BIOCHEMICAL CHARACTERIZATION OF 2,4,6-TRICHLOROPHENOL

DEGRADATION IN BACTERIUM *CUPRIAVIDUS*

NECATOR JMP134

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of SARA

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Abstract

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2,4,6-Trichlorophenol (TCP) is a priority pollutant introduced into the environment

through numerous sources including preservations of wood and leather as well as kraft

pulp mill bleaching. The removal of TCP from the environment can be accomplished

through bioaugmentation, a bioremediation approach involving addition of specific

microorganisms to minimize the contaminant. *Cupriavidus necator* JMP134 is an

attractive bioaugmentation agent as it is not only able to metabolize TCP but also utilizes

it as a sole carbon and energy source. The degradation pathway has been elucidated and

the enzymes responsible have been localized to the *tcpRXABCYD* gene cluster on the

chromosome. While the functional roles of *tcpR, tcpA, tcpC,* and *tcpD* were previously

identified in the metabolism of TCP, the roles of the *tcpX, tcpB*, and *tcpY* gene products

had not been determined and were the focus of our studies. The first enzyme of the

degradation, the $FADH_2$-utilizing monooxygenase TcpA, requires reduced flavin to

convert TCP to 6-chloro-*p*-hydroxyquinone. Sequence analysis indicated both *tcpX* and

tcpB were potential flavin reductases that would satisfy the partner flavin reductase role. Both proteins were purified and their enzymatic activities were determined. TcpX was found to be the active NADH:flavin oxidoreductase. In coupled assays, TcpX acts as the partner flavin reductase to TcpA. Purified TcpB could not reduce flavin, instead acting to reduce quinone substrates. In coupled assays, TcpB reduces the TcpA reaction product, 6-chloro-*p*-hydroxyquinone, to 6-chloro-*p*-hydroxyquinol. This enzymatic reduction was further studied and found to minimize the detrimental side reactions of the 6-chloro-*p*-hydroxyquinone with cellular thiols. Computational analysis indicates TcpY is a β-barrel outer membrane protein. Constitutive expression of *tcpY* increases the uptake of TCP in two *C. necator* species. TcpY is thus characterized as an outer membrane transporter for TCP. Our work completes the identification of the enzymes in the TCP degradative pathway for *C. necator* JMP134, and the mechanism for TCP entry into the cell has been determined. Our findings have the potential to be utilized in the bioengineering of bacteria for bioremediation of TCP.

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DEDICATION

This work is dedicated to my entire family whether by blood or by choice. I have been

extremely lucky to receive unconditional love and support from too many people to

individually name. I would like to single out my partner, Athena Harper, for her love and

her laughter.

CHAPTER ONE

INTRODUCTION AND BACKGROUND

Bioremediation

A xenobiotic is a chemical considered foreign to the environment in which it is found (see Figure 1 for xenobiotics discussed here). A large increase in the production and use of xenobiotics in the past 60 years has created numerous sites of environmental contamination that need to be restored to their pre-contamination state (17, 27). The treatment of these sites is complicated by the fact that many of these xenobiotics are recalcitrant to microbial breakdown (27). Thus, the persistence of numerous organic chemicals in the environment is of significant public concern due to potential toxicity, mutagenicity, and bioconcentration in higher organisms. Cleanup strategies traditionally employ physical means to remove the contaminant, but often result only in the transport of the contaminant to another medium or location and not its total elimination.

In contrast to physical remediation, bioremediation often results in the complete eradication of the xenobiotic. This method utilizes biological activities to reduce or completely eliminate xenobiotics. Thus, it can be viewed as a more cost effective and desirable cleanup technology. In particular, bioaugmentation is an approach for bioremediation that has shown success in various ecosystems. This process refers to the deliberate addition of natural or engineered microorganisms with the desired catalytic activities for xenobiotic breakdown (17). Successful bioaugmentation efforts include the use of *Pseudomonas vesicularis* to significantly increase the dechlorination rate of the insecticide α-hexachlorocyclohexane (6) and the studies of Crawford and Mohn (14) who

bioaugmented contaminated soil samples with *Sphingobium chlorophenolicum* (formerly

Flavobacterium sp.) resulting in complete elimination of the contaminating

pentachlorophenol.

The studies presented here focus on the bacterium *Cupriavidus necator* JMP134

and its degradation pathway for the xenobiotic 2,4,6-trichlorophenol (TCP). The

degradation pathway (Figure 2) was partially characterized when these studies were

initiated (12, 37, 41, 49, 54, 64). We report here the isolation and characterization of

several proteins participating in this TCP mineralization increasing the attractiveness of

this strain for use as a bioaugmentation agent against TCP.

2,4,6-Trichlorophenol

In general, chlorophenols (CPs) consist of the benzene ring, a hydroxyl group and

chlorine. CPs encompass many compounds with significantly different molecular

structures and thus diverse physical and chemical properties (15). The origins of CPs

range from biological production, such as tick production of 2,6-dichlorophenol as a sex

pheromone (7, 42), to the synthetic pentachlorophenol molecule first produced in the

1930s (43). The higher chlorinated CPs are considered substances of high environmental

impact due to their toxicity and persistence.

Of particular interest is TCP, a hazardous substance listed as a priority pollutant

by the US Environmental Protection Agency (EPA) (20). TCP exists in the environment

due to its use since the 1950s as an antiseptic, a wood, leather, and glue preservative, and

an anti-mildew treatment (22, 33, 46).   It can form during treatment of industrial

wastewaters, and during chlorination of drinking water (46). It is also a major component

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of bleached kraft mill pulp effluents (35). Degradation of prochloraz, a widely used

broad spectrum fungicide, results in accumulation of TCP, a decomposition intermediate

(8). TCP can also exist as an impurity in 2,4-dichlorophenoxyacetic acid (2,4-D, another

widely used pesticide (20).

Although TCP production was discontinued in most countries in the 1980s, it is

still found in numerous ecosystems, including rivers throughout the world (39, 47, 60).

For example, 30 μg/L was detected in the St. Maurice River in Canada (31) and up to

26.6 μg/L is present in the Isipingo River in South Africa (26). Fish present in rivers can

accumulate TCP (47, 60) with a bioconcentration factor of 250-310 (23), indicating that

levels of TCP are 250-310 times higher in fish than the surrounding waters. The soils

around sawmill and waste sites can be saturated with TCP (33), in turn causing the

groundwater to contain from 0.03 to 91.3 μg/L of TCP (62). Even human urine is not

free from contamination, as 1.3 μg/L of TCP was detected in 84% of the American

population sampled by Bravo et al. (9).

TCP toxicity

TCP can be toxic to humans because it is absorbed into the body through drinking

water, food, or inhalation of contaminated air (5, 46). Occupational exposure is

associated with respiratory problems including cough, chronic bronchitis, chest

wheezing, altered pulmonary function, and pulmonary lesions (5). No data are available

for carcinogenicity in humans, but animal studies showed male F344 rats subjected to a

prolonged exposure of 10,000 μg/L of TCP developed lymphomas and leukemias, and

B6C3F1 mice subjected to prolonged exposure of 5,000 μg/L developed hepatocellular

adenomas and carcinomas (44). TCP is mutagenic in *Escherichia coli* (18) and

Saccharomyces cerevisiae (21). Genotoxic effects have also been identified in *Bacillus subtilis* (48). Pregnant mice injected with TCP displayed increased frequency of spotted coats in the offspring, suggesting TCP causes genetic alterations in mammals (21). TCP also possesses DNA-damaging activity in human leukemic (HL) cell line-60 cells (48), and it induces both structural and numerical chromosome aberrations in Chinese hamster ovary cells and V79 cells (4, 32). Recently, TCP was found to induce point mutations in the *P53* gene of zebrafish at as little as 5 μg/L exposure (65).

Although the mechanism of toxicity is not known, the cell culture and animal data above contributes to TCP being listed in the class 2B group of possible human carcinogens (63). The World Health Organization has determined the maximum admissible concentration in drinking water for TCP was 200 μg/L (63) while the US EPA Clean Water Act sets the limit much lower, at 2.4 μg/L (46). Due to its toxicity and widespread contamination, the US EPA has identified TCP as a harmful pollutant (20) with at least 20 sites in the US requiring immediate cleanup (Figure 3).

Biodegradation of TCP

Under aerobic conditions, most CPs are resistant to biodegradation due to chlorine substituents interfering with the action of oxygenase enzymes that initiate degradation (13). Fortunately, TCP degradation can be mediated aerobically by various microorganisms (8, 25, 34, 36, 52). The first TCP degrading bacterium, strain KC3, was identified in 1973 (11). KC3 was initially characterized for its ability to degrade pentachlorophenol, but later discovered to also degrade other CPs including TCP. TCP

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degradation continued to be identified in other pentachlorophenol-degrading bacteria including *Arthrobacter* sp. (55), *Sphingobium chlorophenolicum* (56), and *Rhodococcus chlorophenolicus* (3). The TCP degradation appeared to be performed by pentachlorophenol degradation enzymes, and not enzymes specific for TCP. The first bacterium characterized for its ability to specifically degrade TCP, and not pentachlorophenol, was *Azotobacter* strain GP1 (36). Other TCP-degraders include *Streptomyces rochei* 303 (25) and *Alcaligenes eutrophus* TCP (2). The latter bacterium is particularly interesting, as it successfully removed TCP from contaminated soil samples, indicating its potential as a bioaugmentation organism (2). The subject of our study, *C. necator* JMP134, is closely related to this strain.

Cupriavidus necator JMP134

C. necator JMP134 (ex *Alcaligenes eutrophus*; ex *Ralstonia eutropha*; ex *Wautersia eutropha*) is a Gram-negative bacterium whose genome has been sequenced. It was isolated from Australian soil for its ability to utilize the herbicide 2,4-D as a sole carbon and energy source using degradative enzymes encoded on the pJP4 plasmid (19, 50). In fact, the strain has successfully remediated soils contaminated with 2,4-D in studies focusing on its bioaugmentation potential (16, 30, 40, 53). *C. necator* JMP134 is also able to consume TCP as a sole energy and carbon source (12). Valenzuela et al. (61) successfully bioaugmented *C. necator* JMP134 into bleached kraft mill effluents to eliminate TCP. No significant removal of TCP was observed in the absence of this strain. As studies have shown *C. necator* JMP134 to be a successful bioaugmentation

agent, the full characterization of the TCP degradation pathway in *C. necator* JMP134 is

highly desirable for the advancement of TCP bioremediation.

TCP degradation and the *tcpRXABCYD* gene cluster

Clement et al. (12) determined that TCP degradation was encoded separately from

the 2,4-D degradative enzymes encoded on the pJP4 plasmid. Louie et al. (37) localized

the genes responsible for degradation to the chromosome, identifying a portion of the

tcpRXABCYD gene cluster (Figure 4) that was further defined by Matus et al. (41). Prior

to the studies we conducted, only sequence homology data existed for the *tcpX*, *tcpB*, and

tcpY genes, thus resulting in a partially characterized degradation pathway (Figure 2).

Our efforts sought to complete the elucidation of the TCP biodegradation pathway in *C.*

necator JMP134.

The TCP-degradative enzymes were found to be inducible and subject to

catabolite repression by Louie et al. (37). Matus et al. (41) determined that the *tcpR* gene

shared significant identity with the *pcpR* gene of *S. chlorophenolicum*. The *pcpR* gene

encodes a LysR-type regulator that acts as a positive transcriptional regulator of

pentachlorophenol degradation (10). Transcriptional fusion studies demonstrated that

TcpR activates the expression of *tcpXABCYD* by responding specifically to TCP (54).

TcpR was thus identified as the positive regulator of the operon.

Louie et al. (37) first identified the *tcpA* gene as being absolutely required for

growth on TCP, and partial purification identified TcpA as a TCP 4-monooxygenase.

Further analysis of purified TcpA by Xun and Webster (64) indicated it first catalyzes an

oxidative conversion of TCP to 2,6-dichloro-*p*-benzoquinone, followed by a hydrolytic

dechlorination to 6-chloro-*p*-hydroxyquinone. The oxidative reaction requires reduced flavin adenine dinucleotide ($FADH_2$), indicating TcpA is an $FADH_2$-utilizing monooxygenase. These enzymes are often closely associated with partner flavin reductases. For example, TftD is a $FADH_2$-utilizing monooxygenase from *Burkholderia cepacia* AC1100 that converts 2,4,5-trichlorophenol to 5-chloro-*p*-hydroxyquinone (24). TftC is the partner flavin reductase to TftD, and the genes encoding the two enzymes are directly linked (24, 29). The *tcpX* gene directly upstream and the *tcpB* gene directly downstream of *tcpA* share sequence homology with flavin reductases (54). However, a *tcpB* inactivation mutant still degraded TCP (37). Sanchez and Gonzalez (54) compared TCP degradation rates by *tcpR* mutants of *C. necator* JMP134 (no native TCP degradation) containing *tcpA*, *tcpXA* or *tcpAB*. Recombinants of *tcpA* and *tcpAB* transformed TCP at similar rates, indicating that TcpB is not the partner flavin reductase. The *tcpXA* vector, on the other hand, significantly increased the transformation of TCP, suggesting that *tcpX* was likely the partner flavin reductase. Chapter two describes our purification and characterization of TcpX to further explore its functionality.

The direct product of the TcpA-catalyzed reaction, 6-chloro-*p*-hydroxyquinone, must be reduced to 6-chloro-*p*-hydroxyquinol before ring cleavage can occur (64). This reduction can occur chemically (64), but chlorinated quinones can also chemically react with thiols including glutathione, an abundant cellular molecule. The thiol-conjugate products are often more toxic than the substrate and cannot be further degraded by the pathway (38, 45). Therefore, enzymatic reduction is a viable route for minimizing detrimental side reactions. The *tcpB* gene has sequence homology to nitroreductases, but, as discussed above, it is not essential for TCP degradation under laboratory conditions

(37, 54). As nitroreductases often have broad substrate ranges, we believed TcpB might utilize 6-chloro-*p*-hydroxyquinone as a quinone substrate. Chapter two describes our exploration of the functional role of TcpB and its possible role in TCP degradation *in vivo*.

6-Chloro-*p*-hydroxyquinol is the substrate for the TcpC protein, 6-chloro-*p*-hydroxyquinol 1,2-dioxygenase. This enzyme catalyzes the opening of the aromatic ring (Figure 2). The product of this reaction is 2-chloromaleylacetate (37). The last gene in the cluster, *tcpD*, encodes a maleylacetate reductase that converts 2-chloromaleylacetate to β-ketoadipate (54). The β-ketoadipate can then be converted to the tricarboxylic acid cycle metabolites acetyl-CoA and succinyl-CoA through the actions of the enzymes encoded by the *pcaIJF* gene cluster (51), a pathway common in several *Proteobacteria* (28).

While the degradation pathway discussed above appears complete, the function of *tcpY*, an unidentified gene within the operon, remained unknown. The gene was peculiar for its lack of homologous genes in the current BLAST database (1). A search utilizing the predicted amino acid sequence returned no significant identity to any known proteins, but it did return as a member within the cluster of orthologous group (COG) 4313 (57-59). Although the function of this COG4313 is defined as proteins involved in the *meta*-pathway of phenol degradation, no experimental evidence exists for this prediction. Sanchez and Gonzalez (54) determined that the *tcpY* inactivation mutant was able to grow and remove TCP at rates similar to the wild type of *C. necator* JMP134. Chapter three details our journey to discover the functional role for TcpY.

The significance

Our studies have contributed significant advances towards understanding the biodegradation of TCP by *C. necator* JMP134. They help to complete our knowledge of the TCP degradation pathway, including the analysis of potentially toxic thiol-conjugate intermediates (Chapter two) and the first identification of a protein involved in transport of TCP into the cell (Chapter three). Detailed analysis of TCP biodegradation by *C. necator* JMP134 suggests that potential biodegradative problems (including buildup of the mentioned thiol-conjugates) may be avoided, thus enhancing bioremediation. As *C. necator* JMP134 has already shown success in bioaugmentation studies, both for 2,4-D (16, 53) and TCP (61), our findings will enhance the likelihood of the inclusion of this species in future TCP bioremediation strategies, including those employed at TCP-contaminated sites in the US (Figure 3).

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Figure 1. Chemical structures of relevant xenobiotics. IUPAC name of prochloraz is *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide.

α–hexachlorocyclohexane   pentachlorophenol   2,4,6–trichlorophenol

prochloraz   2,4–dichlorophenoxyacetic acid
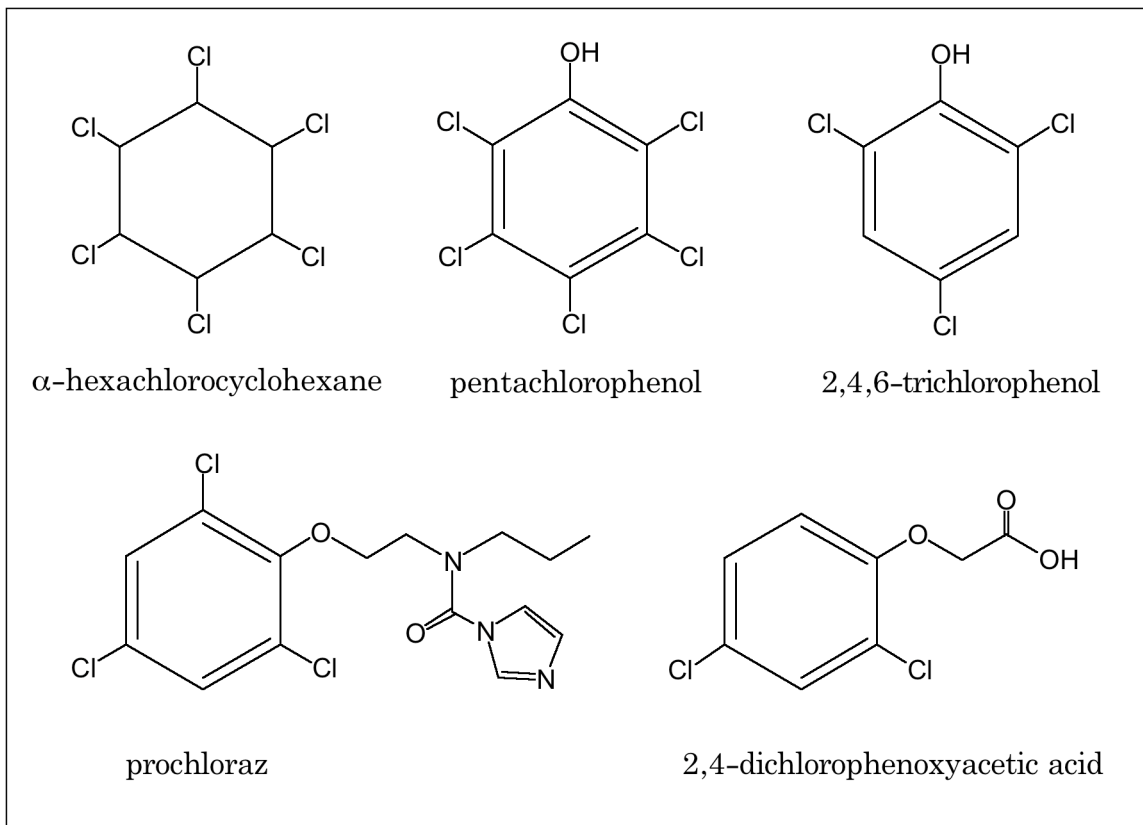
Figure 2. TCP degradative pathway in *C. necator* JMP134. TcpA initiates the

degradation through conversion of TCP to 2,6-dichloro-*p*-hydroxyquinone and then 6-

chloro-*p*-hydroxyquinone, a compound which must be reduced to 6-chloro-*p*-

hydroxyquinol (64) before TcpC cleaves the ring to produce 2-chloromaleylacetate (37).

TcpD removes the final chlorine to form maleylacetate and converts the latter to β-

ketoadipate (41). The enzymes PcaI, PcaJ, and PcaF convert this to acetyl-CoA and

succinyl-CoA (28).
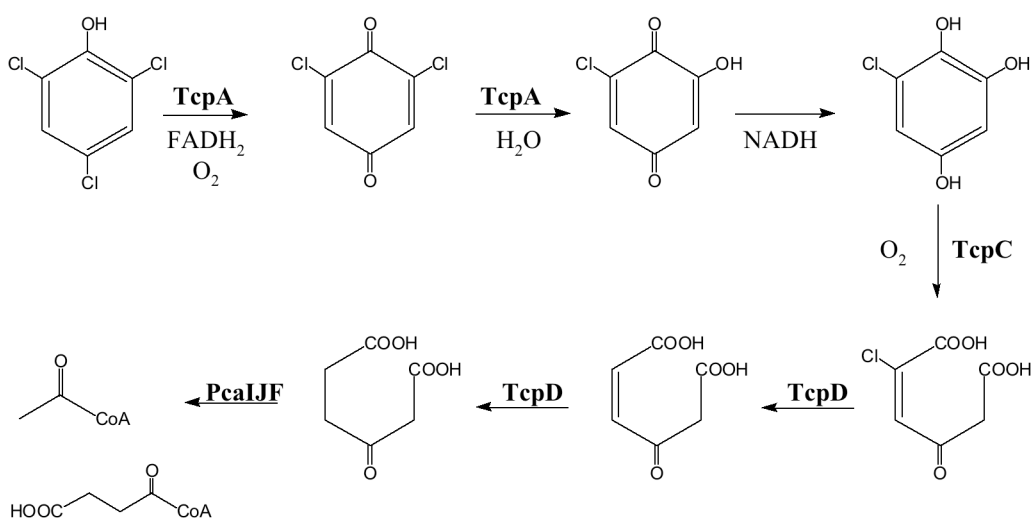
Figure 3. TCP-contaminated sites in the United States. Location of 20 SuperFund sites

in the US where TCP is a major contaminant. Image generated using Google Maps.

Figure 4. Genetic organization of the *tcpRXABCYD* gene cluster. White genes are biochemically or genetically characterized (37, 41, 54, 64). Grey genes are explored in this work.
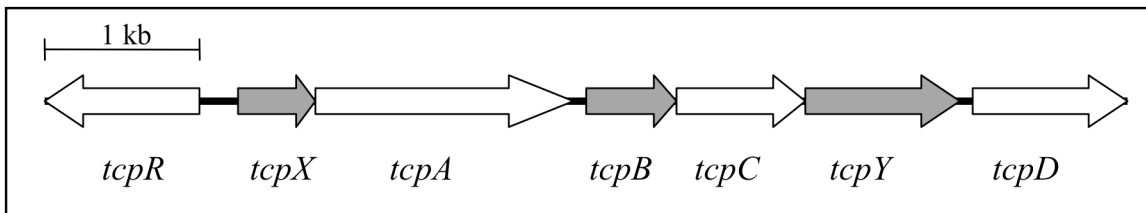
CHAPTER TWO

FUNCTIONS OF FLAVIN REDUCTASE AND QUINONE REDUCTASE IN 2,4,6-

TRICHLOROPHENOL DEGRADATION BY *CUPRIAVIDUS NECATOR* JMP134

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ABSTRACT

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, over-expressed, 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 towards NADPH or riboflavin. Coupling TcpX$_H$ and TcpA demonstrated 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 can enhance TcpA activity. Further, we showed that TcpB was more effective to reduce 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.

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INTRODUCTION

Trichlorophenols are anthropogenic environmental contaminants known to cause harm to humans (1, 3). These chemicals have been used in wood and leather preservation and as biocides and herbicides (1, 18). The aerobic bacterium *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134 (22) completely degrades 2,4,6-trichlorophenol (2,4,6-TCP) (15, 17). The key enzymes converting 2,4,6-TCP to 3-ketoadipate have been identified and characterized and the corresponding *tcpRXABCYD* operon has been reported (15, 20, 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 ($FADH_2$), and ascorbate (15) or potentially enzymatically reduced to the corresponding quinol. 6-Chlorohydroxyquinol is the substrate for the *tcpC* gene product, a dioxygenase that cleaves the ring (15, 17).

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 and its gene location proximity with *tcpA* (6, 12, 16). Further evidence is provided by the increased 2,4,6-TCP degradation upon co-expression of cloned *tcpA* and *tcpX* when compared to *tcpA* expression alone (20). TcpB belongs to PF00881 (2, 5), a nitroreductase family with broad substrate specificities (10, 14, 26, 27). Its gene location between *tcpA* and *tcpC* suggests a possible role in 2,4,6-TCP metabolism. As 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 over-produced 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 (15). *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 specified.

Gene cloning and protein expression. *C. necator* JMP134 genomic DNA was isolated using PureGene DNA Isolation Kit (Gentra, Minneapolis, MN). The *tcpX* gene was amplified from JMP134 with primers TcpXF (gaggagatccatatgtcgtcc) and TcpXR (cttcaagtcggaattcgcggcgac) and cloned between the NdeI and EcoRI sites of 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 (gcaaggaggaattcatgcaaaccaatg) and TcpBR-stop (gccttggtaagcttatgctggtcatactc) and cloned between EcoRI and HindIII sites of pET-30 LIC vector as a non-fusion 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 QIAprep Spin MiniPrep Kit (QIAGEN, Valencia, CA). The correct clone was electroporated into *E. coli* BL21(DE3) for recombinant protein production.

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 (15). His-tagged TcpX (TcpX$_H$) and TcpB were purified from *E. coli* cells over-producing the proteins. Cells were grown in 1 liter LB medium at 37°C to turbidity of 0.5 at 600 nm, induced with 300 μM isopropsyl-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 final concentration of 0.5 mM. The cells were disrupted by passing 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 Ni-NTA agarose resin (10 mg protein per 2 ml resin) (QIAGEN) for binding for 1 h. Resin was packed into 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, 1mM dithiothreitol (DTT), and 20 mM imidazole, and the elution buffer contained all of the above except 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 (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 phenyl agarose column (1.5 by 18 cm; Sigma) equilibrated

with 20% saturation of ammonium sulfate in the KPi buffer (pH 7.0) containing 1 mM

DTT. The proteins were eluted with a 100 ml of linear gradient of ammonium sulfate

(20% to 0% saturation) in KPi buffer (pH 7.0) with 1 mM DTT. TcpB was eluted off

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 subject to dialysis in the KPi

buffer containing 1 mM DTT. After 4 hours, the sample was centrifuged to remove any

precipitated proteins and the supernatant injected onto a Bio-Scale UNO-QR1 column (7

x 35 mm; Bio-Rad, Hercules, CA) equilibrated with the KPi buffer. TcpB did not bind to

the column and came off in 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 NADH and 20 μM

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 auto-

oxidation. Ferricyanide and nitrofurazone were also tested as alternative substrates.

Assays included 300 μM NADH, 20 μM FAD or 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 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 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 as menadione undergoes auto-oxidation at pH 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 acetonitrile-acetic acid mixture (vol/vol, 9:1), centrifuged, and analyzed with high performance liquid chromatography (HPLC) equipped with a C-18 column and photodiode array detector, as previously described (15). Samples contained 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.

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. Reactions were incubated at 30°C for 20 min and analyzed with HPLC as 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 (15).

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 SWIFT program (Pharmacia Biotech) and transferred to Microsoft (Redmond, WA) Excel format for analysis. A HPLC system (Waters, Milford, MA) was utilized with a Biosep Sec-S3000 size exclusion column (7.8 × 300 mm; Phenomenex, Torrance, CA) to estimate native molecular weights of pure proteins (7). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a reported method (13) and gels were stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL). Protein concentrations were determined by using 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 pET30-LIC vector to generate pTcpX$_H$ and pTcpB, respectively. A C-terminal His-tag fusion TcpX$_H$ was produced for ease of purification. Since a C-terminal His-tag fusion TcpB$_H$ was insoluble in *E. coli* cells, a non-fusion TcpB 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 (Figure 1), and the purified proteins were colorless. TcpX$_H$ migrated to 21 kDa on SDS-PAGE, agreeing with the calculated molecular weight of 21,344. TcpB appeared to be a 21 kDa protein on 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 one liter of culture. The enzyme stocks were stored at -80ºC with no apparent loss of activity after several months.

Enzyme characterization. Gel filtration chromatography indicated that TcpX$_H$ was 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 pH 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 activities, 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 activities towards the artificial electron acceptors.

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. As menadiol undergo auto-oxidation at pH levels above 6.0, the ferricyanide assay was utilized to determine optimal pH range. The highest enzymatic activity occurred at pH 6.0 with 90, 79, 81, 74, and 79% activity retained at pH 5.8, 6.2, 6.4, 6.8 and 7.0, respectively. The optimal temperature was observed at 32ºC with 69, 91, 76, and 66% activity retained at 30, 36, 38, and 40ºC, respectively. Optimal ionic strengths tested in KPi buffer were observed at concentrations between 20 to 90 mM KPi. The kinetic parameters of TcpB reduction of menadione were determined at 25ºC 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} was 350.7 ± 28.8 s^{-1}.

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 (15, 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

(Figure 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 (15). 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

organo-thiols, e.g. GSH and 2-mercaptoethanol, which form conjugates with chlorine

substituted quinones but not chlorine substituted quinols (the reduced form) (11, 19). If

TcpB could reduce 6-chlorohydroquinone faster than 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 $TcpA/TcpX_H$ (Table 3). A new peak at 5.8 min

with absorption maximum at 315 nm by HPLC analysis was likely the GS-

hydroxyquinone conjugate. When 2-mercaptoethanol was used instead of GSH, the

conjugate peak was eluted off the HPLC as 6.6 min with 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 (Table 3). Most interestingly, in

reactions containing only TcpA and $TcpX_H$ only 41 μM 6-chlorohydroxyquinol was

produced from completely consumption of 100 μM 2,4,6-TCP. A new HPLC peak was

observed at 6.2 min with absorption maximum at 317 nm. The new peak was likely a conjugate with 0.25 mM DTT present in the reaction mixture carried over from enzyme solutions.

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 (7) 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 (15, 25). TcpX is likely the flavin reductase to provide 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 (20). 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 compared to FMN (7), while HpaC uses FMN with higher efficiency than FAD (6). With regards 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 (Figure 3).

The function of TcpB in 2,4,6-TCP degradation has been investigated by genetic analysis (15, 20). 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 PF00081 enzyme family, and related enzymes often reduce nitrofurazone (10, 14, 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 k_{cat} as high as 350 s^{-1}. Since 6-chlorohydroxyquinone is a metabolic intermediate of 2,4,6-TCP degradation, TcpB played a critical role to reduce 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 (9). 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 transferring the pathway genes 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 (4). Clearly, TcpB also enhanced 2,4,6-

TCP degradation when included in the reaction mixture containing TcpA/TcpX$_H$ (Figure 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 reductases (ferredoxin-NADPH reductases) family 1, containing a bound FAD and a [2Fe-2S] center. TcpB belongs to COG0778: nitroreductase family (21). 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 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, as GSH conjugates with chlorine substituted quinones but not the corresponding quinols (11, 19). Without TcpB, GSH formed conjugate with no production of 6-chlorohydroxyquinone. TcpB minimized the conjugate formation (Table 3).

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 GS-quinone conjugate. *In vivo*, the quinone would be exposed to multiple thiols, including GSH, but also free

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cysteine and cysteine residues of proteins. These cellular covalently-linked thiol

conjugates are detrimental to the cell and can cause toxicity (8). Further, the formation of

conjugates may prevent complete degradation of 2,4,6-TCP.

ACKNOWLEDGEMENTS

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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 25ºC. 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.

Table 2: Electron acceptor specificity of TcpB[a]

Substrate	Specific Activity[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 25ºC. Values are means of three experiments with standard deviations.

[b] Specific activity as μmol NADH per minute per mg of protein.

Table 3: The effects of GSH on production

of 6-chlorohydroxyquinol[a]

	6-chlorohydroxyquinol (μM)	
Thiol	TcpX/A	TcpX/A/B
None	41.5 ± 1.5	100.0 ± 1.9
GSH[b]	not detected	81.4 ± 1.4

[a] Complete degradation of 100 μM

2,4,6-TCP by TcpA and TcpX$_H$ with

or without TcpB was all completed

within 20 min incubation. DTT was

present at about 0.25 mM in all

reactions as a carryover from

enzyme solutions and was required

to stabilize the enzymes.

[b] GSH was added to 1 mM.

Figure 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$_H$.
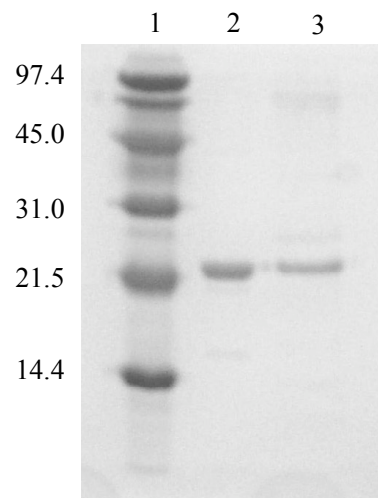
Figure 2. TcpB enhanced 2,4,6-TCP degradation by TcpA and TcpX$_H$. When TcpB was added to the reaction mix containing 2 μg of TcpX$_H$ and 10 μg of TcpA, a clear increase in total TcpA activity occurred. Total TcpA activity was expressed as μmol 2,4,6-TCP consumed per min; TcpB activity was μmol menadione reduced per min.
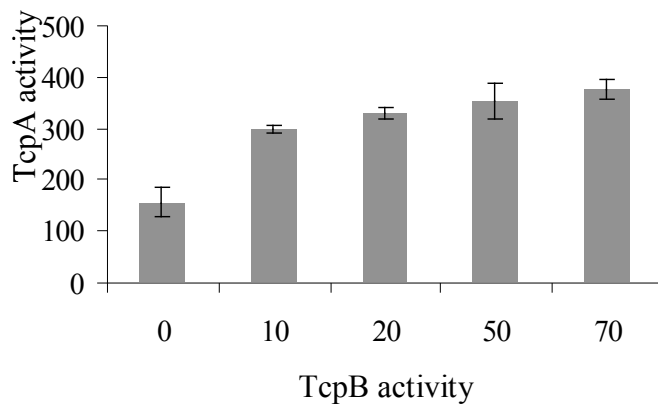
Figure 3. Proposed functions of TcpA, TcpX, and TcpB in 2,4,6-TCP degradation. I, 2,4,6-TCP; II, 2,6-dichloroquinone; III, 6-chlorohydroxyquinone; IV, 6-chlorohydroxyquinol; and V, thiol-quinone conjugate. Note: 2,6-Dichloroquinone is a transitory metabolite that remained with TcpA (25).
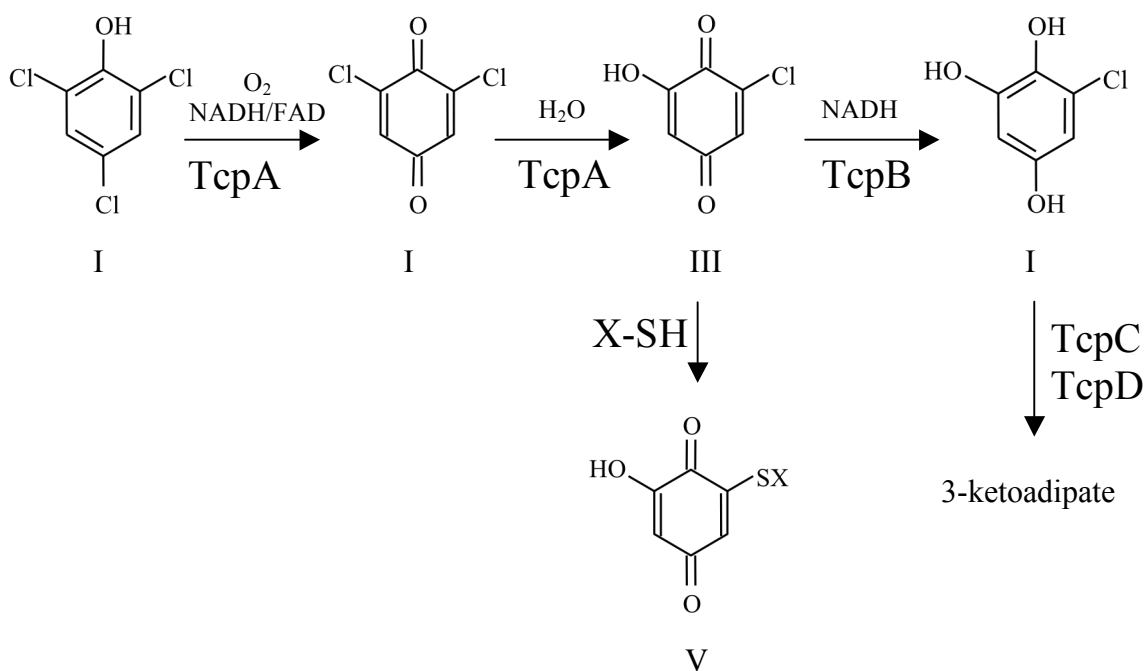
CHAPTER THREE

IDENTIFICATION OF A β-BARREL OUTER MEMBRANE PROTEIN FOR CELLULAR UPTAKE OF POLYCHLOROPHENOLS IN THE GRAM-NEGATIVE BACTERIUM *CUPRIAVIDUS NECATOR*

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ABSTRACT

The *tcpXABCYD* operon of *Cupriavidus necator* JMP134 is involved in the degradation of 2,4,6-trichlorophenol (TCP), a toxic pollutant. All of the gene products except TcpY have been identified as enzymes participating in TCP metabolism. A BLASTP identified TcpY as a member of COG4313, a group defined as proteins involved in the meta-pathway of phenol degradation. The definition stems from the location of the COG4313 genes in gene clusters involved in the degradation of aromatic compounds. Further sequence analysis of TcpY identified a signal peptide indicating TcpY is a membrane or secreted protein. Secondary structure and topology analysis indicated TcpY as a β-barrel outer membrane protein. A structural homology search showed that TcpY is structurally similar to FadL, an *E. coli* outer membrane transporter of hydrophobic long chain fatty acids. Constitutive expression of *tcpY* within two strains of *C. necator* rendered the cells more sensitive towards TCP as well as other polychlorophenols. Our data indicated that TcpY is an outer membrane transporter for polychlorophenols across the outer membrane of *C. necator*. The function of TcpY implies the transporting role of other OCG4313 proteins for aromatic compounds across the outer membrane of gram-negative bacteria.

INTRODUCTION

2,4,6-Trichlorophenol (TCP), a major environmental pollutant, has been used extensively as a preservative and a biocide (8). *Cupriavidus necator* JMP134, a versatile degrader of aromatic compounds, can use TCP as a carbon and energy source (7, 26). A gene cluster, *tcpRXABCYD*, is involved in TCP degradation (21, 23, 27), and all the encoded proteins have been characterized except TcpY (Fig. 1). TcpA (TCP 4-monooxygenase) is an interesting enzyme because it first oxidizes TCP to 2,6-dichloro-*p*-benzoquinone and then hydrolyzes the latter to 6-chloro-2-hydroxy-*p*-benzoquinone (34). Further, TcpA is a reduced flavin adenine dinucleotide ($FADH_2$)-dependent monooxygenase. Because $FADH_2$ is not stable, TcpX (NADH:FAD oxidoreductase) supplies $FADH_2$ in coupled reactions (5). TcpB (NADH:quinone oxidoreductase) reduces the quinone to 6-chloro-2-hydroxy-*p*-quinone (5). A dioxygenase (TcpC) catalyzes the ring-cleavage of the latter to produce 2-chloromaleylacetate (21). TcpD is chloromaleylacetate reductase that removes the last chlorine and then converts maleylacetate to 3-ketoadipate, a common metabolic intermediate of microbial degradation of aromatic compounds (11). The *tcpR* gene codes for a regulatory protein controlling the *tcpXABCYD* operon expression (27).

The *tcpY* gene is in the middle of the *tcp* gene cluster, but the function of TcpY is unknown. TcpY does not share significant sequence similarity to any known proteins, and a *tcpY* mutant is normal in TCP degradation (27). However, a structural homology search described below indicated TcpY displayed significant homology with FadL, an *E. coli* outer membrane transporter protein required for long-chain fatty acid (LCFA) transport (31). Gram-negative bacteria have hydrophilic outer membranes that act as a

barrier against hydrophobic molecules and thus require transporters to facilitate diffusion into the cytoplasm (30). Like LCFAs, TCP is a hydrophobic molecule. Therefore, TcpY may function as a transporter to facilitate TCP diffusion into the cell. Here, we report evidence supporting TcpY as an outer membrane transporter that facilitates the diffusion of polychlorophenols into *C. necator*.

RESULTS

Sequence analysis of the *tcpY* revealed a β-barrel outer membrane protein.
BLASTP search with TcpY sequence returned only hypothetical proteins that are
grouped together in the cluster of orthologous groups (COG) 4313 (22). Although no
experimental data exists for this particular COG, members are thought to be involved in
the meta-pathway of phenol degradation due to their location in close proximity to genes
responsible for phenol degradation (33). Thus, although sequence similarity did not
reveal the function of TcpY, its inclusion in COG4313 suggested involvement in TCP
degradation. SignalP indicated that TcpY had a signal peptide and a predicted cleavage
site between amino acid residues 38 and 39. The estimated molecular mass of the mature
polypeptide was 31,715.24 Da. The signal peptide suggested that TcpY was a membrane
or secreted protein. *C. necator* is a gram-negative bacterium with cytoplasmic and outer
membranes. The integral membrane proteins of cytoplasmic membranes usually consist
of α-helices for the transmembrane portions , while the outer membrane proteins are
composed of antiparallel β-strands for transmembrane segments that form a channel in
the outer membrane, e.g. porins (28). PRED-TMBB, a webserver capable of predicting
and discriminating β-barrel outer membrane proteins, was utilized to analyze TcpY (4).
PRED-TMBB scored TcpY with a value of 2.877 that is less than the threshold value of
2.965. Values below this threshold correctly predict β-barrel membrane proteins 95% of
the time using a database of known β-barrel outer membrane proteins (4). Since the
TcpY value was smaller than the threshold value, the chance that TcpY was a β-barrel
outer membrane protein was higher than 95%. PRED-TMBB predicted the topology of
TcpY with fourteen transmembrane β-strands (Fig. 2A). Structural homology modeling

using Phyre predicted a TcpY structure with 95% estimated precision relatedness to the three-dimensional structure of FadL from *E. coli* (Fig. 2B). FadL is a transporter that facilitates the diffusion of hydrophobic LCFAs across hydrophilic outer membranes of *E. coli* and related gram-negative bacteria (31).

Phylogenetic analysis compared TcpY and related proteins to outer membrane hydrophobic molecule transporters. FadL transports hydrophobic LCFAs and FadL homologs have been linked to the uptake of hydrophobic molecules for biodegradation (30). XylN, TodX, StyE, and TbuX were identified as outer membrane transporters for the uptake of xylene (17), toluene (32), styrene (24), and toluene (16), respectively. To determine how TcpY is related to hydrophobic molecule transporters, a multiple alignment was utilized. The proteins are listed in Table 1 and the phylogenetic tree created from the alignment is shown in Fig. 3. Bootstrap values of 100 indicated the proteins fell into three separate clades: LCFA transporters, xenobiotic transporters, and COG4313 hypothetical proteins.

Knockout of *tcpY* caused no apparent difference in degradation or sensitivity to TCP. The *tcpY* gene was disrupted via homologous integration of a suicidal plasmid that carried an internal fragment of the *tcpY* gene in *C. necator* JMP134. The integration of pKOY at *tcpY* resulted in a kanamycin resistant mutant that contained two truncated copies of *tcpY* on the chromosome. Integration of pKOY was confirmed by PCR using one primer located on the chromosome TcpYKOF (gtattccggcctgatgttgc) and one located on the plasmid pCR-R (gttttcccagtcacgacgtt) (data not shown). The *tcpY* mutant and *C. necator* JMP134 wild type degraded TCP at comparable rates using TCP concentrations from 10 to 400 μM. Also, the mutant and wild type showed similar sensitivity to TCP

(100 to 400 µM) as well as other polychlorophenols both in the mineral salt liquid medium and on agar plates (data not shown).

Constitutively expressed *tcpY* was toxic to *C. necator*. To evaluate whether TcpY facilitated the diffusion of TCP into *C. necator* JMP134 cells, we compared the sensitivity to TCP with and without the presence of constitutively expressed *tcpY*. If TcpY functions as an outer membrane transporter for TCP, constitutive expression of *tcpY* should produce TcpY at higher concentrations than would exist in the wild type where the gene is respressed in the presence of glutamate (27). Without the operon expressing the proteins required to degrade TCP, the toxic chemical would result in decreased growth. The decreased growth would produce small or no colonies on agar plates inoculated with serially diluted cells. On agar plates, *C. necator* JMP134 containing pBBR1MCS2 (empty vector) displayed decreased growth starting at 200 µM TCP but was still able to grow at 400 µM TCP (Fig. 4A). On the other hand, the strain containing pTcpY, which constitutively expressed *tcpY,* exhibited decreased growth at 100 µM and almost a complete abolishment of growth at 400 µM TCP (Fig. 4A). The results indicated that TcpY allowed more TCP to enter the cells.

Constitutive expression of *tcpY* in *C. necator* H16 also increased TCP toxicity. To study the function of TcpY in a closely related bacterium, pBBR1MCS2 and pTcpY were electroporated into *C. necator* H16, a bacterium not capable of TCP degradation. Overall, *C. necator* H16 had a higher sensitivity to TCP in comparison to *C. necator* JMP134. On agar plates, *C. necator* H16 control did not display decreased growth until 100 µM TCP while the strain carrying pTcpY started to exhibit decreased growth at 50 µM TCP and almost a complete inhibition of growth at 200 µM (Fig. 4B). The results

indicated that TcpY also brought more TCP into the cells and made them more sensitive to TCP in comparison to the control carrying the empty vector.

TcpY increased toxicity towards other polychlorophenols. As FadL is specific for its substrate, we tested the ability of *tcpY* to transport polychlorophenols other than TCP in *C. necator* JMP134. It is important to note that the *tcp* operon can only be induced by TCP, and not other polychlorophenols (27). Also, glutamate was used as the carbon source for this experiment ensuring the repression of the *tcp* operon. We determined the sensitivity of *C. necator* JMP134 towards DiCP, TCP, 2,3,4,6-TeCP, 2,3,5,6-TeCP, and PCP. DiCP did not decrease the growth of *C. necator* JMP134 carrying pTcpY compared to the empty vector control (Fig. 5), indicating it is likely not a substrate for TcpY. The two tested TeCPs and PCP all caused decreased growth in the empty vector control when compared to a control with no polychlorophenols in the medium demonstrating toxicity of the TeCPs and PCP. However, *C. necator* JMP134 carrying pTcpY displayed increased sensitivity to TCP, the two TeCPs, and PCP compared to the empty vector control (Fig. 5).

DISCUSSION

β-Barrel outer membrane proteins share similar structures but are difficult to predict based on sequence analysis alone due to the lack of evolutionary constraints for loop regions that connect the β-sheets (10, 13, 14). TcpY does not share apparent sequence identity with any known proteins, but computational analysis revealed a signal peptide, scored TcpY as a β-barrel outer membrane protein, and showed structural similarity to characterized β-barrel outer membrane proteins, including FadL. FadL is specific for the hydrophobic LCFAs (31). FadL structural homologs are involved in transport of hydrophobic xenobiotic compounds in gram-negative bacteria (30), including toluene transported by TodX in *P. putida* F1 (32) and TbuX in *R. pickettii* PKO1 (16). The resolved structures of TodX and TbuX are similar to FadL (12).

Outer membrane transporters may play important roles in the biodegradation of hydrophobic compounds by gram-negative bacteria. When the xylene transporter, XylN, is inactivated in *P. putida* KT2440, the mutant grows on higher xylene concentrations compared to the wild type, indicating xylene transport into the cell is significantly decreased in the absence of XylN (17). On the other hand, the inactivation of *tcpY* in *C. necator* JMP134 showed no difference in TCP degradation or TCP toxicity. Sanchez and Gonzalez have also reported that a *tcpY* knockout mutant can grow the same as the wild type with TCP as the sole carbon source (27). Thus, it is consistent that TcpY does not affect TCP degradation in *C. necator* JMP134 under tested conditions. It is unknown if TcpY affects TCP transport in the environment, as different growth conditions alter the permeability of the outer membrane of gram-negative bacteria (25).

StyE is an outer membrane transporter for styrene in *P. putida* CA-3 (24). When *styE* is overexpressed in *P. putida* CA-3, the transcription level of the styrene degradation genes increases. This increase is attributed to higher amounts of styrene entering the cell. Since TCP is toxic, increased amounts of TCP inside the bacterial cell will reduce growth. The constitutive expression of *tcpY* in *C. necator* JMP134 and H16 made the cells more sensitive to TCP (Fig. 4). Further, TcpY makes *C. necator* JMP134 more sensitive to other polychlorophenols (Fig. 5). Lipopolysaccharides of the outer membrane are hydrophilic (25), which can slow down the passing of hydrophobic polychlorophenols. Our toxicity data suggest that TcpY is a β-barrel outer membrane protein specific for polychlorophenol transport across the outer membrane of *C. necator* (Fig. 6). Once in the periplasmic space, they can readily diffuse across the cytoplasmic membrane, causing toxicity or being degraded in the cytoplasm (20). COG4313 members are all hypothetical proteins with the exception of TcpY. The assignment of TcpY as an outer membrane polychlorophenol transporter suggests COG4313 proteins are β-barrel outer membrane transporters. The COG4313 proteins used for phylogenetic analysis (Fig. 3) were computationally analyzed (Table 2). Like TcpY, the four hypothetical proteins all contained signal peptides, scored as β-barrel outer membrane proteins, and shared high structural homology with FadL. As COG4313 proteins are within gene clusters responsible for biodegradation of aromatic compounds, the location of the COG4313 genes indicates potential substrates. The *orf8* gene is located near meta-pathway phenol degradation genes in *Acinetobacter calcoaceticus* PHEA-2, indicating a potential phenol transport (33). The *P. putida* GB-1 protein (GB-1-

Hypo) gene is adjacent to predicted phenol degradation genes, suggesting another

possible outer membrane phenol transporter.

EXPERIMENTAL PROCEDURES

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 and *C. necator* H16 were grown in a mineral salt medium with 0.2% glutamate as the carbon source at 30°C (21). *E. coli* strains DH5α and TOP10 were grown on Luria-Bertani (LB) medium at 37°C. Kanamycin was used at a final concentration of 30 μg/ml. *E. coli* strain TOP10 was used to host plasmids constructed from the TOPO TA cloning vector pCR2.1 (Invitrogen), which was also used as a suicidal plasmid for *C. necator* JMP134.

Sequence analysis. Protein sequence similarity searches were carried out using the BLASTP program (1, 2) from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/cgi-bin/blast). Signal sequence at the N-terminal region of membrane proteins was predicted by using SignalP http://www.cbs.dtu.dk/services/SignalP/) (9).

TcpY modeling. The likelihood of proteins to form β-strands in the outer membrane of gram-negative bacteria was calculated using the PRED-TMBB (http://bioinformatics.biol.uoa.gr/PREDTMBB) method based on a hidden markov model (4). The three-dimensional model of TcpY was constructed using a structural homology search carried out using Protein Homology/analogY Recognition Engine (Phyre version

0.2; http://www.sbg.bio.ic.ac.uk/phyre/) (6). Phyre calculates the best fit towards a

solved crystal structure with estimated precision predicting likelihood of fit.

Phylogenetic analysis. Protein sequences were aligned using CLUSTALW (20).

MEGA version 4.0.2 (29) was used to generate a phylogenetic tree. The percentage of

replicate trees in which the associated taxa clustered together in the bootstrap test (2000

replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths

in the same units as those of the evolutionary distances used to infer the phylogenetic

tree. The evolutionary distances were computed using the Jones-Taylor-Thornton (15)

method and are in units of the number of amino acid substitutions per site.

Chromosomal disruption of *tcpY* in *C. necator* JMP134. A 493-bp internal

fragment of *tcpY* was amplified from *C. necator* JMP134 DNA by PCR using primer pair

TcpY-internalF (gtggtaccgatcgtgtctgc) and TcpY-internalR (actgatactgggtggcttcg). The

PCR products were cloned into pCR2.1-TOPO, forming plasmid pKOY. The plasmid

was amplified in *E. coli* and isolated. The purified plasmid DNA (50 ng) was

electroporated into *C. necator* JMP134 cells prepared as previously described (21).

Recombinant strains were selected on LB agar plates containing kanamycin.

Constitutive expression of TcpY in *C. necator* JMP134.. The full length *tcpY*

gene was expressed in the plasmid pTrc99a (Pharmacia, Piscataway, NJ), where *tcpY* was

under the control of the *trc* promoter that contains a LacI binding site (3). The *tcpY* gene

was PCR amplified with primers TcpYR (gctttcatcgaagcttctcccgtc) and TcpYF-pTrc99

(gttcgacagatgccatggaggtcc). The product was digested with HindIII and NcoI, ligated

into restriction-digested pTrc99a and cloned into E. coli DH5α for plasmid propagation.

As the pTrc99a-tcpY plasmid cannot replicate in *C. necator* JMP134, pBBR1MCS2 (18,

19) was a more desirable host for the *tcpY* gene as it can replicate in a broad host range. The pTrc99a-tcpY plasmid was used as a template for a second round of PCR using pTrc99-F (gttctggataagctttttttgcgcc) engineered with a HindIII site and pTrc99-R (atcttctctcatccgcca). This PCR product was digested with HindIII and ligated into pBBR1MCS2 to create pTcpY, in which *tcpY* is under the control of the *trc* promoter from pTrc99a. Electroporation was utilized to generate transformants containing pTcpY into *C. necator* JMP134 and *C. necator* H16. In *C. necator*, the *trc* promoter is constitutively expressed due to the lack of repressor, LacI. pBBR1MCS2 was also electroporated into these strains as the empty vector control.

Agar plate toxicity assays for TCP. To determine the effects of constitutive *tcpY* expression when TCP is present, we utilized mineral salt medium agar plates with 0.2% glutamate and varying amounts of TCP (from 0 μM to 400 μM). Glutamate represses the *tcp* operon in *C. necator* JMP134, so no degradation occurs (21). Briefly, cells were cultured overnight in LB medium, harvested and washed with mineral salt medium containing no carbon source, and resuspended in the same medium to a turbidity of 1.0 at 600 nm (~1 x 10^7 cells/ml). Serial dilutions were performed in the medium, and 3 μl was spotted onto the agar plates. The plates were incubated for 3 days at 30ºC and pictures were taken with a digital camera. If the cells are sensitive to the TCP concentrations, the cells at higher dilutions would have small or no colonies due to slower growth.

Liquid culture toxicity assays for polychlorophenols. Several polychlorophenols were tested to determine if TcpY is specific to TCP. *C. necator* JMP134 was cultured overnight in LB, harvested, and resuspended in the mineral salt

medium with no carbon source to a turbidity of 1.0 at 600 nm. A 1% inoculum was then

transferred to the mineral salt medium containing 0.2% glutamate and 100 μM 2,6-

dichlorophenol (DiCP), TCP, 2,3,4,6-tetrachlorophenol (TeCP), 2,3,5,6-TeCP, or

pentachlorophenol (PCP). Cultures were shaken for 18 hours at 30ºC and a final turbidity

of 600 nm was performed.

Analytical methods. An Ultrospec 4000 (Pharmacia) UV/visible

spectrophotometer was used to analyze optical density of cultures. Data were recorded

using the SWIFT program (Pharmacia). Plasmid purification was performed using a

Qiagen kit (Valencia, CA) following the recommended protocol.

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Table 1: TcpY and related proteins used in the phylogenetic analysis.

Protein	Organism	Accession No.	Pairwise score[a]	Proposed Function
TcpY	*Cupriavidus necator* JMP134	YP_295798	100%	Conserved hypothetical protein
383-Hypo	*Burkholderia* sp. 383	YP_373710	38%	Meta-pathway phenol degradation-like protein[b]
GB-1-Hypo	*Pseudomonas putida* GB-1	YP_001669533	35%	Meta-pathway phenol degradation-like protein[b]
SB-Hypo	*Syntrophus aciditrophicus* SB	YP_461556	32%	Conserved hypothetical protein
Orf8	*Acinetobacter calcoaceticus* PHEA-2	CAD92317	31%	Meta-pathway phenol degradation-like protein[b]
TbuX	*Ralstonia pickettii* PKO1	AAF03168	12%	Transport of toluene across the cell membrane
XylN	*Pseudomonas putida* KT2440	BAA09665	9%	Transport of xylene across the cell membrane
StyE	*P. putida* CA-3	AAR24508	9%	Transport of styrene across the cell membrane
FadL-ET1/99	*Erwinia tasmaniensis* Et1/99	YP_001907087	9%	LCFA outer membrane transporter
FadL-TTO1	*Photorhabdus luminescens subsp. laumondii* TTO1	NP_930431	9%	LCFA outer membrane transporter
TodX	*P. putida* F1	AAC43318	7%	Transport of toluene across the cell membrane
FadL	*Escherichia coli* W3110	AP_002944	6%	LCFA outer membrane transporters
FadL-SCRI1043	*Pectobacterium atrosepticum* SCRI1043	YP_051171	6%	LCFA outer membrane transporter

[a] Percentage sequence identity to TcpY divided by the number of residues in the alignment by MEGA4 (29).
[b] No specific information was given.

Table 2: COG4313 proteins computational analysis.

	Signal peptide cleavage site[a]	PRED-TMBB score[b]	Estimated precision with FadL structure[c]
Orf8	24	2.876	90%
383-Hypo	27	2.904	95%
GB-1-Hypo	27	2.871	95%
SB-Hypo	34	2.883	95%

[a] As determined by Signal P (9).

[b] A score lower than the cutoff of 2.965 indicates a β-barrel outer membrane protein (4).

[c] As determined by Phyre server (6).

Figure 1. (A) Degradation pathway of *C. necator* JMP134. TcpX and TcpA catalyze the conversion of TCP to 6-chloro-2-hydroxy-*p*-benzoquinone. TcpB reduces 6-chloro-2-hydroxy-*p*-benzoquinone to 6-chloro-2-hydroxy-*p*-hydroquinone. TcpC converts the latter to 2-chloromaleylacetate, which is reduced by TcpD to maleylacetate and then to 3-ketoadipate. (B) The *tcp* operon in *C. necator* JMP134. The bar represents 1 kb.
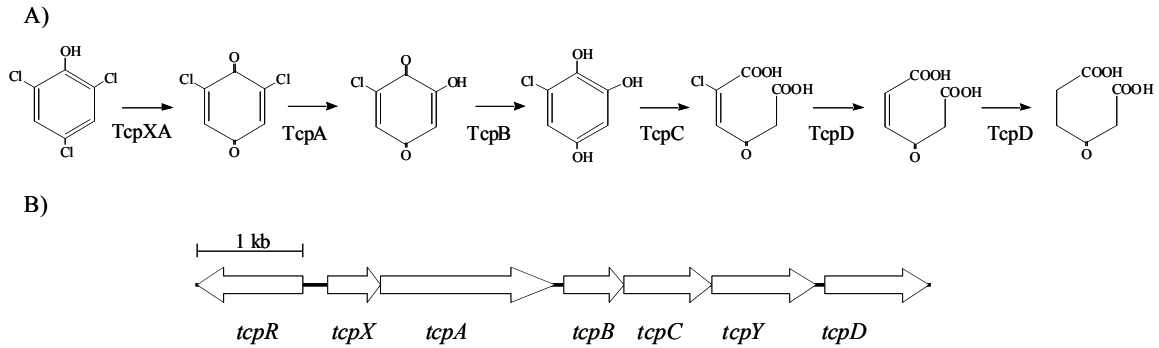
A)

B)

Figure 2. Topology and structure modeling of TcpY. Both models display the
extracellular environment at the top of the figure and periplasm at the bottom. (A)
PRED-TMBB predicted fourteen transmembrane domains of TcpY. (B) Predicted
structure of TcpY using the solved structure of FadL (31) as the template.
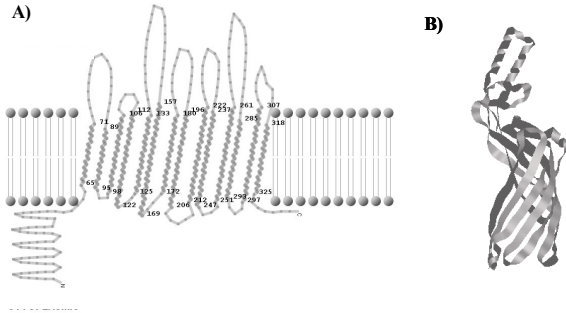
Figure 3. Phylogenetic relationship of β-barrel outer membrane transporters as determined using MEGA4 (29). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The proteins, species, GenBank accession number, pairwise score, and proposed function are listed in Table 1.
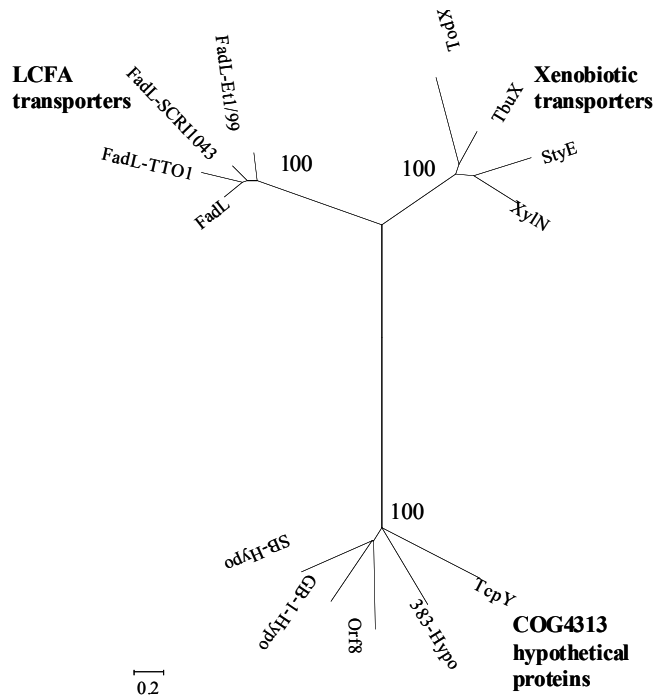
Figure 4. Sensitivity of *C. necator* strains on agar plates with TCP. Cultures were grown

overnight, harvested, washed, and resuspended to a turbidity of 1.0 in the mineral salt

medium. Three µl of serial diluted cells were dropped onto the mineral salt agar plates

with 0.2% glutamate and various concentrations of TCP. The plates were incubated at

30°C for 3 days. (A) *C. necator* JMP134; (B) *C. necator* H16. Ctl refers to clone

containing pBBR1MCS2, and pTcpY refers to the clones containing constitutively
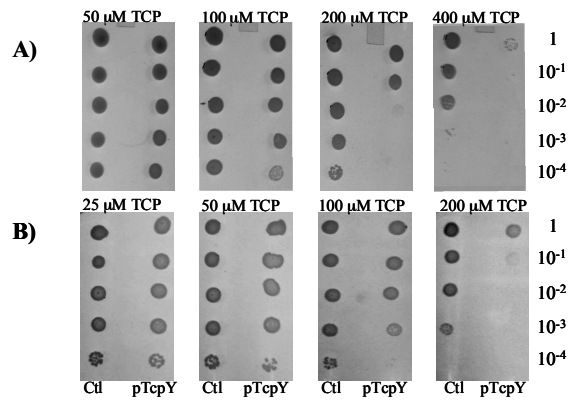
expressed *tcpY*.

Figure 5. Toxicity of chlorophenols to *C. necator* JMP134. A 1% inoculum from overnight cultures was added to the minimal salt medium with 0.2% glutamate containing 100 μM of DiCP, TCP, 2,3,4,6-TeCP, 2,3,5,6-TeCP, or PCP. Cultures were incubated with shaking at 30ºC for 18 hours before turbidities were taken at 600 nm. *C. necator* JMP134 containing pBBR1MCS2 or pTcpY was tested.
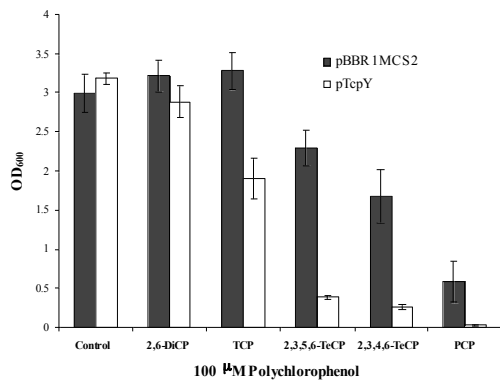
Figure 6. Schematic representation of TCP uptake in *C. necator*. The grey box represents TcpY facilitating the diffusion of TCP across the outer membrane (OM). TCP may also diffuse through OM without the aid of transporters. The lipopolysaccharide (LPS) layer that comprises the outer leaflet of OM is indicated. Once in the periplasm, TCP should diffuse through the inner membrane (IM) unaided. Inside the cytoplasm, TCP is degraded by other *tcp* gene products.
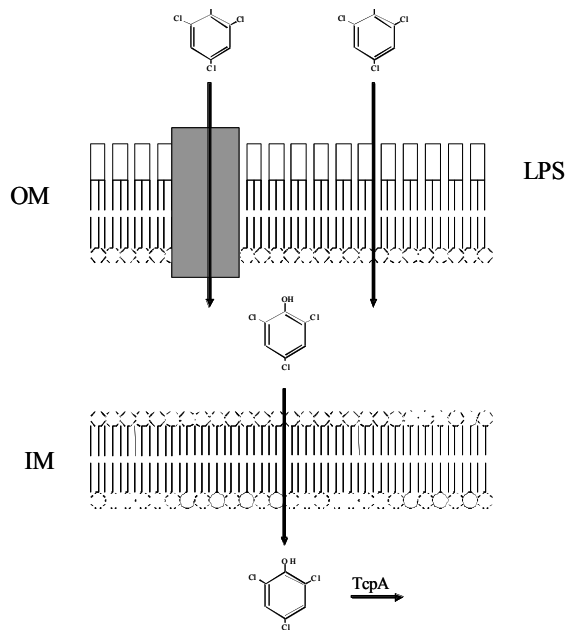
CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

Bioremediation is a desirable method to remove toxic, persistent xenobiotics from the environment. Specifically, bioaugmentation relies on the addition of microorganisms into a polluted environment to reduce or completely eliminate the contaminant. We have focused on biodegradation pathways in specific microorganisms to help identify bioaugmentation candidates for field use. The work presented here centers on the degradation of TCP, a priority pollutant present in many ecosystems. Specifically, we spotlight the degradation in *C. necator* JMP134, a bacterium successfully utilized in field bioaugmentation studies. Through completion of the characterization of the degradation pathway and the components involved, we solidify its desirability as a bioaugmentation agent. The completed research opens the door for future applications of the bacterium in TCP contaminated sites.

The work in chapter two details the characterization of the products of *tcpX* and *tcpB* located in the *tcpRXABCYD* gene cluster in *C. necator* JMP134. TcpX was characterized as a NADH:flavin oxidoreductase that can be grouped with other flavin reductases partnered with flavin-utilizing monooxygenases. Our research demonstrated that TcpX was able to directly supply TcpA with the $FADH_2$ needed to initiate TCP metabolism. TcpB was identified as a nitroreductase by sequence similarity, but had limited activity towards traditional nitroreductase substrates. The possible role of TcpB in quinone reduction first came from our experiments on direct quinone reduction using the artificial quinone substrate menadione. A functional role for TcpB as a quinone

reductase was further confirmed in a coupled assay including TcpX and TcpA where the direct product of TcpA, 6-chloro-*p*-hyroxyquinone, was reduced to 6-chloro-*p*-hydroxyquinol. Formation of the reduced chlorinated quinol was indirectly measured through the production of thiol-conjugates. Thiol conjugates are readily created when thiols, such as glutathione, chemically react with the oxidized form of chlorinated quinone. Since the reduced chlorinated quinol cannot react with the thiols, thiol conjugation formation is an indirect measurement of the formation; less thiol conjugate indicates a faster quinone reduction. Addition of TcpB significantly decreased thiol conjugate formation indicating an enhanced reduction of the chlorinated quinone implying an enzymatic reduction. TcpB was thus characterized as a NADH:quinone reductase that reduces 6-chloro-*p*-hydroxyquinone to 6-chloro-p-hydroxyquinol. Our functional characterization of TcpX and TcpB enhances the understanding of the TCP degradation pathway in *C. necator* JMP134, completing the journey from the initial TCP molecule to the β-ketoadipate end product. Furthermore, as TcpB was found to minimize detrimental side reactions with thiol *in vitro*, the possible toxicity of these thiol conjugates *in vivo* should be explored. TcpB may be absolutely required during TCP biodegradation *in vivo* for efficient utilization of TCP as a carbon and energy source.

Chapter three details the computational and biochemical characterization of *tcpY*. For the majority of the evidence, we used computational analysis of the gene and its predicted amino acid sequence. TcpY is a member of COG4313, a group proposed to be involved in the *meta*-pathway of phenol degradation. As no experimental evidence existed for this prediction, the group membership gave little indication of the function of TcpY. The first indication of its role resulted from analysis of the predicted three-

dimensional structure of TcpY, which indicated significant homology to β-barrel outer membrane proteins. Specifically, TcpY is structurally homologous to members of the FadL family, a family of outer membrane transporters involved in transportation of hydrophobic molecules in Gram-negative bacteria. Our biochemical data supported the role of TcpY as an outer membrane transporter of TCP in *C. necator*, as *tcpY* constitutive expression increased the toxicity of TCP in *C. necator* JMP134 and closely related *C. necator* H16. This increase in sensitivity indicated an increase in the uptake of TCP that could cause detrimental effects including decreased cell growth and even death. We thus characterized TcpY as a β-barrel outer membrane protein functioning in TCP transport in *C. necator* JMP134. From our analysis, we predicted the role of COG4313 members to be involved in the transport of hydrophobic molecules in their respective hosts.

While the overall goal of bioremediation appears much more broad than the scope of this work, no system can be successful without understanding the underlying mechanism. Therefore, the work presented here creates a significant advance in TCP bioremediation by completing the characterization of the TCP degradation pathway in *C. necator* JMP134. Future work can address the rate of flux through the degradation pathway. The TCP biodegradation pathway in *C. necator* JMP134 is an efficient system under laboratory conditions, but the rate of degradation *in situ* may be significantly lower. For example, the quinone thiol conjugates discussed in chapter two may present significant carbon loss and TCP transport discussed in chapter three may be severely inhibited in field conditions. Therefore, future studies with *C. necator* JMP134 should focus upon the application of this strain in TCP-contaminated sites.

In addition to bioaugmentation with this particular organism, the potential to transfer the *tcpRXABCYD* gene cluster to another bacterium can also be pursued. A TCP-contaminated site may have environmental conditions that inhibit the growth of *C. necator* JMP134 but favor the growth of another species of bacteria. The favored organism could be engineered with the *tcpRXABCYD* gene cluster resulting in enhanced TCP bioremediation. Since the functional roles of the gene products have been identified by our research, the studies could focus on the bioaugmentation of the TCP by the engineered organism.