with YM10 membrane (M_r 10,000 cut-off, Amicon). The concentrate was applied to a Sephacryl S-100 HR gel filtration column (1.6×90 cm) that had been previously equilibrated with buffer A. Subsequent elution of the enzyme activities was carried out at 12 ml/h and fractions of 2 ml were collected. Fractions containing the highest catechol 1,2-dioxygenase activities were combined. FPLC was performed using a Pharmacia FPLC system which consisted of a LCC-500 Plus Liquid-Chromatography Controller, P-500 pumps and a LKB Bromma 2212 Helirac fraction collector. All FPLC operations were carried out at room temperature and collected fractions were immediately put on ice. The pooled active fractions from the gel filtration step were loaded onto a Mono Q (HR 10/10) anion exchange column (Pharmacia) that had been pre-equilibrated with buffer A and the column was then washed with 30% buffer B (buffer A containing 1 M NaCl). The enzyme was eluted with a linear gradient of 30–60% buffer B. Fractions (1.5 ml) were collected at 3 ml/min and fractions containing the highest enzyme activities were combined and stored at −20°C until required. This enzyme preparation from extract of benzoate-grown cells was used for the characterization of catechol 1,2-dioxygenase unless otherwise stated.

Thermal Inactivation of Enzyme

Aliquots of enzyme were placed in duplicate eppendorf tubes and incubated in a water bath set at the desired temperature. Samples were removed at various time intervals and immediately cooled in ice-water for 5 min prior to being assayed.

RESULTS

Purification of Catechol 1,2-Dioxygenase from *R. rhodochrous* N75

Purification of catechol 1,2-dioxygenase was attempted from both benzoate-grown and *p*-toluate-grown cells of *R. rhodochrous* N75 in order to determine if any isofunctional enzymes were induced when the cells were grown at the expense of *p*-toluate as a sole carbon source. All the chromatographic methods attempted resulted in very similar chromatograms for the extracts from both cell types and always produced a single peak of catechol 1,2-dioxygenase activity. The overall purification obtained was 43-fold from benzoate-grown cells and 82-fold from *p*-toluate-grown cells. The results are summarized in Table 1.

Table 1. Purification of catechol 1,2-dioxygenase from *R. rhodochrous* N75.

Benzoate-grown cells

Purification step	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Purification fold	Yield (%)	Ratio[a]
Crude extract	150	964	101.7 (75.5)[b]	0.106 (0.078)	1	100	0.74
$(NH_4)_2SO_4$	34	310	77.7 (63.2)	0.250 (0.204)	2.4 (2.6)	76 (84)	0.82
DEAE Toyopearl	20	62	52.1 (43.2)	0.840 (0.696)	7.9 (8.9)	51 (57)	0.83
Sephacryl S-100	14	42	39.3 (32.3)	0.929 (0.764)	8.8 (9.8)	39 (43)	0.82
Mono Q	6	2.6	11.9 (9.8)	4.598 (3.796)	43.4 (48.7)	12 (13)	0.83

p-Toluate-grown cells

Purification step	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Purification fold	Yield (%)	Ratio[a]
Crude extract	148	1,018	92.5 (83.8)[b]	0.091 (0.082)	1	100	0.90
$(NH_4)_2SO_4$	152	391	59.6 (58.7)	0.152 (0.150)	1.7 (1.8)	65 (70)	0.99
DEAE Toyopearl	35	116	95.5 (89.6)	0.826 (0.764)	9.1 (9.3)	103 (107)	0.93
Sephacryl S-100	8	21.5	42.5 (39.2)	1.973 (1.787)	21.7 (21.8)	46 (47)	0.91
Mono Q	6	2.8	20.9 (19.0)	7.481 (6.795)	82.2 (82.8)	23 (23)	0.91

[a]Ratios are given as relative activities for 4-methylcatechol to catechol.
[b]Figures in parentheses are activities for 4-methylcatechol.

When samples of purified enzyme obtained from the Mono Q chromatography step from both benzoate- and *p*-toluate-grown cells were combined, desalted, and re-applied to a Mono Q column, only a single peak of catechol 1,2-dioxygenase activity was observed. In addition, almost identical elution profiles were found for all the chromatographic steps attempted when the enzyme activity was assayed with 4-methylcatechol rather than catechol (data not shown). The ratio of catechol 1,2-dioxygenase activity against catechol to 4-methylcatechol remained constant throughout all the purification steps though the enzyme purified from *p*-toluate-grown cells showed higher relative activities against 4-methylcatechol (Table 1). These results imply that the enzyme activity against 4-methylcatechol was not due to that of an isozyme and hence a single catechol 1,2-dioxygenase was induced during the growth on benzoate or *p*-toluate as a sole carbon source.

M_r and Subunit Composition

The purity of purified catechol 1,2-dioxygenase was examined by SDS-PAGE, which showed one major band with a M_r of 35,000 from either benzoate- or *p*-toluate-grown cells though there were a few contaminating proteins (Fig. 1). In order to verify the identity of the enzyme subunit, gel slices excised from non-denaturing polyacrylamide gel were tested for enzyme activity and then re-applied to SDS-PAGE. The major band in the non-denaturing polyacrylamide gel displayed catechol 1,2-dioxygenase activity when excised from the gel. SDS-PAGE analysis of the gel slice containing catechol 1,2-dioxygenase activity resulted in a single band which also possessed a M_r of 35,000.

The native molecular weight of active catechol 1,2-dioxygenase was estimated by gel filtration chromatography, with reference to standard proteins of known M_r. Catechol 1,2-dioxygenase eluted with an apparent M_r of 71,000,

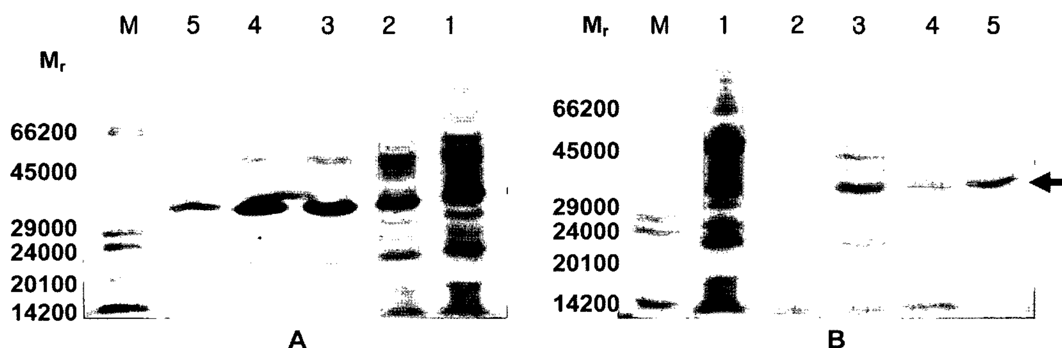

Fig. 1. SDS-PAGE analysis of purification of catechol 1,2-dioxygenase.
Purification from benzoate-grown cells (**A**) and *p*-toluate-grown cells (**B**): Lane M, M_r markers; lane 1, crude extract; lane 2, $(NH_4)_2SO_4$ step; lane 3, DEAE-Toyopearl step; lane 4, Sephacryl S-100 step; lane 5, Mono Q step.

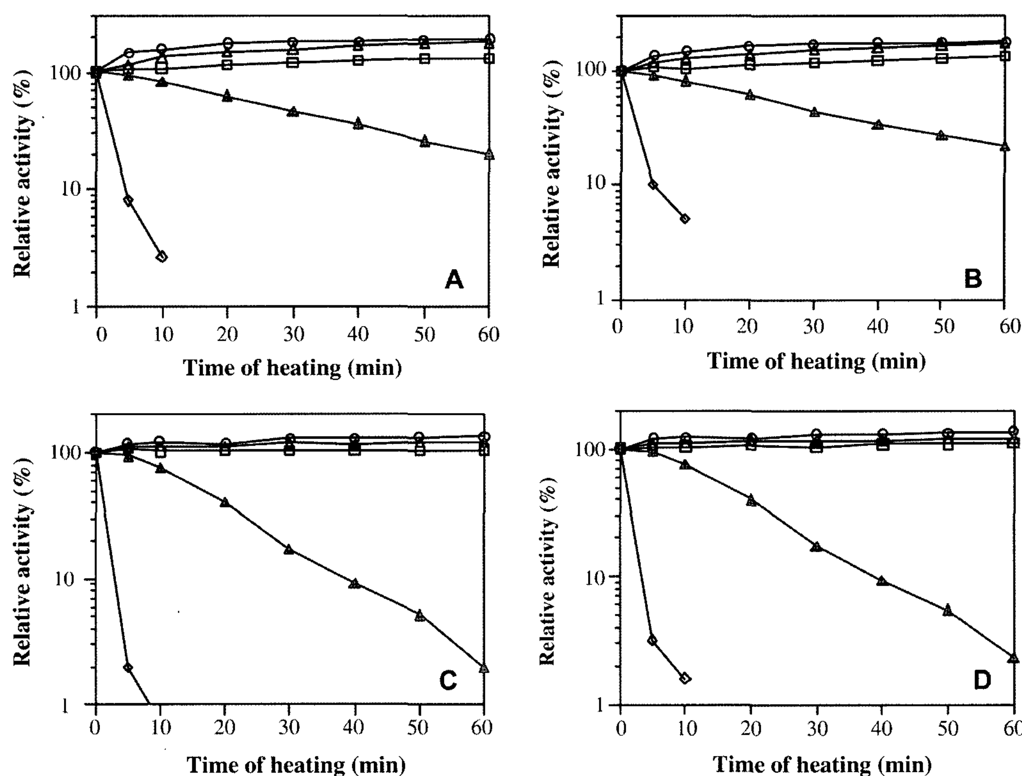

Fig. 2. Time course of thermal inactivation of catechol 1,2-dioxygenases.
Purified samples of enzyme from benzoate-grown cells (**A** and **B**) and *p*-toluate-grown cells (**C** and **D**) were incubated as described in Materials and Methods at 30°C (□), 40°C (△), 50°C (○), 55°C (▲) and 60°C (◇). Catechol 1,2-dioxygenase activities were measured with catechol (**A** and **C**) and 4-methylcatechol (**B** and **D**).

suggesting that the active enzyme exists as a dimer of two identical subunits.

Thermal Inactivation of Catechol 1,2-Dioxygenase

The purified catechol 1,2-dioxygenases from benzoate- and *p*-toluate-grown cells were subjected to thermal inactivation. The inactivation profiles were similar for both sources of the purified enzyme (Fig. 2). When the enzyme activity was assayed with 4-methylcatechol as substrate, the inactivation profiles were almost identical to those for catechol, further suggesting that no isozyme of catechol 1,2-dioxygenase was induced. Surprisingly, the enzyme activities increased during the course of incubations peaking at 50°C. The half-life of the enzyme activity at 55°C was 27 min for benzoate-grown cells and 14 min for *p*-toluate-grown cells.

pH Optimum

The pH optimum of the catechol 1,2-dioxygenase reaction was determined by measuring activity, with substrate levels as in the standard assay, in 50 mM MOPS, 50 mM Tris-HCl, or 50 mM Bis-tris-propane with the pH adjusted with NaOH or HCl to various levels between 6.5 and 9.5. The pH profile for activity of the purified catechol 1,2-dioxygenase is shown in Fig. 3. The maximum activity was observed at pH 8 in 50 mM Bis-tris-propane buffer and 50 mM Tris-HCl buffer. The enzyme displayed high activity over a broad pH range (pH 6.5 to 9.5).

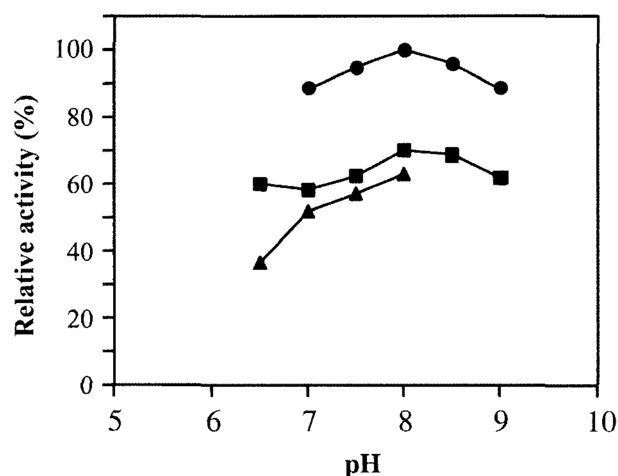

Fig. 3. pH Profile of catechol 1,2-dioxygenase activity.
The enzyme activity was determined in the following buffers; 50 mM Bis-tris-propane (■), 50 mM MOPS (▲), and 50 mM Tris-HCl (●).

Table 2. Effect of enzyme-inactivating agents on catechol 1,2-dioxygenase.

Reagent	Activity[a] (%)
Control	100
Sodium dithionite (1 mM)	64
Reduced Glutathione (1 mM)	116
$HgCl_2$ (1 mM)	48
$AgNo_3$ (1 mM)	92
$CuSO_4$ (0.1 mM)	98
Iodoacetamide (0.1 mM)	88
p-CMB (0.1 mM)	84
α,α'-Dipyridyl (0.1 mM)	106
o-Phenanthroline (0.05 mM)	101
Tiron[b] (0.5 mM)	80

[a]Activities are represented as a percentage of the activity of a control which was incubated in the same buffer without inhibitors.
[b]Tiron is disodium-1,2-dihydroxybenzene-3,5-disulphonate.

Effect of Various Enzyme-Inhibiting Agents

Dilute catechol 1,2-dioxygenase (approximately 3.5 µg/ml) was incubated for 5 min in 50 mM Tris-HCl buffer (pH 8.0) containing the compounds tested, and the activity was then measured by the addition of 0.3 mM catechol. The results of inhibition studies are summarized in Table 2. Sodium dithionite significantly inhibited the enzyme activity as it reduced the concentration of molecular oxygen. Low concentrations of chelating agents for ferrous ion such as α,α'-dipyridyl and *o*-phenanthroline did not show any inhibition. In contrast, a slight inhibition by Tiron, a chelating agent for ferric iron, was observed, suggesting that catechol 1,2-dioxygenase from *R. rhodochrous* N75 also contained Fe^{3+} like other catechol 1,2-dioxygenases. Sulfhydryl agents such as iodoacetamide and *p*-CMB showed only a small degree of inhibition. Amongst the heavy metals tested, only Hg^{2+} inhibited the enzyme activity.

Table 3. Substrate specificity of catechol 1,2-dioxygenases.

Substrate	Activity[a] (%)
Catechol (control)	100
3-Methylcatechol	64
4-Methylcatechol	76
4-Ethylcatechol	42
4-*n*-Propylcatechol	17
4-*iso*-Propylcatechol	23
4-*tert*-Butylcatechol	9
4-Hexylcatechol	2
3,4-Dimethylcatechol	21
3,5-Dimethylcatechol	36
3,4,5-Trimethylcatechol	5
3,4,6-Trimethylcatechol	8
3-Chlorocatechol	6
4-Chlorocatechol	6
4-Bromocatechol	4
4-Fluorocatechol	65

[a]Enzyme activity was assayed polarographically by measuring the oxygen consumption using Clarke oxygen electrode, as described in materials and methods. Activities are quoted as a percentage of the activity of a control in which catechol was used as substrate.

Table 4. Kinetic parameters of catechol 1,2-dioxygenase.

Substrate	Apparent V_{max} (U/mg)	Apparent K_m[a] (iM)	V_{max}/K_m
Catechol	5.392±0.161	0.690±0.067	7.814
4-Methylcatechol	4.437±0.035	1.346±0.042	3.296
4-Ethylcatechol	nd[b]	49.4±15.7[a]	nd
4-*iso*-Propylcatechol	nd	27.0±11.1[a]	nd
4-Chlorocatechol	0.306±0.007	0.210±0.033	1.457
4-Bromocatechol	nd	0.581±0.058	nd
4-Fluorocatechol	3.492±0.122	0.604±0.104	5.781

[a]K_m values for 4-ethylcatechol and 4-*iso*-propylcatechol were determined by polarographic assays using 5–50 µM substrate. The other parameters were determined by spectrophotometric assays using 0.3–30 µM substrate.
[b]not determined.

Substrate Specificity

A wide range of catecholic substrates were tested as substrates (Table 3), and catechol 1,2-dioxygenase from *R. rhodochrous* N75 was found to exhibit broad substrate specificity. The enzyme retained its activity against a range of alkyl-substituted catechols though steric hindrance by side chain or bulky alkyl-substituents was evident. Poor activity was observed against the halocatechols with the exception of 4-fluorocatechol.

Kinetic Properties

Michaelis-Menten kinetic parameters were determined for a range of catechols using the Grafit software package (Erithacus Software Ltd., Staines, U.K.) (Table 4). Alkyl-substituted catechols exhibited higher apparent K_m values than that of catechol, suggesting that alkyl-substituents caused steric influences upon enzyme-substrate binding. Although halocatechols were very poor substrates with the exception of 4-fluorocatechol, they were bound even better than catechol itself, showing lower apparent K_m values. This was also confirmed by inhibition studies, which indicated that halocatechols acted as powerful competitive inhibitors (data not shown).

Extradiolic Activity of Catechol 1,2-Dioxygenase from *Rhodococcus rhodochrous* N75

Catechol 1,2-dioxygenase from *R. rhodochrous* N75 was also found to exhibit catechol 2,3-dioxygenase activity with 4-methylcatechol whereby the enzyme cleaved the aromatic ring extradiolically. Catechol 2,3-dioxygenase activity was assayed spectrophotometrically by means of increase in A_{382} using the same assay mixture as that for catechol 1,2-dioxygenase activity described in Materials and Methods with the omission of EDTA. The molar absorption

coefficient for 4-methylcatechol at 382 nm was 28100 (litre·mol^{-1}·cm^{-1}). The extradiolic activity was approximately 0.6% of intradiolic activity. Attempts with 3-methylcatechol, which has been known to be extradiolically cleaved by other catechol 1,2-dioxygenases, did not produce any activity. In the absorption spectrum of the reaction product of catechol 1,2- dioxygenase with 4-methylcatechol, a major peak observed at 255 nm can be attributed to 3-methyl-*cis,cis*-muconate and a very small peak observed at 384 nm of which the absorption increased at alkaline pH values is thought to be 2-hydroxy-5-methylmuconic semialdehyde, the product from extradiolic activity of the enzyme. The same spectra were observed with culture supernatants following the growth of this organism at the expense of *p*-toluate.

DISCUSSION

Catechol 1,2-dioxygenase, which initiates the catabolism of aromatic compounds, has been shown to exist in some bacteria and fungi as isozymes. Three isozymes of catechol 1,2-dioxygenase were found in *P. arvilla* C-1 though their physiological role was not explained [24]. The isozymes, αα, ββ and αβ, which were composed by different combinations of two non-identical subunits, α and β, were separated by DEAE-Toyopearl anion exchange chromatography. Isozymes of catechol 1,2-dioxygenases are more commonly known to be induced for the degradation of halo-substituted catechols which were poor substrates for the classical catechol 1,2-dioxygenase induced by non-substituted aromatic compounds [3, 9, 17, 29]. Another type of isozyme of catechol 1,2-dioxygenase was found to be induced in the fungus *T. cutaneum* when the fungus was grown at the expense of *p*-cresol [27]. The isozyme, referred to as 4-methylcatechol 1,2-dioxygenase, showed a higher activity against 4-methylcatechol than catechol and consisted of two non-identical subunits both of which were different from the subunits of the enzyme induced with phenol. The discovery of 4-methylcatechol 1,2-dioxygenase indicated that this enzyme might play a major role in the cleavage of methyl-substituted aromatic rings and further implied that a different enzymic route might be present for the catabolism of methyl-substituted aromatic compounds in this organism.

The genus *Rhodococcus* has been well known not only to degrade a range of xenobiotic compounds [1, 7, 15], but to metabolize alkyl-substituted catechols [5, 6]. *R. rhodochrous* N75 is similar to *T. cutaneum* in that it is capable of growth on an analogous range of methyl-substituted aromatic compounds, which raised the possibility that isozymes of catechol 1,2-dioxygenase might be also induced in *R. rhodochrous* N75. The catechol 1,2-dioxygenase from *R. rhodochrous* N75 was, therefore, purified from benzoate- and *p*-toluate-grown cells and characterized. Purification profiles and characterisation of the enzymes from both cultures implied that a single catechol 1,2-dioxygenase was probably induced at the expense of either benzoate or *p*-toluate. Enzyme preparations from both cultures could not be separated on Mono Q chromatography and showed an identical enzyme subunit and native M_r. Although these results imply that a single catechol 1,2-dioxygenase is produced by this strain, some slight differences were observed in the ratio of activity for catechol to 4-methylcatechol and in the half-life of the enzyme from both preparations, which may be due to some impurities present in enzyme preparations.

Catechol 1,2-dioxygenase from *R. rhodochrous* N75 exhibited some interesting properties. The enzyme showed broad specificity for its substrate, especially towards alkyl-substituted catechols. Interestingly, the enzyme was observed to display extradiolic activity with 4-methylcatechol but not with 3-methylcatechol unlike a number of other catechol 1,2-dioxygenases [9–11]. The extradiol activity displayed by catechol 1,2-dioxygenase of *R. rhodochrous* N75 is presumed to be a common feature for the enzymes of a number of nocardioform actinomycetes [20].

Although catechol 1,2-dioxygenases from other *Rhodococcus* strains have been studied [22, 28, 30], the enzyme from *R. rhodochrous* N75 was found to differ in certain aspects from the enzymes of other organisms reported so far. Other catechol 1,2-dioxygenases induced by non-substituted aromatic growth substrates generally display a narrow substrate specificity, whereas Rhodococcal catechol 1,2-dioxygenase is an exception since it showed a relatively broad substrate specificity. It also exhibited lower K_m values for halocatechols than that of catechol. In addition, activity against 4-fluorocatechol was relatively high. High affinities for halocatechols and broad substrate specificities are known to be characteristic of chlorocatechol 1,2-dioxygenases (catechol 1,2-dioxygenase II) [3, 9, 17, 25, 29]. However, catechol 1,2-dioxygenase from *R. rhodochrous* N75 apparently showed dissimilarities to chlorocatechol 1,2-dioxygenases since it possessed poor activities for halocatechols and a lower affinity and activity for 4-methylcatechol than those for catechol. It is noteworthy that catechol 1,2-dioxygenase from *R. rhodochrous* N75 differs in M_r, subunit composition and relative affinity for 4-chlorocatechol, even from those of the enzyme from chlorophenol-degrading *R. erythropolis* 1CP, where catechol 1,2-dioxygenase I (C12D I) and II (C12D II) are induced when this strain is grown at the expense of benzoate and 4-chlorophenol, respectively. In *R. rhodochrous* N75, the enzyme consists of a single subunit of 35 kDa, whereas C12D I has two subunits of 33.5 kDa and 35 kDa, and C12D II a single subunit of 26.5 kDa in *R. erythropolis* [17]. Therefore, in terms of classification, catechol 1,2-dioxygenase from *R. rhodochrous* N75 appears to

Table 5. Different types of catechol 1,2-dioxygenases.

Organism	Type	Substrate specificity	Relative activity[a]	Relative K_m values[b]	Subunit M_r	Reference
Pseudomonas B 13	type I	narrow	4CC (96), 4FC (148), 4MC (316)	C<4CC<4MC	ns[c]	[8]
Pseudomonas B 13	type II	broad	4CC (11), 4FC (30), 4MC (92)	4CC<C<4MC	ns	[8]
Pseudomonas putida	type II	broad	4CC (50), 4MC (187)	C<4MC<4CC	33,000	[20]
A. eutrophus B9	type I	narrow	4CC (7), 4FC (23), 4MC (41)	C<4CC<4MC	ns	[8]
R. erythropolis 1CP	type I	narrow	4CC (3)	C<4CC	33,500 and 35,000	[12]
R. erythropolis 1CP	type II	broad	4CC (95), 4FC (212), 4MC (242)	4CC<4MC<C	26,500	[12]
R. rhodochrous N75		broad	4CC (6), 4FC (65), 4MC (76)	4CC<C<4MC	35,000	This study

[a]Relative activities are quoted as a percentage of the activity for catechol; 4CC=4-chlorocatechol, 4FC=4-fluorocatechol and 4MC=4-methylcatechol.
[b]C=K_m value for catchcol, 4CC=K_m value for 4-chlorocatechol and 4MC=K_m value for 4-methylcatechol.
[c]not shown in reference.

stand in-between catechol 1,2-dioxygenase (type I) and chlorocatechol 1,2-dioxygenase (type II) (Table 5). The complete nucleotide sequence and structural studies would reveal its mechanical, structural and evolutionary relatedness to other catechol 1,2-dioxygenases.

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REFERENCES

1. Ahn, T.-S., G.-H. Lee, and H.-G. Song. 2005. Biodegradation of phenanthrene by psychrotrophic bacteria from lake Baikal. *J. Microbiol. Biotechnol.* **15:** 1135–1139.
2. An, H. R., H. H. Park, and E. S. Kim. 2001. Cloning and expression of thermophilic catechol 1,2-dioxygenase gene (*catA*) from *Streptomyces setonii. FEMS Microbiol. Lett.* **195:** 17–22.
3. Broderick, J. B. and T. V. O'Halloran. 1991. Overproduction, purification, and characterization of chlorocatechol dioxygenase, a non-heme iron dioxygenase with broad substrate tolerance. *Biochemistry* **30:** 7349–7358.
4. Bruce, N. C. and R. B. Cain. 1988. Beta-methylmuconolactone, a key intermediate in the dissimilation of methylaromatic compounds by a modified 3-oxoadipate pathway evolved in nocardioform actinomycetes. *FEMS Microbiol. Lett.* **50:** 233–239.
5. Cha, C. J. and N. C. Bruce. 2003. Stereo- and regiospecific *cis,cis*-muconate cycloisomerization by *Rhodococcus rhodochrous* N75. *FEMS Microbiol. Lett.* **224:** 29–34.
6. Cha, C. J., R. B. Cain, and N. C. Bruce. 1998. The modified beta-ketoadipate pathway in *Rhodococcus rhodochrous* N75: Enzymology of 3-methylmuconolactone metabolism. *J. Bacteriol.* **180:** 6668–6673.
7. Choi, J.-H., T.-K. Kim, Y.-M. Kim, W.-C. Kim, G.-J. Joo, K. Y. Lee, and I.-K. Rhee. 2005. Cloning and characterization of cyclohexanol dehydrogenase gene from *Rhodococcus* sp. TK6. *J. Microbiol. Biotechnol.* **15:** 1189–1196.
8. Dorn, E. and H. J. Knackmuss. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. *Biochem. J.* **174:** 85–94.
9. Dorn, E. and H. J. Knackmuss. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. *Biochem. J.* **174:** 73–84.
10. Fujiwara, M., L. A. Golovleva, Y. Saeki, M. Nozaki, and O. Hayaishi. 1975. Extradiol cleavage of 3-substituted catechols by an intradiol dioxygenase, pyrocatechase, from a Pseudomonad. *J. Biol. Chem.* **250:** 4848–4855.
11. Hou, C. T., R. Patel, and M. O. Lillard. 1977. Extradiol cleavage of 3-methylcatechol by catechol 1,2-dioxygenase from various microorganisms. *Appl. Environ. Microbiol.* **33:** 725–727.
12. Jang, J. Y., D. Kim, H. W. Bae, K. Y. Choi, J. C. Chae, G. J. Zylstra, Y. M. Kim, and E. Kim. 2005. Isolation and characterization of a *Rhodococcus* species strain able to grow on *ortho-* and *para*-xylene. *J. Microbiol.* **43:** 325–330.
13. Kim, J. S., J. H. Kim, E. K. Ryu, J. K. Kim, C. K. Kim, I. G. Hwang, and K. Lee. 2004. Versatile catabolic properties of Tn4371-encoded *bph* pathway in *Comamonas testosteroni* (formerly *Pseudomonas* sp.) NCIMB 10643. *J. Microbiol. Biotechnol.* **14:** 302–311.
14. Klecka, G. M. and D. T. Gibson. 1981. Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. *Appl. Environ. Microbiol.* **41:** 1159–1165.
15. Larkin, M. J., L. A. Kulakov, and C. C. Allen. 2005. Biodegradation and *Rhodococcus*-masters of catabolic versatility. *Curr. Opin. Biotechnol.* **16:** 282–290.
16. Maltseva, O. V., I. P. Solyanikova, and L. A. Golovleva. 1991. Catechol 1,2-dioxygenases of a chlorophenol-degrading strain of *Rhodococcus erythropolis*: Purification and properties. *Biochemistry (Mosc)* **56:** 1548–1555.
17. Maltseva, O. V., I. P. Solyanikova, and L. A. Golovleva. 1994. Chlorocatechol 1,2-dioxygenase from *Rhodococcus erythropolis* 1CP. Kinetic and immunochemical comparison

with analogous enzymes from gram-negative strains. *Eur. J. Biochem.* **226:** 1053–1061.
18. Mars, A. E., J. Kingma, S. R. Kaschabek, W. Reineke, and D. B. Janssen. 1999. Conversion of 3-chlorocatechol by various catechol 2,3-dioxygenases and sequence analysis of the chlorocatechol dioxygenase region of *Pseudomonas putida* GJ31. *J. Bacteriol.* **181:** 1309–1318.
19. Matsumura, E., S. Ooi, S. Murakami, S. Takenaka, and K. Aoki. 2004. Constitutive synthesis, purification, and characterization of catechol 1,2-dioxygenase from the aniline-assimilating bacterium *Rhodococcus* sp. AN-22. *J. Biosci. Bioeng.* **98:** 71–76.
20. Miller, D. J. 1979. Aromatic metabolism in nocardioform actinomycetes. PhD Thesis, University of Kent.
21. Moiseeva, O. V., I. P. Solyanikova, S. R. Kaschabek, J. Groning, M. Thiel, L. A. Golovleva, and M. Schlomann. 2002. A New modified *ortho* cleavage pathway of 3-chlorocatechol degradation by *Rhodococcus opacus* 1CP: Genetic and biochemical evidence. *J. Bacteriol.* **184:** 5282–5292.
22. Murakami, S., N. Kodama, R. Shinke, and K. Aoki. 1997. Classification of catechol 1,2-dioxygenase family: sequence analysis of a gene for the catechol 1,2-dioxygenase showing high specificity for methylcatechols from Gram$^+$ aniline-assimilating *Rhodococcus erythropolis* AN-13. *Gene* **185:** 49–54.
23. Murakami, S., C. L. Wang, A. Naito, R. Shinke, and K. Aoki. 1998. Purification and characterization of four catechol 1,2-dioxygenase isozymes from the benzamide-assimilating bacterium *Arthrobacter* species BA-5-17. *Microbiol. Res.* **153:** 163–171.
24. Nakai, C., K. Horiike, S. Kuramitsu, H. Kagamiyama, and M. Nozaki. 1990. Three isozymes of catechol 1,2-dioxygenase (pyrocatechase), alpha alpha, alpha beta, and beta beta, from *Pseudomonas arvilla* C-1. *J. Biol. Chem.* **265:** 660–665.
25. Ngai, K. L. and L. N. Ornston. 1988. Abundant expression of *Pseudomonas* genes for chlorocatechol metabolism. *J. Bacteriol.* **170:** 2412–2413.
26. Park, D. W., K. Lee, J. C. Chae, K. Kudo, and C. K. Kim. 2004. Genetic structure of *xyl* gene cluster responsible for complete degradation of (4-chloro)benzoate from *Pseudomonas* sp. S-47. *J. Microbiol. Biotechnol.* **14:** 483–489.
27. Powlowski, J. B., J. Ingebrand, and S. Dagley. 1985. Enzymology of the beta-ketoadipate pathway in *Trichosporon cutaneum*. *J. Bacteriol.* **163:** 1136–1141.
28. Solyanikova, I., E. Golovlev, O. Lisnyak, and L. Golovleva. 1999. Isolation and characterization of catechol 1,2-dioxygenases from *Rhodococcus rhodnii* strain 135 and *Rhodococcus rhodochrous* strain 89: Comparison with analogous enzymes of the ordinary and modified *ortho*-cleavage pathways. *Biochemistry (Mosc)* **64:** 824–831.
29. Solyanikova, I. P., O. V. Maltseva, and L. A. Golovleva. 1992. Purification and properties of catechol 1,2-dioxygenase II from *Pseudomonas putida* 87. *Biochemistry (Mosc)* **57:** 1310–1316.
30. Strachan, P. D., A. A. Freer, and C. A. Fewson. 1998. Purification and characterization of catechol 1,2-dioxygenase from *Rhodococcus rhodochrous* NCIMB 13259 and cloning and sequencing of its *catA* gene. *Biochem. J.* **333:** 741–747.