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Cloning and Sequence Analysis of Hydroxyquinol 1,2-Dioxygenase Gene in 2,4,6-Trichlorophenol-Degrading *Ralstonia pickettii* DTP0602 and Characterization of Its Product

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A gene encoding hydroxyquinol 1,2-dioxygenase was cloned from 2,4,6-trichlorophenol-degrading *Ralstonia* (*Pseudomonas*) *pickettii* strain DTP0602. Cell-free extracts of *Escherichia coli* containing a cloned 1.4-kb *Stu*I-*Xho*I DNA fragment of *R. pickettii* DTP0602 hydroxyquinol 1,2-dioxygenase converted hydroxyquinol into maleylacetate and also degraded 6-chlorohydroxyquinol. The 1.4-kb DNA fragment contained one open reading frame (designated *hadC*) composed of 948 nucleotides. The molecular mass of 34,591 deduced from the gene product (HadC) was in agreement with the size (35 kDa) of the purified HadC protein determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amino acid sequence of HadC exhibited high homology to that of the hydroxyquinol 1,2-dioxygenase of 2,4,5-trichlorophenoxyacetic acid-degrading *Burkholderia cepacia* AC1100 (Daubaras, D. L. *et al.*, Appl. Environ. Microbiol., 61, 1279–1289, 1995). The active enzyme had a molecular mass of 68 kDa, suggesting that it is functional as a homodimer. The enzyme also catalyzed the oxidation of pyrogallol and 3-methylcatechol, possible intermediates in the degradation of 2,4,6-trichlorophenol, in addition to 6-chlorohydroxyquinol and hydroxyquinol. The dioxygenase catalyzed both *ortho*- and *meta*-cleavage of 3-methylcatechol.

[**Key words:** *Ralstonia pickettii*, 2,4,6-trichlorophenol, chlorophenol, hydroxyquinol, hydroxyquinol 1,2-dioxygenase]

Polychlorinated phenols, including pentachlorophenol and 2,4,6-trichlorophenol (2,4,6-TCP) are used as preservatives in the wood industry. Because of their resistance to microbial degradation and their broad toxicity, they have become widespread environmental pollutants. Several microorganisms with the ability to degrade these compounds have been isolated (1–7).

We have isolated *Ralstonia* (*Pseudomonas*) *pickettii* strain DTP0602, which is capable of mineralizing 2,4,6-TCP, and identified 2,6-dichrolohydroquinone as a first metabolic intermediate (4). In a previous study, we cloned two 2,4,6-TCP 4-dechlorinase (hydroxylase) genes (*hadAB*) from the chromosome of DTP0602 which is responsible for the first 2,4,6-TCP degradation step (8). Recently, we found that the combination of purified HadA and HadB proteins catalyzes the conversion of 2,4,6-TCP to 6-chlorohydroxyquinol (6-CHQ) (unpublished data). These findings suggest that 6-CHQ is an intermediate in the degradation of *R. pickettii* DTP0602. We also found that a derivative of *R. pickettii* DTP0602, into which Tn*5* was inserted just downstream of the *hadB* gene, did not degrade 2,4,6-TCP. This mutant with the Tn*5* insert accumulated a bright brown compound which was conjectured to be an oxidized form of 6-CHQ or hydroxyquinol (HQ) (9). These observations suggest that the region into which Tn*5* was inserted encodes an enzyme that is responsible for the degradation of HQ derivative.

HQ and its derivatives are known to be key intermediates prior to ring fission in the microbial degradation of aromatic compounds, including 2,4,6-TCP (10, 11), pentachlorophenol (12), 2,4,5-trichlorophenoxyacetate (2,4,5-T) (4, 13), 2,4-dichlorophenoxyacetate (2,4-D) (13), vanillate, 2,4-dinitrotoluene, 2,4-dichlorophenol (14), salicylate (15), 4-nitroanisole (16), phenol (17), and 4-nitorophenol (18). It is believed that HQ-metabolizing enzymes play an important role in the microbial degradation of various aromatic compounds.

HQ 1, 2-dioxygenases have been purified and characterized from the 2,4,6-TCP degraders *Azotobacter* sp. GP1 (10) and *Streptomyces rochei* 303 (11), and from a 2,4-D degrader, *Nocardioides simplex* 3E (13), but genetic studies on the HQ 1,2-dioxygenases of these degraders have not been reported. In 2,4,5-T-degrading *B. cepacia* AC1100, the *tftH* gene encoding HQ 1,2-dioxygenase was cloned and analyzed (19), and the enzyme was characterized (20). In fungi, HQ 1,2-dioxygenase of *Phanerochaete chrysosporium* (14) and *Trichosporon cutaneum* (15), which are involved in the metabolism of vanillate and salicylate, respectively, have been purified and characterized.

In this study, we found that the gene encoding HQ 1,2-dioxygenase (*hadC*) was located just downstream of the *hadAB* genes in the 2,4,6-TCP degrader *R. pickettii* DTP0602. We describe the characterization of the *hadC* gene and the encoded enzyme involved in 2,4,6-TCP degradation in *R. pickettii* DTP0602.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The isolation and characterization of *R. pickettii* DTP0602 have been described previously (4). *E. coli* JM109 *recA1, endA1, gryA96, thi, hsdR17, supE44,*

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relA1, Δ(*lac-proAB*), /F'[*traD36, proAB*$^+$, *lacI*^{q}, *lacZ* ΔM15] was used as a host strain for DNA manipulation and for overproducing the gene product. *E. coli* strains were routinely grown at 37°C in Luria broth (LB) (10 g Bacto-tryptone [Difco], 5 g yeast extract, and 5 g NaCl per liter) containing 100 μg ampicillin per ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used at concentrations of 110 and 40 μg/ml, respectively.

DNA manipulation and cloning of the *hadC* gene Total DNA of *R. pickettii* DTP0602 was prepared as described by Marmur (21). Plasmid DNA was purified by alkaline lysis followed by polyethylene glycol precipitation, as described by Sambrook *et al.* (22).

The total DNA of DTP0602 was digested with *Ban*III and the resulting approximately 8-kb fragments, which were recovered by agarose gel electrophoresis, were ligated with pBluescript II KS$^+$ and introduced into *E. coli* JM109 cells by the $CaCl_2$ procedure. Transformants lacking β-galactosidase activity were selected on LB agar plates containing ampicillin, X-Gal, and IPTG. Clones containing the *hadB* gene were selected by dot blot hybridization with the 0.6-kb *Sal*I-*Eco*RI fragment containing *hadB* as a probe. Southern blot analysis was performed as described by Southern (23) using a Hybond N$^+$ nylon membrane filter (Amersham International, Buckinghamshire, UK). DNA sequencing was carried out by the dideoxy chain-termination method of Sanger *et al.* (24).

Preparation of cell-free extracts and enzyme assays The plasmid pTC14, which was constructed by ligating a 1.4-kb *Stu*I–*Xho*I fragment containing the *hadC* gene into a multicopy *tac* promoter vector, pTTQ18 (Amersham), was introduced into *E. coli* JM109. The cells were grown to an optical density at 600 nm of 1.0 in a 2-*l* LB containing 250 μg ampicillin per ml. Expression of the *hadC* gene was induced with IPTG. After 4-h induction, cells were collected by centrifugation at 5000×*g* for 10 min. The collected cells were washed twice with and resuspended in 25 mM phosphate buffer (pH 7.5) containing 7.5% *n*-propanol, 10% glycerol, and 1 mM mercaptoethanol (buffer A). The cell suspension was passed twice through a French pressure cell (Aminco, Urbana, IL, USA) at 90 MPa. The product was centrifuged at 12,000×*g* for 30 min and then at 105,000×*g* for 60 min. The supernatant was used as a cell-free extract for assaying HQ 1,2-dioxygenase. Enzymatic activity was measured by following the formation of *ortho*-cleavage reaction products at 243 nm with a Shimadzu (Tokyo) UV 160A spectrophotometer. The activity was measured at 25°C in 10 mM phosphate buffer (pH 7.2) containing 0.2 mM hydroxyquinol, 0.5 mM $FeSO_4$, and enzyme solution. One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate per min. The molar extinction coefficient of the product under the assay condition was taken to be 4440 $cm^{-1} \cdot M^{-1}$ (25).

The relative enzyme activities were determined by the extinction coefficients of the *ortho*-ring cleavage products of 6-chlorohydroxyquinol (λ_{max}, 253; ε, 9470 $cm^{-1} \cdot M^{-1}$) (25), the *ortho*-ring cleavage products of 3-methylcatechol (λ_{max}, 260 nm; ε, 18,000 $cm^{-1} \cdot M^{-1}$) (26), the *meta*-ring cleavage product of 3-methylcatechol (λ_{max}, 388 nm; ε, 32,000 $cm^{-1} \cdot M^{-1}$) (27), and pyrogallol (λ_{max}, 280 nm; ε, 575 $cm^{-1} \cdot M^{-1}$).

Purification of HQ 1,2-dioxygenase

DEAE Toyopearl chromatography Crude extract was applied onto a DEAE-Toyopearl 650M (Tosoh, Tokyo) column (50 by 20 cm) previously equilibrated with buffer A. After the column was washed with 600 ml of the same buffer, the enzyme was eluted with 2000 ml of a linear gradient from 0 to 0.4 M KCl. The enzyme was eluted around 0.35 M KCl.

Mono Q chromatography The fractions containing HQ 1,2-dioxygenase activity eluted from the DEAE-Toyopearl column were pooled and dialyzed against buffer A. The resulting solution was applied onto a Mono Q HR 16/10 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer A. After the column was washed with 60 ml of buffer A, the enzyme was eluted with 400 ml of a linear gradient from 0 to 0.5 M KCl. The enzyme was eluted around 0.4 M KCl.

Gel filtration chromatography The fractions containing enzyme activity eluted from the Mono Q column were pooled, concentrated, and injected into a Superdex HR 10/30 column (Pharmacia Biotech) which was equilibrated with buffer A containing 0.1 M NaCl. The enzyme was eluted with the same buffer, and HQ 1,2-dioxygenase activity was associated with a major protein peak. The fractions containing the enzyme activity were pooled, concentrated, and stored at −80°C until use.

Analytical methods Protein concentration was determined by the Bradford method (28) using bovine gamma globulin as a standard.

The molecular weight was estimated by gel filtration chromatography using a Superdex 200 HR10/30 column (Pharmacia Biotech) on a FPLC system. Elution was carried out using 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.5 ml/min. The molecular weight was estimated based on a standard linear regression curve of reference proteins with molecular weights from 25,000 to 450,000.

The enzyme purity and molecular weight of the subunits were determined by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmili (29). The proteins were stained with Coomassie brilliant blue R250. The molecular weight was calculated from a standard linear regression curve of reference proteins with molecular weights from 14,400 to 94,000.

The NH_2-terminal sequence was determined on a protein sequencer (model 477A; Applied Biosystems, Foster City, CA, USA).

Gas chromatography-mass spectrometry (GC-MS) analysis was performed to identify the reaction products of HQ 1,2-dioxygenase using a model 5971A GC-MS (Hewlett-Packard Co., Palo Alto, CA, USA) with an Ultra-2 capillary column (50 m by 0.2 mm; Hewlett Packard). To separate the metabolites by gas chromatography, the column temperature was increased from 60 to 250°C at a rate of 5 degrees per min.

Identification of reaction products The purified HQ 1,2-dioxygenase (10 units) solution was added to 200 ml 0.01 M potassium phosphate buffer (pH 7.0) containing 100 μmol HQ. This mixture was incubated for 10 min at 30°C. The reaction was stopped by acidification to pH 2 with HCl. The mixture was saturated with NaCl and extracted three times with an equal volume of ethyl acetate, as described by Rieble *et al.* (14). To generate trimethylsilyl (TMS) derivatives, samples were treated with N,O-Bis (trimethylsilyl)-acetamide. The resultant derivatives were subjected to GC-MS analysis as de-

scrived above.

Chemicals 6-CHQ was synthesized following the method described by Lautus *et al.* (10). Hydroxyquinol, catechol, 3-methylcatechol, and pyrogallol were obtained from Wako Pure Chemical Ind. (Osaka). All other chemicals used were of reagent grade.

Nucleotide sequence accession number The nucleotide sequence determined in this study has been submitted to the DDBJ, EMBL, and GenBank databases and will appear under the accession number D86544.

RESULTS

Cloning of the HQ 1,2-dioxygenase gene To obtain the DNA fragment containing the gene specifying an enzyme metabolizing 6-CHQ, an intermediate of 2,4,6-TCP, the DNA fragment containing the *hadB* gene and its downstream region was cloned and analyzed. DTP0602 DNA was digested with *Ban*III, and the digests were hybridized with a *hadB* probe. DNA fragments approximately 8 kb in length were recovered by agarose gel electrophoresis and inserted into pBluescript II KS$^+$. The resulting plasmids were introduced into *E. coli* JM109. Among ampicillin-resistant and white transformants, a single *E. coli* clone (pTC77), which contained a 7.7-kb DNA fragment carrying *hadB*, was selected by dot blot hybridization (Fig. 1).

The 2.5-kb *Sal*I segment of the 7.7-kb *Ban*III DNA was subcloned into pUC18 to obtain pTC25. The DNA sequence of the 2.5-kb *Sal*I fragment showed that it is composed of 2582 nucleotides and includes a part (377 bp) of the *hadB* gene (591 bp); there is only one entire open reading frame (ORF3) in the downstream region. ORF3 is composed of 948 nucleotides and is predicted to encode a protein with a molecular weight of 34,591 and composed of 316 amino acid residues. The guanine and cytosine content of ORF3 is 67.1%, which is close to that of the *hadAB* genes (65.6%) (8). Codon usage in ORF3 resembles that of the *hadAB* gene products. A database search was conducted for proteins homologous to the ORF3 gene product. As indicated in Fig. 2, the amino acid sequence of the ORF3 gene product showed 56.6% identity to that of HQ 1,2-dioxygenase (TftH) of 2,4,5-T-degrading *B. cepacia* AC1100 (19). Other HQ 1,2-dioxygenases have been purified from the 2,4,6-TCP-degraders *Azotobacter* sp. GP1 (10) and *Streptomyces rochei* 303 (11), and their NH_2-terminal amino acid sequences were determined for 40 and 21 residues, respec-

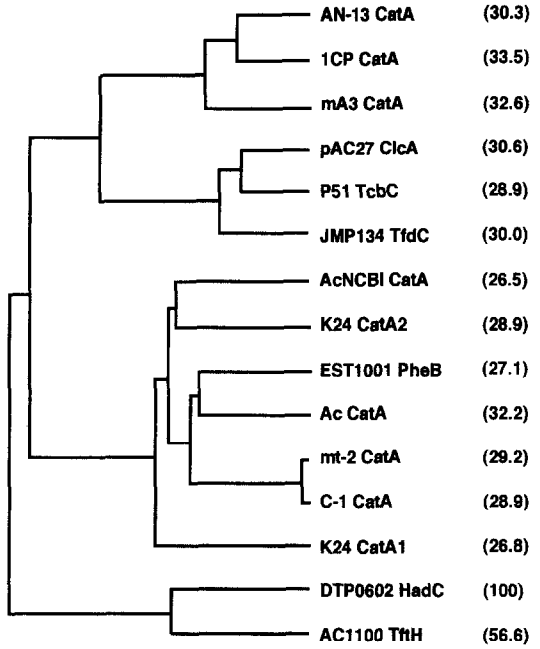

FIG. 2. Phylogenetic tree of HadC and related catechol 1,2-dioxygenases. The tree was deduced from pairwise alignments of amino acid sequences by using the unweighted pair group method. Enzyme abbreviations: AN-13 CatA, *catA* product of *Rhodococcus erythropolis* AN-13 (32); 1CP CatA, *catA* product of *R. erythropolis* 1CP (33); mA3 CatA, *catA* product of *Arthrobacter* sp. mA3 (34); pAC27 ClcA, *clcA* product of *P. putida* (35); P51 TcbC, *tcbC* product of *Pseudomonas* sp. P51 (36); JMP134 TfdC, *tfdC* product of *Alcaligenes eutrophus* JMP134 (37); Ac NCBI CatA, *catA* product of *Acinetobacter calcoaceticus* NCIB8250 (38); K24 CatA2, *catA2* product of *Acinetobacter lwoffii* K24 (39); EST1001 PheB, *pheB* product of *Pseudomonas* sp. EST1001 (40); Ac CatA, *catA* product of *Acinetobacter calcoaceticus* (41); mt-2 CatA, *catA* product of *Pseudomonas putida* mt-2 (42); C-1 CatA, *catA* product of *Pseudomonas arvilla* C-1 (42); K24 CatA1, *catA1* product of *Acinetobacter lwoffii* K24 (39); DTP0602 HadC, *hadC* product of *R. pickettii* DTP0602; AC1100 TftH, *tftH* product of *B. cepacia* AC1100 (19). The percentage of amino acid sequence identity of each enzyme with HadC is shown in parentheses.

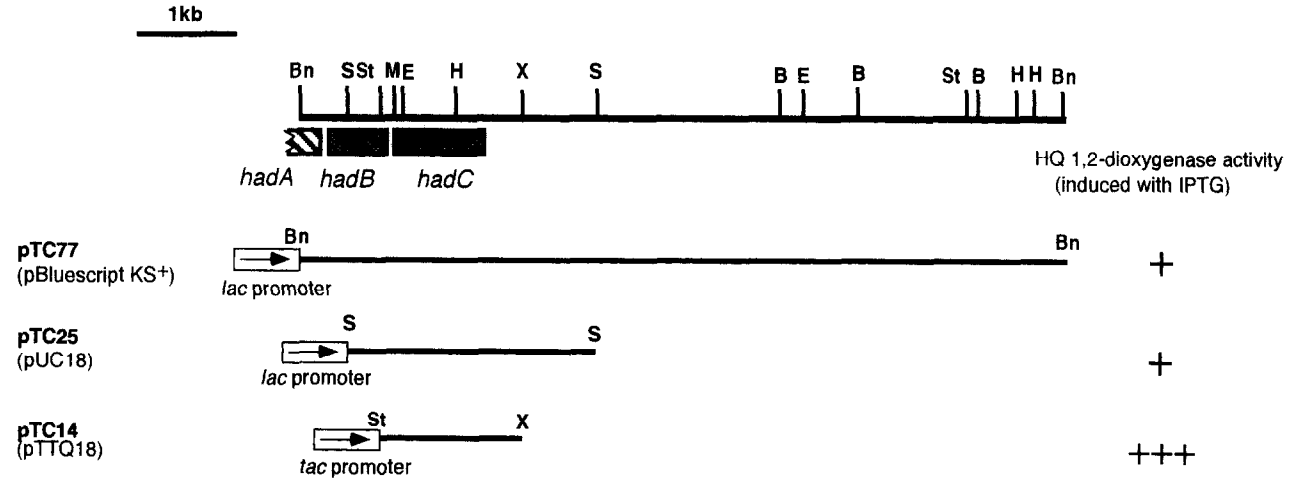

FIG. 1. Subcloning of the gene for HQ 1,2-dioxygenase. The open boxes indicate *lac* and *tac* promoters. B, *Bam*HI; Bn, *Ban*III; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; S, *Sal*I; St, *Stu*I; X, *Xho*I.

tively. The NH_2-terminal amino acid sequence of the ORF3 gene product showed 70.0 and 47.6% identity to those of the *Azotobacter* GP1 and *S. rochei* 303 enzymes, respectively. These observations strongly suggested that the ORF3 gene encodes an HQ 1,2-dioxygenase.

Purification and characterization of HQ 1,2-dioxygenase The cell-free extract of *E. coli* JM109 containing pTC14 rapidly changed the UV spectra of HQ (data not shown). The absorption peak of HQ at 278 nm disappeared quickly and a new peak was observed at 243 nm, which is consistent with that of the *ortho*-cleavage product of HQ (10). Also, when 6-CHQ was used as a substrate, the absorption peak at 278 nm disappeared and gave rise to a peak at 253 nm. The TMS derivatives of the reaction product of HQ was subjected to GC-MS. The mass spectrum of the derivative was consistent with the TMS derivative of maleylacetic acid reported by Rieble *et al.* (14), this observation indicating that the protein encoded by the ORF3 gene is HQ 1,2-dioxygenase. We named the ORF gene *hadC*.

The HQ 1,2-dioxygenase activity in the cell-free extract was unstable and was reduced to less than 15% after 24 h in 10 mM phosphate buffer at 4°C. The enzyme activity was stabilized by adding 7.5% *n*-propanol to the buffer, and 90% of the activity was retained at 4°C for two weeks. The enzyme was purified 28.3-fold, with a yield of 22% (Table 1). The purified enzyme showed a single band on SDS-PAGE, and the molecular weight of the protein was estimated to be 35 kDa (data not shown); the native molecular mass of the enzyme was estimated to be 68 kDa by gel filtration. These results indicate that the native enzyme is a homodimer.

The 20 amino acid residues from the amino terminus of the purified enzyme were determined by Edman degradation to be Arg-Asn-Leu-Asp-Glu-Asp-Thr-Ile-Thr-Gln-Ala-Val-Leu-Ala-Arg-Leu-Ala-Asp-Thr-Pro. This sequence is identical to that deduced from the nucleotide sequence of the *hadC* gene except that the first methionine is absent, indicating that the NH_2-terminal residue methionine is post-transcriptionally removed.

At high concentrations, the enzyme showed a light red color, and a peak at 280 nm and a faint shoulder around 420 nm were found in the absorption spectrum. The purified enzyme was activated 16-fold by incubation with 0.5 mM $FeSO_4$.

Compared to other known HQ 1,2-dioxygenases (10, 11, 13–15, 20), the HQ 1,2-dioxygenase of *R. pickettii* exhibited relatively broad substrate specificity. The enzyme catalyzed the oxidation of 3-methylcatechol and pyrogallol in addition to HQ and 6-CHQ, possible intermediates in 2,4,6-TCP degradation (Table 2), but was inactive towards catechol, 3- and 4-chlorocatechol, 4-methylcatechol, protocatechuate, and 2,3-dihydroxybiphenyl. Incubation of the enzyme with 3-methylcatechol produced a yellow-colored reaction product which showed two absorption peaks, one at 260 nm and the other at 388 nm. These two peaks are in agreement with those of the *ortho*- and *meta*-cleavage products of 3-methylcatechol, respectively. The absorbance at 388 nm was intensified by alkalization of the reaction mixture (data not shown). The yellow product from 3-methylcatechol may be 2-hydroxy-6-oxohepta-2,4-dienoate (30). The HQ 1,2-dioxygenase of *R. pickettii* DTP0602 simultaneously catalyzed both the *ortho*- and *meta*-cleavage of 3-methylcatechol, similar to the catechol 1,2-dioxygenases of some bacteria (26, 31).

TABLE 2. Kinetic parameters of HQ 1,2-dioxygenase of *R. pickettii*

Substrate[a]	K_m (μM)	V_{max} (U/mg)	k_{cat} (1/s)	k_{cat}/K_m (1/s·μM)
HQ	11	21.3	12.4	1.13
6-CHQ	37	5.96	3.48	0.094
Pyrogallol	83	2.96	1.73	0.021
3-Methylcatechol				
ortho cleavage	5.5	1.42	0.83	0.15
meta cleavage	9.5	2.22	1.30	0.14

[a] HQ, Hydroxyquinol; 6-CHQ, 6-chlorohydroxyquinol.

DISCUSSION

The *hadC* gene encoding HQ 1,2-dioxygenase, which is responsible for 2,4,6-TCP degradation, was found to be located just downstream of the *hadAB* genes encoding 2,4,6-TCP-4-hydroxylase (Fig. 1). Although a promoter sequence (σ^{54} recognition sequence) was found upstream of the *hadA* gene (8), no similar sequence was obserbed upstream of the *hadC* gene. Thus, the *hadABC* genes seem to form an operon.

The deduced amino acid sequence of the *hadC* gene exhibited 56.6% identity to that of the *tftH* gene of *B. cepacia* AC1100 (19). The NH_2-terminal amino acid sequence of the HQ 1,2-dioxygenase of *R. pickettii* exhibited 47.6 and 70.0% similarity to the NH_2-terminal amino acid sequences of the 6-CHQ 1,2-dioxygenase of *S. rochei* 303 (11) and HQ 1,2-dioxygenase of *Azotobacter* sp. GP1 (10), respectively. Phylogenetic analysis revealed that HadC belongs to a group which includes TftH, but it is not grouped with catechol 1,2-dioxygenases (Fig. 2).

The following evidence supports the contention that HQ 1,2-dioxygenase is an enzyme responsible for the degradation of 2,4,6-TCP in *R. pickettii* DTP0602: (i) the enzyme catalyzed the ring-fission of HQ and 6-CHQ, possible intermediates in the degradation of 2,4,6-TCP (Fig. 3); (ii) the enzyme was induced during bacterial growth only in the presence of 2,4,6-TCP; and (iii) the gene that encodes the enzyme, *hadC*, apparently forms an operon with the *hadAB* genes.

The HQ 1,2-dioxygenases of *Azotobacter* sp. GP1 (10), *S. rochei* 303 (11), *N. simplex* 3E (13), *T. cutaneum* (15), and *B. cepacia* AC1100 (20) have been reported

TABLE 1. Purification of HQ 1,2-dioxygenase from *E. coli*

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)[a]	Yield (%)
Crude extract	140	1938	1620	0.836	100
DEAE-Toyopearl 650M	80	208	992	4.77	61
Mono Q	6	26.7	514	19.3	32
Superdex 200HR	10	15.3	362	23.7	22

[a] One unit (U) is defined as the amount of protein that converts 1 μmol of hydroxyquinol per min.

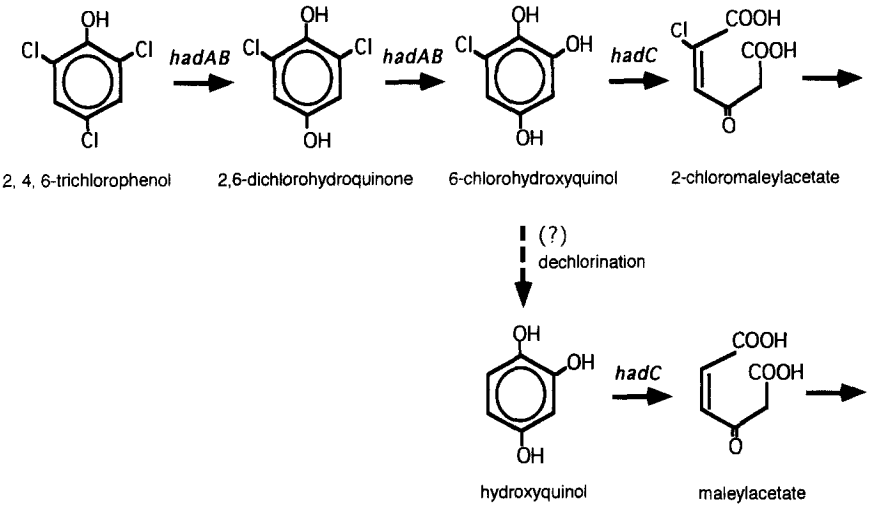

FIG. 3. Proposed pathway of 2,4,6-TCP degradation in *R. pickettii* DTP0602. The genens encode the following enzymes: *hadAB*, chlorophenol *p*-hydroxylase; *hadC*, hydroxyquinol 1,2-dioxygenase.

to be highly specific for HQ or 6-CHQ, while the HQ 1,2-dioxygenase of *P. crysosporium* has weak activity against catechol (14). Compared with these HQ 1,2-dioxygenases, the HQ 1,2-dioxygenase of *R. pickettii* has a relatively broad substrate specificity, showing activity against pyrogallol and 3-methylcatechol in addition to HQ and 6-CHQ (Table 2). Interestingly, the HQ 1,2-dioxygenase of *R. pickettii* catalyzed both the *ortho*- and *meta*-cleavage of 3-methylcatechol simultaneously.

Some bacterial catechol 1,2-dioxygenases have been reported to catalyze the *ortho*- and *meta*-cleavage of 3-methylcatechol (26, 31), but none of the HQ 1,2-dioxygenases reported so far has both activities toward 3-methylcatechol (10, 11, 13, 15, 20). Even though the HQ 1,2-dioxygenase of *R. pickettii* did not cleave catechol, it catalyzed both the *ortho*- and *meta*-cleavage of 3-methylcatechol. Catechol 1,2-dioxygenases that have both activities show stronger *ortho*-cleavage than *meta*-cleavage activity toward 3-methylcatechol (26, 31). In contrast, the HQ 1,2-dioxygenase of *R. pickettii* exhibited stronger *meta*-cleavage activity (Table 2).

Some intradiol dioxygenases, including the HQ 1,2-dioxygenase of *Azotobacter* sp. GP1 and protocatechuate 3,4-dioxygenase of *P. aeruginosa*, have been reported to contain ferric iron (10, 13, 43). In protocatechuate 3, 4-dioxygenase, ferric iron is coordinated by four ligands, Tyr-118, Tyr-147, His-160, and His-162, in the catalytic center (43). The HQ 1,2-dioxygenase of *R. pickettii* also has the same amino acid residues, Tyr-118, Tyr-147, His-160, and His-162, at similar intervals. In addition, these ligands are different from those for ferrous iron in extradiol dioxygenases (44). In its active form, the enzyme of *R. pickettii* may contain ferric iron like other *ortho*-cleavage enzymes.

It remains unclear whether 6-CHQ is cleaved immediately or after dechlorination by a 6-CHQ dechlorinase particular to the mineralization (Fig. 3). However, it was clarified that HQ 1,2-dioxygenase is responsible for the metabolism of 2,4,6-TCP in *R. pickettii* DTP0602.

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