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Cloning and sequence analysis of a catechol 2,3-dioxygenase gene from the nitrobenzene-degrading strain *Comamonas* sp JS765

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Comamonas **sp strain JS765 utilizes nitrobenzene as a carbon and nitrogen source. The initial attack on nitrobenzene is carried out by nitrobenzene 1,2-dioxygenase, which converts nitrobenzene to an unstable nitrohydrodiol that spontaneously decomposes to form catechol and nitrite. Catechol is then degraded via a *meta* cleavage pathway. We now report the cloning of a DNA fragment carrying a catechol 2,3-dioxygenase gene from JS765. Nucleotide sequence analysis revealed three open reading frames (ORFs) predicted to encode proteins of 33.6, 13.0, and 35.0 kDa. Homology searches of the deduced amino acid sequences of three proteins suggested that ORF1 encodes a LysR-type transcriptional regulator, ORF2 encodes a XylT-type ferredoxin, and ORF3 encodes a catechol 2,3-dioxygenase. The putative regulatory gene, designated *cdoR*, is divergently transcribed from the ferredoxin and catechol dioxygenase genes, *cdoT* and *cdoE*, respectively. The catechol 2,3-dioxygenase is most similar in amino acid sequence to the I.2.C subfamily of extradiol dioxygenases which include 3-methylcatechol 2,3-dioxygenase from the aniline- and toluidine-degrading *Pseudomonas putida* UCC2, TbuE from the toluene monooxygenase pathway of *Pseudomonas pickettii* PKO1 and catechol 2,3-dioxygenase II from the TOL plasmid pWW15. The substrate range of the catechol 2,3-dioxygenase produced by the recombinant *E. coli* strains was very similar to that of the enzyme present in nitrobenzene-grown JS765, suggesting that we have cloned the catechol 2,3-dioxygenase gene required for nitrobenzene degradation.**

Keywords: catechol; dioxygenase; *meta* cleavage; nitrobenzene; biodegradation; aromatic

Introduction

Nitroaromatic compounds are widely used in the chemical industry for the production of explosives, dyes, polymers, pesticides and solvents. Many of these compounds are highly toxic even at low concentrations and have proven to be recalcitrant in nature. For these reasons, release of nitroaromatic compounds into the environment has caused concern. Nitrobenzene is one of seven nitroaromatic compounds currently on the US Environmental Protection Agency's list of priority pollutants [20].

Comamonas sp strain JS765 was isolated for its ability to grow with nitrobenzene as sole carbon, nitrogen and energy source [30]. JS765 degrades nitrobenzene by an initial dioxygenation reaction catalyzed by nitrobenzene 1,2-dioxygenase. The resulting nitrohydrodiol intermediate is unstable and spontaneously decomposes to catechol with the release of nitrite (Figure 1) [30]. This type of dioxygenase-catalyzed removal of nitro groups has also been observed in the oxidative pathways for the biodegradation of 2-nitrotoluene [1], 2,4-dinitrotoluene [42], 1,3-dinitrobenzene [10] and 3-nitrobenzoate [26]. In the second enzymatic step of the nitrobenzene degradation pathway, catechol 2,3-dioxygenase catalyzes a *meta* ring cleavage to yield 2-hydroxymuconic semialdehyde (HMS), which is further metabolized by HMS dehydrogenase and HMS hydrolase (Figure 1) [30].

We have initiated a genetic analysis of JS765 to further characterize the nitrobenzene degradation pathway and report here the cloning and sequence analysis of a gene cluster that contains the catechol 2,3-dioxygenase gene from JS765.

Materials and methods

Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown at 30°C or 37°C in LB [7] or TB [23] medium with ampicillin (150 μg ml^{-1}) or kanamycin (100 μg ml^{-1}) added as appropriate for plasmid maintenance. When appropriate, 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) and isopropyl-β-d-thiogalactopyranoside (IPTG) were added to solid media to final concentrations of 40 μg ml^{-1} and 100 μM, respectively. JM109 was grown in LB supplemented with 2% maltose and 10 mM $MgSO_4$ prior to λ infections. JS765 was grown in modified LB medium containing no NaCl.

Recombinant DNA techniques

Cosmids and plasmids were purified using the Qiagen Midi Kit (Qiagen, Chatsworth, CA, USA) or by the method of

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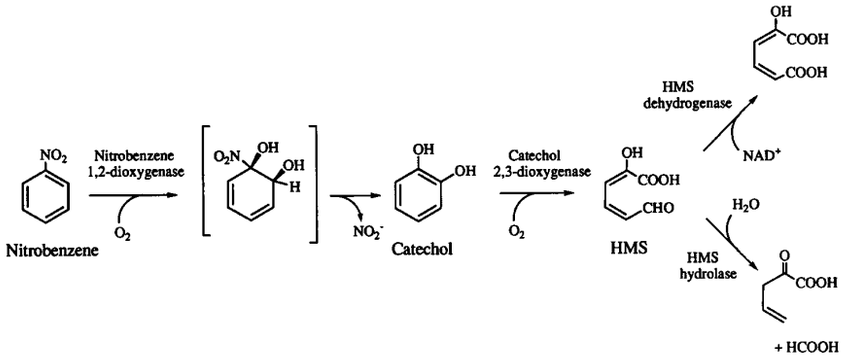

Figure 1 Initial reactions in the degradation of nitrobenzene by *Comamonas* sp strain JS765. HMS, 2-hydroxymuconic semialdehyde.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
Comamonas sp JS765	Nitrobenzene-degrading isolate	[30]
Escherichia coli DH5α	Δ(*lacZYA-argF*)U169, *hdsR*17, *relA*1, *supE*44, *endA*1, *recA*1, *thi*, *gyrA*96, ϕ80d*lac*ZΔM15	Life Technologies, Gaithersburg, MD
Escherichia coli JM109	*endA*1, *recA*1, *gyrA*96, *thi*, *hdsR*17, *relA*1, *supE*44, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}ZΔM15]	[46]
Plasmids		
pHC79	Ap^r, Tc^r, cosmid cloning vector	[18]
pK19	Km^r, ColE1 origin, *lac* promoter	[35]
pUC19	Ap^r, Co1E1 origin, *lac* promoter	[46]
pDTG900	pHC79 containing a 34-kb *Sau*3AI partial fragment from JS765	This study
pDTG901	pK19 containing a 7.0-kb *Sac*I fragment from pDTG900	This study
pDTG902	pDTG901 with a deletion of the 4.5-kb *Sma*I fragment	This study
pDTG903	pUC19 containing the 4.5-kb *Sma*I fragment from pDTG901	This study
pDTG904	pUC19 containing the 1.3-kb *Hinc*II fragment from pDTG901	This study
pDTG905	pUC19 containing the 1.7-kb *Hinc*II fragment from pDTG901	This study
pDTG906	pK19 containing the 0.8-kb *Pst*I fragment from pDTG904	This study
pDTG907	pK19 containing the 0.5-kb *Pst*I fragment from pDTG904	This study
pDTG908	pDTG901 with a 5.5-kb *Pst*I deletion	This study
pDTG909	pDTG901 with a 4.0-kb *Pst*I deletion	This study
pDTG911	pDTG905 with the 0.5-kb *Pst*I fragment deleted	This study
pDTG917	pDTG905 with a 0.5-kb *Sma*I-*Msc*I deletion	This study

Lee and Rasheed [23]. Total genomic DNA from JS765 was purified as described previously [14]. Restriction enzyme digestions and ligation reactions were performed under the conditions recommended by the suppliers (Promega Corp, Madison, WI, USA; New England Biolabs, Beverly, MA, USA). DNA fragments were purified from gel slices using the Geneclean Spin Kit (Bio 101, Vista, CA, USA). Transformation of *E. coli* strains and standard molecular biology techniques were as previously described [2].

Cloning of the JS765 catechol 2,3-dioxygenase gene

JS765 genomic DNA was partially digested with *Sau*3AI, dephosphorylated, and ligated into *Bam*HI-digested pHC79 by a previously described method [36]. Ligated DNA was packaged into bacteriophage λ particles using a Gigapack II Packaging Extract according to the manufacturer's instructions (Stratagene Cloning Systems, La Jolla, CA, USA). JM109 was infected with the cosmid library [36] and plated onto LB agar plates containing ampicillin. Ampicillin-resistant colonies were sprayed with a solution of 10 mM catechol using Spra-Tool aerosol propellant (Fisher Scientific, Pittsburg, PA, USA). Recombinants expressing catechol 2,3-dioxygenase turned yellow due to the accumulation of 2-hydroxymuconic semialdehyde [12,28].

Nucleotide sequence analysis

Prior to sequencing, plasmid DNA isolated using the Qiagen Midi Kit was further purified using a Centricon-100 filter unit (Amicon, Beverly, MA, USA). Nucleotide sequencing was carried out by the University of Iowa DNA Facility using an Applied Biosystems 373A Automated DNA Sequencer. Custom oligonucleotides were obtained from Genosys Biotechnologies (Midland, TX, USA). Sequence analysis was carried out using Gene Jockey software (Biosoft, Cambridge, UK) and the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI, USA [9]). The BLAST network service at the National Center for Biotechnology Information (Bethesda, MD, USA) was used to identify similar sequences in the SWISS-PROT and GenBank databases.

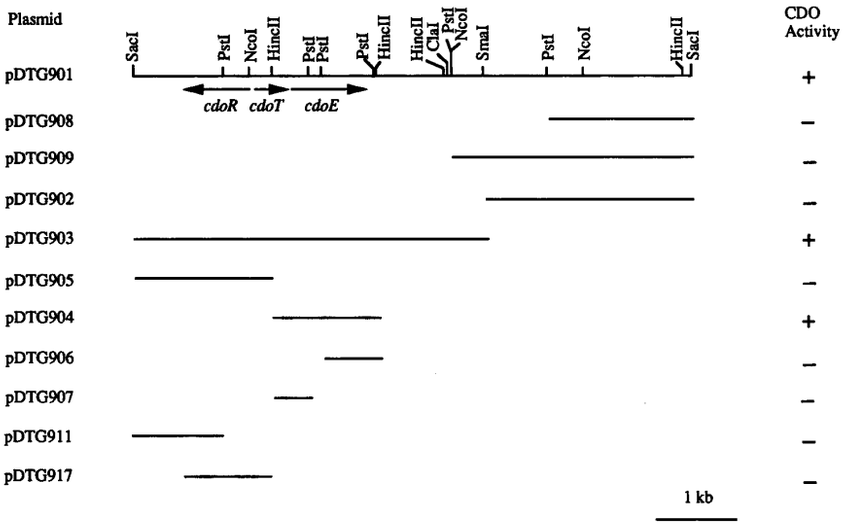

Figure 2 Subcloning of the catechol 2,3-dioxygenase gene. Shown is the restriction map of pDTG901 and the catechol 2,3-dioxygenase (CDO) activity of subclones determined by the catechol spray plate assay. Subclones are described in Table 1.

Preparation of cell extracts

E. coli JM109(pDTG900), DH5α(pDTG901) and DH5α(pDTG904) were grown in LB medium containing ampicillin at 30°C. DH5α(pDTG901) and DH5α(pDTG904) were induced with 100 μM IPTG for 2.5 h when culture turbidity at 660 nm reached 0.6. JM109(pDTG900) was allowed to continue growing until the culture turbidity reached 0.9. Cells were harvested by centrifugation and stored at –20°C. Frozen cells were thawed and suspended in 50 mM Tris-HCl (pH 7.5). Cells were broken by passing twice through a chilled French pressure cell at 20 000 lb in^{-2}. Cell debris and membranes were removed by centrifugation at 150 000 $\times$ *g* for 1 h at 4°C.

Enzyme assays

Oxygen uptake by catechol 2,3-dioxygenase (EC 1.13.11.2) with various substituted catechols was determined at 30°C with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK). Reactions (1 ml total volume) were carried out in air-saturated 50 mM Tris-HCl buffer (pH 7.5) containing an appropriate amount of cell extract to give a linear O_2 uptake rate. Reactions were initiated by the addition of substrate (1 mM final concentration). All rates were corrected for endogenous respiration.

Catechol 2,3-dioxygenase activity was measured spectrophotometrically as previously described [31]. Reactions were carried out in 50 mM Tris-HCl buffer (pH 7.5) containing catechol at a final concentration of 1 mM. Reactions were initiated by the addition of cell extract. The formation of 2-hydroxymuconic semialdehyde was monitored at 375 nm ($\epsilon = 33\,400$ mol^{-1} cm^{-1} [4]). One unit of catechol 2,3-dioxygenase activity was defined as the amount of enzyme required to form one μmole of 2-hydroxymuconic semialdehyde per min. Protein concentrations were determined by the method of Bradford [5] with bovine serum albumin as standard.

Materials

Catechol was obtained from Aldrich Chemical Co, Milwaukee, WI, USA. Protocatechuate was from Sigma Chemical Co, St Louis, MO, USA. 3-Methylcatechol and 4-methylcatechol were obtained from Pfalz and Bauer, Waterbury, CT, USA. 3-Chlorocatechol was provided by Sol Resnick, Department of Microbiology, University of Iowa.

Results and discussion

Gene localization and subcloning

A cosmid library was generated by ligating a partial *Sau*3AI digest of JS765 genomic DNA with *Bam*HI-digested pHC79 cosmid. Of 490 colonies screened on catechol spray plates, one turned yellow. Cosmid DNA was isolated from this recombinant and designated pDTG900. Restriction mapping indicated that approximately 34 kb of DNA was inserted in the 6.4-kb vector. Digestion with *Sac*I revealed the presence of five fragments (17.0, 7.2, 6.7, 6.2 and 2.7 kb) which were subcloned into *Sac*I-digested pK19. The subclone carrying the 7.2-kb *Sac*I fragment was shown to have catechol 2,3-dioxygenase activity, and was designated pDTG901. The restriction map of pDTG901 is shown in Figure 2. Construction of the deletion clones and subclones described in Table 1 and subsequent analyses for catechol 2,3-dioxygenase activity localized the gene to the 1.3-kb *Hin*cII fragment on pDTG904 (Figure 2).

Sequence analysis

Both strands of the 1.3-kb *Hin*cII fragment of pDTG904 were completely sequenced using a combination of subclones (pDTG904, pDTG906, pDTG907) and oligonucleotide primers. Portions of pDTG901, pDTG905, pDTG911, and pDTG917 were sequenced in order to complete the sequence of the two upstream open reading frames (ORFs) in the gene cluster. The G+C content of the sequenced

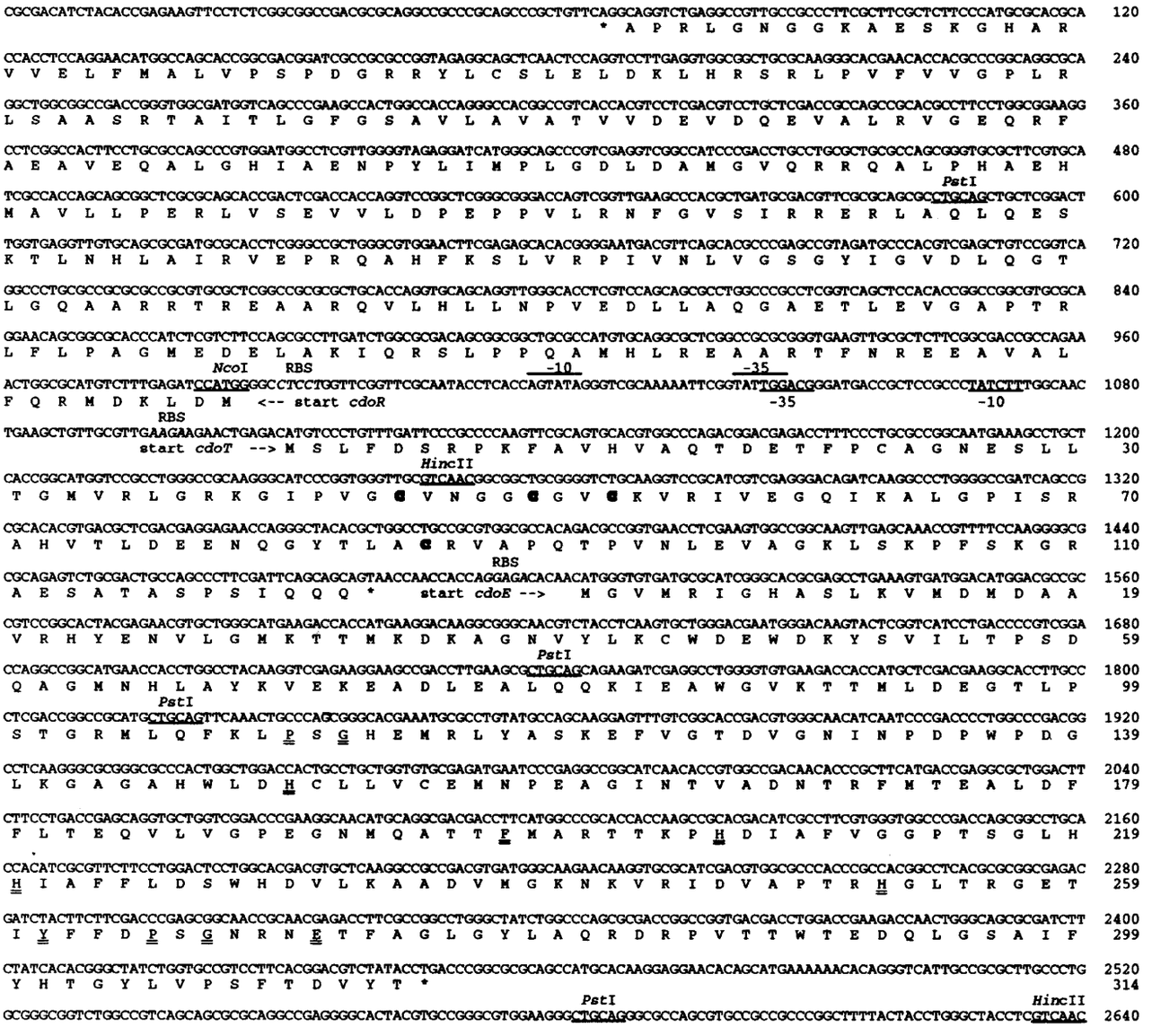

Figure 3 Nucleotide sequence of the *cdoRTE* gene cluster from JS765 which encodes the LysR-type regulator, plant-type ferredoxin, and catechol 2,3-dioxygenase (GenBank accession number U93090). Deduced amino acid sequences are shown below the nucleotide sequence. Asterisks (*) indicate stop codons. Restriction sites are underlined and labeled above. Potential ribosome-binding sites are italicized and labeled RBS. The putative promoter for the *cdoT* gene is underlined and the -10 and -35 hexamers labeled below. The putative promoter for the *cdoR* gene is overlined and the -10 and -35 hexamers labeled above. Conserved cysteines (Cys-45, Cys-50, Cys-53, Cys-86) in the plant-type ferredoxin are indicated by **C**. In the catechol 2,3-dioxygenase, conserved metal-binding residues (His-150, His-220, Glu-271), active site residues (His-206, His-252, Tyr-261), and conserved residues involved in domain–domain interactions (Pro-110, Gly-112, Pro-265, Gly-267) and substrate binding (Phe-198) are double underlined.

region was 64%, typical of *Comamonas* sp genes which range from 61–68% G+C [8,44].

Nucleotide sequence analysis revealed three ORFs which were designated *cdoRTE* (Figure 3). The *cdoR* gene encodes a 306-amino acid protein. Homology searches with the amino acid sequence deduced from *cdoR* suggested that the 33 639 molecular weight protein is a member of the LysR family of transcriptional regulators. Pairwise comparisons revealed the highest amino acid identities (34–36%) with activators of the *ortho* or modified-*ortho* cleavage pathway genes (Table 2). These regulators include ClcR from plasmid pAC27 [6], CatR from *Pseudomonas putida* PRS2000 [37], TfdR from *Ralstonia eutropha* JMP134 [24], and TcbR from *Pseudomonas* sp P51 [45]. Residues 18–37 of the CdoR form a helix-turn-helix motif very similar to the LysR consensus sequence [39]. The *cdoR* gene is divergently transcribed from the *cdoT* gene, and putative sigma-70 type promoters for the *cdoR* and *cdoT* genes were identified (Figure 3). These observations suggest that CdoR regulates transcription of *cdoTE*, although this has not yet been tested.

The *cdoT* gene encodes a protein with a calculated molecular weight of 12 983. Analysis of the amino acid sequence deduced from *cdoT* revealed strong homology to plant-type ferredoxins, especially the XylT-type ferredoxins which are associated with aromatic compound degradation

Table 2 Pairwise comparisons of deduced amino acid sequences with those of similar proteins

Gene	Predicted product (No. amino acids)	Similar protein (No. amino acids)	Amino acid identity[a] (%)	GenBank accession No. [Reference]
cdoR	Regulatory protein (306)	TfdR (295)	35.9	P10086 [24]
		ClcR (294)	35.3	Q05840 [6]
		BphR (314)	34.8	D38633
		CatR (289)	34.4	P20667 [37]
		TcbR (294)	33.7	P27102 [45]
		LysR (311)	22.0	P03030 [43]
		NahR (300)	18.6	P10183 [47]
cdoT	Plant-type ferredoxin (123)	TbuW (119)	43.7	U20258 [21]
		NahT (108)	37.7	J05317 [16, 48]
		PhlG (101)	34.7	X80765 [17]
		PhhQ (101)	34.7	X79063 [29]
		XylT (112)	34.2	M64747 [16]
		ORF-KF715 (112)	32.7	S78585 [22]
		DmpQ (112)	31.2	X60835 [41]
cdoE	Catechol 2,3-dioxygenase (314)	TdnC (314)	83.8	X59790
		CDOII (314)	68.5	U01826
		TbuE (314)	60.6	U20258 [21]
		XylE (307)	45.5	V01161 [27]
		DmpB (307)	45.2	M33263 [3]
		PhhB (307)	45.2	X77856 [29]
		NahH (307)	44.2	X06412 [13]
		PhlH (307)	43.9	X80765 [17]

[a]% Amino acid identities were obtained using the GAP program from the Wisconsin Package. The gap weight was 3.0 and the gap length weight was 0.1.

(Table 2). Homologous proteins include TbuW from the toluene/benzene monooxygenase gene cluster of *P. pickettii* PKO1 [21], XylT from the TOL plasmid [16], NahT from the NAH7 plasmid [16,48], and DmpQ, PhhQ, and PhlG from the phenol hydroxylase gene clusters of *Pseudomonas* sp CF600 [41], *P. putida* P35X [29], and *P. putida* H [17], respectively. Conserved cysteines characteristic of plant-type ferredoxins [16] were identified in the deduced amino acid sequence and are marked in Figure 3. These amino acids are potential ligands for plant-type [2Fe-2S] clusters.

Each of the plant-type ferredoxin genes mentioned above is located directly upstream of a catechol 2,3-dioxygenase gene. Only *xylT* has been characterized in detail. Mutations in *xylT* from the TOL plasmid prevented growth on *para*-substituted aromatic compounds. The associated catechol 2,3-dioxygenase (XylE) was shown to be sensitive to 4-methylcatechol, and *in vivo* studies demonstrated that XylT was able to reactivate XylE that had been inactivated by 4-methylcatechol [33]. In *Pseudomonas* sp CF600, DmpQ was shown to be required for growth with *para*-substituted phenols [34], and based on this, DmpQ is predicted to play a role similar to that of XylT: modulation of catechol 2,3-dioxygenase (in this case DmpB) activity. By analogy, it is possible that CdoT may expand the substrate range of CdoE by modifying its activity in the presence of suicide substrates.

A 314-amino acid protein with a molecular weight of 35 020 is encoded by *cdoE*. The deduced amino acid sequence had high homology with several catechol 2,3-dioxygenases (Table 2), especially those of the I.2.C subfamily of extradiol dioxygenases [11]. The most similar protein is TdnC (84% amino acid identity) from the aniline- and toluidine-degrading *P. putida* UCC2 [38]. CDOII from the TOL plasmid pWW15 [19], and TbuE from *P. pickettii* PKO1 [21] are also very similar. The deduced amino acid sequence was found to contain conserved residues involved in metal binding, catalysis, and dimerization that were identified in crystallization studies of 2,3-dihydroxybiphenyl 1,2-dioxygenases [15,40]. These are indicated in Figure 3.

Catechol 2,3-dioxygenase activity in cell extracts

Extracts of recombinant strains were tested for the ability to convert catechol to 2-hydroxymuconic semialdehyde (HMS). The extract from JM109(pDTG900) had very low activity (Table 3). This result was not surprising, since the cosmid does not carry a strong promoter for expression and the copy number of the cosmid is low. Activities in extracts of DH5α carrying pDTG901 or pDTG904 were significantly higher. As seen with the catechol 2,3-dioxygenase from nitrobenzene-induced JS765 [30], heat treatment of the DH5α(pDTG901) extract (60°C for 10 min) did not significantly reduce enzyme activity (Table 3).

In contrast to results with JS765 [30], the yellow color produced from catechol by DH5α(pDTG901) extracts did

Table 3 Catechol 2,3-dioxygenase activity in cell extracts

Extract source	Catechol 2,3-dioxygenase specific activity (μmol min^{-1} mg^{-1} protein)
JS765[a]	3.04
DH5α(pDTG900)	0.005
DH5α(pDTG901)	0.55 (0.47)[b]
DH5α(pDTG904)	0.26

[a]Data from [30].
[b]Activity after incubation of crude cell extract at 60°C for 10 min.

not fade with time, even when catechol was provided at a reduced concentration (100 μM) or when NAD (500 μM) was added. This result suggests that the HMS hydrolase and HMS dehydrogenase are not encoded on the DNA fragment.

Substrate range of catechol 2,3-dioxygenase

The substrate range of catechol 2,3-dioxygenase was investigated by measuring O_2 uptake by cell extracts with various substituted catechols. No O_2 uptake by extracts of JM109(pDTG900) was detected. Although the relative rates were not absolutely proportional, the pattern of substrate oxidation by extracts of DH5α carrying either pDTG901 or pDTG904 was identical to that of the enzyme from nitrobenzene-induced JS765 (Table 4). High O_2 uptake rates with catechol, 3-methylcatechol, and 4-methylcatechol indicated that these compounds were good substrates for the enzyme. The initial O_2 consumption rate with 3-chlorocatechol was high, but rapidly decreased, suggesting that the enzyme is inactivated in the presence of this substrate. No O_2 uptake was observed with 4-nitrocatechol or protocatechuate, suggesting that these compounds do not serve as substrates. This substrate range is similar to that of the catechol 2,3-dioxygenase from *P. pickettii* PKO1 [21], an observation made by Nishino and Spain [30] based on studies of the enzyme present in extracts of nitrobenzene-induced JS765. These results provide further evidence that CdoE and TbuE belong to the same subfamily.

Conclusions

We have identified an interesting and unique gene cluster from JS765. The gene organization (*xylT*-type ferredoxin gene followed by catechol 2,3-dioxygenase gene) is conserved in the *cdo*, *xyl*, *nah*, *tbu*, *dmp*, *phh*, and *phl* gene clusters. However, only in the *cdo* gene cluster described here is a regulatory gene located directly upstream of and divergently transcribed from the ferredoxin gene. The regulatory protein encoded by *cdoR* is most like those of *ortho*-cleavage pathways, and the coinducers associated with these pathways have been shown to be the *ortho* cleavage products *cis,cis*-muconate [32] or 2-chloro-*cis,cis*-muconate [25,45], compounds which would not be formed by a *meta* cleavage pathway such as the one described here. It will be interesting to identify the inducer associated with the *cdo* gene cluster.

Table 4 Oxygen uptake by cell extracts

Assay substrate	O_2 uptake (μmol min^{-1} mg^{-1} protein)		
	DH5α (pDTG901)[a]	DH5α (pDTG904)[a]	JS765[b]
Catechol	2.07	0.10	0.35
3-Chlorocatechol	1.22	0.07	0.27
3-Methylcatechol	1.11	0.07	0.26
4-Methylcatechol	0.84	0.05	0.25
4-Nitrocatechol	<0.01	<0.01	<0.01
Protocatechuate	<0.01	<0.01	<0.01

[a]Substrate concentration, 1 mM.
[b]Data from [30], substrate concentration, 100 μM.

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