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Novel Gene Clusters and Metabolic Pathway Involved in 3,5,6-Trichloro-2-Pyridinol Degradation by *Ralstonia* sp. Strain T6

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3,5,6-Trichloro-2-pyridinol (TCP) is a widespread pollutant. Some bacteria and fungi have been reported to degrade TCP, but the gene clusters responsible for TCP biodegradation have not been characterized. In this study, a fragment of the reduced flavin adenine dinucleotide ($FADH_2$)-dependent monooxygenase gene *tcpA* was amplified from the genomic DNA of *Ralstonia* sp. strain T6 with degenerate primers. The *tcpA* disruption mutant strain T6-Δ*tcpA* could not degrade TCP but could degrade the green intermediate metabolite 3,6-dihydroxypyridine-2,5-dione (DHPD), which was generated during TCP biodegradation by strain T6. The flanking sequences of *tcpA* were obtained by self-formed adaptor PCR. *tcpRXA* genes constitute a gene cluster. TcpR and TcpX are closely related to the LysR family transcriptional regulator and flavin reductase, respectively. T6-Δ*tcpA*-com, the complementation strain for the mutant strain T6-Δ*tcpA*, recovered the ability to degrade TCP, and the strain *Escherichia coli* DH10B-*tcpRXA*, which expressed the *tcpRXA* gene cluster, had the ability to transform TCP to DHPD, indicating that *tcpA* is a key gene in the initial step of TCP degradation and that TcpA dechlorinates TCP to DHPD. A library of DHPD degradation-deficient mutants of strain T6 was obtained by random transposon mutagenesis. The fragments flanking the Mariner transposon were amplified and sequenced, and the *dhpRIJK* gene cluster was cloned. DhpJ could transform DHPD to yield an intermediate product, 5-amino-2,4,5-trioxopentanoic acid (ATOPA), which was further degraded by DhpI. DhpR and DhpK are closely related to the AraC family transcriptional regulator and the MFS family transporter, respectively.

TCP (3,5,6-trichloro-2-pyridinol) is the main degradation product of the herbicide triclopyr and the insecticides chlorpyrifos and chlorpyrifos-methyl (1–4), which have been widely used in agriculture because of their high efficiency and moderate toxicity. TCP is classified as persistent and mobile by the U.S. EPA, with a half-life ranging from 65 to 360 days in soil (5), and is more migratory than its parent molecule due to its greater water solubility, which causes widespread contamination of soils and aquatic environments (3, 6, 7). TCP was detected in >95% of the indoor floor dust and hard-floor surface wipe, solid food, and indoor air samples in the everyday environments of preschool children in six North Carolina counties, posing a potential hazard to children (8). TCP has high affinity to the DNA molecule and can bind to the DNA groove, which might cause the metabolite to act as a potential health hazard (9).

Some bacteria, bacterial consortia, and fungi have been reported to degrade TCP and can grow using it as their sole carbon source; these organisms include *Alcaligenes faecalis* DSP3 (1), *Burkholderia* sp. strain KR100 (4), *Pseudomonas* sp. strain ATCC 700113 (7), *Paracoccus* sp. strain TRP (10), *Pseudomonas* sp. strains 1 to 4 (11), *Agrobacterium* sp. strains 5 and 6 (11), *Bacillus* sp. strain 7 (11), *Trichosporon* sp. strain TCF (12), *Bacillus pumilus* C2A1 (13), *Pseudomonas* strain M285 (14), and *Ralstonia* sp. strain T6 (15). However, the genes and metabolic pathways responsible for TCP biodegradation have rarely been reported, and the gene clusters affecting TCP biodegradation have not been well characterized. There are only two reports on the genes of TCP biodegradation. A novel gene involved in TCP degradation, *tcp3A*, was isolated from a cow rumen metagenomic library and was functionally verified in *Escherichia coli* (16). The genome sequence of the chlorpyrifos and TCP degrader *Paracoccus* sp. TRP was completed, but no genes related to TCP degradation were identified (17). A reductive dechlorination mechanism was proposed in the TCP degrader *Pseudomonas* sp. ATCC 700113 (18), which is the well-known metabolic pathway of TCP biodegradation.

Ralstonia sp. T6 degrades TCP more effectively than do other TCP degraders (1, 4, 7, 10, 11, 12, 13, 14, 15), and discovering the genes and metabolic pathway for TCP degradation is valuable. We report here the cloning and functional characterization of two gene clusters and the partial metabolic pathway responsible for TCP degradation in strain T6.

MATERIALS AND METHODS

Materials, bacterial strains, cultures, and plasmids. TCP (99.3%) was obtained from the Gu'an Enkang Medicine Chemical Raw Material Co. Ltd., Langfang, China. A concentrated stock solution of TCP (10 g/liter) was prepared in water. All solvents were of pure analytical grade, except for chromatographically pure methanol. PCRs were performed with *Taq*, LA *Taq*, or PrimeSTAR HS DNA polymerase (TaKaRa Biotechnology, Dalian, China), and the primers were purchased from the Invitrogen Co. (Shanghai, China). Restriction endonucleases were purchased from TaKaRa Biotechnology Co. (Dalian, China). The minimal salts medium (MSM) contained (per liter) 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.03 g $MgSO_4$, and 1.0 g NaCl, pH 7.0. An appropriate con-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Ralstonia sp.		
T6	Wild type, TCP degrader, Gm^- Tc^r	Lab stock
T6-Δ*tcpA*	*tcpA* insertion mutant of T6	This study
T6-Δ*tcpA*-com	Complementation for the *tcpA* insertion mutant of T6	This study
Cupriavidus necator JMP134	Wild type, 2,4,6-trichlorophenol degrader	24
E. coli		
DH5α	Host strain for cloning vectors	Lab stock
DH10B	Host strain for cloning vectors	Lab stock
$SM10_{\lambda pir}$	Conjugation strain	Lab stock
BL21(DE3)	Host strain for expressing vectors	Lab stock
Plasmids		
pMD19-T	T-A cloning vector, Amp^r	TaKaRa
pMD19-RXA	Vector for *tcpRXA* heterologous expression	This study
pMD19-RXA-JMP134	Vector for heterologous expression of *tcpRXA* from *C. necator* JMP134	This study
pUC19	Parent vector of pMD19-T digested with EcoRV	TaKaRa
pJQ200SK	Suicide vector, Gm^r	22
pJQ-TY1	Vector for the *tcpA* insertion mutant of T6	This study
pRK2013	Helper plasmid, mob^+ tra^+ Km^r	Lab stock
pSC123	Vector for transposon-mediated mutagenesis of bacteria, Km^r	25
pBBR1MCS-2	Broad-host-range cloning vector, Km^r	23
pBB-XA	pBBR1MCS-2 derivative carrying *tcpX* and *tcpA* genes	This study
pET-29a(+)	Expression vector, Km^r	Lab stock
pET-I	pET-29a(+) derivative carrying *dhpI*	This study
pET-J	pET-29a(+) derivative carrying *dhpJ*	This study

centration of TCP was added to the MSM to prepare the TMM medium. The Luria-Bertani (LB) medium contained (per liter) 10.0 g tryptone, 5.0 g yeast extract, and 10.0 g NaCl, pH 7.0. Solid medium plates were prepared by adding 16 g/liter agar into the above liquid media. The bacterial strains and plasmids used in this study are listed in Table 1. *Ralstonia* sp. T6 and its mutant derivatives were grown at 30°C in LB medium or on LB agar. All *E. coli* strains were routinely cultivated at 37°C in LB medium or on LB agar, except *E. coli* BL21(DE3) and *E. coli* DH10B, which were cultivated at 30°C when used to express enzyme proteins and to transform TCP to 3,6-dihydroxypyridine-2,5-dione (DHPD), respectively. Ampicillin (Amp), kanamycin (Km), tetracycline (Tc), and gentamicin (Gm) were used at 100, 50, 30, and 30 mg/liter in culture medium, respectively.

Cloning of *tcpA* and the *tcpRXA* gene cluster by SEFA PCR. The partial *tcpA* gene was amplified from *Ralstonia* sp. T6 with the degenerate primers TftD-S and TftD-A (19) (Table 2). The flanking sequences of *tcpA* were obtained by self-formed adaptor PCR (SEFA PCR) (20), and the primers used are presented in Table 2. The primers TftDR-Sp1, TftDR-Sp2, and TftDR-Sp3 were used to amplify the 3′ flanking sequence of *tcpA*. The primers TftDF-Sp1, TftDF-Sp2, and TftDF-Sp3 were used to amplify the 5′ flanking sequence of *tcpA*. The genomic DNA of strain T6 and its mutant was extracted using a high-concentration salt precipitation method (21). All the amplified nucleotide sequences were confirmed by DNA sequencing with an ABI Genetic Analyzer 3730 (Invitrogen Bio Inc., Shanghai, China).

RT-PCR analyses of the common promoter for *tcpX* and *tcpA*. The total RNA of strain T6 cells grown in LB medium was extracted with the RNAprep Pure Cell/Bacteria kit (Tiangen Biotech Co., Ltd., Beijing, China). Reverse transcriptase PCR (RT-PCR) analyses were carried out using the GoScript reverse transcription system (Promega Corp., Madison, WI) according to the manufacturer's instructions. Three primer sets were used for RT-PCR. Primer set A (forward, 5′-TCGTCCGCAGTATC CATC-3′; reverse, 5′-ACCATCGCGGATTTGCTG-3′) amplified the internal 356 bp of *tcpX*. Primer set B (forward, 5′-ACCTGGAATCGCTCA ACG-3′; reverse, 5′-GCCGATAGTCTGCGTTTC-3′) amplified the internal 392 bp of *tcpA*. Primer set C (forward, 5′-GCCATACAGCAAAT CCGC-3′; reverse, 5′-GCCGATAGTCTGCGTTTC-3′) amplified 663 bp of the *tcpX-tcpA*-spanning region. All the amplified fragments were confirmed by DNA sequencing with an ABI Genetic Analyzer 3730 (Invitrogen Bio Inc., Shanghai, China).

Disruption and complementation of *tcpA* in *Ralstonia* sp. T6. Homologous recombination was used to disrupt *tcpA*. The homologous recombination-directing sequence was amplified with the primers TY-S and TY-A (Table 2), using strain T6 genomic DNA as a template. The purified PCR product was ligated to the pMD19-T vector (TaKaRa Biotechnology, Dalian, China), which was then recovered by digestion with PstI and SacI and ligated into the suicide vector pJQ200SK (22), yielding pJQ-TY. The plasmid pJQ-TY was transformed into *Ralstonia* sp. T6 by triparental conjugation. The donor, helper, and recipient strains were *E. coli* $SM10_{\lambda pir}$ containing plasmid pJQ-TY, *E. coli* pRK2013, and *Ralstonia* sp. T6, respectively. All of the strains were cultured in LB medium to an optical density at 600 nm (OD_{600}) of 1.0. Then, 1 ml of each culture was collected by centrifugation at 2,500 × *g* for 5 min and washed three times with fresh LB medium to remove any residual antibiotics. Cells were resuspended in 50 μl LB medium. The mixture was spread onto a nitrocellulose filter (with an average pore size of 0.45 μm) on an LB plate and incubated at 30°C for 24 h. The mixtures were then resuspended in 1 ml of LB medium and spread onto LB plates (200 μl per plate) supplemented with Gm and Tc. After incubation at 30°C for 3 days, colonies with single recombination events were selected on LB plates containing Gm and Tc. The disruption of *tcpA* was confirmed by the presence/absence of the expected PCR products in the wild-type and mutant strains using the primers TftD-S and TftD-A. One of the selected mutant strains was designated T6-Δ*tcpA*.

To complement the disrupted *tcpA* gene of T6-Δ*tcpA*, a fragment carrying both *tcpX* and *tcpA* was amplified from the genomic DNA of strain T6 with the primers TCPXA-S and TCPXA-A (Table 2) because of their common promoter. The fragment (89% identity to *tcpXA* from the 2,4,6-trichlorophenol degrader *Cupriavidus necator* JMP134) was inserted into the broad-host-range plasmid pBBR1MCS-2 (23), yielding

TABLE 2 Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)[a]	Purpose	Position
TftD-S	AGTACCTGGAGTCSCTSAACGAC	To amplify part of sequence of *tcpA*	1955–1977 in *tcp* gene cluster
TftD-A	CGGSGTSCCGTTGAATTCTCGAA	To amplify part of sequence of *tcpA*	3285–3262 in *tcp* gene cluster
TY-S	CCGTGGGAAACGCAGACTATCG	To amplify the homologous sequence of *tcpA*	2290–2311 in *tcp* gene cluster
TY-A	TGCACTGCGGGAATCTTGTTGG	To amplify the homologous sequence of *tcpA*	2906–2885 in *tcp* gene cluster
TftDR-Sp1	CTCACAGGGCGACGAACTGGACG	To amplify the flanking sequence of *tcpA*	2676–2698 in *tcp* gene cluster
TftDR-Sp2	GTCGCACGTGTTCCACCTAGGCA	To amplify the flanking sequence of *tcpA*	2733–2755 in *tcp* gene cluster
TftDR-Sp3	CACCAACAAGATTCCNNNNNNNNNGACTCG	To amplify the flanking sequence of *tcpA*	2883–2912 in *tcp* gene cluster
TftDF-Sp1	CGCACAGTTCAGGTCATGCTGCTTG	To amplify the flanking sequence of *tcpA*	2388–2364 in *tcp* gene cluster
TftDF-Sp2	TCAGCGCCGATAGTCTGCGTTTC	To amplify the flanking sequence of *tcpA*	2318–2296 in *tcp* gene cluster
TftDF-Sp3	CGTCGATGTAGGTCTNNNNNNNNNAGTTGT	To amplify the flanking sequence of *tcpA*	2281–2252 in *tcp* gene cluster
TCPXA-S	GGTACCGACGCGAGGTGATTAGCAAA (KpnI)	Complementation for the *tcpA* insertion mutant	1132–1151 in *tcp* gene cluster
TCPXA-A	GGGCCCTGCACTCCTTCCTTGGTTCA (ApaI)	Complementation for the *tcpA* insertion mutant	3556–3537 in *tcp* gene cluster
TCPRXA-S	GGTACCGACTATAACCGGCGCTCATC (KpnI)	Heterologous expression of the *tcpRXA* gene cluster of T6	145–164 in *tcp* gene cluster
TCPRXA-A	GGGCCCTGCACTCCTTCCTTGGTTCA (ApaI)	Heterologous expression of the *tcpRXA* gene cluster of T6	3556–3537 in *tcp* gene cluster
ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	To amplify the flanking sequence of Mariner transposon	None
ARB2	GGCCACGCGTCGACTAGTACNNNNNNNNNNNACGCC	To amplify the flanking sequence of Mariner transposon	None
ARB3	GGCCACGCGTCGACTAGTAC	To amplify the flanking sequence of Mariner transposon	None
F(1)-SP1	AGCCAGGGATGTAACGCACT	To amplify the flanking sequence of Mariner transposon	198–179 in Mariner transposon
F(1)-SP2	TAACGGCTGACATGGGAATT	To amplify the flanking sequence of Mariner transposon	128–109 in Mariner transposon
F(2)-SP1	GCAAGAATCCCAGCAATCG	To amplify the flanking sequence of Mariner transposon	1721–1703 in *dhp* gene cluster
F(2)-SP2	GACGCCACGGTGACAAGAT	To amplify the flanking sequence of Mariner transposon	1632–1614 in *dhp* gene cluster
F(3)-SP1	CAGGCTCTGGTACAAACACG	To amplify the flanking sequence of Mariner transposon	404–385 in *dhp* gene cluster
F(3)-SP2	CATGAAGCTGAAGATGCGGT	To amplify the flanking sequence of Mariner transposon	361–342 in *dhp* gene cluster
R(1)-SP1	TTGAAGGATCAGATCACGCA	To amplify the flanking sequence of Mariner transposon	3322–3341 in Mariner transposon
R(1)-SP2	GGTATCGCTCTTGAAGGGAACT	To amplify the flanking sequence of Mariner transposon	3463–3484 in Mariner transposon
R(2)-F	GAACTCGTCGTTGCTATCGG	To amplify the flanking sequence of Mariner transposon	2610–2629 in *dhp* gene cluster
R(2)-R	CAGTGTCAGGAAACTACCGC	To amplify the flanking sequence of Mariner transposon	5361–5342 in *dhp* gene cluster
R(3)-SP1	ACCTTGCTATCGGTCTTCGG	To amplify the flanking sequence of Mariner transposon	5192–5211 in *dhp* gene cluster
R(3)-SP2	TGTCGTTGCCTGTGCGTAAG	To amplify the flanking sequence of Mariner transposon	5247–5266 in *dhp* gene cluster
R(4)-SP1	CTTCTTCGGCTAAGCGTGC	To amplify the flanking sequence of Mariner transposon	5491–5509 in *dhp* gene cluster
R(4)-SP2	CGCATTGTGCCATGGTTG	To amplify the flanking sequence of Mariner transposon	5575–5592 in *dhp* gene cluster
DHPI-S	CATATGCCTAGAGCCAGTGCGGA (NdeI)	Expression of *dhpI*	2340–2359 in *dhp* gene cluster
DHPI-A	CTCGAGGGACTCAGACGATCTCGACC (XhoI)	Expression of *dhpI*	3060–3041 in *dhp* gene cluster
DHPJ-S	CATATGAAACCACTCGAACGCA (NdeI)	Expression of *dhpJ*	3081–3099 in *dhp* gene cluster
DHPJ-A	CTCGAGTGCACGGATCCGGCTATGA (XhoI)	Expression of *dhpJ*	3972–3954 in *dhp* gene cluster

[a] Restriction sites of primers are underlined.

pBB-XA. The plasmid pBB-XA was transformed into T6-Δ*tcpA* by triparental conjugation to generate the complementation strain T6-Δ*tcpA*-com, and the resulting transconjugants were selected on LB plates with Gm, Tc, and Km. Complementation of *tcpA* was confirmed by extracting the plasmid pBB-XA.

Heterologous expression of the *tcpRXA* gene cluster in *E. coli* DH10B. The gene cluster of *tcpRXA* (88% identity to *tcpRXA* from *C. necator* JMP134) was cloned from the genomic DNA of strain T6 with the primers TCPRXA-S and TCPRXA-A (Table 2). Then, the fragment was ligated into the pMD19-T vector, producing the plasmid pMD19-RXA, which was transformed into *E. coli* DH10B, yielding the *tcpRXA* gene cluster expression strain DH10B-*tcpRXA*. Meanwhile, the strain DH10B-pUC19 containing the plasmid pUC19 (TaKaRa Biotechnology, Dalian, China) was taken as a control, and the heterologous expression strain *E. coli* DH10B containing *tcpRXA* from *C. necator* JMP134 (24) was constructed for comparison with DH10B-*tcpRXA*.

Screening for green metabolite degradation-deficient mutants. In the process of TCP degradation by *Ralstonia* sp. T6, a green intermediate metabolite was detected (15) and was used as a selection marker for strain T6 mutants losing part of their TCP degradation ability. A mutant library of strain T6 was generated by Mariner transposon mutagenesis. *E. coli* $SM10_{\lambda pir}$ containing the plasmid pSC123 (25) was used as the donor strain. The conjugation procedure was the same as that described in the literature (26), except that the antibiotics used were Km and Tc. After incubation at 30°C for 3 days, conjugants were transferred onto numbered LB plates supplemented with Km and Tc. All of the colonies on numbered LB plates were then transferred into 96-well plates with filter-sterilized MSM containing the green intermediate metabolite, Km, and Tc. Colonies that grew on numbered LB plates but did not make the green color fade were selected and streaked to yield single colonies.

Cloning of the fragments flanking the Mariner transposon. The sequences flanking the Mariner transposon in the T6 mutant were obtained by thermal asymmetric interlaced PCR (TAIL PCR) (27) and sequence homology to *Ralstonia* sp. strain H16 with the primers presented in Table 2. The primers ARB1, ARB2, ARB3, F(1)-SP1, and F(1)-SP2 were used for the first round of amplifying the 5′ flanking sequence of the transposon. Besides ARB1, ARB2, and ARB3, the primer pairs F(2)-SP1 and F(2)-SP2 and F(3)-SP1 and F(3)-SP2 were used for the second and third rounds of amplifying the 5′ flanking sequence, respectively. The primers ARB1, ARB2, ARB3, R(1)-SP1, and R(1)-SP2 were used for the first round of amplifying the 3′ flanking sequence of the transposon, while the primers R(2)-S and R(2)-A were used for the second round of amplifying the 3′

flanking sequence, according to sequence homology. Besides ARB1, ARB2, and ARB3, the primer pairs R(3)-SP1 and R(3)-SP2 and R(4)-SP1 and R(4)-SP2 were used for the third and fourth rounds of amplifying the 3′ flanking sequence, respectively. The conditions for PCR with the primers R(2)-S and R(2)-A were as follows: 5 min of denaturation at 94°C, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 2.5 min, and a final extension at 72°C for 10 min.

After purification by agarose gel electrophoresis, PCR fragments were ligated into the pMD19-T vector and transformed into *E. coli* DH5α for sequencing.

Expression of *dhpI* and *dhpJ* in *E. coli*. DNA fragments containing *dhpI* and *dhpJ* were independently amplified by PCR from genomic DNA of strain T6 using two primer sets, DHPI-S and DHPI-A for *dhpI* and DHPJ-S and DHPJ-A for *dhpJ*. The amplified PCR fragments were sequenced and separately inserted into the expression vector pET29a with NdeI and XhoI to produce plasmids pET-I and pET-J. The pET29a constructs were then transformed into *E. coli* BL21(DE3) for expression with induction of 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) at 30°C for 3.5 h after it was grown in LB with 50 mg/liter kanamycin at 37°C to an optical density at 600 nm (OD_{600}) of 0.5.

The cells were harvested and suspended in 8 ml of 20 mM phosphate-buffered saline (PBS) buffer (pH 7.0) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After the cells were disrupted by ultrasonication (Kutota; Insonator 201 M) on ice, the lysate was centrifuged at 15,000 × *g* for 15 min, and the supernatant was obtained as the cell extracts. The expressed proteins were identified by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28).

TCP degradation or transformation by *Ralstonia* sp. T6 and *E. coli* DH10B derivatives. The inocula for all of the biodegradation or transformation experiments by *Ralstonia* sp. T6 and *E. coli* DH10B derivatives were prepared by growing bacteria in 50 ml LB medium overnight on a rotary shaker (180 rpm) at 30°C and 37°C, respectively. Cells were collected by centrifugation at 3,800 × *g* for 10 min at room temperature. The pellets of *Ralstonia* sp. T6 and DH10B derivatives were washed twice and resuspended with MSM to give OD_{600}s of 2.1 and 1.1, respectively. The cells were inoculated at 5% (vol/vol) into a 50-ml flask containing 20 ml TMM (50 mg/liter, pH 7.0) for T6 derivatives and 20 ml TMM (10 mg/liter, pH 7.0) for DH10B derivatives and incubated at 30°C in a rotary shaker (180 rpm). TCP was measured by a previously described method (15). All experiments were performed in triplicate.

TCP and 2,4,6-trichlorophenol conversion by *E. coli* DH10B derivatives, *Ralstonia* sp. T6, and *C. necator* JMP134. All of the TCP and 2,4,6-trichlorophenol conversion experiments by *E. coli* DH10B derivatives, *Ralstonia* sp. T6, and *C. necator* JMP134 were carried out as mentioned above. *Ralstonia* sp. T6 and *C. necator* JMP134 were grown at 30°C, and OD_{600} was adjusted to 2.1; *E. coli* DH10B derivatives were grown at 37°C, and OD_{600} was adjusted to 1.1. The initial concentration of 2,4,6-trichlorophenol was 20 mg/liter, and those of TCP for *E. coli* DH10B derivatives and *C. necator* JMP134 were 11 mg/liter and 10 mg/liter, respectively.

Enzyme assay of cell extracts. The crude cell extract enzyme assay was demonstrated by detecting the depletion of the substrate. To detect DphJ, activity against 3,6-dihydroxypyridine-2,5-dione (DHPD) was determined by monitoring the disappearance of absorbance at 390 nm (UV-2450; Shimadzu). Enzymatic activity was measured in MSM (pH 7.0) containing 0.2 mM DHPD and 0.02 mg protein/ml of DphJ cell extracts. The product formed from complete conversion of DHPD was prepared as the substrate of DhpI. The reaction mixture of DhpI contained 0.03 mg protein/ml of the cell extracts and 0.2 mM substrate. The reaction was followed by monitoring the loss of absorbance at 332 nm. Those reactions were carried out in a 3.05-ml reaction mixture for DhpJ and a 3.1-ml reaction mixture for DhpI, both of which were incubated at 30°C for 40 min until substrates completely vanished in a UV-visible scanning spectrum. All assays were also conducted with strains carrying only vectors as controls. One unit of enzyme activity was defined as the amount required for the disappearance of 1 nmol substrate per min. Specific activities are expressed as units per milligram of protein. All experiments were performed in triplicate.

Analysis of the metabolites during TCP degradation by strain T6. A preliminary analysis of the green metabolite produced during TCP biodegradation by T6 was previously reported (15). To further identify the green metabolite, it was analyzed by tandem mass spectrometry (MS/MS) (Finnigan TSQ Quantum Ultra AM; Thermal, USA) with direct injection. The green metabolite was purified with a preparative liquid chromatograph equipped with a YMC-Pack Pro C_{18} column (20 by 150 mm, 5-μm grain size) for nuclear magnetic resonance (NMR) analysis to further verify its structure, and the mobile phase was acetonitrile-water (30:70, vol/vol). ^{1}H NMR measurements were conducted on a Bruker Avance III 400 spectrometer operating at 300.18 MHz with dimethyl sulfoxide (DMSO) inside at room temperature.

Another metabolite in TCP biodegradation by T6 was named Met_{332} for its UV absorbance at 332 nm. Met_{332} was analyzed by MS/MS (Agilent 6410; triple quadrupole liquid chromatograph/mass spectrometer [QQQ LC/MS]) and ionized by electrospray with a negative polarity. The metabolite was identified from 15 ml of the reaction mixtures of the DhpJ crude extracts, which were dried by lyophilization and dissolved in 2 ml methanol. The mobile phase was methanol-water (60:40, vol/vol), and the flow rate was 0.5 ml/min.

Nucleotide sequence accession numbers. The DNA sequences obtained in this study have been deposited in the GenBank database (accession no. KC294622 for the *tcpRXA* gene cluster and accession no. KC294623 for the *dhpRIJK* gene cluster).

RESULTS

Disruption of *tcpA* in *Ralstonia* sp. T6 and cloning of the *tcpRXA* gene cluster. Considering the structural similarity of 3,5,6-trichloropyridinol and 2,4,6-trichlorophenol, we deduced that 2,4,6-trichlorophenol monooxygenase TcpA from *C. necator* JMP134 might be involved in the degradation of 3,5,6-trichloropyridinol by *Ralstonia* sp. T6. To test our hypothesis, an approximately 1,330-bp fragment of *tcpA* was amplified from the genomic DNA of *Ralstonia* sp. T6, and it showed 91% identity to *tcpA* of strain JMP134. The *tcpA* gene of T6 was then disrupted with the suicidal plasmid pJQ-TY, which carried a 617-bp internal fragment of *tcpA*, to confirm its function in the biodegradation of TCP. The wild-type strain of T6 could turn the medium green and degrade TCP. In contrast, the mutant strain T6-Δ*tcpA* could not degrade TCP (Fig. 1A) and no green intermediate was produced, but it could degrade the green metabolite DHPD generated by strain T6 (data not shown).

Disruption of *tcpA* from strain T6 indicated that *tcpA* and its flanking genes function in the initiation step of TCP biodegradation. A 6,412-bp fragment was amplified by SEFA PCR and contained three putative genes that constitute the *tcpRXA* gene cluster (Fig. 2). All *tcp* genes start with the ATG codon. The genes *tcpR*, *tcpX*, and *tcpA* are 972 bp, 552 bp, and 1,554 bp in length, respectively. Gene *tcpR* is located approximately 150 bp upstream of *tcpX*, which is spaced from *tcpA* by a sequence of 104 bp. RT-PCR analyses were performed to clarify the transcriptional features of these genes. Total RNA extracted from strain T6 was used as the template, and three oligonucleotide sets were used as the PCR primers. RT-PCR with each of the primer sets amplified DNA fragments of the expected sizes (see Fig. S1 in the supplemental material). These results indicated that *tcpX* and *tcpA* were transcribed from a common promoter. The best matches with existing database sequences for the *tcp* genes are shown in Table 3. TcpA and TcpR are closely related to 2,4,6-trichlorophenol monooxy-

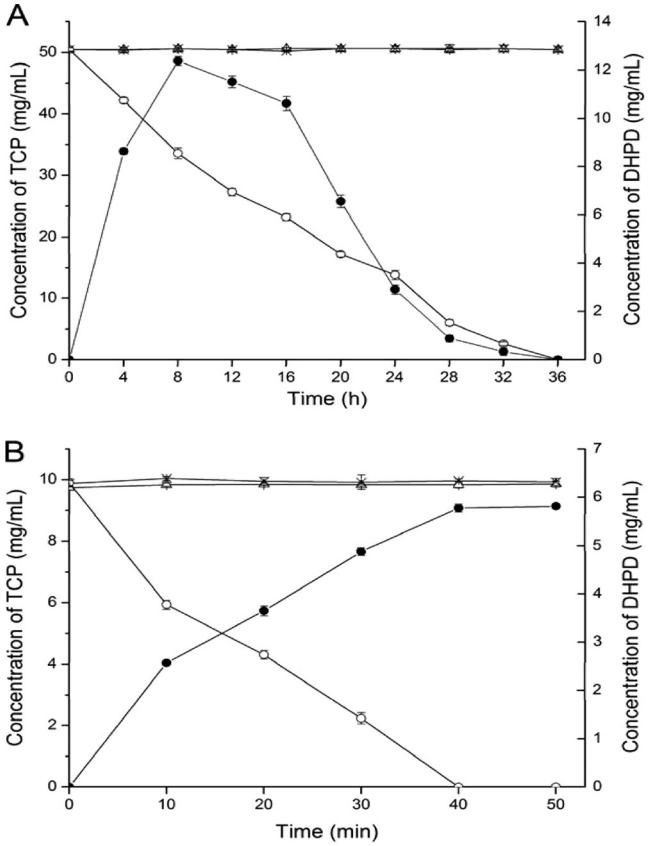

FIG 1 (A) Degradation of 3,5,6-trichloro-2-pyridinol (TCP) by the derivatives of *Ralstonia* sp. T6. △, concentration of TCP not inoculated; ×, concentration of TCP inoculated with the mutant T6-Δ*tcpA*; ○, concentration of TCP inoculated with the complementation strain T6-Δ*tcpA*-com; ●, concentration of DHPD inoculated with T6-Δ*tcpA*-com. (B) Transformation of 3,5,6-trichloro-2-pyridinol (TCP) by the derivatives of *E. coli* DH10B. △, concentration of TCP not inoculated; ×, concentration of TCP inoculated with DH10B-pUC19; ○, concentration of TCP inoculated with DH10B-*tcpRXA*; ●, concentration of DHPD inoculated with DH10B-*tcpRXA*.

genase and LysR family transcriptional regulator, respectively, and TcpX is closely related to a flavin reductase that can deliver H from NAD(P)H to flavin adenine dinucleotide (FAD) to produce reduced FAD ($FADH_2$). The proteins encoded by *orf1* and *orf2* show high identity to NADH-dependent maleylacetate reductase TcpD from *C. necator* JMP134, which is involved in 2-chloromaleylacetate reduction, and the GntR family transcriptional regulator, respectively.

Function of *tcpA* in TCP biodegradation. To verify the function of *tcpA* in TCP biodegradation, a DNA fragment of 2,437 bp, including *tcpXA* with its original promoter, was amplified using the primers TCPXA-S and TCPXA-A. The strain T6-Δ*tcpA*-com recovered the ability to degrade TCP and showed an accumulation of DHPD comparable to strain T6 (Fig. 1A); the degradation rate was 33.3 mg $liter^{-1}$ day^{-1} (22% of strain T6). Additionally, the *tcpRXA* gene cluster expression strain, DH10B-*tcpRXA*, could completely transform 10 mg/liter TCP to DHPD in 40 min, but the control strain DH10B-pUC19 could not transform TCP to DHPD (Fig. 1B). Strain DH10B-*tcpRXA* could not further transform DHPD (data not shown). These data indicate that *tcpA* is the key gene in TCP degradation by strain T6, the protein of which can

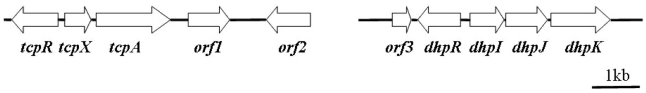

FIG 2 Organization of 3,5,6-trichloro-2-pyridinol catabolic clusters in *Ralstonia* sp. T6. The primers for generating the *tcpRXA* and *dhpRIJK* gene clusters are listed in Table 2. The homology of *Ralstonia* sp. T6 gene products is shown in Table 3.

dehalogenate TCP to yield DHPD. In addition, in contrast to *E. coli* DH10B with *tcpRXA* (DH10B-*tcpRXA*), *E. coli* DH10B with *tcpA* alone could not transform TCP to DHPD (data not shown); it indicated that monooxygenase TcpA needs flavin reductase TcpX to supply $FADH_2$ when transforming TCP.

Cloning and sequence analysis of the *dhpRIJK* gene cluster from T6. A library of DHPD degradation mutants of T6 was obtained by random transposon mutagenesis. Four clones that could not degrade DHPD (data not shown) were selected from more than 6,000 mutants, and one of them was designated DHPD-RM-1 and used to amplify the flanking sequences of the Mariner transposon.

The genomic DNA of the mutant DHPD-RM-1 was extracted for PCR amplification. Subsequently, a 5,942-bp fragment containing the mutated gene and its flanking sequences was obtained after several rounds of genome walking by PCR. Sequence analysis showed that the Mariner transposon inserted in a putative AraC family transcriptional regulator gene. Four putative genes were identified and annotated as the *dhpRIJK* gene cluster, on the basis of BLAST analysis (Fig. 2). Most *dhp* genes start with an ATG codon, except for *dhpK*, which starts with a GTG codon. The genes *dhpR*, *dhpI*, *dhpJ*, and *dhpK* are 900 bp, 717 bp, 879 bp, and 1,251 bp in length, respectively. The spacing between *dhpR* and *dhpI* is 211 bp, the spacing between *dhpI* and *dhpJ* is 24 bp, and the spacing between *dhpJ* and *dhpK* is 62 bp. The best database matches with existing sequences for the *dhp* genes are shown in Table 3. DhpR, DhpI, DhpJ, and DhpK are highly similar to AraC family transcriptional regulator, putative fumarylpyruvate hydrolase, chitooligosaccharide deacetylase, and MFS family transporter of the *Ralstonia eutropha* H16 genome (accession number AM260480), respectively.

Functional analysis of *dhpI* and *dhpJ* in TCP biodegradation. IPTG-induced expression of DhpI and DhpJ was confirmed by SDS-PAGE (see Fig. S2 in the supplemental material). The molecular mass of DhpI is approximately 30 kDa, and that of DhpJ is approximately 37 kDa, which agree well with the molecular masses deduced from their amino acid sequences. Cell extracts of *E. coli* BL21-pETJ were found to contain DhpJ with a specific activity of 250 U/mg. The characteristic absorption peak of DHPD at 390 nm vanished completely in UV scanning, and a new absorption peak at 332 nm appeared, which indicated that a new metabolite was produced (Fig. 3A). The metabolite was named Met_{332} according to its absorption at 332 nm. Neither DHPD consumption nor Met_{322} release was detected in the negative control. This result indicated that *dhpJ* is the key gene in the biodegradation of DHPD.

The metabolite Met_{332} generated a deprotonated prominent molecular ion at $m/z = 158$ $[M\text{-}H]^-$ in the first-order MS that is 18 Da more than its parent molecule of DHPD. This finding indicates that Met_{332} is the product generated by the hydrolysis of DHPD. DhpJ is highly similar to chitooligosaccharide deacetylase

TABLE 3 BLAST results for deduced amino acid sequences of TCP catabolic gene clusters

T6 protein	Representative homolog	Source	GenBank accession no.	% identity
TcpR	TcpR, LysR family transcriptional regulator	*Cupriavidus necator* JMP134	YP_295793	89
	LysR family transcriptional regulator	*Cupriavidus necator* N-1	YP_004687804	89
TcpX	TcpX, putative flavin reductase	*Cupriavidus necator* JMP134	YP_295794	87
	RutF, NAD(P)H-flavin reductase	*Cupriavidus necator* N-1	YP_004687805	87
TcpA	TcpA, 2,4,6-trichlorophenol monooxygenase	*Cupriavidus necator* JMP134	YP_295795	96
	2,4,6-Trichlorophenol monooxygenase	*Cupriavidus necator* N-1	YP_004687806	96
Orf1	TcpD, maleylacetate reductase	*Cupriavidus necator* JMP134	YP_295799	91
	TftE, maleylacetate reductase	*Cupriavidus necator* N-1	YP_004687810	92
Orf2	GntR family transcriptional regulator	*Cupriavidus necator* JMP134	YP_295790	82
	GntR family transcriptional regulator	*Cupriavidus necator* N-1	YP_004687751	83
DhpR	AraC family transcriptional regulator	*Ralstonia eutropha* H16	YP_728590	99
	AraC family transcriptional regulator	*Burkholderia dolosa* AUO158	ZP_04947452	41
DhpI	Putative fumarylpyruvate hydrolase	*Ralstonia eutropha* H16	YP_728591	97
	Fumarylacetoacetate (FAA) hydrolase family protein 14	*Achromobacter xylosoxidans* A8	YP_003982846	70
DhpJ	Chitooligosaccharide deacetylase	*Ralstonia eutropha* H16	YP_728592	99
	Polysaccharide deacetylase	*Methylobacterium radiotolerans* JCM 2831	YP_001757215	50
DhpK	MFS family transporter	*Ralstonia eutropha* H16	YP_728593	99
	Major facilitator transporter	*Achromobacter xylosoxidans* AXX-A	EGP45397	43
Orf3	Hypothetical protein	*Ralstonia eutropha* H16	YP_728589	95
	Glutathione-dependent formaldehyde-activating protein	*Marinobacter aquaeolei* VT8	YP_960185	76

that hydrolyzes the amide bond and has a structure similar to the amide bond in DHPD. Met_{332} is preliminarily identified as 5-amino-2,4,5-trioxopentanoic acid (ATOPA), which shows characteristic fragment ion peaks of the second-order MS at m/z = 140 $[M-18]^-$, 86 $[M-72]^-$, indicating the results of losing H_2O, CO + CO + NH_2 (Fig. 4B).

The hydrolysis product of DHPD, ATOPA, was used as the substrate for DhpI. The characteristic absorption peak at 332 nm

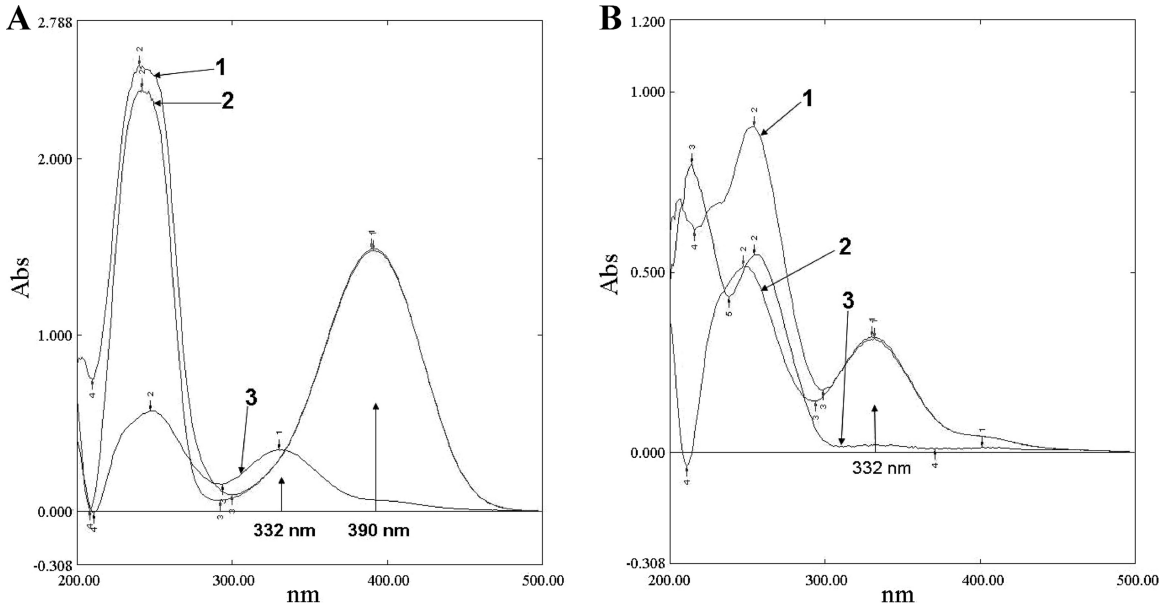

FIG 3 (A) UV-visible spectral changes in DHPD conversion. Line 1, added with cell extracts of BL21-pET29a; line 2, added with MSM; line 3, added with cell extracts of BL21-pET-J. (B) UV-visible spectral changes in ATOPA conversion. Line 1, added with cell extracts of BL21-pET29a; line 2, added with MSM; line 3, added with cell extracts of BL21-pET-I.

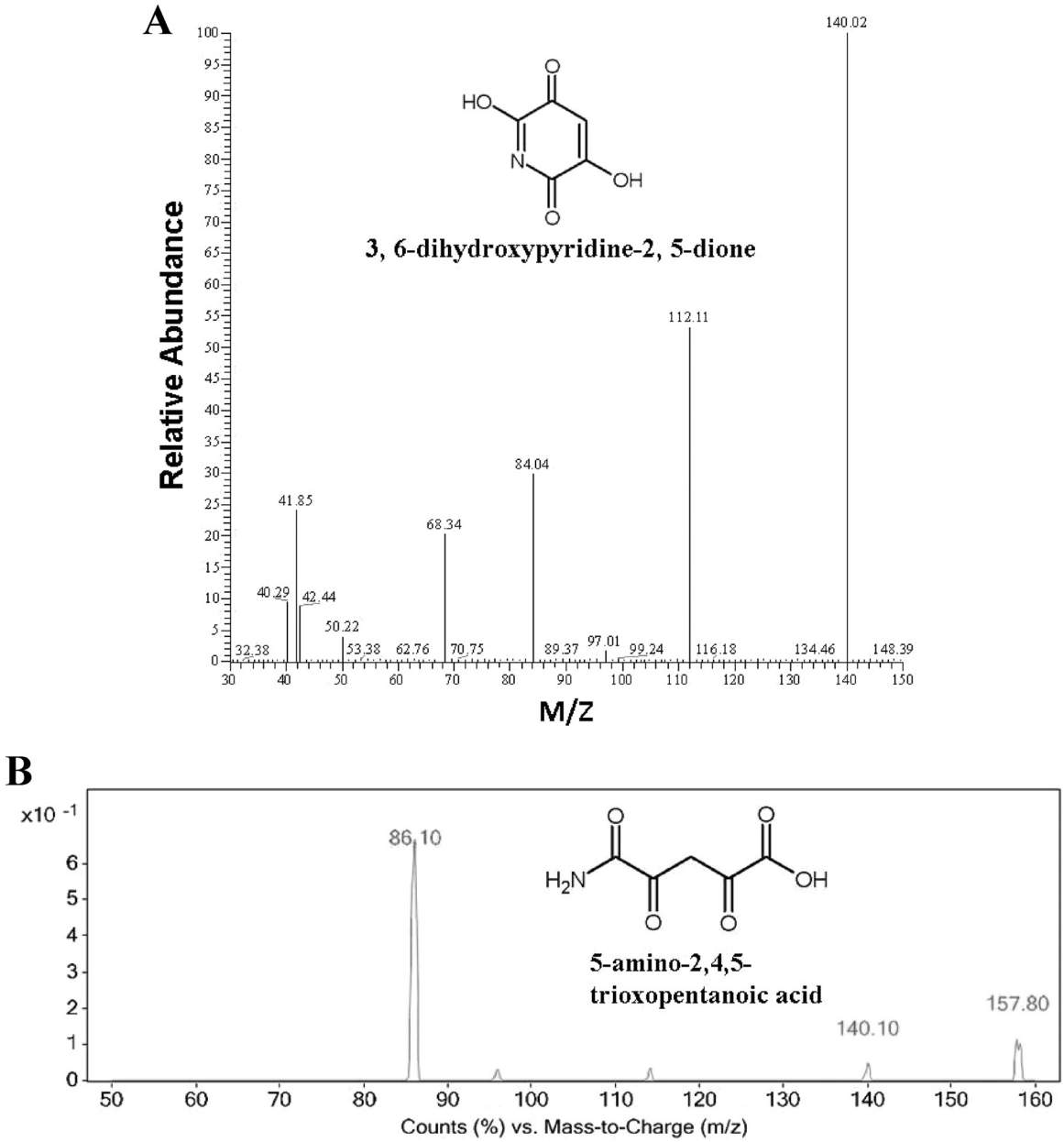

FIG 4 Mass spectra of the intermediate metabolites in 3,5,6-trichloro-2-pyridinol degradation by *Ralstonia* sp. T6. (A) Negative-ion mass spectra of the metabolite 3,6-dihydroxypyridine-2,5-dione; (B) negative-ion mass spectra of the metabolite 5-amino-2,4,5-trioxopentanoic acid.

of ATOPA vanished after incubation with DhpI for 40 min at 30°C (Fig. 3B). ATOPA was transformed completely by DhpI, which showed a specific activity of 167 U/mg. These results indicated that DhpI is the key enzyme in the degradation of ATOPA.

DISCUSSION

The environmental fate of 3,5,6-trichloro-2-pyridinol (TCP) has been widely investigated because of its potential toxicity to the environment (29–33). The complete degradation of TCP in soil is believed to be microbially mediated (7), but the bacteria that degrade TCP are difficult to screen for because of the antibiotic activity of TCP (6). Most TCP degraders isolated degraded TCP with a relatively low efficiency; 3 to 12 days was required to degrade less than 100 mg/liter of TCP with the concentration of 10^8 cells/ml, and some of them lost the ability when the TCP concentration was higher than 200 mg/liter (11). Strain T6 was a highly effective TCP degrader and could degrade 100 mg/liter TCP in 12 h by equivalent numbers of cells and even retain the degradative ability at high concentrations of TCP up to 700 mg/liter (15). Strain T6 is a good model system for researching the molecular mechanism of TCP biodegradation.

The *tcpRXA* gene cluster was cloned from strain T6 and showed significant identity with the *tcpRXA* gene cluster involved in 2,4,6-trichlorophenol degradation from *C. necator* JMP134. *tcpB* (quinone reductase gene), *tcpC* (6-chlorohydroxy-quinol 1,2-dioxygenase gene), *tcpY* (unknown protein gene), and *tcpD*

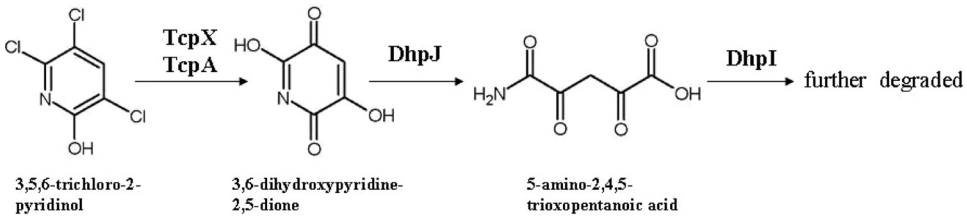

FIG 5 Proposed metabolic pathway for 3,5,6-trichloro-2-pyridinol in *Ralstonia* sp. T6.

(maleylacetate reductase gene) were downstream of *tcpRXA* in *C. necator* JMP134, but they were all missed in *Ralstonia* sp. T6; however, *tcpD* showed significant identity to *orf1* (34). The insertion mutation strain T6-Δ*tcpA* could not degrade TCP but could degrade the green metabolite DHPD, and the complementation strain T6-Δ*tcpA*-com recovered the ability to degrade TCP. The *tcpRXA* gene cluster expression strain, *E. coli* DH10B-*tcpRXA*, had the ability to transform TCP to DHPD. These results indicated that *tcpA* is the key gene for the initiation step of TCP degradation by strain T6.

TcpA of strain T6 was completely different from Tcp3A derived from the cow rumen metagenomic library (16). It has been reported that *E. coli* DH5α harboring *tcp3A* could use TCP as the sole source of carbon for its growth, but the reaction catalyzed by Tcp3A has not been identified (16). Strain T6 could also use trichlorophenol as the substrate, but transformation activity of *C. necator* JMP134 was not observed when TCP was used as the substrate (see Fig. S3 in the supplemental material). However, when the *tcpRXA* genes from *C. necator* JMP134 and *Ralstonia* sp. T6 were expressed in *E. coli*, both of them could use TCP and trichlorophenol as the substrates (see Fig. S4 in the supplemental material). This outcome implied the lack of transporter of TCP in *C. necator* JMP134. In addition, it is reasonable to speculate that TcpA from *Ralstonia* sp. T6 confers a reaction mechanism similar to that proposed for TcpA from *C. necator* JMP134. TcpA of strain JMP134 catalyzes sequential dechlorinations of 2,4,6-trichorophenol by oxidative and hydrolytic reactions (19, 35, 36). TcpA of strain T6 performs a function toward dehalogenase from the pyridine ring similar to that of TcpA of strain JMP134. We failed to detect any dehalogenation intermediates during the degradation of TCP by strain T6, and the 3 chlorides were released simultaneously, as determined by chloride titration during the degradation process (15). The green intermediate was preliminarily identified as 3,6-dihydroxypyridine-2,5-dione (15). To further clarify its structure, the green metabolite, with a molecular ion at *m/z* 140 $[M]^-$ in the first-order MS, was analyzed by MS/MS. The green metabolite showed characteristic fragment ion peaks of the second-order MS at *m/z* = 112 $[M-28]^-$, 84 $[M-56]^-$, 68 $[M-72]^-$, 42 $[M-98]^-$, indicating that CO, CO + CO, CO + CO + O, and CO + CO + CH≡COH were lost (Fig. 4A). We further verified its structure with NMR. In the NMR spectrum of DHPD (see Fig. S5 in the supplemental material), only one hydrogen atom on the pyridine ring (7.30 ppm) could be detected. The hydrogens of the hydroxyl group were too active to be detected. According to the MS and NMR results, the structure of DHPD was proved to be right. These results indicated that 2,4,6-trichlorophenol monooxygenase TcpA of strain T6 might have adopted novel dehalogenation mechanisms for the degradation of TCP.

The products of *tcpRXA* from strain T6 could not further utilize DHPD. It can be expected that diverse genes are involved in the degradation of TCP. The *dhpRIJK* gene cluster was cloned after random transposon insertion, which is closely related to a conceptually annotated gene cluster from *R. eutropha* H16 on the basis of BLAST analysis. This 5,341-bp (bp 271 to 5611) fragment showed 98% similarity to the genome sequence of *R. eutropha* H16, and a 265-bp sequence in the 5′ terminus was similar to a transposase from *Ralstonia oxalatica* transposon Tn*437*. No significant similar sequences were identified for the 331-bp sequence at the 3′ terminus. Such an organized structure implies that strain T6 perhaps acquired this genomic region through bilateral transfer. DhpJ (H16_B0428) from strain H16 is a putative chitooligosaccharide deacetylase, which catalyzes the hydrolysis of carbon-nitrogen bonds. DHPD also has a similar bond structure. Based on the structure of the product, 5-amino-2,4,5-trioxopentanoic acid (ATOPA), we deduced that DhpJ of strain T6 could cleave the pyridine ring of DHPD by hydrolysis. This is different from the ring fission of other hydroxylated pyridines (37). Dioxygenase and monooxygenase were proposed to be involved in the transformation of hydroxylated pyridines (37). ATOPA could be degraded further by DhpI. 3,6-Dihydroxypyridine-2,5-dione hydrolase DhpJ and 5-amino-2,4,5-trioxopentanoic acid hydrolase DhpI could collaboratively degrade DHPD. Additionally, it was deduced that DhpR and DhpK play a regulatory role and a transporter role, respectively, in the *dhpRIJK* gene cluster by analysis of protein sequence homology. Insertion mutations in *dhpR* or *dhpJ* caused strain T6 to be deficient in DHPD degradation.

A proposed pathway of TCP biodegradation by strain T6 is shown in Fig. 5. 2,4,6-Trichlorophenol monooxygenase TcpA of strain T6 transforms TCP to DHPD first, which is then cleaved by the hydrolysis reaction of 3,6-dihydroxypyridine-2,5-dione hydrolase DhpJ to produce ATOPA. ATOPA is further degraded by 5-amino-2,4,5-trioxopentanoic acid hydrolase DhpI. One well-known report on the TCP biodegradation metabolic pathway addresses the combination of photolytic and microbiological degradation by *Pseudomonas* sp. ATCC 700113. The resting cells of *Pseudomonas* sp. ATCC 700113 degraded the reductive dechlorination products of TCP generated by photolysis, suggesting that *Pseudomonas* sp. ATCC 700113 utilizes a reductive dechlorination pathway in degrading TCP (18). However, we propose that TCP is dechlorinated by oxidative and hydrolytic reactions in strain T6, to generate DHPD. The metabolic pathway of TCP biodegradation in strain T6 has never been reported before.

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