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NK8**

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**Mutational analysis of the inducer recognition sites of the LysR-type
transcriptional regulator TfdT of *Burkholderia* sp. NK8**

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Abstract

TfdT is a LysR-type transcriptional regulator that activates the transcription of the chlorocatechol degradative gene operon *tfdCDEF* of the chlorobenzoate-degrading bacterium *Burkholderia* sp. NK8. To identify the amino acids involved in the effector recognition by TfdT, a polymerase-chain-reaction-based random mutagenesis protocol was applied to introduce mutations into the *tfdT* gene. Nine types of TfdT mutant bearing a single-amino-acid substitution at positions, Lys-129, Arg-199, Val-226, Val-246 and Pro-267, were obtained on the basis of their altered effector profiles and enhanced responses particularly to 2-chlorobenzoate, 2-aminobenzoate, and 2,6-dichlorobenzoate. All the TfdT mutants showed enhanced response to the effectors with a chloro-group in C-2 of benzoic acid. A homology model of wild-type TfdT was built on the basis of the crystal structure of CbnR with SwissModel. In this model, residues corresponding to the mutation sites of isolated TfdT mutants were located at the interface between the domains RD-I and RD-II. The findings that these TfdT mutants expressed altered effector specificities and enhanced responses to specific effectors suggest that these ~~four~~ five residues are involved in effector binding by TfdT.

Introduction

The chlorocatechol *ortho*-cleavage pathway (or the modified *ortho*-cleavage pathway) is the central pathway channeling chlorocatechols derived from the degradation of various chlorinated

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4 1 aromatic compounds to the energy-generating tricarboxylic acid cycle (Pieper and Reineke 2004;
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7 2 Reineke 1998). Chlorocatechol *ortho*-cleavage pathway genes are clustered into operons and are
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9
10 3 often found on plasmids (Ogawa et al. 2004; ~~Pieper and Reineke 2004; Reineke 1998~~). Most
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13 4 chlorocatechol operons are regulated by LysR-type transcriptional regulators (LTTRs) that are
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16 5 typically divergently transcribed from the genes encoding degrading enzymes (Coco, et al. 1993;
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19 6 Leveau and van der Meer 1996; McFall, et al. 1998; Tropel and van der Meer 2004; van der Meer,
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22 7 et al. 1991).

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26 8 LTTRs comprise the largest family of prokaryotic regulatory proteins that have been identified
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29 9 so far, which control the expression of genes of various metabolic pathways (Henikoff, et al. 1988;
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32 10 Maddocks and Oyston, 2008; Schell 1993). Most LTTRs act as transcriptional activators of the
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35 11 regulated metabolic genes, and ~~are presumed to~~ repress their own expression (Maddocks and
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38 12 Oyston, 2008). The transcriptional activation requires an effector or inducer, which is usually a
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41 13 substrate or an intermediate of the metabolic pathway that is regulated. Members of the LTTR
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44 14 family share a conserved domain organization. The N-terminal region of ca. 50 amino acids
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47 15 contains a helix-turn-helix (HTH) DNA-binding motif with relatively conserved sequence. The less
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50 16 conserved central and the C-terminal regions are ~~suggested to be~~ involved in effector recognition
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53 17 (Maddocks and Oyston, 2008; Schell 1993). The binding of an LTTR to the promoter region under
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56 18 non-induced conditions results in the bending of DNA, which is relieved to a certain extent by the
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59 19 binding of the effector to the LTTR in most cases (~~Ogawa, et al. 1999; Maddocks and Oyston, 2008;~~

Tropel and van der Meer 2004). This conformational change seems to be connected with transcriptional activation. Thus far, full-length CbnR and DntR have been crystallized as tetramers and their three-dimensional structures have been solved (Muraoka, et al. 2003; Smirnova, et al. 2004). Although the mechanism by which effector binding ultimately results in transcriptional activation has not been elucidated, these structural models fit previous biochemical experimental results on LTTRs well.

~~The divergence of the central and the C-terminal regions of LTTRs probably reflects structural variations to accommodate the variety of effectors that exert transcriptional activation.~~ Mutational analyses have indicated that changes in several amino acid residues in ~~these regions~~ the central and the C-terminal region of the well studied LTTRs, including DntR, NahR, CysB, OccR, NodD and CbbR, can markedly alter their effector profiles. ~~These residues are supposed to be involved in effector recognition~~ (Akakura and Winans 2002; Cebolla, et al. 1997; Dangel, et al. 2005; Horvath, et al. 1987; Lochowska, et al. 2001; Smirnova, et al. 2004; Tyrrell, et al. 1997). Likewise, the differences in only five amino acids ~~between~~ in the C-terminal regions of NtdR and NagR result in an altered effector specificity (Lessner, et al. 2003). ~~The divergence of the central and the C-terminal regions of LTTRs probably reflects structural variations to accommodate the variety of effectors that exert transcriptional activation.~~ Recently, the effector binding domains of BenM and CatM of the degradation pathway of benzoate and catechol have been crystallized with biologically relevant effector bound to the effector-binding sites (Ezezika, et al. 2007). However, there has been

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1 no report on protein sites involved in the effector recognition by LTTRs of the chlorocatechol
2 *ortho*-cleavage pathway that is central for the complete degradation of chloroaromatics.

3 Most LTTRs involved in the transcriptional activation of the chlorocatechol *ortho*-cleavage
4 pathway genes can recognize only chloromuconate(s) or both of chloromuconate(s) and muconate
5 as inducers, which are intermediate products converted from chlorocatechol(s) or catechol by the
6 first enzyme of the pathway, (chloro)catechol 1,2-dioxygenase (Filer and Harker 1997; McFall, et al.
7 1997; Ogawa, et al. 1999). The very limited range of effector specificity hindering hinders the study
8 analysis of protein the relationship of the effector and the structure sites involved in effector
9 recognition by of the LTTRs of the chlorocatechol *ortho*-cleavage pathways.

10 TfdT is a LTTR from the chlorocatechol *ortho*-cleavage gene operon *tfdT-CDEF* of the
11 chlorobenzoate-degrading bacterium *Burkholderia* sp. NK8. Unlike effectors of LTTRs of other
12 chlorocatechol *ortho*-cleavage pathways, 2-chlorobenzoate (2CB) and 3-chlorobenzoate (3CB), the
13 chlorinated aromatic compounds *per se*, can act as effectors of transcriptional activation regulated
14 by TfdT, although TfdT retains certain homologies similarities with other LTTRs of chlorocatechol
15 *ortho*-cleavage pathway genes (Liu, et al. 2001). Using the combination of TfdT and a
16 *Pseudomonas* background, we constructed a screening system and identified several amino acid
17 residues whose mutations changed the effector profile and enhanced the transcriptional activation
18 by TfdT. This is the first report of amino acids involved in the effector recognition by an LTTR
19 engaged in the transcriptional activation of the chlorocatechol *ortho*-cleavage pathway genes, which

are critical for the degradation of various chlorinated aromatic compounds.

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Material and methods

Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium, 1/3 diluted LB medium, 2×YT medium, and M9 minimal medium (Sambrook et al. 1989) with glucose were used to maintain the bacterial strains or to screen for mutant *tfdT* candidates and were supplemented, where required, with 50 µg/ml kanamycin (Km), 40 µg/ml gentamicin (Gm), or 50 µg/ml ampicillin (Ap). In addition, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) was used at 40 µg/ml in agar plates where required.

DNA manipulations and sequencing

DNA manipulations were performed by standard methods (Sambrook et al. 1989). Plasmid DNA was purified with a QIAprep Spin Miniprep Kit or a Plasmid Midi Kit (QIAGEN, Tokyo, Japan). Restriction endonucleases were purchased from Nippon Gene (Tokyo, Japan) or TaKaRa (Tokyo, Japan). T4 polynucleotide kinase was from TaKaRa, and alkaline phosphatase (*Escherichia coli*) was from TOYOBO (Tokyo, Japan). All these enzymes were used according to the manufacturers' instructions. DNA ligation experiments were performed using a DNA ligation kit Ver2.1 (TaKaRa). KOD DNA polymerase from TOYOBO was used for Polymerase chain reaction (PCR) amplification of the *tfd* genes. DNA sequencing was carried out with a BigDye Terminator

V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3100 DNA sequencer (Applied Biosystems).

Construction of *Ptfd::lacZ* fusion plasmid and triparental mating

The DNA region including the intergenic region between *tfdT* and *tfdC*, eight codons of *tfdT* and 83 codons of *tfdC*, which contained the *tfdC* promoter, was designated as *Ptfd*. A 289-bp nucleotide sequence encompassing N-terminal region of *tfdT*, intergenic promoter region of *tfdT* and *tfdC*, and N-terminal region of *tfdC* was designated as *Ptfd*. The PCR primers used to amplify *Ptfd* are as follows: forward primer 5'-GCGGCCGC***GGTACC***AGTATCTCAACTGCCTCATTTCCA-3' (the *NotI* site underlined; the *KpnI* site shown in bold italics) and reverse primer 5'-GCGGCCGC***GGATCC***GACTCCGTAATCTCCTTTTCATCG-3' (the *NotI* site underlined; the *BamHI* site shown in bold italics). The PCR product was cloned into the *PvuII* site of pUC18, in which an original 322-bp *PvuII* fragment was deleted. To place the β -galactosidase gene under the control of the *tfdC* promoter, a *BamHI* fragment of pKOK6.1 containing a promoterless *lacZ* and a kanamycin resistance marker gene was inserted into the *BamHI* site of the above mentioned pUC18-based plasmid. The *NotI* fragment including the transcriptional fusion of *Ptfd* and the promoterless *lacZ* gene together with the kanamycin resistance marker gene (simplified as *Ptfd::lacZ*) from the resulting plasmid was then excised and placed into the *NotI* site of a Tn5-based minitransposon delivery plasmid, pJMS11. This gave rise to two delivery plasmids, pJtfd1 and pJtfd2, bearing the *Ptfd::lacZ* fusion inserted to pJMS11 in the different orientation. The constructed minitransposon

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4 1 harboring *Ptfd::lacZ* was inserted into the chromosome of *Pseudomonas putida* KT2440 by
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7 2 transferring pJtfd1 and pJtfd2 from the host strain *E. coli* CC118 λ pir to *P. putida* KT2440 using a
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10 3 triparental mating strategy (de Lorenzo and Timmis 1994) with the helper strain *E. coli* HB101
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13 4 (RK600). Transconjugants were screened for resistance to Km in M9 minimal medium with 10 mM
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16 5 citrate and verified upon turning yellow after spraying with 1% (W/V) catechol. Primarily isolated
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19 6 transconjugants were further confirmed by detection of the *Ptfd* region from chromosome DNA by
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22 7 PCR analysis.
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26 8 **Construction of broad-host-range plasmids containing *tfdT***

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29 9 A broad-host-range vector, pBBR1MCS-5 (Kovach, et al. 1995), was employed for the cloning
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32 10 of *tfdTC'* or *tfdTCD'* with the wild type or mutagenized *tfdT*. DNA fragments of *tfdT-CDEF* were
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35 11 excised from pHP11 (Liu, et al. 2001). A 1.45 kb *Bam*HI-*Sal*I fragment from pHP11 containing *tfdT*,
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38 12 the promoter region, and the truncated *tfdC* gene (TfdC was truncated at position 86 out of 251
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41 13 amino acids) was inserted into the *Bgl*III-*Sal*I site of pBBR1MCS-5 to generate pBB*tfdTC'*. To
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44 14 express the *tfdC* gene as well as the *tfdT* gene in *P. putida* KT2440 *Ptfd::lacZ*, pBB*tfdTCD'* was
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47 15 constructed by subcloning the 2.1 kb *Bam*HI-*Ssp*I fragment of pHP11 into the *Bam*HI-*Sma*I site of
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50 16 pBBR1MCS-5. TfdD was truncated at position 69 out of 371 amino acids.
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54 17 **Random mutagenesis of the *tfdT* gene and screening for mutants**

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57 18 Random mutations were introduced into the *tfdT* gene using a GeneMorph II Random
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60 19 Mutagenesis kit (Stratagene, La Jolla, CA). pBB*tfdTC'* was used to provide DNA templates for

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4 1 random mutagenesis reaction. A DNA region of 987 bp including 860 bp nucleotide of *tfdT*
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7 2 corresponding to 286 amino acids of the C-terminus of TfdT was amplified by mutagenesis PCR
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10 3 using oligonucleotides MupBB-F (5'-GCCGCTCTAGAACTAGTGGATC-3') and Mutfd-R (5'-
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13 4 CCGCTGAAAGAATGCATATCTC-3'). Seven hundred and fifty nanograms of pBB*tfdTC*' was
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16 5 supplemented in every 100 µl of reaction mixture. PCR was performed on an iCycler (Bio-Rad
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19 6 Laboratories, Hercules, CA) with an initial denaturation at 98°C for 2 min followed by 35 cycles of
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22 7 denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 min,
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25 8 and a final extension at 72°C for 10 min. A DNA fragment of 935 bp was cut out from 987-bp
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28 9 random mutagenesis PCR products with *Bam*HI and *Mlu*I, then purified and used to replace the
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31 10 equivalent fragment of the plasmid pBB*tfdTCD*'. The resulting plasmid mixture was purified by
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34 11 ethanol precipitation and was transformed into *P. putida* KT2440 *Ptfd::lacZ* by electroporation.
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37 12 Transformants were picked up from LB agar plates containing Km and Gm, and then replicated
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40 13 onto 1/3 diluted LB agar plates amended with Km, Gm, X-gal, and with or without one of the
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43 14 aromatic compounds (5 mM) including benzoate, 2CB, 3CB, 4-chlorobenzoate (4CB),
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46 15 2-hydroxybenzoate (salicylate), 3-hydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate
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49 16 (anthranilate) (2NHB), 3-aminobenzoate, and dichlorobenzoates. After incubation at 30°C for 3
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52 17 days, TfdT mutants were screened for altered effector specificity or enhanced transcriptional
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55 18 activity of the *tfdC* promoter by comparison of the color intensity with that of the simultaneously
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58 19 cultured *P. putida* KT2440 *Ptfd::lacZ* harboring pBB*tfdTCD*'. The clones that retained their
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4 1 inducible nature and exhibited higher intensities of the blue color than the clones containing the
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7 2 wild-type *tfdT* after induction were considered as the candidate clones containing TfdT mutants
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10 3 with altered effector specificity. After sequence analysis and verification of the altered specificity by
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13 4 measuring β -galactosidase activity using liquid culture, the plasmids with one amino acid
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16 5 substitution in the *tfdT* gene were named as a series of pBBMut*tfdTCD'* with increasing number
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19 6 (from pBBMut*tfdTCD'*1 to pBBMut*tfdTCD'*8). To examine the transcriptional activity of
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22 7 wild-type TfdT (wtTfdT) or TfdT mutant (K129M) in *E. coli* DH5 α , the *Ptfd* region together with
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25 8 the wild-type *tfdT* or a *tfdT* mutant was fused to the *lacZ* gene by inserting the 1.45 kb *Bam*HI-*Sal*I
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28 9 fragment of pHP11 or isolated pBBMut*tfdTCD'*1 into the *Bgl*II-*Sal*I site of pQF50. The resulting
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31 10 plasmids, named as pQF*tfdTC'* or pQFMut*tfdTC'*1, were transformed into *E. coli* DH5 α , and
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34 11 β -galactosidase activity was analyzed.

38 12 **Site-directed mutagenesis**

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41 13 Site-directed mutagenesis was carried out by overlap extension using PCR according to the method
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44 14 described by Horton (Horton 1995). Oligonucleotides employed for the mutation A203V were
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47 15 5'-AAACAACCTTATCAACGAAACTCGGCCGG-3' and
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50 16 5'-AGTTTCGTTGATAAGGTTGTTTCCCTGTTC-3' (The replaced nucleotide was underlined),
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53 17 and for G300D were 5'- GGATGAATCTCGTCTTCGAGACCCCCACC-3' and
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56 18 5'-GGGTCTCGAAGACGAGATTCATCCGTCCAG-3' (The replaced nucleotide was underlined).
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59 19 Two external primers for overlap extensions were the same as used in random mutagenesis. PCR
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products from overlap extensions were digested with *Bam*HI and *Mlu*I, and used to replace the equivalent fragment of the plasmid pBB*tfdTCD*'. Constructed mutants were confirmed by sequencing.

β-galactosidase assay

The transcriptional activity of wt*TfdT* or *TfdT* mutants was measured quantitatively by analyzing their abilities to cause accumulation of β-galactosidase in *P. putida* KT2440 *Ptfd::lacZ* or in *E. coli* DH5α by methods described previously (Liu, et al. 2001; Miller 1972). The *P. putida* KT2440 *Ptfd::lacZ* strains harboring pBB*tfdTCD*' or pBB*MutfdTCD*' were cultured overnight in LB medium with Km and Gm. The preculture was diluted 100-fold with basal synthetic medium (Aldrich, et al. 1987) supplemented with 10 mM glucose, Km, Gm, and with or without an aromatic compound (5 mM) and incubated for 15 h at 30°C. *E. coli* DH5α harboring pQF*tfdTC*' or pQF*MutfdTC*'¹ was cultured overnight in LB medium with Ap. The preculture was diluted 100-fold with 2×YT medium supplemented with Ap and with or without an aromatic compound (5 mM) and cultivated for 15 h at 28°C. Cells were harvested by centrifugation at 2500 g for 15 min at 4°C and washed with Z buffer (Miller 1972). Harvested cells were disrupted by sonication and cell debris was removed by centrifugation at 15000 g for 30 min at 4°C. The specific activity of β-galactosidase in the extract was expressed as Miller unit (nmol/min/mg protein) (Hefferon and Miller 2002; Miller 1972). β-Galactosidase activity values given throughout this paper represent the average of triplicate cultures.

1 Construction of structural model

2 Homology modeling of the wild type TfdT was performed in the SWISS-MODEL server (Guex
3 and Peitsch 1997). The PDB record 1ixcB (2.20 Å) (Parent PDB entry is 1ixc. The crystal structure
4 of chain B of CbnR) was identified as an appropriate modeling template. The predicted structural
5 model of TfdT was generated and displayed by MolFeat (version 2.2, FiatLux Co. Tokyo, Japan).

7 Results

8 Construction of reporter strains with *P. putida* KT2440

9 The two Tn5-based minitransposon delivery plasmids, pJtfd1 and pJtfd2, were mobilized
10 into *P. putida* KT2440. On 1/3 diluted LB agar plates supplemented with Km and X-gal, colonies of
11 transconjugants derived from the mobilization of pJtfd1 showed slightly higher color intensity than
12 those derived from the mobilization of pJtfd2. One of the transconjugants obtained derived from the
13 mobilization of pJtfd2 was designated *P. putida* KT2440 *Ptfd::lacZ* and was used in the following
14 TfdT mutant selection and analysis. The expression of β galactosidase was proved to be inducible
15 by 3CB in *P. putida* KT2440 *Ptfd::lacZ* transformed with pBB*tfdTCD*'. Clones of *P. putida*
16 KT2440 *Ptfd::lacZ* transformed with pBB*tfdTCD*' showed increased blue color when induced with
17 3CB on 1/3 diluted LB agar. The strain *P. putida* KT2440 *Ptfd::lacZ* was used for selection of
18 colonies containing TfdT mutants.

19 Isolation of *tfdT* mutants with altered effector specificity

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4 1 Colonies of *P. putida* KT2440 *Ptfd::lacZ* harboring *tfdT* mutants that showed increased color
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7 2 intensity on plates supplemented with aromatic compounds compared with the clones expressing
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10 3 the wtTfdT were considered as candidate clones that contained TfdT with an altered function. From
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13 4 about 5000 colonies, 76 clones were primarily isolated based on their increased color intensity
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16 5 induced by 2CB and/or 2NHB. The putative mutagenized *tfdTs* of the clones were subjected to
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19 6 DNA sequencing. As a result, ~~26 clones were excluded because they harbored plasmids containing~~
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22 ~~truncated *tfdTs*, or *tfdTs* mutated at sites outside expected effector binding region~~ 26 clones were
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26 8 excluded because of the following reasons. Four clones among them harbored plasmids containing
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29 9 truncated *tfdTs*. The mutation sites of the remaining 22 clones were located in the N-terminal region
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32 10 of TfdT, which was the DNA binding domain and the hinge region predicted by the SMART server
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35 11 (<http://smart.embl-heidelberg.de/>) as well as judged by the sequence similarity of TfdT to a related
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38 12 LTTR, CbnR (52.7% amino acid identity), for which the three-dimensional structure has been
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41 13 solved at full length (Muraoka, et al. 2003). The response of the remaining clones to the induction
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44 14 by 2CB, 3CB and 2NHB were verified by LacZ assay using liquid culture. Eight types of mutants
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47 15 that carried a single amino acid change in TfdT were confirmed to show an enhanced response to
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50 16 2CB and/or 2NHB (~~Table 2~~ Table 3; Except P267A, which was constructed by site-directed
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53 17 ~~mutagenesis~~). The mutation of K129M was found in three independent clones. Each of the
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56 18 mutations, R199Q and R199L, was found in two independent clones. On the other hand, a type of
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59 19 TfdT mutant bearing changes at two positions, P267A and G300D, was found in four independent
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colonies and showed increased response to the induction by 2CB and 2,6-dichlorobenzoate (2,6DCB). Another TfdT mutant bearing two amino acid substitutions, R199Q and A203V, was also found to give increased response to the induction by 2CB and 2,6DCB. To evaluate the effect of the respective residues substituted in TfdT mutants bearing two amino acid substitutions, corresponding single-site *tfdT* mutants were prepared by ~~sited-directed~~ site-directed mutagenesis method. Results of β -galactosidase assay proved that the increasing responses to the induction by 2CB and 2,6DCB of the two double sites mutants were attributed to the presence of mutations in residue 267 and 199, which had been isolated from single site mutants, and not caused by amino acid replacement at the other position. The response of the constructed single site mutant P267A to aromatic compounds is shown in Table 3. The remaining 34 colonies, which showed higher constitutive expression levels and ~~uneobvious~~ no obvious change of relative transcriptional activity in response by the examined effectors, were excluded.

Characterization of TfdT mutants

Transcriptional activities of the *tfdC* promoter activated by wtTfdT and the selected TfdT mutants in response to aromatic compounds were measured by determination of β -galactosidase activity in *P. putida* KT2440 *Ptfd::lacZ* cells (Table 3). wtTfdT exhibited the strongest response to 3CB among the tested aromatic compounds, which showed 9.6-fold β -galactosidase activity compared with the non-induced control. Induction by 2CB, 2NHB and 2,6DCB resulted in 4.2-fold, 3.6-fold, and 1.8-fold β -galactosidase activity, respectively. Compared with wtTfdT, all TfdT mutants acquired

altered effector profiles, and particularly showed enhanced response to effectors possessing a chloro (or an amino) -group in C-2 position of benzoic acid. To the compounds that do not have a substituent in C-2 of benzoic acid, i.e. benzoate, 4-chlorobenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 3-aminobenzoate, and 3,4-dichlorobenzoate, TfdT mutants did not show markedly different response change, as the ratio of β -galactosidase value against that of non-induced cells, when compared with the response of wtTfdT to the respective compound (data not shown). In contrast to wtTfdT that exhibited the strongest response to 3CB and faint response to 2,6DCB, all the TfdT mutants, except for mutant V226F, exhibited the strongest response to 2CB and significantly enhanced response to 2,6DCB while they showed decreased activation pattern to the induction by 3CB compared with wtTfdT. They also showed a tendency of enhanced response to 2,4-dichlorobenzoate and 2,5-dichlorobenzoate. The mutant V226F showed the highest transcriptional activity in the presence of ~~2,3-dichlorobenzoate~~ 2,3-dichlorobenzoate and similar transcriptional activation in the presence of 2CB, 3CB, 2NHB and 2,6DCB. Among the isolated mutants, K129M showed the biggest change in the response pattern to the inducers compared to that of wtTfdT. K129M showed 17.7-fold and 17.6-fold response to 2,6DCB and 2CB, respectively, while it exhibited only 1.5-fold response to 3CB compared with the non-induced control.

Affinity of TfdT mutant for effector compounds

To gain some insights into the mechanism by which the TfdT mutant altered their effector profile, the apparent affinities of wtTfdT and TfdT mutant K129M for 2CB and 2,6DCB, were

1 determined. To this end, we employed the procedure developed by Ramos et al. (Ramos, et al.
2 1990) to estimate in vivo apparent affinity constant (K_s') values of effectors for prokaryotic
3 transcriptional regulators. *E. coli* DH5 α harboring pQF $tfdTC'$ or pQFM $utfdTC'$ 1 was used to
4 analyze the transcriptional activities of wtTfdT and TfdT mutant K129M. The K_s' of wtTfdT or
5 TfdT mutants for effectors was estimated by measuring their activation of transcription from *Ptfd* in
6 *E. coli* DH5 α . The data points showed hyperbolic-like curves, and K_s' was expressed as the effector
7 concentration when the β -galactosidase level reached half maximum in the curves. The constructed
8 strains were subjected to induction experiments with the concentrations of the aromatic compounds
9 ranging from 0.3 mM to 6 mM, and β -galactosidase levels were determined 15 hours later. Fig. 1
10 shows that β -galactosidase levels depended on the concentration of effectors and the β -galactosidase
11 levels in TfdT mutant K129M were higher than those in wild type. The shape of the dose-dependent
12 curves indicated that the mutation did increase the affinity of TfdT to the tested aromatic
13 compounds. As shown in Fig. 1A, the apparent affinities of mutant K129M for 2CB increased,
14 which was reflected by a decrease of K_s' from 2.5 mM (wtTfdT) to 1.4 mM. Moreover, wtTfdT
15 almost had no affinity to 2,6DCB, while mutant K129M had a K_s' value of 0.9 mM (Fig. 1B). These
16 results suggested that ~~the amino acids at 129 were directly~~ the mutation of K129M affected amino
17 acid residue(s) involved in effector recognition and binding.

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Discussion

TfdT is a LTTR from the chlorocatechol degradative gene cluster *tfdT-CDEF* of the chlorobenzoate-degrading bacterium *Burkholderia* sp. NK8. We examined the amino acids critical for effector binding in TfdT by introducing random mutation into the *tfdT* gene and screening for altered specificities for aromatic effectors. Eight TfdT mutants with a single-residue replacement at five positions were isolated based on their altered effector profiles and increased activities to initiate transcription on the *tfdC* promoter, and one mutant (P267A) was constructed and confirmed with its altered specificity for effectors. Stability of the mutant proteins in the cell could be altered compared to that of wtTfdT. However, because the transcriptional activities exhibited by each of the tested nine mutant TfdTs were reproducible and consistent throughout the experiments, they were considered to be stable as the transcriptional activator.

Among the aromatic compounds in Table 3, *P. putida* KT2440 is able to convert 3CB to 3-chlorocatechol and 4-chlorocatechol by its chromosomally-encoded benzoate 1,2-dioxygenase (Klemba et al. 2000, Nelson et al. 2002). 3-Chlorocatechol and 4-chlorocatechol then can be converted to 2-chloro-*cis,cis*-muconate and 3-chloro-*cis,cis*-muconate, respectively, by chlorocatechol 1,2-dioxygenase (TfdC) when the full-length *tfdC* gene is expressed. 3CB and its intermediates could act as effectors to induce transcriptional activation by TdfT (Liu et al. 2001), thus the β -galactosidase activity of *P. putida* KT2440 *Ptfd::lacZ* strains harboring pBB*tfdTCD*' or pBBMut*tfdTCD*' indicated the comprehensive induction effects of 3CB and its intermediates when

3CB was supplemented. In contrast, other aromatic compounds in Table 3 including 2CB and 2,6DCB cannot be converted in *P. putida* KT2440, so that they act as effectors by themselves. Mutant K129M ~~greatly altered transcriptional activity under the induction by 2CB and 2,6DCB~~ acquired the ability to give response to 2,6DCB and showed increased response to 2CB and 2NHB in *P. putida* KT2440 *Ptfd::lacZ*. In vivo affinity assay in *E. coli* DH5 α showed that the affinity of mutant K129M to 2CB and 2,6DCB was largely increased. ~~These results suggested strongly that the amino acid residue at position 129 could be directly involved in effector binding.~~ The substitution of Lys, a polar and positively charged amino acid, with a hydrophobic amino acid, Met, at position 129 might have affected the conformation of adjacent amino acids that directly interact with Cl at C-2 position of benzoic acid, thereby indirectly increasing the affinity for the effectors with Cl at C-2 (or C-6) position. Moreover, since mutant K129M showed increased response to all benzoate analogs in Table 3 except 3CB, this may reflect a potential flexibility of the effector pocket in TfdT.

Thus far, full-length CbnR and DntR, and effector-binding domains of CysB, BenM and CatM have been crystallized and respective structural models have been solved (Ezezika, et al. 2007; Muraoka, et al. 2003; Smirnova, et al. 2004; Tyrrell, et al. 1997; Verschueren, et al. 1999). Biochemical and genetic studies have provided strong supporting evidence that the effector-binding sites of LTTRs are located at the interface between the regulatory domains of each subunit (Akakura and Winans 2002; Dangel, et al. 2005; Lochowska, et al. 2001; Schell 1993; Smirnova, et al. 2004; Tyrrell, et al. 1997). While the effector-binding sites of BenM and CatM from the degradative gene

1 cluster for benzoate and catechol of *Acinetobacter baylyi* ADP1 keep the characteristics of LTTR in
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4 1 cluster for benzoate and catechol of *Acinetobacter baylyi* ADP1 keep the characteristics of LTTR in
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7 2 that the (primary) binding sites for the non-aromatic effector *cis,cis*-muconate are located between
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10 3 the regulatory domain I and II in each regulator, BenM has a secondary binding site for the effector
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13 4 benzoate in domain I. In BenM, the two effectors have the synergistic effect to activate the
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16 5 regulated genes (Ezezika, et al. 2007). Similar to CbnR (Ezezika, et al. 2007), in TfdT, the region
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19 6 corresponding to the secondary effector-binding site of BenM is fully occupied by hydrophobic
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22 7 residues. Particularly, the residues corresponding to Arg-160 and Tyr-293 in BenM, which
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25 8 interacted with the carboxyl group of benzoate in the secondary binding site of BenM are occupied
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28 9 by hydrophobic amino acids Gly-161 and Ile-292 in TfdT, respectively. It suggests that a secondary
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31 10 effector-binding site might not exist in TfdT and that TfdT might recognize the aromatic effectors
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34 11 with a different mechanism from that of BenM. Amino acid sequence similarities show that TfdT,
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37 12 together with CbnR (48.7% 52.7% amino acid identity with TfdT) from the chlorocatechol
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40 13 *ortho*-cleavage pathway genes of *Ralstonia eutropha* NH9, forms a different branch from that of
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43 14 BenM and CatM (60.5% amino acid identity to each other, and 34.8% and 31.2% identity with TfdT,
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46 15 respectively) in the subgroup of LTTR that regulate the expression of the catabolic genes for
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49 16 aromatic compounds (Tropel and van der Meer 2004). This is consistent with the fact that the
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52 17 former group regulates the expression of chlorocatechol chlorocatechol *ortho*-cleavage pathway
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55 18 genes and the latter group regulates the expression of benzoate/catechol degradative genes. A
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58 19 homology model of wtTfdT was built on the basis of the crystal structure of CbnR with SwissModel
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(Guex and Peitsch 1997; Muraoka, et al. 2003; Schwede, et al. 2003). The homology model of wtTfdT exhibits domains and regions similar to the reference crystal structure of CbnR, including two supposed effector recognition domains (RD-I and RD-II), the DNA binding domain, hinge 1, hinge 2 and hinge 3. The residues in wtTfdT that correspond to the substituted residues in the isolated mutants were assigned to the three-dimensional structural model of TfdT (Fig. 2). In the homology model of TfdT, Lys-129 was located in RD-I, while Arg-199, Val-226 and Val-246 were located in RD-II. ~~Substitution of Lys with Met in position 129 resulted in the acquirement of the ability to be induced by 2,6DCB, and showed increased response to 2CB and 2NIIB. These results imply that the residue in position 129 could directly bind with the substituent in the C-2 position of benzoic acid.~~ Similar to the crystal structure of CbnR, hinge 3 of the structure model of TfdT is composed of two cross-over regions between RD-I and RD-II, which is suggested to be the point for conformational change between the compact and extended forms of the LTTR monomer (Dangel, et al. 2005; Muraoka, et al. 2003; Smirnova, et al. 2004). Pro-267 is located within hinge. Three TfdT mutants with substitution in Pro-267 obtained altered effector profile, but showed a little increase in basal transcriptional activity. This phenomenon suggested that Pro-267 might be mainly involved in effector binding and these three kinds of mutation almost gave no effect to ~~confirmation~~ ~~conformation~~ change. Of particular interest is the location of the five residues, Lys-129, Arg-199, Val-226, Val-246 and Pro-267, all of which are located at the interface between RD-I and RD-II. These findings suggest that effector binding of TfdT occurs in the interface

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4 1 between RD-I and RD-II, and that the amino acids at positions highlighted in this study might join
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7 2 to outline the effector-binding cavity. Although the LTTRs that activate the transcription of
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10 3 chlorocatechol *ortho*-cleavage pathway genes have been characterized in terms of their DNA
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13 4 binding and response to effectors, our present study provides evidence for the critical amino acids
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16 5 involved in the effector-binding cavity and specificities of the LTTR of the chlorocatechol
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19 6 *ortho*-cleavage pathway for the first time.
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23 7 The results of this study highlighted five amino acid residues that were critical for effector
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26 8 recognition by TfdT. Among the five residues, Lys-129, Arg-199, Val-246 and Pro-267, of wtTfdT
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29 9 are conserved among all reported LTTR proteins of chlorocatechol *ortho*-cleavage pathways, while
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32 10 these residues are not conserved among members of the entire LTTR family. Another residue,
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35 11 Val-226, is conserved in most LTTR proteins of chlorocatechol *ortho*-cleavage pathways. These
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38 12 residues might be involved in effector binding of the LTTRs of other chlorocatechol *ortho*-cleavage
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41 13 pathways as well as of TfdT. Further analysis of TfdT and related LTTR members of the
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44 14 chlorocatechol *ortho*-cleavage pathway may provide insights into the unique inducer-recognizing
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47 15 properties of TfdT and the basis for the different inducer-recognizing properties of the LTTRs of the
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50 16 chlorocatechol *ortho*-cleavage pathways.
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57 18 **Acknowledgements**
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Figure legends

Fig. 1. Effect of the concentration of chloroaromatic effectors on *Ptfd* activity regulated by wtTfdT and TfdT mutant K129M.

Wild-type *tfdT* or *tfdT* mutant were fused to the promoterless *lacZ* gene on a broad-host-range vector pQF50, as described in “Materials and methods”. *E. coli* DH5 α bearing these plasmids were subjected to induction experiments using chloroaromatic effectors of 2CB (A) and 2,6DCB (B) at various concentrations as indicated. The transcriptional activities caused by wtTfdT (●) and TfdT mutant K129M (■) were measured.

Fig. 2. Structural model of TfdT monomer (based on the structure of a related LTTR CbnR).

Residues relative to the mutation sites of isolated TfdT mutants are indicated in a ball-stick structure. α -Helices are magenta, β -sheets are yellow and turns are blue. RD-I and RD-II indicate two regulatory domains. Lys-129 is within RD-I, while Arg-199 and Val-226 are within RD-II. Pro-267 is within hinge 3.

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Table 1 Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
Strains		
<i>Pseudomonas putida</i> KT2440	BA ⁺ , 3CB ⁻	Bagdasarian et al. 1981
<i>P. putida</i> KT2440 <i>Ptfd::lacZ</i>	KT2440 with <i>tfdC</i> promoter- <i>lacZ</i> inserted into the chromosome	This study
<i>Escherichia coli</i> DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 endA1 gyrA96 thi-I relA1</i>	Takara, Tokyo, Japan
<i>E. coli</i> CC118 <i>λpir</i>	<i>Δ(ara-leu) araD ΔlacX74 galE galK phoA phoA20 thiI rpsE rpoB</i>	
	<i>argE(AM) recA1</i> , lysogenized with <i>λ pir</i> phage	de Lorenzo and Timmis 1994
<i>E. coli</i> HB101(RK600)	Sm ^r <i>recA thi pro leu hsdR⁻ M⁺</i>	de Lorenzo and Timmis 1994
Plasmids		
pUC18	Ap ^r	Takara.
pQF50	Ap ^r ; Broad-host-range <i>lacZ</i> promoter probe vector	Farinha and Kropinski 1990
pKOK6.1	Ap ^r Km ^r Tc ^r ; promoterless <i>lacZ</i>	Kokotek and Lotz 1989
pJMS11	Km ^r Ap ^r ; Delivery vector for the minitransposon	Panke et al. 1998
pHP11	Ap ^r ; 3.2 kb <i>EcoRV</i> - <i>HpaI</i> fragment of pSL1 inserted into the <i>EcoRV</i> - <i>HincII</i> sites of pBluescript KS II	Liu et al. 2001
pBBR1MCS-5	Gm ^r , mob ⁺	Kovach et al. 1995
pJtfd2	Km ^r , <i>Ptfd::lacZ</i> -Km ^r cassette was inserted into the <i>NotI</i> site of pJMS11, Km ^r gene was adjacent to the <i>xylE-npt</i> cassette on pJMS11.	This study
pBB <i>tfdTC</i> '	Gm ^r ; 1.45 kb <i>BamHI</i> - <i>SalI</i> fragment of pHP11 inserted into the <i>BglII</i> - <i>SalI</i> site of pBBR1MCS-5; <i>tfdC</i> is truncated at amino acid 86 out of 251 amino acids	This study
pBB <i>tfdTCD</i> '	Gm ^r ; 2.1 kb <i>BamHI</i> - <i>SspI</i> fragment of pHP11 inserted into the <i>BamHI</i> - <i>SmaI</i> site of pBBR1MCS-5; <i>tfdD</i> is truncated at amino acid 69 out of 371 amino acids	This study
pBB <i>MutfdTCD</i> '	The same structure as that of pBB <i>tfdTCD</i> ' except that pBB <i>MutfdTCD</i> ' series (pBB <i>MutfdTCD</i> ' 1 to pBB <i>MutfdTCD</i> ' 8) encode TfdT mutants with one-amino-acid substitution from wild-type TfdT	This study
pQF <i>tfdTC</i> '	Ap ^r ; 1.45 kb <i>BamHI</i> - <i>SalI</i> fragment of pHP11 inserted into the <i>BglII</i> - <i>SalI</i> site of pQF50; <i>tfdC</i> is truncated at amino acid 86 out of 251 amino acids	
	The same structure as that of pQF <i>tfdTC</i> ' except that pQF <i>MutfdTC</i> ' series contain <i>tfdT</i> mutants from the plasmids, pBB <i>MutfdTCD</i> ' 1.	This study
pQF <i>MutfdTC</i> ' 1		This study

BA: benzoate; 3CB: 3-chlorobenzoate

Table 2 Properties of the TfdT mutants

Plasmid		Mutant	Codon change
pBBMutfdTCD'1	pQFMutfdTC'1	K129M	AAG → ATG
pBBMutfdTCD'2		R199Q	CGG → CAG
pBBMutfdTCD'3		R199L	CGG → CTG
pBBMutfdTCD'4		R199W	CGG → TGG
pBBMutfdTCD'5		V226F	GTC → TTC
pBBMutfdTCD'6		V246I	GTC → ATC
pBBMutfdTCD'7		P267A	CCC → GCC
pBBMutfdTCD'8		P267H	CCC → CAC
pBBMutfdTCD'9		P267S	CCC → TCC

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Table 3

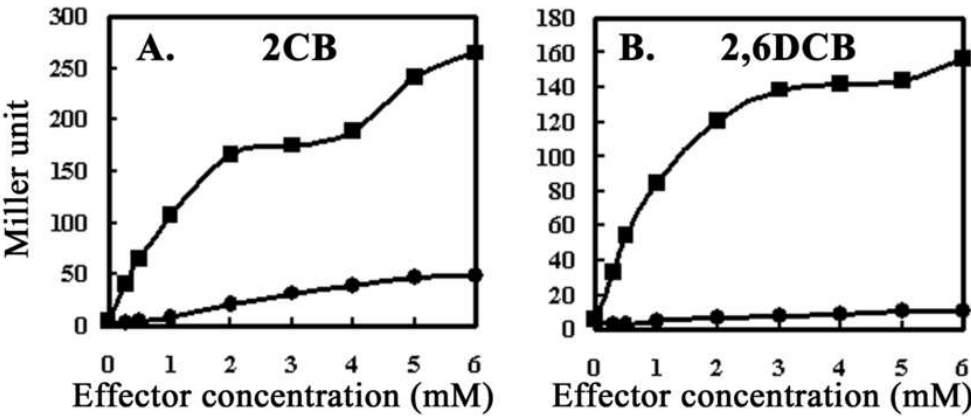
Transcriptional activity of wild-type TfdT and TfdT mutants induced by aromatic compounds

Transcriptional activity ^a of <i>Ptfd :: lacZ</i> plus										
Effector	Mutant									
	wtTfdT	K129M	R199Q	R199L	R199W	V226F	V246I	P267A	P267H	P267S
None	34 ± 2.6	59 ± 6.2	101 ± 11.8	99 ± 11.4	63 ± 3.5	52 ± 1.8	45 ± 2.9	42.6 ± 1.9	61 ± 4.1	50 ± 2.8
2CB	144 ± 16.7	1039 ± 35.8	679 ± 37.5	478 ± 17.7	322 ± 10.5	266 ± 2.9	209 ± 8.7	214.3 ± 41.1	383 ± 5.0	214 ± 5.5
	(4.2)	(17.6)	(6.7)	(4.8)	(5.1)	(5.1)	(4.6)	(5.0)	(6.3)	(4.3)
3CB	325 ± 17.3	89 ± 4.2	177 ± 11.2	208 ± 2.9	127 ± 6.7	267 ± 3.0	107 ± 4.8	150.7 ± 15.9	287 ± 15.5	176 ± 3.6
	(9.6)	(1.5)	(1.8)	(2.1)	(2.0)	(5.1)	(2.4)	(3.5)	(4.7)	(3.5)
2NHB	121 ± 7.5	450 ± 11.1	472 ± 18.7	316 ± 23.1	220 ± 7.0	265 ± 18.5	120 ± 5.9	115.5 ± 5.6	303 ± 13.2	128 ± 2.2
	(3.6)	(7.6)	(4.7)	(3.2)	(3.5)	(5.1)	(2.7)	(2.7)	(5.0)	(2.6)
2,3DCB	129 ± 9.5	546 ± 35.4	321 ± 19.9	344 ± 19.9	215 ± 8.7	379 ± 22.0	159 ± 15.1	166.9 ± 25.2	303 ± 27.9	149 ± 10.5
	(3.8)	(9.3)	(3.2)	(3.5)	(3.4)	(7.3)	(3.5)	(3.9)	(5.0)	(3.0)
2,4DCB	29 ± 4.1	289 ± 6.1	146 ± 8.0	119 ± 5.2	81 ± 10.4	118 ± 19.1	68 ± 8.3	70.7 ± 2.7	91 ± 16.8	55 ± 1.6
	(0.9)	(4.9)	(1.4)	(1.2)	(1.3)	(2.3)	(1.5)	(1.6)	(1.5)	(1.1)
2,5DCB	58 ± 8.2	176 ± 6.7	239 ± 23.2	275 ± 9.2	147 ± 10.3	182 ± 8.5	100 ± 3.6	84.5 ± 1.4	142 ± 9.0	95 ± 5.7
	(1.7)	(3.0)	(2.4)	(2.8)	(2.3)	(3.5)	(2.2)	(2.0)	(2.3)	(1.9)
2,6DCB	61 ± 4.9	1043 ± 14.3	394 ± 18.4	330 ± 20.2	177 ± 11.1	282 ± 9.8	103 ± 5.5	162.9 ± 23.5	187 ± 18.8	165 ± 3.3
	(1.8)	(17.7)	(3.9)	(3.3)	(2.8)	(5.4)	(2.3)	(3.8)	(3.1)	(3.3)

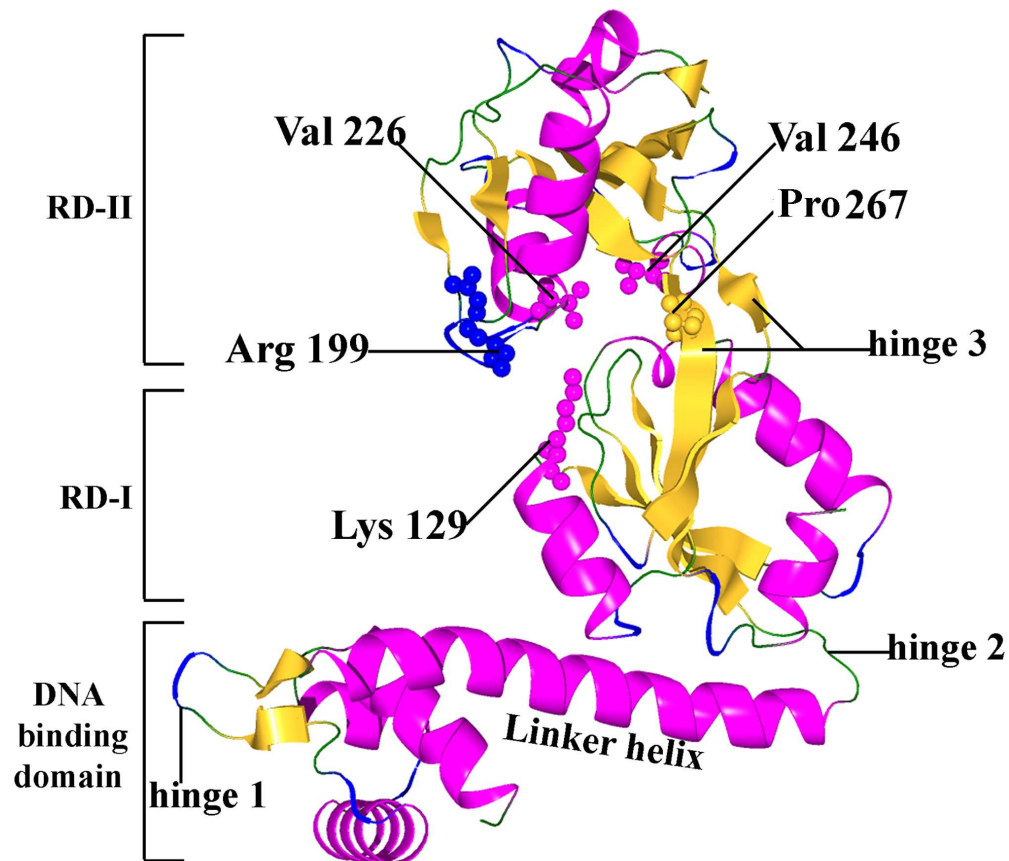
^a Transcriptional activity was measured as the amount of β-galactosidase accumulated in *Pseudomonas putida* KT2440 *Ptfd::lacZ* transformed with pBB*tfdTCD*' or pBB*Mu*tfdTCD**'. Miller units of β-galactosidase

(nmol/min/mg protein) assayed in *P. putida* KT2440 *Ptfd::lacZ* cultured in basal salts medium supplemented with 10 mM glucose and 5 mM effectors at 30°C for 15 h. All values are indicated as means \pm S.D. of three separate cultures. The ratio of each value to that of the non-induced cells is indicated in parenthesis.

Abbreviations: 2CB: 2-chlorobenzoate; 3CB: 3-chlorobenzoate; 2NHB: 2-aminobenzoate (anthranilate); 2,3DCB: 2,3-dichlorobenzoate; 2,4DCB: 2,4-dichlorobenzoate; 2,5DCB: 2,5-dichlorobenzoate; and 2,6DCB: 2,6-dichlorobenzoate.



36x15mm (600 x 600 DPI)



85x76mm (600 x 600 DPI)