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Functions of Flavin Reductase and Quinone Reductase in 2,4,6-Trichlorophenol Degradation by *Cupriavidus necator* JMP134[▽]

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The *tcpRXABCYD* operon of *Cupriavidus necator* JMP134 is involved in the degradation of 2,4,6-trichlorophenol (2,4,6-TCP), a toxic pollutant. TcpA is a reduced flavin adenine dinucleotide ($FADH_2$)-dependent monooxygenase that converts 2,4,6-TCP to 6-chlorohydroxyquinone. It has been implied via genetic analysis that TcpX acts as an FAD reductase to supply TcpA with $FADH_2$, whereas the function of TcpB in 2,4,6-TCP degradation is still unclear. In order to provide direct biochemical evidence for the functions of TcpX and TcpB, the two corresponding genes (*tcpX* and *tcpB*) were cloned, overexpressed, and purified in *Escherichia coli*. TcpX was purified as a C-terminal His tag fusion ($TcpX_H$) and found to possess NADH:flavin oxidoreductase activity capable of reducing either FAD or flavin mononucleotide (FMN) with NADH as the reductant. $TcpX_H$ had no activity toward NADPH or riboflavin. Coupling of $TcpX_H$ and TcpA demonstrated that $TcpX_H$ provided $FADH_2$ for TcpA catalysis. Among several substrates tested, TcpB showed the best activity for quinone reduction, with FMN or FAD as the cofactor and NADH as the reductant. TcpB could not replace $TcpX_H$ in a coupled assay with TcpA for 2,4,6-TCP metabolism, but TcpB could enhance TcpA activity. Further, we showed that TcpB was more effective in reducing 6-chlorohydroxyquinone than chemical reduction alone, using a thiol conjugation assay to probe transitory accumulation of the quinone. Thus, TcpB was acting as a quinone reductase for 6-chlorohydroxyquinone reduction during 2,4,6-TCP degradation.

Trichlorophenols are anthropogenic environmental contaminants known to cause harm to humans (2, 21). These chemicals have been used in wood and leather preservation and as biocides and herbicides (17, 21). The aerobic bacterium *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134 (22) completely degrades 2,4,6-trichlorophenol (2,4,6-TCP) (14, 16). The key enzymes converting 2,4,6-TCP to 3-ketoadipate have been identified and characterized, and the corresponding *tcpRXABCYD* operon has been described (14, 19, 25). The *tcpA* gene encodes the monooxygenase responsible for the initial steps of dechlorination. The product of TcpA is 6-chlorohydroxyquinone, a potentially detrimental chemical. This quinone may be chemically reduced by a reducing agent, such as NADH, reduced flavin adenine dinucleotide ($FADH_2$), or ascorbate (14) or potentially enzymatically reduced to the corresponding quinol. 6-Chlorohydroxyquinol is the substrate for the *tcpC* gene product, a dioxygenase that cleaves the ring (14, 16).

Since TcpA is an $FADH_2$-dependent monooxygenase, it requires a flavin reductase to provide $FADH_2$. Sequence analysis indicates that both *tcpX* and *tcpB* encode potential flavin reductases. However, TcpX is likely to generate $FADH_2$ for TcpA due to its similarity to several partner flavin reductases of $FADH_2$-dependent monooxygenases (5, 11, 15) and its gene location proximity with *tcpA*. Further evidence is provided by the increased 2,4,6-TCP degradation upon coexpression of cloned *tcpA* and *tcpX* compared to that with *tcpA* expression alone (19). TcpB belongs to PF00881 (1, 4), a nitroreductase family with broad substrate specificities (9, 13, 26, 27). Its gene location between *tcpA* and *tcpC* suggests a possible role in 2,4,6-TCP metabolism. Since the product of TcpA is 6-chlorohydroxyquinone (25), TcpB possibly reduces this product to 6-chlorohydroxyquinol for further metabolism. In order to provide direct evidence on the function of TcpX and TcpB, the proteins were overproduced in *Escherichia coli* and characterized.

MATERIALS AND METHODS

Chemicals and enzymes. All reagents used were of the highest purity available and were purchased from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI), or Fisher Scientific Co. (Pittsburgh, PA). PCRs were performed with *Taq* DNA polymerase and primers purchased from Invitrogen (Carlsbad, CA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA).

Bacterial strains and culture conditions. *C. necator* JMP134 was grown at 30°C in a mineral salt medium described previously (14). *E. coli* strains DH5α and BL21(DE3) were grown on Luria-Bertani (LB) medium or on LB agar with kanamycin (30 μg/ml) at 37°C or as specified.

Gene cloning and protein expression. *C. necator* JMP134 genomic DNA was isolated using the PureGene DNA isolation kit (Gentra, Minneapolis, MN). The *tcpX* gene was amplified from JMP134 with primers TcpXF (GAGGAGATCC ATATGTCGTCC) and TcpXR (CTTCAAGTCGGAATTCGCGGCGAC) and cloned between the NdeI and EcoRI sites of the pET-30 LIC vector (Novagen, Madison, WI) to contain a C-terminal six-His tag fusion gene. The *tcpB* gene was amplified with PCR primers TcpBF (GCAAGGAGGAATTCATGCAAACCA ATG) and TcpBR-stop (GCCTTGGTAAGCTTATGCTGGTCATACTC) and cloned between the EcoRI and HindIII sites of the pET-30 LIC vector as a nonfusion gene. The ligation products were then electroporated into *E. coli* DH5α. The correct clones were identified by colony PCR and sequencing, and the plasmid was isolated using the QIAprep Spin MiniPrep kit (Qiagen, Valencia, CA). The correct clone was electroporated into *E. coli* BL21(DE3) for recombinant protein production.

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Protein purifications. All purification steps were performed at 4°C. $TcpA_H$, the 2,4,6-TCP monooxygenase from *C. necator* JMP134, was purified as previously reported (14). His-tagged TcpX ($TcpX_H$) and TcpB were purified from *E. coli* cells overproducing the proteins. Cells were grown in 1 liter LB medium at 37°C to a turbidity of 0.5 at 600 nm, induced with 300 μM isopropyl-β-D-thiogalactopyranoside, and then incubated at room temperature for 4 h. The cells were harvested by centrifugation and suspended in 20 mM potassium phosphate (KPi) buffer (pH 7.0). Freshly prepared phenylmethylsulfonyl fluoride in absolute ethanol was added to a final concentration of 0.5 mM. The cells were disrupted by passage through a French pressure cell (model FA-030; Aminco, Urbana, IL) three times at 260 MPa. The lysate was centrifuged at 10,000 × *g* for 10 min at 10°C to remove cell debris. The supernatants were further ultracentrifuged at 50,000 × *g* for 1 h to remove membranes. The supernatant of $TcpX_H$ was further purified by mixing nickel-nitrilotriacetic acid agarose resin (10 mg protein per 2 ml resin) (Qiagen) for binding for 1 h. Resin was packed into a small column by gravity and washed with 10 ml wash buffer. $TcpX_H$ was then eluted with 4 ml of the elution buffer. The wash buffer contained 20 mM KPi (pH 7.0), 0.3 M NaCl, 1 mM dithiothreitol (DTT), and 20 mM imidazole, and the elution buffer contained all of the above except that imidazole was added to a final concentration of 200 mM. The buffer was exchanged with 20 mM KPi (pH 7.0) containing 10% glycerol and 1 mM DTT using a Centriprep Ultracel YM-10 centrifugal filter device (Millipore, Bedford, MA). The sample was aliquoted and stored at −80°C.

TcpB purification was similar to $TcpX_H$ purification through the ultracentrifugation step. The supernatant contained approximately 25 mg of protein. The sample was brought to 30% saturation of ammonium sulfate and centrifuged. The supernatant was loaded onto a phenyl agarose column (1.5 by 18 cm; Sigma) equilibrated with a 20% saturation of ammonium sulfate in the KPi buffer (pH 7.0) containing 1 mM DTT. The proteins were eluted with 100 ml of a linear gradient of ammonium sulfate (20% to 0% saturation) in KPi buffer (pH 7.0) with 1 mM DTT. TcpB was eluted around 12% saturation of ammonium sulfate. Fractions containing TcpB were pooled and precipitated with ammonium sulfate (70% saturation). After centrifugation, the pellet was resuspended in 5 ml 20 mM KPi (pH 7.0) and subjected to dialysis in the KPi buffer containing 1 mM DTT. After 4 h, the sample was centrifuged to remove any precipitated proteins and the supernatant injected onto a Bio-Scale UNO-QR1 column (7 by 35 mm; Bio-Rad, Hercules, CA) equilibrated with the KPi buffer. TcpB did not bind to the column and came off in a 2-ml wash with the equilibrating buffer. The samples were brought to 10% glycerol and stored at −80°C.

Enzyme assays. Flavin reductase activity was monitored by following the disappearance of the NADH absorbance at 340 nm (ε_{340} = 6,220 M^{-1} cm^{-1}) in 60 mM KPi (pH 6.7) at 25°C. Concentrations of the components were 300 μM for NADH and 20 μM for FAD. The reaction was initiated by adding enzyme.

Quinone reductase activity was measured by using menadione as the quinone substrate, because the proposed native substrate, 6-chlorohydroxyquinone, was not commercially available. The pH was lowered to pH 6.0 to avoid menadiol autooxidation. Ferricyanide and nitrofurazone were also tested as alternative substrates. Assays included 300 μM NADH, 20 μM FAD or flavin mononucleotide (FMN), and 100 μM of the electron acceptor (quinone, ferricyanide, or nitrofurazone) in 90 mM KPi buffer at 25°C. The consumption of NADH was followed spectrophotometrically for the quinone reductase assay. Ferricyanide reductase activity was monitored by following the disappearance of the ferricyanide absorbance at 420 nm (ε_{420} = 12,960 M^{-1} cm^{-1}). Nitrofurazone reductase activity was monitored by following the consumption of nitrofurazone at 400 nm (ε_{400} = 17,800 M^{-1} cm^{-1}).

For kinetic analysis, three independent sets of experiments were run with substrate concentrations from 1/2 K_m to 4 K_m. Data were fitted to a Michaelis-Menten equation using KaleidaGraph software (Synergy, Reading, PA).

pH, ionic strength, and temperature optima. $TcpX_H$ activity was tested using the flavin reductase assay. TcpB activity was measured using the ferricyanide assay, since menadione undergoes autooxidation at pHs above 6.0. Activities were tested at pH levels ranging from 6.0 to 7.6 in 60 mM KPi buffer for $TcpX_H$ and 90 mM KPi for TcpB at 25°C. The effect of ionic strength was tested in KPi buffer (pH 7.0), ranging from 10 to 500 mM. Enzyme activity was also tested at temperatures from 25 to 45°C in 40 mM KPi (pH 7.0).

Enzyme complementation. The 40-μl assay mixture consisted of 20 mM KPi (pH 7.0), 10 μg of TcpA, 2 μg of $TcpX_H$, and 2 μg of TcpB if included, 100 μM 2,4,6-TCP, 1 mM ascorbic acid, 10 U (μmol/min) of catalase (Sigma), and 5 μM FAD. The addition of 2.5 mM NADH initiated the reaction and incubation at 30°C for 5 min or longer. Reactions were terminated by the addition of 40 μl of an acetonitrile-acetic acid mixture (vol/vol, 9:1), and products were centrifuged and analyzed with high-performance liquid chromatography (HPLC) using a C_{18} column and photodiode array detector, as previously described (14). Samples contained a fixed amount of TcpA where $TcpX_H$ and TcpB concentrations were varied. Enzymatic rates were calculated from the decrease of 2,4,6-TCP, which was eluted off the column at 8.9 min with an absorption maximum of 292 nm.

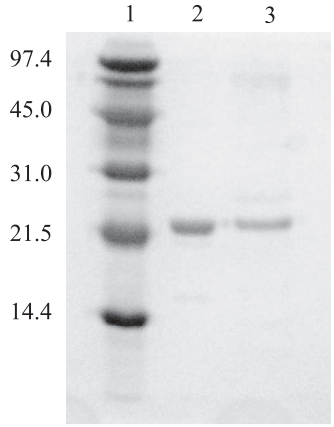

FIG. 1. SDS-PAGE of TcpB and $TcpX_H$. Lane 1, molecular mass standards in kDa (Bio-Rad); lane 2, 2 μg of purified TcpB; lane 3, 1 μg of purified $TcpX_H$.

Thiol conjugation experiments. The 40-μl assay mixture included 20 mM KPi (pH 7.0), 100 μM 2,4,6-TCP, 1 mM ascorbic acid, 10 U of catalase, 10 μg of TcpA, 2 μg of $TcpX_H$, and/or 2 μg of TcpB, 5 μM FAD, and 1 mM glutathione (GSH) or 2-mercaptoethanol. NADH was added to initiate the reaction. Reaction mixtures were incubated at 30°C for 20 min and analyzed with HPLC as described above for the disappearance of 2,4,6-TCP and appearance of 6-chlorohydroxyquinol, which came off the column at 5.8 min with a maximum absorption at 290 nm (14).

Analytical methods. An Ultrospec 4000 (Pharmacia Biotech, Piscataway, NJ) UV-visible spectrophotometer was used to analyze absorption changes during enzymatic assays. Data were recorded using the SWIFT program (Pharmacia Biotech) and transferred to Microsoft (Redmond, WA) Excel format for analysis. An HPLC system (Waters, Milford, MA) was utilized with a Biosep Sec-S3000 size exclusion column (7.8 by 300 mm; Phenomenex, Torrance, CA) to estimate native molecular weights of pure proteins (6). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a reported method (12), and gels were stained with GelCode blue stain reagent (Pierce, Rockford, IL). Protein concentrations were determined by using a Bio-Rad protein assay with bovine serum albumin as the standard.

RESULTS

Overproduction and purification of $TcpX_H$ and TcpB. For ease of purification, the genes *tcpX* and *tcpB* were cloned into the pET30-LIC vector to generate $pTcpX_H$ and pTcpB, respectively. A C-terminal His tag fusion $TcpX_H$ protein was produced for ease of purification. Since a C-terminal His tag fusion $TcpB_H$ protein was insoluble in *E. coli* cells, a nonfusion TcpB protein was produced in *E. coli* for purification and characterization. Strain BL21(DE3) carrying $pTcpX_H$ or pTcpB produced large quantities of soluble and active $TcpX_H$ or TcpB. Both were purified to apparent homogeneity (Fig. 1), and the purified proteins were colorless. $TcpX_H$ migrated to 21 kDa on an SDS-PAGE gel, agreeing with the calculated molecular weight of 21,344. TcpB appeared to be a 21-kDa protein by SDS-PAGE, in good agreement with the calculated weight of 21,532. For a typical purification, 8 mg of pure $TcpX_H$ and 10 mg of TcpB were purified from 1 liter of culture. The enzyme stocks were stored at −80°C with no apparent loss of activity after several months.

TABLE 1. Kinetic parameters of $TcpX_H$[a]

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
FMN_{NADH}[b]	25.0 ± 2.5	570.2 ± 14.3	2.3×10^7
$NADH_{FMN}$[d]	64.8 ± 6.6	559.9 ± 16.9	8.7×10^6
FAD_{NADH}[b]	10.8 ± 1.2	76.8 ± 2.6	7.1×10^6
$NADH_{FAD}$[c]	244.1 ± 48.8	94.5 ± 7.8	4.0×10^5

[a] Experiments were done in 60 mM KPi (pH 6.7) at 25°C. Values are averages of three measurements with standard deviations. No activities were detected for riboflavin and NADPH.
[b] Determined with fixed NADH concentration at 300 μM.
[c] Determined with fixed FAD concentration at 20 μM.
[d] Determined with fixed FMN concentration at 50 μM.

Enzyme characterization. Gel filtration chromatography indicated that $TcpX_H$ was a monomer. $TcpX_H$ used NADH to reduce either FAD or FMN, and no activity was observed when NADPH or riboflavin was used. The kinetic parameters of $TcpX_H$ were determined (Table 1). Although $TcpX_H$ had a lower K_m value for FAD, it had a higher catalytic efficiency for FMN (Table 1). The highest enzyme activity was observed at pH 7.0 in 60 mM KPi buffer, with 73, 81, 89, and 85% activity retained at pHs 6.6, 6.8, 7.2, and 7.4, respectively. The optimal temperature was seen at 38°C, with 80, 86, 97, and 96% activity at 32, 34, 36, and 40°C, respectively. The optimal ionic strength was 60 mM KPi, with similar activities between 10 and 80 mM, which was reduced to 80% at 100 mM KPi.

Gel filtration chromatography indicated TcpB behaved as a dimeric protein. TcpB used NADH but not NADPH for minimal FMN reduction, and no activity was observed when FAD or riboflavin was directly used as an electron acceptor (Table 2). However, TcpB effectively used several electron acceptors, including menadione and ferricyanide, in the presence of FMN (Table 2). When FMN was replaced by FAD, TcpB showed significantly lower levels of activity toward the artificial electron acceptors.

The highest activities were observed with menadione as the electron acceptor in the presence of FMN, whereas ferricyanide was a less-efficient substrate. Nitrofurazone was inert as an electron acceptor under all tested conditions. Since menadiol undergoes autooxidation at pH levels above 6.0, the ferricyanide assay was utilized to determine the optimal pH range. The highest enzymatic activity occurred at pH 6.0, with 90, 79, 81, 74, and 79% activity retained at pHs 5.8, 6.2, 6.4, 6.8, and 7.0, respectively. The optimal temperature was observed at 32°C, with 69, 91, 76, and 66% activity retained at 30, 36, 38, and 40°C, respectively. Optimal ionic strengths tested in KPi buffer were observed at concentrations between 20 and 90 mM KPi. The kinetic parameters of TcpB reduction of menadione were determined at 25°C and pH 6.0. The K_m values of NADH, FMN, and menadione were 231.1 ± 66.6 μM, 9.6 ± 2.3 μM, and 86.3 ± 16.6 μM; the k_{cat} value was 350.7 ± 28.8 s^{-1}.

TABLE 2. Electron acceptor specificity of TcpB[a]

Substrate	Sp act[b]	Relative activity (%)
Menadione + FMN	911.4 ± 64.5	100
Ferricyanide + FMN	179.1 ± 2.1	20
Menadione + FAD	35.3 ± 0.6	4
Ferricyanide + FAD	8.1 ± 0.6	2
FMN	3.5 ± 0.5	0.4
FAD	0	0

[a] Assays performed in 90 mM KPi buffer (pH 6.0) at 25°C. Values are means from three experiments with standard deviations.
[b] Specific activity is expressed as μmol NADH per minute per mg of protein.

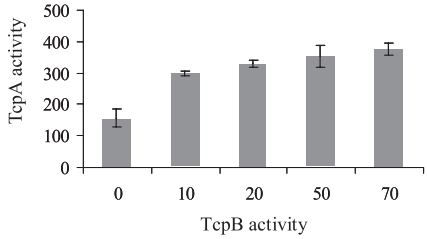

FIG. 2. TcpB enhanced 2,4,6-TCP degradation by TcpA and $TcpX_H$. When TcpB was added to the reaction mixture containing 2 μg of $TcpX_H$ and 10 μg of TcpA, a clear increase in total TcpA activity occurred. Total TcpA activity was expressed as μmol 2,4,6-TCP consumed per min; TcpB activity was μmol menadione reduced per min.

Functional roles of $TcpX_H$ and TcpB. TcpA is an $FADH_2$-dependent monooxygenase that requires an FAD reductase to generate $FADH_2$ during in vitro assays (14, 25). $TcpX_H$ but not TcpB provided $FADH_2$ to TcpA for 2,4,6-TCP oxidation in a reaction mixture containing 2,4,6-TCP, NADH, and FAD under aerobic conditions. Including TcpB in the reaction mixture enhanced the rate of 2,4,6-TCP degradation (Fig. 2). Since TcpA converts 2,4,6-TCP to 6-chlorohydroxyquinone (25), the likely explanation for the enhanced activity was that TcpB reduced 6-chlorohydroxyquinone to 6-chlorohydroxyquinol, possibly preventing product inhibition. In the absence of TcpB, 6-chlorohydroxyquinone was chemically reduced to 6-chlorohydroxyquinol by NADH, $FADH_2$, or ascorbate present in the assay mixture (14). Therefore, 6-chlorohydroxyquinone was only transitorily accumulated in the assay mixture with or without TcpB and could not be detected by our normal HPLC analysis.

The transitory accumulation of 6-chlorohydroxyquinone could be probed with organothiols, e.g., GSH and 2-mercaptoethanol, which form conjugates with chlorine-substituted quinones but not chlorine-substituted quinols (the reduced form) (10, 18). If TcpB could reduce 6-chlorohydroquinone faster than was the case with chemical reduction alone, less 6-chlorohydroxyquinone should be accumulated. Consequently, TcpB should significantly reduce the thiol-hydroxyquinone conjugate formation. When tested, 1 mM GSH in the reaction mixture completely eliminated the production of 6-chlorohydroxyquinol when 2,4,6-TCP was completely oxidized by TcpA/$TcpX_H$. A new peak at 5.8 min with an absorption maximum at 315 nm by HPLC analysis was likely the glutathionyl-hydroxyquinone conjugate. When 2-mercaptoethanol was used instead of GSH, the conjugate peak was eluted off the HPLC column at 6.6 min with an absorption maximum at 317 nm. In reactions containing TcpA, $TcpX_H$ and TcpB without additional thiols, 2,4,6-TCP was quantitatively converted to 6-chlorohydroxyquinol (100.0 μM ± 1.9 μM). Most interestingly, in reactions containing only TcpA and $TcpX_H$, only 41 μM 6-chlorohydroxyquinol was produced from complete consumption of 100 μM 2,4,6-TCP (41.5 ± 1.5 μM 6-chlorohydroxyquinol). A new HPLC peak was observed at

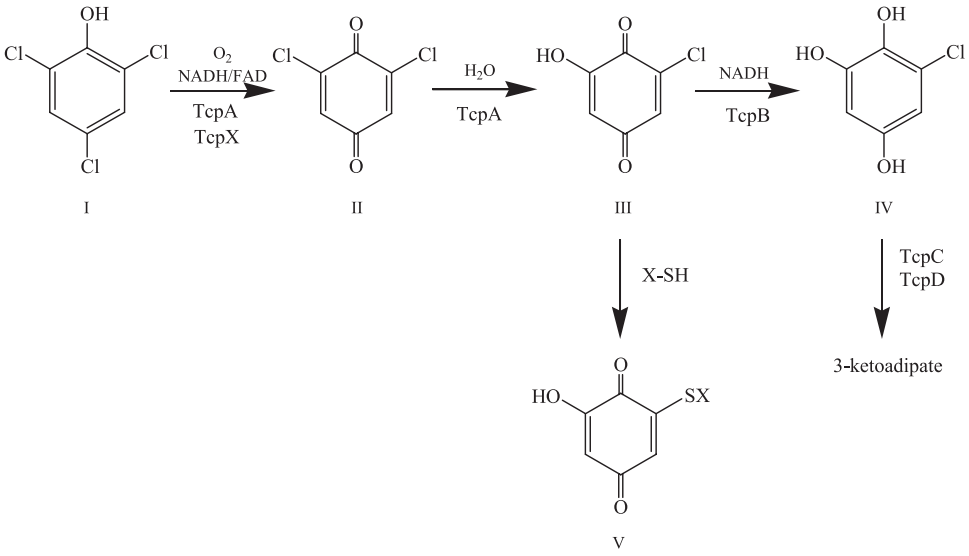

FIG. 3. Proposed functions of TcpA, TcpX, and TcpB in 2,4,6-TCP degradation. I, 2,4,6-TCP; II, 2,6-dichloroquinone; III, 6-chlorohydroxyquinone; IV, 6-chlorohydroxyquinol; V, thiol-quinone conjugate, which can be reduced to the corresponding quinol conjugate. Note: 2,6-dichloroquinone is a transitory metabolite that remained with TcpA (25).

6.2 min, with an absorption maximum at 317 nm. The new peak was likely a conjugate with 0.25 mM DTT, which was present in all reaction mixtures as a carryover from enzyme solutions and was required to stabilize the enzymes. In reactions containing TcpA, $TcpX_H$ and TcpB with 1 mM GSH, 81.4 ± 1.4 μM 6-chlorohydroxyquinol was produced.

DISCUSSION

TcpA is the $FADH_2$-dependent monooxygenase of the 2,4,6-TCP degradation pathway. It is related to other $FADH_2$-dependent monooxygenases, including TftD of *Burkholderia cepacia* AC1100 (6) and HpaB of *E. coli* W (24). TftD and HpaB require their partner flavin reductases, TftC and HpaC, respectively, for producing $FADH_2$. TcpA also has this requirement for a flavin reductase (14, 25). TcpX is likely the flavin reductase providing TcpA with $FADH_2$, because TcpX shares 53% amino acid sequence identity with TftC and 34% identity with HpaC. Genetic analysis indicates that TcpX is likely the partner flavin reductase for TcpA (19). The functional role of TcpX was directly demonstrated with our biochemical analysis. $TcpX_H$ reduced both FAD and FMN using NADH as the electron donor (Table 1), behaving similarly to TftC and HpaB. TftC has a higher catalytic efficiency for FAD than for FMN (6), while HpaC uses FMN with higher efficiency than FAD (5). With regard to the flavin preference, $TcpX_H$ is similar to HpaC. The fact that $TcpX_H$ was able to supply TcpA with $FADH_2$ for 2,4,6-TCP metabolism provided direct evidence that TcpX was the partner FAD reductase for TcpA (Fig. 3).

The function of TcpB in 2,4,6-TCP degradation has been investigated by genetic analysis (14, 19). However, *tcpB* inactivation mutants of *C. necator* still oxidize 2,4,6-TCP, suggesting that TcpB is not required to provide TcpA with $FADH_2$. In fact, TcpB did not reduce FAD (Table 2) and did not provide TcpA with $FADH_2$ in coupled assays containing only TcpA and TcpB. Our data showed that TcpB was a quinone reductase. TcpB belongs to the PF00081 enzyme family, and related enzymes often reduce nitrofurazone (9, 13, 26, 27). TcpB was unable to reduce nitrofurazone under the tested conditions, but it reduced ferricyanide and menadione in the presence of either FMN or FAD. The best activity was for menadione reduction, with a k_{cat} value as high as 350 s^{-1}. Since 6-chlorohydroxyquinone is a metabolic intermediate of 2,4,6-TCP degradation, TcpB played a critical role in reducing it. First, it enhanced TcpA activity, possibly by removing the enzyme product and preventing product inhibition; second, it reduced the production of thiol-quinone conjugates between 6-chlorohydroxyquinone and cellular thiols. Thus, it is clear that TcpB played an important role in 2,4,6-TCP degradation by reducing 6-chlorohydroxyquinone to 6-chlorohydroxyquinol, which is the ring cleavage substrate of TcpC.

The role of quinone reductases in the microbial degradation of substituted aromatic compounds was first demonstrated with 4-methyl-5-nitrocatechol metabolism in *Pseudomonas* sp. strain DNT (8). A monooxygenase converts 4-methyl-5-nitrocatechol to 2-hydroxy-5-methylquinone with the removal of the nitro group. This quinone is reduced to a quinol by a quinone reductase. The *ortho*-nitrophenol degradation pathway of *Alcaligenes* sp. strain NyZ215 also requires a monooxygenase to oxidize the substrate to create *o*-benzoquinone. OnpB of this pathway is thought to reduce the quinone to a catechol. The *onpB* gene is required when the pathway genes are transferred to *Pseudomonas putida* PaW340, indicating OnpB catalyzes a critical step in *o*-nitrophenol degradation in vivo (23). For pentachlorophenol degradation in *Sphingobium chlorophenolicum*, pentachlorophenol monooxygenase oxidizes pentachlorophenol to tetrachloro-*p*-quinone. The quinone can be chemically reduced to tetrachloro-*p*-quinol by NADH, but a quinone reductase (PcpD) can facilitate the reduction and can enhance the activity of pentachlorophenol monooxygenase (3). Clearly, TcpB also enhanced 2,4,6-TCP degradation when in-

cluded in the reaction mixture containing TcpA/TcpX$_H$ (Fig. 2). Although OnpB, PcpD, and TcpB all function as quinone reductases in biodegradation pathways, they are different types of enzymes. PcpD and OnpB belong to COG1018:flavodoxin reductase (ferredoxin-NADPH reductases) family 1, containing a bound FAD and a [2Fe-2S] center. TcpB belongs to the COG0778:nitroreductase family (20). Further, none are related to mammalian quinone reductases involved in general detoxification. Thus, it appears that microorganisms can use a variety of reductases to fulfill quinone reductase roles.

To further demonstrate that TcpB was involved in the reduction of 6-chlorohydroxyquinone, we used thiols to probe its activity. TcpA and TcpX$_H$ oxidized 2,4,6-TCP to 6-chlorohydroxyquinone, which was reduced to 6-chlorohydroxyquinol with or without TcpB in an enzymatic reaction. However, TcpB was more efficient for the reduction than chemical reaction by NADH, FADH$_2$, or ascorbate present in the reaction mixture. This conclusion was derived from GSH probing, since GSH conjugates with chlorine-substituted quinones but not the corresponding quinols (10, 18). Without TcpB, GSH formed a conjugate with no production of 6-chlorohydroxyquinol. TcpB minimized the conjugate formation (see above).

Although the thiol experiment was designed to demonstrate that 6-chlorohydroxyquinone reduction was more efficient by TcpB catalysis than by chemical reduction alone, the results also suggested that TcpB is important in 2,4,6-TCP degradation. Our in vitro tests showed that TcpB minimized reaction between 6-chlorohydroxyquinone and GSH that leads to the formation of glutathionyl-quinone conjugate. In vivo, the quinone would be exposed to multiple thiols, including GSH, but also free cysteine and cysteine residues of proteins. These cellular covalently linked thiol conjugates are detrimental to the cell and can cause toxicity (7). Further, the formation of conjugates may prevent complete degradation of 2,4,6-TCP.

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