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ORIGINAL PAPER

# A β-barrel outer membrane protein facilitates cellular uptake of polychlorophenols in *Cupriavidus necator*

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**Abstract** The *tcpRXABCYD* operon of *Cupriavidus necator* JMP134 is involved in the degradation of 2,4,6-trichlorophenol (TCP). All of the gene products except TcpY have assigned functions in TCP metabolism. Sequence comparison identified TcpY as a member of COG4313, a group of hypothetical proteins. TcpY has a signal peptide, indicating it is a membrane or secreted protein. Secondary structure and topology analysis indicated TcpY as a β-barrel outer membrane protein, similar to the *Escherichia coli* outer membrane protein FadL that transports hydrophobic long-chain fatty acids. Constitutive expression of *tcpY* in two *C. necator* strains rendered the cells more sensitive to TCP and other polychlorophenols. Further, *C. necator* JMP134 expressing cloned *tcpY* transported more TCP into the cell than a control with the cloning vector. Thus, TcpY is an outer membrane protein that facilitates the passing of polychlorophenols across the outer membrane of *C. necator*. Similarly, other COG4313 proteins are possibly outer membrane transporters of hydrophobic aromatic compounds.

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## Introduction

2,4,6-Trichlorophenol (TCP), a major environmental pollutant, has been used extensively as a preservative and a biocide (Czaplicka 2004). The gram negative bacterium *Cupriavidus necator* JMP134, a versatile degrader of aromatic compounds, can use TCP as a carbon and energy source (Clement et al. 1995; Padilla et al. 2000). A gene cluster, *tcpRXABCYD*, is involved in TCP degradation (Louie et al. 2002; Matus et al. 2003; Sanchez and Gonzalez 2007), and all the encoded proteins have been characterized except TcpY (Fig. 1). First, TcpA (TCP 4-monooxygenase) oxidizes TCP to 2,6-dichloro-*p*-benzoquinone and then hydrolyzes the latter to 6-chloro-2-hydroxy-*p*-benzoquinone (Xun and Webster 2004). Since TcpA is a reduced flavin adenine dinucleotide ($FADH_2$)-dependent monooxygenase, TcpX (NADH:FAD oxidoreductase) supplies $FADH_2$ to TcpA in coupled reactions (Belchik and Xun 2008). Second, TcpB (NADH:quinone oxidoreductase) reduces the quinone to 6-chloro-2-hydroxy-*p*-hydroquinone (Belchik and Xun 2008). Third, TcpC (dioxygenase) catalyzes the ring-cleavage of the latter to produce 2-chloromaleylacetate (Louie et al. 2002). Fourth, TcpD (maleylacetate reductase) removes the last chlorine and then converts

maleylacetate to 3-ketoadipate, a common metabolic intermediate of microbial degradation of aromatic compounds (Harwood and Parales 1996). The *tcpR* gene codes for a regulatory protein controlling the expression of *tcpXABCYD* (Sanchez and Gonzalez 2007).

The *tcpY* gene is located in the middle of the *tcp* gene cluster, but the function of TcpY remains unknown. TcpY does not share significant sequence similarity to any known proteins, and a *tcpY* mutant is normal in TCP degradation (Sanchez and Gonzalez 2007). However, a structural homology search described below indicated TcpY displayed significant homology with FadL, an *Escherichia coli* outer membrane transporter protein required for long-chain fatty acid (LCFA) transport (van den Berg et al. 2004). 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 (van den Berg 2005). 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*.

## Materials and methods

### Chemicals and enzymes

Reagents used were purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). Enzymes were purchased from Invitrogen (Carlsbad, CA) or New England Biolabs (Beverly, MA).

### Bacterial strains and culture conditions

*Cupriavidus necator* JMP134 and *C. necator* H16 were grown in a mineral salt medium with 0.2% glutamate as the carbon source at 30°C (Louie et al. 2002). *E. coli* strains were grown in Luria–Bertani (LB) medium at 37°C. Kanamycin was used at a final concentration of 30 μg $ml^{-1}$.

### Sequence analysis

The BLASTP program (Altschul et al. 1997) was used to search sequence similarity. Signal sequence of membrane proteins was predicted by using SignalP http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson et al. 2007).

### TcpY modeling

The likelihood of proteins to form $\beta$-strands in the outer membrane of gram-negative bacteria was calculated using the PRED-TMBB (http://bioinformatics.biol.uoa.gr/PREDTMBB) (Bagos et al. 2004). The three-dimensional model of TcpY was constructed using Phyre version 0.2 (http://www.sbg.bio.ic.ac.uk/phyre/) (Bennett-Lovsey et al. 2008). Phyre calculates the best fit towards a solved crystal structure with estimated precision predicting likelihood of fit.

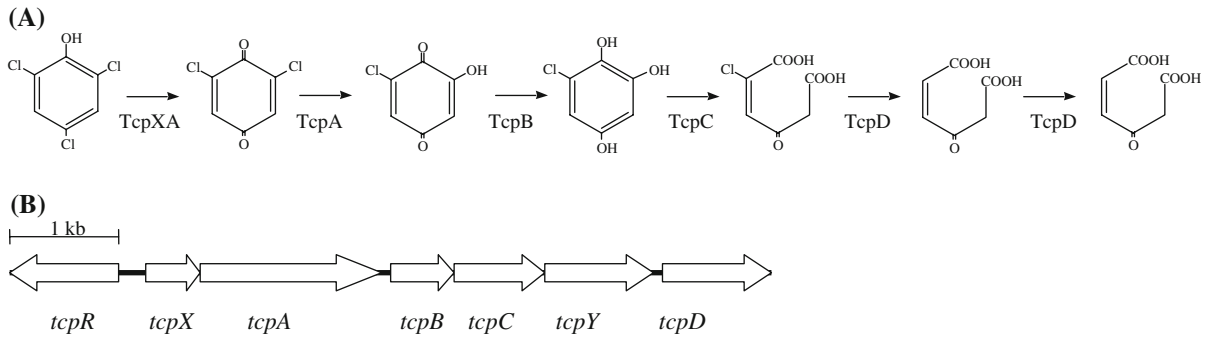


**Fig. 1** **a** Degradation pathway of *C. necator* JMP134. TcpX and TcpA catalyze the conversion of TCP to 6-chloro-2-hydroxy-*p*-benzoquinone. TcpB reduces 6-chloro-2-hydroxy-*p*-benzoquinone to 6-chloro-2-hydroxy-*p*-hydroquinone. TcpC converts the latter to 2-chloromaleylacetate, which is reduced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

### Phylogenetic analysis

Protein sequences were aligned by using CLUSTALW (Larkin et al. 2007), and a phylogenetic tree was generated by using MEGA version 4.0.2 (Tamura et al. 2007). The evolutionary distances were computed using the Jones et al. (1992) 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 (Louie et al. 2002). Recombinant strains were selected on LB agar plates containing kanamycin.

### Constitutive expression of TcpY in *C. necator*

The full length *tcpY* gene was cloned in the plasmid pTrc99a (Pharmacia, Piscataway, NJ), where *tcpY* was under the control of the *trc* promoter that contains a LacI binding site (Amann et al. 1988). The *tcpY* gene was PCR amplified with primers TcpYR (gctttcatcgaagcttctcccgtc) and TcpYF-pTrc99 (gttcgacagatgccatggaggtcc), digested with HindIII and NcoI, and ligated into pTrc99a to generate pTrc99a-tcpY. As the pTrc99a-tcpY plasmid cannot replicate in *C. necator* JMP134, the cloned tcpY is moved to pBBR1MCS2 that can replicate in a broad host range (Kovach et al. 1994, 1995). The pTrc99a-tcpY plasmid was used as a template for PCR using pTrc99-F (gttctgga*taagctt*ttttgcgcc) 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 transfer 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 assay 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 to 400 μM). Glutamate represses the *tcp* operon in *C. necator* JMP134, so no degradation occurs (Louie et al. 2002). Briefly, cells were cultured overnight in LB medium, harvested and resuspended in the mineral salt medium without carbon source at a turbidity of 1.0 at 600 nm ($\sim 1 \times 10^7$ cells $ml^{-1}$). Serial dilutions were performed in the medium, and 3 μl was spotted onto the agar plates. The plates were incubated for 3 days at 30°C and pictures were taken. 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 assay for polychlorophenols

Several polychlorophenols were tested to determine if TcpY was 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 h at 30°C and a final turbidity of 600 nm was measured.

### TCP uptake

Cells of *C. necator* JMP134 carrying pTcpY or pBBR1MCS2 were cultured for 5 h to a turbidity of 2 at 600 nm in LB medium, harvested and washed with mineral salt medium containing 0.2% glutamate, and resuspended in the same medium to a turbidity of 2.0 at 600 nm. Protein concentration was determined to be about 0.4 mg per ml by using modified Lowry protein assay (Pierce Biotechnology, Rockford, IL). One ml of culture was transferred to 1.7-ml microfuge tube, to which varying amounts of TCP (12.5–100 μM TCP) was added. The tubes were shaken for 1 min at room temperature, and then were centrifuged at 12,000×*g* for 1 min. First, the pellets were

washed with 1 ml of 50 mM KPi (pH 7.0) without suspending the cells. Then, they were washed twice with 1 ml of 50 mM KPi (pH 7.0) by suspension and centrifugation. After the washing, each pellet was resuspended in 100 µl of the KPi buffer with 1% sodium dodecyl sulfate. The sample was vortexed and incubated at 50°C for 5 min. About 100 µl of hexane:1-propanol (vol/vol, 4:1) was added, and the mixture was vortexed for 1 min. After centrifugation, TCP in the organic phase was analyzed with high performance liquid chromatography equipped with a C-18 column and photodiode array detector, as previously described (Louie et al. 2002). The volume of the organic phase was assumed to be 100 µl for calculations.

## Results and discussion

### 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 (Marchler-Bauer et al. 2007). 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 gene location with genes responsible for phenol degradation (Xu et al. 2003). 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* has both cytoplasmic and outer membranes. Integral membrane proteins of cytoplasmic membranes usually consist of α-helices for the transmembrane portions; whereas, the outer membrane proteins are composed of antiparallel β-strands for transmembrane segments that form a barrel in the outer membrane, e.g., porins (Schulz 2002). PRED-TMBB, a web-server capable of predicting and discriminating β-barrel outer membrane proteins, was utilized to analyze TcpY (Bagos et al. 2004). 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 with 95% accuracy using a database of known β-barrel outer membrane proteins (Bagos et al. 2004). PRED-TMBB predicted the topology of TcpY with fourteen transmembrane β-strands. Structural homology modeling using Phyre predicted TcpY structure with an estimated 95% precision related to the three-dimensional structure of FadL from *E. coli*. FadL is a transporter that facilitates the diffusion of hydrophobic LCFAs across hydrophilic outer membranes of *E. coli* and related gram-negative bacteria (van den Berg et al. 2004). Thus, bioinformatic analysis indicated TcpY as a β-barrel outer membrane protein, possibly involved in TCP uptake.

### Phylogenetic analysis of TcpY and structural homologs

Other structural homologs of FadL have been linked to the uptake of hydrophobic molecules for biodegradation (van den Berg 2005). XylN is identified as an outer membrane transporter for the uptake of xylene (Kasai et al. 2001); TodX and TbuX are for toluene uptake (Wang et al. 1995; Kahng et al. 2000); StyE is for styrene uptake (Mooney et al. 2006). The resolved structures of TodX and TbuX are similar to FadL (Hearn et al. 2008). To determine how TcpY is related to these proteins, a multiple alignment was utilized. The proteins are listed in Table 1 and the phylogenetic tree created from the alignment is shown in Fig. 2. Bootstrap values of 100 indicated the proteins fell into three separate clades: LCFA transporters, xenobiotic transporters, and COG4313 hypothetical proteins. TcpY was grouped with the COG4313 proteins. The substrates for the three groups are also different: hydrophobic LCFA, aromatic compounds without hydrophilic groups, and aromatic compounds with hydroxyl groups, respectively.

The COG4313 proteins used for phylogenetic analysis (Fig. 2) were further 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. COG4313 proteins are within gene clusters responsible for biodegradation of phenolic compounds, which are likely the substrates of COG4313 proteins. The *orf8* gene is located near meta-pathway of phenol degradation genes in *Acinetobacter*

**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 (Tamura et al. 2007)

[b] No specific information was given

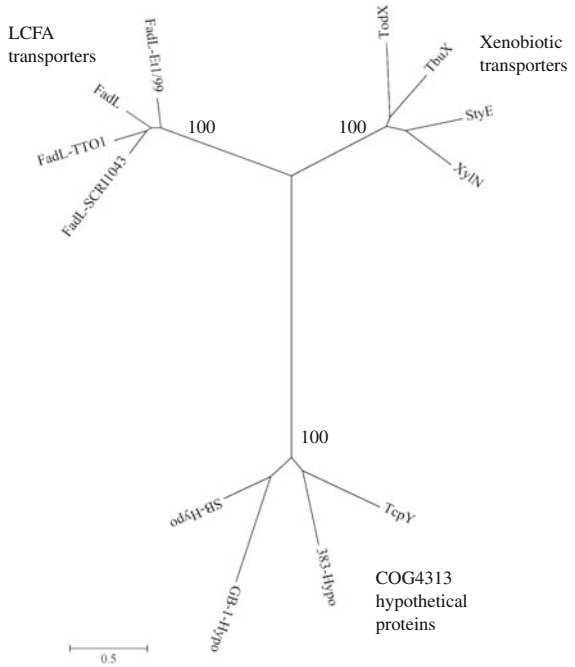


**Fig. 2** Phylogenetic relationship of $\beta$-barrel outer membrane transporters as determined using MEGA4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The proteins, species, GenBank accession number, pairwise score, and proposed function are listed in Table 1

**Table 2** COG4313 proteins computational analysis

| Protein[a] | Signal peptide cleavage site[a,b] | PRED-TMBB score[c] | Estimated precision with FadL structure (%)[d] |
|---|---|---|---|
| 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] Accession numbers are given in Table 1

[b] As determined by SignalP 3.0 Server (Emanuelsson et al. 2007)

[c] A score lower than the cutoff of 2.965 indicates a $\beta$-barrel outer membrane protein with 95% confidence (Bagos et al. 2004)

[d] As determined by Phyre server (Bennett-Lovsey et al. 2008)

*calcoaceticus* PHEA-2 (Xu et al. 2003). The *P. putida* GB-1-Hypo protein gene is adjacent to phenol degradation genes.

## 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 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 (100–400 μM) as well as other polychlorophenols both in the mineral salt liquid medium and on agar plates (data not shown). Sanchez and Gonzalez have also reported that a *tcpY* knockout mutant can grow at the same rate as the wild type with TCP as the sole carbon source (Sanchez and Gonzalez 2007). Thus, TCP is able to pass the outer membrane of *C. necator* JMP134 without the aid of TcpY under the culturing conditions. However, 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 (Nikaido 2003).

## Constitutively expressed *tcpY* was toxic to *C. necator* JMP134

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 in the wild type where the gene is repressed in the presence of glutamate (Louie et al. 2002). Without the operon expressing the genes required to degrade TCP, TCP would slow down cell 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. 3). On the

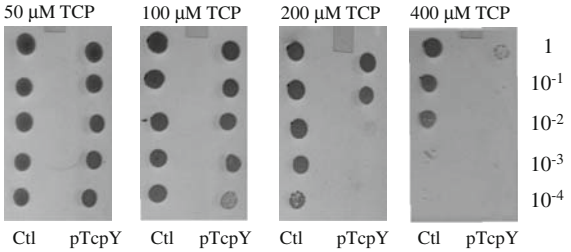


**Fig. 3** Sensitivity of *C. necator* JMP134 on agar plates with TCP. Cultures were grown overnight, harvested, washed, and resuspended to a turbidity of 1.0 in the mineral salt medium. Three micro litres of serial diluted cells were dropped onto the mineral salt agar plates with 0.2% glutamate and various concentrations of TCP. The plates were incubated at 30°C for 3 days. *Ctl* refers to clone containing pBBR1MCS2, and *pTcpY* refers to the clones containing constitutively expressed *tcpY*

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. 3). 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 unable to degrade TCP. 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 (data not shown). 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

Since FadL transports various LCFAs, we tested the ability of *tcpY* to transport other polychlorophenols. The experiments were done by growing the cells with glutamate that suppresses the expression of the *tcp* operon. Further, the *tcp* operon is only induced by TCP and not by other polychlorophenols (Sanchez and Gonzalez 2007). 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 have any apparent effect on the growth of *C. necator* JMP134 carrying pTcpY compared to the empty vector control (Fig. 4), indicating it is likely not a substrate for TcpY. The two TeCPs and PCP all reduced growth when compared to without polychlorophenols in the medium for cells carrying the empty vector, demonstrating toxicity of the TeCPs and PCP. Moreover, *C. necator* JMP134 cells carrying pTcpY displayed more sensitivity to TCP, 2,3,4,6-TeCP, 2,3,5,6-TeCP, and PCP than the cells with the empty vector (Fig. 4).

### TcpY facilitated the uptake of TCP into *C. necator* JMP134 cells

2,4,6-Trichlorophenol uptake by JMP134 cells carrying pTcpY or pBBR1MCS2 was determined after 1 min incubation in the presence of glutamate. JMP134 cells constitutively expressing *tcpY* from the plasmid pTcpY transported more TCP into the cells than the cells carrying the vector pBBR1MCS2 at various TCP concentrations (Fig. 5). The method was taking advantage of TCP accumulation in lipid membranes so that quick washes did not result in any significant loss of the transported TCP. Further, glutamate inhibits TCP metabolism (Louie et al. 2002). Because centrifugation and wash both took time, the calculated rate may not be accurate. However, the measured rates reflected the difference mediated by TcpY. The data provided direct evidence that TcpY facilitated TCP transportation into *C. necator* cells.

## Conclusion

*β*-Barrel outer membrane proteins share similar structures but limited sequence homology due to the lack of evolutionary constraints for loop regions that connect the *β*-sheets (Jeanteur et al. 1991). Some *β*-barrel outer membrane proteins can facilitate the transport of hydrophobic substrates across the outer membrane, which is often hydrophilic due to lipopolysaccharides (Nikaido 2003). TcpY is identified as

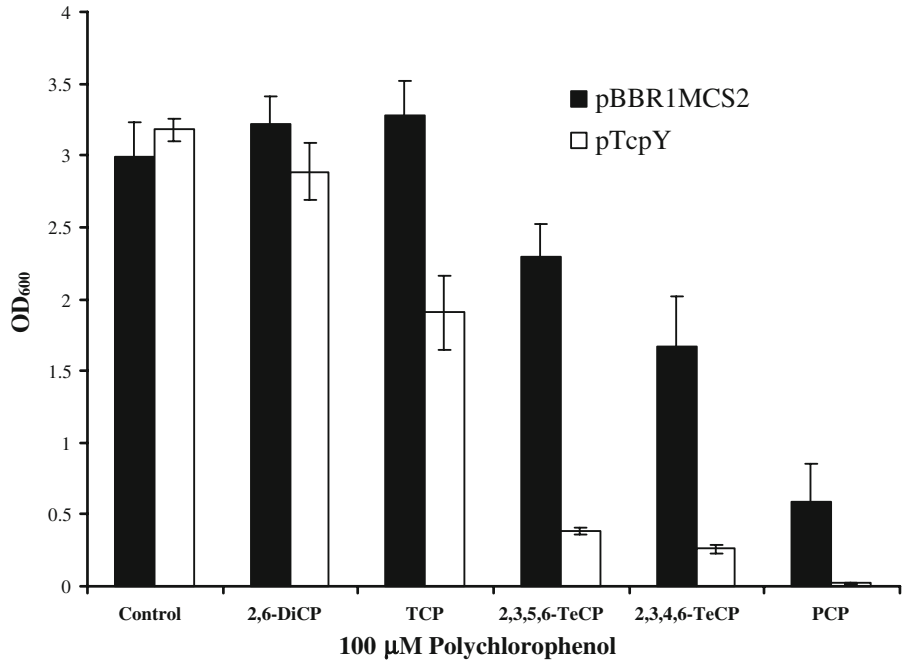


**Fig. 4** Toxicity of chlorophenols to *C. necator* JMP134. A 1% inoculum from overnight cultures was added to the minimal salt medium with 0.2% glutamate containing 100 μM of DiCP, TCP, 2,3,4,6-TeCP, 2,3,5,6-TeCP, or PCP. Cultures were incubated with shaking at 30°C for 18 h before turbidities were taken at 600 nm. *C. necator* JMP134 containing pBBR1MCS2 or pTcpY was tested

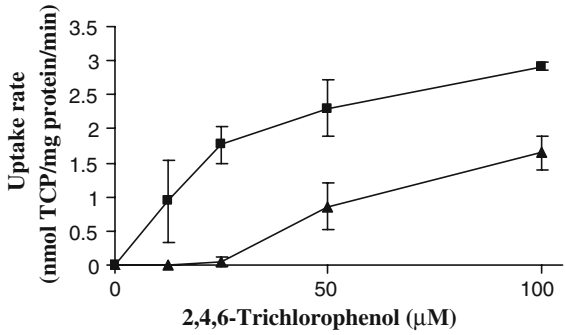


**Fig. 5** TCP uptake by JMP134 cells carrying pTcpY (*filled square*) or pBBR1MCS2 (*filled triangle*). Late log-phase cells were suspended in mineral medium with glutamate and various amounts of TCP. After 1 min shaking, the cells were collected by centrifugation and washed three times with KPi buffer. The TCP transported into the cells were extracted with hexane and determined

a $\beta$-barrel outer membrane protein because of its structural similarity to characterized $\beta$-barrel outer membrane proteins, including FadL, TodX and TbuX (Wang et al. 1995; Kahng et al. 2000; van den Berg 2005). The toxicity and uptake studies support that TcpY facilitates the transport of polychlorophenols across the outer membrane of gram negative bacterium *C. necator*.

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