Regulatory Mechanism of The 2,4,6-Trichlorophenol Catabolic Operon in *Ralstonia pickettii* DTP0602

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岡山理科大学大学院

工学研究科

システム科学専攻

鳥　居　英　人

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Okayama University of Science

Graduate school of Engineering

System Science

Hideto Torii

Chaper1. Introduction

1-1. INTRODUCTION

Human activities have resulted in the release and introduction into the environment of a plethora of aromatic chemicals. Although the input of these synthetic chemicals may be smaller than the total amount of aromatic compounds released from decaying plant material, their novel structures or their quantities as single pure molecules induce major changes in microbial communities, which are the major recyclers of organic chemicals in nature (Gibson & S Harwood, 2002). Compounds such as chlorinated solvents, herbicides, and pesticides often carry uncommon chemical structures, side chains, or functional groups, which can have toxic effects or provide new carbon sources for bacteria, which have adapted their metabolism to degrade the compounds (Pieper & Reineke, 2000; Reineke & Knackmuss, 1988; van der Meer, 1997). The interest in discovering how bacteria are dealing with hazardous environmental pollutants has driven a large research community and has resulted in important biochemical, genetic, and physiological knowledge about the degradation capacities of microorganisms (Deo & Karanth, 1994; Díaz *et al.*, 2001; Gibson & S Harwood, 2002; Mishra *et al.*, 2001; Pieper & Reineke, 2000; Reineke & Knackmuss, 1988; Reineke, 1998). A large variety of metabolic pathways have been discovered in very different microorganisms, fueling up-to-date online databases such as the Biocatalysis/Biodegradation Database (Ellis *et al.*, 2003). Bioremediation is often the only feasible cleanup option because the remoteness and unique character of these sites preclude conventional physicochemical technologies for soil treatment. Knowledge about the biodegradation capacities of microorganisms is being applied directly in bioremediation practice, and individual biotransformation reactions are potentially very interesting for incorporation into chemical synthesis (Schoemaker *et al.*, 2003).

Less obviously associated with bioremediation applications, green chemistry, or production of pharmacy synthons have been studies of the regulatory mechanisms, which govern the expression of specific catabolic pathways (Keasling, 1999). However, regulation of catabolic pathway expression has attracted the interest of numerous different groups, who have tried to unravel the molecular partners in the regulatory process, the signals triggering pathway expression, and the exact mechanisms of activation and repression. The identification and characterization of such genes and their protein products for catabolite control in would not only reveal one of the diverse molecular mechanisms for the adaptation of bacteria to various environments but also provide useful knowledge for the employment for successful application in various fields, such as bioremediation (Cases & de Lorenzo, 1998). More recently, information about regulatory systems has attracted interest, with the potential of these systems being used as sensory mechanisms in whole-cell living bioreporters, genetically modified bacteria which can be used as sensors to measure the quality of aqueous, soil, and air environments.

The 2,4,6-trichlorophenol (2,4,6-TCP), one of the main components of bleached kraft pulp mill effluents, is widely used as a biocide and preservative and is considered a priority environmental pollutant worldwide (Field & Sierra-Alvarez, 2008). *Ralstonia pickettii* DTP0602 utilizes 2,4,6-TCP as its sole source of carbon and energy, and a catabolic pathway of 2,4,6-TCP has been previously proposed (Hatta *et al.*, 2012; Kiyohara *et al.*, 1989; Kiyohara *et al.*, 1992; Takizawa *et al.*, 1995). Because DTP0602 showed growth on 500-1000 μM 2,4,6-TCP as a sole carbon source, DTP0602 has the possibility of bioremediation applications in 2,4,6-TCP contaminated soil treatment. However, the exact mechanism by which the transcriptional regulator to initiate 2,4,6-TCP degradation is not fully understood.

The aim of this article is to characterize the regulatory mechanism of 2,4,6-trichlorophenol (2,4,6-TCP) catabolic operon expression in *Ralstonia pickettii* DTP0602.

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Chaper2. The characterization of HadR

2-1. SUMMARY

Ralstonia pickettii DTP0602 utilizes 2,4,6-trichlorophenol (2,4,6-TCP) as its sole source of carbon. The expression of catabolic pathway genes (*hadA*, *hadB* and *hadC*) for 2,4,6-TCP has been reported to be regulated by the LysR-type transcriptional regulator (LTTR), HadR. Generally, coinducers are recognized as being important for the function of LTTRs, and alteration of the LTTR-protection sequence and the degree of DNA bending are characteristic of LTTRs with or without a recognized coinducer. In this study, we describe the mechanism by which HadR regulates the expression of 2,4,6-TCP catabolic genes. The 2,4,6-TCP catabolic pathway genes in DTP0602 consist of two transcriptional units: *hadX-hadA-hadB-hadC* and monocistronic *hadR*. Purified HadR binds to the *hadX* promoter and the HadR–DNA complex formation was induced in the presence of sixteen types of substituted phenols, including chloro-, nitro-phenols and tribromo-phenol. In contrast to observations of other well-characterized LTTRs, the tested phenols showed no diversity of the bending angle of HadR binding fragment. The expression of 2,4,6-TCP catabolic pathway genes, which are regulated by HadR, was not influenced by the DNA bending angle of HadR. Moreover, the transcription of *hadX*, *hadA* and *hadB* was induced in the presence of seven types of substituted phenols, whereas the other substituted phenols, which induced the HadR–DNA complex formation, did not induce the transcription of *hadX*, *hadA* and *hadB in vivo*.

2-2. FOOTNOTE

Abbreviations: LTTR, LysR-type transcriptional regulator; DCP, dichlorophenol; TCP, trichlorophenol; TeCP, tetrachorophenol; PCP, pentachlorophenol; NP, mononitrophenol; DNP, dinitrophenol; TNP, trinitrophenol; TBP, tribromophenol; 2-C-4-NP, 2-chloro-4-nitrophenol; bromoxynil, 3,5-dibromo-4-hydroxybenzonitrile; Q-PCR, quantitative-PCR; EMSAs, Electrophoretic mobility shift assays; TSP, The transcription start point; K_Ds, the dissociation constants; MM, minimal medium; HadR^{mt}, HadR protein contained seven additional amino acid residues at C terminus; EBD, effector binding domain

The Genbank/EMBL/DDBJ accession number for the *orf1*, *orf2*, *orf3*, *hadR*, *hadX*, *hadA*, *hadB*, *hadC*, *orf4* and *orf5* sequences of *R.pickettii* DTP0602 examined in this study is AB767276

2-3. INTRODUCTION

A large number of halogenated compounds have been artificially produced and used as industrially manufactured products. Polychlorinated phenols have been widely used as biocides, mainly in wood preservation. Various isomers of chlorophenols are generated in the environment from pentachlorophenol (PCP) and other agricultural biocides such as 2,4-dichloro- and 2,4,5-trichloroacetic acid. 2,4,6-Trichlorophenol (2,4,6-TCP), one of the main components of bleached kraft pulp mill effluents, is widely used as a biocide and preservative and is considered a priority environmental pollutant worldwide (Czaplicka, 2004; Field & Sierra-Alvarez, 2008; McAllister *et al.*, 1996).

We had isolated a *Ralstonia pickettii* strain DTP0602 that utilizes 2,4,6-TCP as its sole source of carbon and energy (Kiyohara *et al.*, 1989; Kiyohara *et al.*, 1992), and a catabolic pathway of 2,4,6-TCP has been previously proposed (Fig. 1a). The genes *hadX*, *hadA*, *hadB* and *hadC* in DTP0602 are clustered and adjacent to seven other open reading frames (ORFs) (*orf1*, *orf2*, *orf3*, *hadR*, *hadX*, *orf4* and *orf5*) (Fig. 1b). The 2,4,6-TCP 4-monooxygenase gene (*hadA*), probable electron transfer protein gene (*hadB*), hydroxyquinol 1,2-dioxygenase gene (*hadC*) and maleylacetate reductase gene (*hadD*) were characterized, previously (Hatta *et al.*, 1999; Takizawa *et al.*, 1995). The *hadX* gene might be the 2,4,6-TCP monooxygenase component of a probable electron transfer gene. The *hadR* gene lies just upstream from *hadX* in the opposite direction, and has a helix–turn–helix DNA binding domain at the N-terminus and a coinducer binding domain at the C-terminus. HadR has similarity to TcpR, NtdR, PcpR and LinR; these proteins belong to the LysR-type transcriptional regulator (LTTR) that sense aromatic compounds (Cai & Xun, 2002; Hatta *et al.*, 2012; Lessner *et al.*, 2003; Miyauchi *et al.*, 2002; Sánchez & González,

2007). Orf2 and Orf3 are located downstream from *hadR* in the opposite direction from *hadX*. Orf2 and Orf3 exhibit homology with a hypothetical protein (accession no. EGE60039.1) in *Rhizobium etli* CNPAF512, an LTTR (accession no. YP_297689.1) in *Cupriavidus necator* JMP134.

LTTRs constitute one of the largest families of transcriptional regulators. They sense an effector molecule as a coinducer and act as transcriptional activators for their target metabolic operons. The binding of a coinducer also changes the DNA binding characteristics of the LTTR protein to its target promoter. Numerous LTTRs have been shown to induce DNA bending upon binding of the protein, which induces a conformational change and typically alters the binding region and DNA bending angle (Maddocks & Oyston, 2008; Schell, 1993; Tropel & van der Meer, 2004).

TcpR regulates the expression of 2,4,6-TCP catabolic genes (*tcpX*, *A*, *B*, *C*, *Y* and *D*) in *C. necator* JMP134 (Sánchez & González, 2007). However, the exact mechanism by which the transcriptional regulator responds to an inducer molecule (2,4,6-TCP or intermediate) to initiate the transcription of inducible 2,4,6-TCP catabolic genes is not fully understood. To characterize the regulatory mechanism underlying 2,4,6-TCP utilization, we examined the control of the expression of *hadX*, *hadA* and *hadB* by HadR. Electrophoretic mobility shift assays (EMSAs), DNase I footprinting analysis, circular permutation analysis and quantitative-PCR (Q-PCR) analysis were performed to investigate the behavior of HadR in the transcription of 2,4,6-TCP catabolic genes.

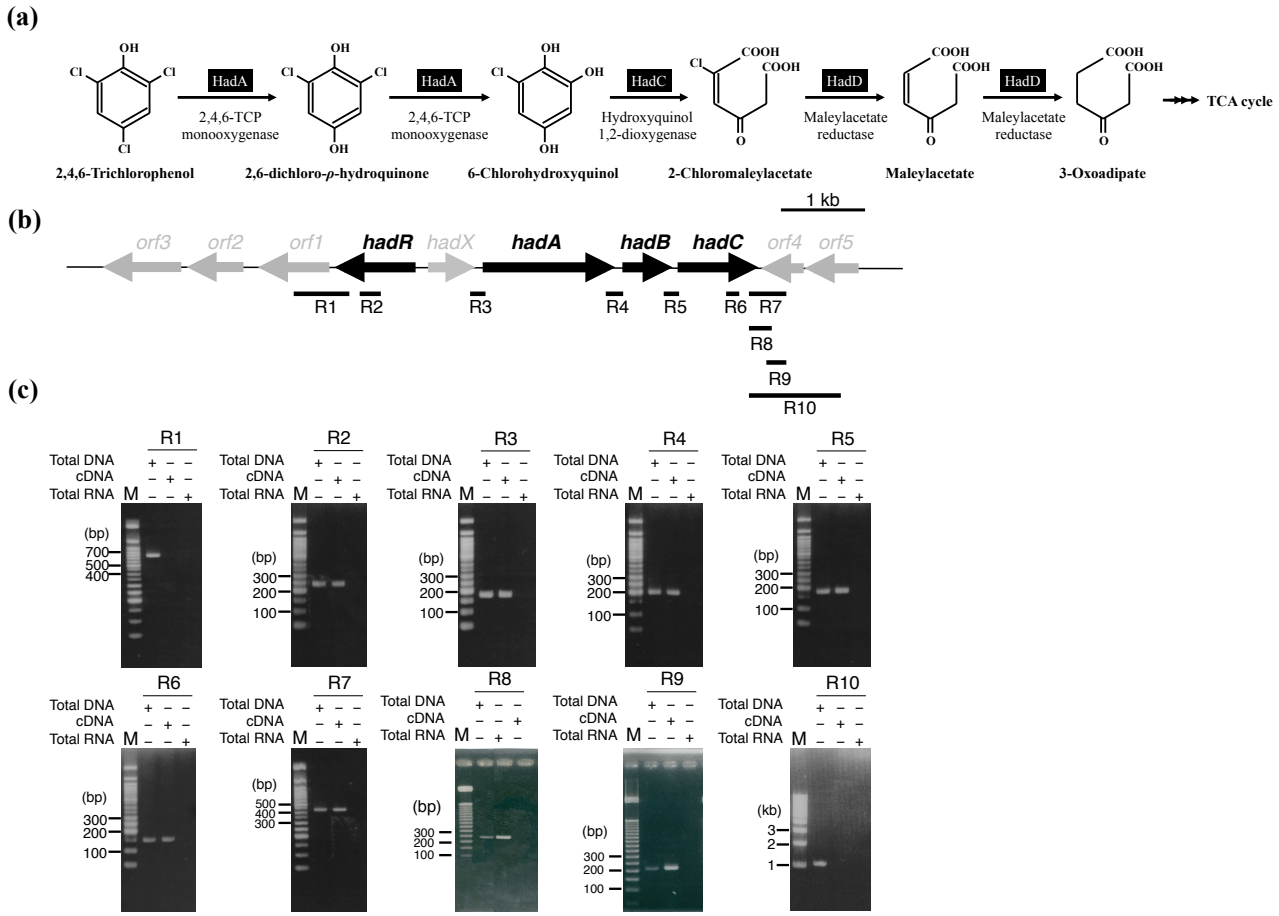

FIG. 1. 2,4,6-TCP catabolic pathway and gene cluster in *Ralstonia pickettii* DTP0602. (a) 2,4,6-TCP catabolic pathway. (b) Identical genetic organization of the *had* genes catalyzing 2,4,6-TCP degradation. Bar represents 1 kb. Bold bars and numbers (R1–10) below the genes in panel b indicate the locations of the amplified RT-PCR products shown in panel c. (c) Agarose gel electrophoresis of products of RT-PCR analysis with primers targeting *orf1-hadR* (expected size, 649 bp), *hadR* (expected size, 247 bp), *hadX-hadA* (expected size, 181 bp), *hadA-hadB* (expected size, 202 bp), *hadB-hadC* (expected size, 181 bp), *hadC* (expected size, 152 bp), *hadC-orf4* (expected size, 438 bp), *hadC-orf4* (expected size, 242 bp) and *orf4* (expected size, 213 bp) and *hadC-orf4-orf5* (expected size, 1072 bp) is shown. Positions of primer pairs and primer sequences are indicated in Table S1. The sizes of the molecular weight markers in lane M are indicated on the left. The numbers (R1 to R10) correspond to the numbers of the amplified regions indicated in panel b. +: presence of template, −: absence of template.

2-4. MATERIALS AND METHODS

2-4-1. Bacterial strains, chemicals and growth conditions

The strains and plasmids used in this study are listed in Table 1. For routine culture, *R. pickettii* DTP0602 and the DTP0602 derivative strain were grown in Luria-Bertani (LB) medium or minimal medium (MM) containing 0.3% (w/v) succinate (Wako Pure Chemical Industry, Osaka, Japan) at 30°C, with shaking (130 r.p.m.) (Kiyohara *et al.*, 1992). *Escherichia coli* JM109 and BL21 (DE3) were used for cloning experiments and protein overproduction. *E. coli* SI7-1 or S17-l λpir were used for biparental filter mating (Miller & Mekalanos, 1988; Simon *et al.*, 1983; Yanisch-Perron *et al.*, 1985). *E. coli* strains were grown in LB at 30 or 37°C with shaking. Antibiotics were used at the following concentrations for *E. coli* strains: 100 μg ml^{-1} of ampicillin, 25 μg ml^{-1} of kanamycin and 25 μg ml^{-1} of chloramphenicol. Antibiotic was used at the following concentration for DTP0602 derivative strain: 100 μg ml^{-1} of kanamycin. Growth was determined by measuring optical density at 600 nm (OD_{600}).

The substituted phenols (coinducers) used in this study were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), Wako Pure Chemical Industry, Lancaster Synthesis (Windham, NH, USA), Cambridge Isotope Laboratories (Andover, MA, USA), Nacalai Tesque (Kyoto, Japan), Aldrich Chemical (Milwaukee, WI, USA) and Acros Organics (Geel, Belgium). 6-chlorohydroxyquinol was synthesized following the method described by Latus *et al.* (Latus *et al.*, 1995). All substituted phenols were dissolved in DMSO and stored at −20°C.

To confirm the growth of DTP0602, *hadR*-null mutant (DTP62dR), *hadA*-null mutant (DTP62dA), DTP0602-pTS1210, DTP62dR-pTS1210, DTP62dR-pTSRl and

DTP62dR-pTSRml on 2,4,6-TCP, the seven strains grown in LB or MM containing succinate and kanamycin were prepared and harvested at an OD_{600} of 0.75–0.80 (a late exponential phase on MMS medium, early exponential phase on LB medium). The cells were centrifuged and the pellet was grown in LB, washed twice with MM, and resuspended in 5 ml of the same medium. The cell suspension was added to 40 ml of MM containing 500 μM 2,4,6-TCP in a 200-ml baffle flask and agitated.

For Q-PCR analysis and β-galactosidase assay, DTP0602 cells or DTP0602 derivative cells grown in LB containing kanamycin were harvested at an OD_{600} of 0.75–0.80. The cell pellet was washed with 50 mM potassium phosphate buffer (pH 7.5) and suspended to an OD_{600} of 1.0 in the same buffer. The cell suspension (15 ml) was placed in a 50-ml test tube and allowed to preincubate for 5 min at 30°C in static culture. Subsequently, 200 μM of substituted phenols were added to the cell suspension and incubated at 30°C in static culture. The mRNA copy number of *hadX-C* operon and β-galactosidase activity peaked at 6 and 3 h, respectively (data not shown). Therefore, incubation time was defined as the point at which the peak of *had* operon transcription (6 h) or β-galactosidase activity (3 h) was observed.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)*	Reference(s) or source
R. pickettii		
DTP0602	Wild type; 2,4,6-TCP^r	Kiyohara *et al.* (1989)
DTP62dA	DTP0602 derivative; *hadA* null-mutant	This study
DTP62dR	DTP0602 derivative; *hadR* null-mutant	This study
DTP62ZA	DTP0602 derivative; *hadA*::*lacZ*; Km^r	This study
DTP0602-pTS1210	DTP0602 harboring pTS1210; Km^r	This study
DTP0602-pJBEC1	DTP0602 harboring pJBEC1; Km^r	This study
DTP0602-pJBEY1	DTP0602 harboring pJBEY1; Km^r	This study
DTP0602-pJBRY1	DTP0602 harboring pJBRY1; Km^r	This study
DTP62dR-pTS1210	DTP62dR harboring pTS1210; Km^r	This study
DTP62dR-pTSR1	DTP62dR harboring pTSR1; Km^r	This study
DTP62dR-pTSRm1	DTP62dR harboring pTSRm1; Km^r	This study
E.coli		
JM109	*recA1 supE44 end A1 hsdR17*($r_K^- m_K^-$)*gyrA96 relA1 thi-1 Δ*(*lac-proAB*) *F'* [*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*]	Yanisch *et al.* (1985)
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (*DE3*)	Novagen
S17-1	*thi pro hsdR hsdM*$^+$ *recA*; integrated plasmid RP4-Tc::Mu-Km::Tn7	Simon *et al.* (1983)
S17-1 λpir	*thi pro hsdR hsdM*$^+$ *recA*; integrated plasmid RP4-Tc::Mu-Km::Tn7 *λpir*	Miller *et al.* (1988)
Plasmids		
pUC19	Cloning vector, Ap^r	Yanisch *et al.* (1985)
pVIK111	Suicide vector for *lacZ* translational fusions	Kalogeraki *et al.* (1997)
pJB861	Expression vector containing *xylS* and the Pm promoter, Km^r Tra$^-$ Mob$^+$ IncP	Blatny *et al.* (1997)
pET52b(+)	Expression vector, T7 promoter, Ap^r	Novagen
pG-KJE8	*dnaK-dnaJ-grpE* and *groES-groEL* overexpression plasmid, Cm^r	Nishihara *et al.* (1998), Nishihara *et al.* (2000)
pTS1210	Broad-host-range vector, pSa *ori* pBR *ori*, Km^r Ap^r	T. Nakazawa
pK18*mobsacB*	Integration vector, *oriT*, *sacB*, Km^r	Simon *et al.* (1983)
pNC2	pUC19 with a 5.3-kb EcoRI-EcoRI fragment carrying *orf1*, *hadR*, *hadX*, *hadA*, and *hadB*	This study
pETHR1	pET52b(+) with a 1.0-kb PCR fragment carrying *hadR* from pNC2	This study
pTSR1	pTS1210 with a 1.2-kb PCR fragment carrying *hadR* from pNC2	This study
pTSRm1	pTS1210 with a 1.2-kb PCR fragment carrying *hadR*^{mt} from pNC2	This study
pK18*mobsacB*Δ*hadA*	pK18*mobsacB* with a 3.0-kb PCR fragment carrying the flanking regions of *hadA* from DTP0602	This study
pK18*mobsacB*Δ*hadR*	pK18*mobsacB* with a 2.0-kb PCR fragment carrying the flanking regions of *hadR* from DTP0602	This study
pVA1	pVIK111 with a 0.7-kb PCR fragment carrying 60-bp 5'end of *hadA* from pNC2	This study
pJBEC1	pJB861 with a 5.3-kb EcoRI-EcoRI fragment carrying *orf1*, *hadR*, *hadX*, *hadA*, and *hadB* from pNC2	This study
pJBEY1	pJB861 with a 5.1-kb PCR fragment carrying *hadX*, *hadA*::*lacZ,* and *lacY* from DTP62ZA	This study
pJBRY1	pJB861 with a 5.9-kb PCR fragment carrying *hadR*, *hadX*, *hadA*::*lacZ*, and *lacY* from DTP62ZA	This study

*Km^r , Ap^r, and Cm^r resistance to kanamycin, ampicillin, and chloramphenicol, respectively.

Table 2. Oligonucleotides used as PCR primers

Primer and fuction(s)	Sequence (5'-3')*
For RT-PCR analysis	
R1-F	AAAATTCTCTCCCACCACCA
R2-F	GTTCCACCGACAGGTGCT
R3-F	CGTTCCCCCTTTGGTCTATT
R4-F	GACACCGAATACCGCAAGAC
R5-F	GACGATCCGGTCGAACTTTA
R6-F	GTACCCGATTCCGGATGAC
R7-F	ATTAGGGGAGGGACAGTTCG
R9-F	AGTTGACAGGAACGCAGCTC
R1-R	GACGAGATGGGACTGCAACT
R2-R	GTCAATTGCGACGCACAG
R3-R	TCAGGGATTCCAGGTACTGC
R4-R	AGGGTAGCAAGGGAAATGGT
R5-R	GATGGTGTCCTCGTCGAGAT
R6-R	CAAGATACTGGTCGCCCTTG
R7-R	CGAGCGTCAACAATGTCAAT
R8-R	CTGCGTTCCTGTCAACTACG
R10-R	TACTACTCGCAGGCGGACTT
For Q-PCR analysis	
qhadR-F	CATCACCTGCAGGTGCTCGACGTG
qhadX-F	GCAGCCAGGACAGAGAAGTTCCCG
qhadA-F	CAGTACCTGGAATCCCTGAACGACG
qhadB-F	CACCATTTCCCTTGCTACCCTGTT
qhadR-R	CATGCGCAAGGCCACGCGCACGA
qhadX-R	CGGGTTGTCGCAGACCGAACAGAC
qhadA-R	TGCAGGTCCGGGCGATGGTGCA
qhadB-R	CTTGGCTTCCATGCTGCGGACGAA
For determination of the HadR binding site and circular permutation analysis	
siteA-F	TGCCGCGCTGACGCTGGACTGC
siteC-F	AGTCCGGCGTTGCAACTAGTCTACG
siteD-F	CGCTGAGGAATAGTGTCCAT
siteE-F	CCAGGTTTCGCCACCGGCGCGATGCGCGCT
siteF-F	GCACGCGTGCGGCCCTGGTCAGGCT
siteG-F	TTTGCCAGCGTCTTGCTCAGCGCCGGCTGC
siteH-F	CGCGCACGAACAGCGGATCGCCAAAGTACG
site1-F	CTTCATGCCGCTGAGGAATAGTGTC
site2-F	CACGTCGAGCACCTGCAGGTGATGCAGGCC
siteB-R	CACGACGAGACGGACAGCGTTC
siteC-R	CTGCCCGACGGCGCCGCGAAGGGAAAT
siteD-R	GTCCTCCTTTGGTGATGACTTCTCGCATCC
siteE-R	TGCAGGTGATGCAGGCCCAGATCGTCTTCA
siteF-R	GCGCGTTCCACGTAGACTAGTTGC
siteG-R	GGACTTCTACTATTGAGACGACGTCATGGA
siteH-R	GGCATGAAGACGATCTGGGC
site3-R	TTCGCCACCGGCGCGATGCGCGCTTGTTGT
site5-R	ACTATTGAGACGACGTCATGGACACTATTC
For primer extension analysis and DNase I footprinting analysis	
hadR-IRD-extension	IRD800-TGATGCAGGCCCAGATCGTC
hadX-IRD-extension	IRD800-CGGGAACTTCTCTGTCCTGG
hadR-IRD-footprint-F	IRD800-TTGAGCACGCGTGCGGCCCT
hadR-footprint-F	TTGAGCACGCGTGCGGCCCT
hadX-footprint-R	CGGGAACTTCTCTGTCCTGG
For construction of the gene disruption strain	
hadA-EcoRI-FF	TCGAGAATTCAGAAGCTGAAG
hadA-XbaI-FR	CGCCTCTAGACGCATGACAG
hadA-XbaI-RF	GCCGTCTAGACGTGTTCGAG
hadA-HindIII-RR	GCCCAAGCTTTGGATCATGAAA
hadR-EcoRI-FF	TTGCGAATTCGCGCCGTCGTAA
hadR-XbaI-FR	GGGCTCTAGATGGCGGACGAGA
hadR-HindIII-RR	ACTGAAGCTTGGTACGAATCAT
For construction of the *hadA*::*lacZ* fusion strain	
hadXz-F	GAAGTCATCACCAAAGGAGGACGACA
hadAz-SalI-R	TCGGTCGACACCCAGACATTGCGGCCGTCGT
For construction of the complementation plasmids of HadR	
phadR-XhoI-F	GCCCCTCGAGCCTGCCTGGCCGTTGGTGGC
hadR-XhoI-R	GTGAGGGCCTCGAGCTATGCCTCGGCCACG
mhadR-XhoI-R	CCATCTCGAGCTAGCGTGGCACCAGAGCGAGCTCTGCCTCGGCCACGGCC
For construction of the promoter probe plasmids	
hadR-EcoRI-F	AGCGGAATTCCCGGCATAGCCACCATAACC
hadX-EcoRI-F	CGTCGAATTCCCAGGACAGAGAAGT
lacY-XbaI-R	ATAAGCTCTAGACCGCATCCGACATTGATT
For construction of the HadR expression plasmid	
hadR-NcoI-F	CGACGCCATGGACACTATTCCTCAGC
hadR-SacI-R	GTGAGGATTTCGAGCTCTGCCTCGGCCACG

* Primers include a restriction site which is underlined.

2-4-2. DNA manipulation and RNA isolation

Plasmid isolation, restriction enzyme digestion, DNA ligation and *E. coli* transformation were performed as described by Sambrook *et al.* (1989). PCR was performed with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The primers used in this study are listed in Table 2 in the supplemental material.

To isolate total RNA, the cells were incubated with the appropriate substituted phenols in a static culture for 6 h and the cell suspension was centrifuged to pellet the cells. The cell pellets were resuspended in RNAlater solution (Ambion, Austin, TX, USA) and stored overnight at 4°C. Total RNA was isolated using the PureLink™ RNA Mini kit (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's instructions, and the RNA was subsequently treated with RNase-free DNase I (Takara Bio, Otsu, Japan).

2-4-3. DNA transformation of *R. pickettii*

Electroporation was performed as described previously using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA, USA) (Dennis & Sokol, 1995; Smith & Iglewski, 1989).

E. coli SI7-1 or S17-l λpir were used for biparental filter mating. *E. coli* strains (donor) and *R. pickettii* DTP0602 (recipients) were cultivated in LB. Sterile 0.45-μm pore-size cellulose acetate membrane filters (Advantec, Tokyo Co., Tokyo, Japan) with mating mixtures (donor and recipients) were placed on LB agar plates overnight at 30°C. After incubation, cells were washed from the filters with P buffer (Kiyohara *et al.*, 1992) and aliquots were plated onto MM agar plates containing succinate and kanamycin.

2-4-4. Recombinant DNA work

To disrupt *hadA* and *hadR*, the flanking region of each gene was amplified using *R. pickettii* DTP0602 chromosomal DNA as the template. The upstream and downstream flanking regions of *hadA* were amplified with primers hadA-EcoRI-FF/hadA-Xbal-FR and hadA-Xbal-RF/hadA-Hindlll-RR and those of *hadR* were amplified with hadR-EcoRI-FF/hadR-Xbal-FR and qhadR-F/hadR-Hindlll-RR. These fragments were digested with *Eco*RI, *Xba*I and *Hind*III, respectively. After digestion, the two DNA fragments were simultaneously ligated into the pK18*mobsacB* (Schäfer *et al.*, 1994). The resulting plasmids were named pK18*mobsacB*Δ*hadA* and pK18*mobsacB*Δ*hadR*.

These suicide plasmids were independently transferred into *R. pickettii* DTP0602 by biparental filter mating using *E. coli* S17-1. Primary recombination of the plasmid was selected by kanamycin resistance. Secondary selection was performed on an MM agar plate containing succinate and 5% (w/v) sucrose, and specific deletions were confirmed by PCR. The organisms belonging to the colonies with the *hadA* and *hadR* gene deletions were named strains DTP62dA and DTP62dR, respectively.

To construct a *hadA*::*lacZ* reporter fusion in DTP0602, the PCR fragment containing the 5′ end of *hadA* was amplified by PCR with the primer hadXz-F/hadAz-Sall-R and was digested with *Sal*I. This fragment was ligated into *Sma*I/*Sal*I-digested pVIK111 (Kalogeraki & Winans, 1997). The resulting plasmid (pVAl) was transferred into DTP0602 by biparental filter mating using *E. coli* SI7-1 λpir as a donor strain. The kanamycin-resistant colony with the *hadA*::*lacZ* reporter fusion was denoted as a colony arising from the strain DTP62ZA.

Plasmid pTS1210 was used for the construction of *hadR* complementation vectors. The flanking region of *hadR* was amplified with primer phadR-XhoI-F/hadR-XhoI-R and the DNA fragment encoding $HadR^{mt}$ (described below) was amplified from pNC2 with primer

phadR-XhoI-F/mhadR-XhoI-R. Two DNA fragments were digested with *Xho*I and ligated into *Sal*I-digested pTS1210, generating the plasmids pTSR1 and pTSRm1. The pJB861 plasmid was used for the construction of LacZ reporter plasmids (Blatny *et al.*, 1997). DNA fragments encoding the *hadA*::*lacZ* fusion were amplified from the DTP62ZA chromosome using primers hadR-EcoRI-F/lacY-XbaI-R and hadX-EcoRI-F/lacY-XbaI-R. These fragments were digested with *Eco*RI and *Xba*I and inserted into *Eco*RI/*Xba*I-digested pJB861, generating pJBRY1 and pJBEY1, respectively. These plasmids were transformed into DTP0602 or DTP62dR by electroporation.

For overexpression and purification of HadR with a C-terminal octahistidine tag (8× His tag), the *hadR* coding region was cloned into the expression vector pET52b(+) (Novagen, Madison, WI, USA). The *hadR* gene was amplified from pNC2 with the primer hadR-NcoI-F/hadR-SacI-R and digested with *Nco*I and *Sac*I. The PCR fragment was inserted into *Nco*I/*Sac*I-digested pET52b(+). The resulting plasmid was named pETHR1 and contained an additional 19 amino acids (ELALVPRGSSAHHHHHHHH) at the C-terminus including a thrombin cleavage site and an octahistidine tag. This plasmid was introduced into *E. coli* BL21 (DE3) (pG-KJE8).

2-4-5. Reverse transcriptase (RT)-PCR, Q-PCR analysis and β-galactosidase assay

Total RNA (3 μg) was reverse transcribed by ReverTra Ace reverse transcriptase (Toyobo) with 51 ng of Random Primers (Invitrogen Life Technologies) according to the manufacturer's instructions. cDNA samples were treated with 0.1 mg/ml RNaseA (Sigma chemical, St. Louis, MO, USA) for 30 min at 37°C.

The intergenic regions R1–R10 were amplified by PCR using primers R1-F/R1-R (R1), R2-F/R2-R (R2), R3-F/R3-R (R3), R4-F/R4-R (R4), R5-F/R5-R (R5), R6-F/R6-R (R6),

R7-F/R7-R (R7), R7-F/R8-R (R8), R9-F/R7-R (R9) and R7-F/R10-R (R10), each of which amplifies the boundaries of *orf1-hadR* and *hadX-hadA-hadB-hadC-orf4-orf5* (Fig. 1b). PCR was performed with 20-µl mixtures containing 50 ng of the cDNA samples. Control samples, in which reverse transcription was omitted in RT-PCR and in which genomic DNA was used as a template for PCR, were run in parallel with RT-PCRs. The products were electrophoresed on 2.0 or 0.7% agarose gels and visualized with ethidium bromide. The rho-independent terminator was predicted with FindTerm (Softberry; http://linux1.softberry.com/berry.phtml).

Q-PCR analysis was performed on a 7500 Real-Time PCR system (Applied Biosystems, Framingham, MA, USA) using *Power* SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The single-stranded cDNA product of the reaction was diluted 1:20 in nuclease-free water and 2 µl was used in a 20-µl Q-PCR reaction. The absolute amount of each specific transcript was quantified by comparison of the cycle threshold values determined for each PCR with a standard curve of cycle threshold values generated using known amounts of DNA for the same target gene; quantification was performed using a 10-fold dilution series (Whelan *et al.*, 2003). The standard curve for *hadR*, *hadX*, *hadA* and *hadB* gave values ranging from 3.0×10^2 to 3.0×10^8 copies/µl. Specific primers were designed to amplify DNA segment of approximately 150 bp from *hadR*, *hadX*, *hadA* and *hadB* (Table 2).

β-galactosidase assay was performed as described previously (Miller, 1972). After 3 h of incubation with substituted phenols, 100 µl of DTP0602 cells was mixed with 900 µl of Z buffer and assayed for β-galactosidase activity.

2-4-6. Primer extension analysis

Total RNAs of 0.2 μg (from DTP0602-pJBEC1) or 2 μg (from DTP0602) were subjected to a reverse transcription reaction with ReverTra Ace reverse transcriptase (Toyobo) and an IRD800-labeled primer, hadR-IRD-extension or hadX-IRD-extension. The primer extension products were purified with phenol, precipitated with ethanol and resuspended with 5 or 25 μl of IR2 stop solution (Li-Cor, Lincoln, NE, USA). The dissolved sample was separated on a 5.5% KB plus gel matrix (Li-Cor) using a Li-Cor 4300 DNA analyzer. The DNA sequencing reaction mixtures were prepared using the same IRD-800-labeled primers and a Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, USA) according to the manufacturer's instructions.

2-4-7. Overexpression and purification of HadR and determination of oligomerization state of HadR

E. coli BL21 (DE3) (pG-KJE8) (pETHR1) was grown at 30°C with vigorous shaking. At an OD_{600} of 0.4, IPTG, L-arabinose and tetracycline were added to a final concentration of 0.05 mM, 10 mg/ml and 50 ng/ml, respectively (Nishihara *et al.*, 1998; Nishihara *et al.*, 2000). Growth was continued for 3 h, and cells from 1,000 ml of culture were centrifuged and resuspended in buffer A [50 mM K_2HPO_4–$KHPO_4$ (pH 7.5), 300 mM NaCl, 2 mM mercaptoethanol and 10% glycerol] at 10 volumes/g of wet weight and then disrupted by a French pressure cell press (Aminco, Urbana IL, USA). After centrifugation at 100,000 × *g* for 60 min at 4°C, HadR-8× His was purified using a Ni-Sepharose 6 fast-flow resin (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. The HadR-8× His was eluted with a 4-ml step gradient of 100–750 mM imidazole. The HadR-containing fractions were concentrated with a Vivaspin-20 concentrator (cutoff 10 kDa; Vivascience, Lincoln, UK). The octahistidine tag was removed with the use of a

thrombin cleavage capture kit (Novagen) according to the manufacturer's instructions. After the cleavage reaction, the biotinylated thrombin was removed and the buffer was again exchanged against buffer B [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 2 mM mercaptoethanol and 10% glycerol]. The resulting HadR protein ($HadR^{mt}$; 323 amino acids, 35.7 kDa) contained seven additional amino acid residues (ELALVPR) from pET52b(+) at the C terminus.

Determination of the $HadR^{mt}$ oligomerization state by gel filtration chromatography was performed as described by Hatta *et al.* (1999). The molecular weights of the subunits and protein concentration were determined by 12.5% SDS-PAGE and Bradford assay, respectively (Bradford, 1976).

2-4-8. EMSAs

EMSAs for HadR were performed with the DIG gel shift kit (second generation) (Roche, Mannheim, Germany) according to the manufacturer's instructions. The DNA fragments for determining the HadR binding region were amplified by PCR using primer pairs siteA-F/qhadA-R, qhadX-F/siteB-R, siteC-F/siteC-R, siteD-F/siteD-R, siteE-F/siteE-R, siteF-F/siteF-R, siteG-F/siteG-R and siteH-F/siteH-R, with pNC2 as the template (Fig. 6a, probes A–H). The DNA–protein binding reactions were carried out at 25°C in a final volume of 10 µl containing 1 ng of DIG-labeled probe, appropriate substituted phenols as coinducers and the HadR tetramer (0.35 µM) for 15 min. After incubation, the samples were separated on an 8% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 80 V at 4°C for 2 h. After electrophoresis, the labeled DNAs were electroblotted onto Biodyne PLUS nylon membranes (Pall Gelman Laboratory, Port Washington, NY, USA) and detected using the ImageQuant™ LAS-4000UV mini CCD

camera system (GE Healthcare). The intensity of chemiluminescence from each well was measured with a densitometer using Multi Gauge version 3.0 (Fujifilm, Tokyo, Japan) and expressed as arbitrary units.

For determination of the dissociation constants (K_Ds) of coinducers, EMSAs were performed using eight concentrations of coinducer (0–100 μM) and 1 ng of DIG-labeled probe E (Fig. 6a). The intensities of the free probe and HadR^{mt}–DNA complex were measured. When the intensity of the free probe at 0 μM was set at 100%, K_D was defined as the coinducer concentration at which 50% of the labeled DNA was bound.

2-4-9. Circular permutation analysis

For circular permutation analysis, DNA fragments that contained the HadR^{mt} protection region at different positions relative to the fragment ends were amplified by PCR using primer pairs site1-F/siteC-R, site2-F/siteD-R, siteF-F/site3-R, siteG-F/siteF-R and siteH-F/site5-R with pNC2 as the template (Fig. 10a, probes 1–5). After three independent EMSAs were performed with these fragments, the mobilities of the HadR^{mt}–DNA complexes were determined by measuring the distance traveled from the well during electrophoresis. The bending angles were calculated as described by Thompson and Landy *et al.* (1988).

2-4-10. DNase I footprinting analysis

DNA fragments containing the *hadX* promoter region were amplified using the following primer pairs: hadR-footprint-F/hadX-IRD-extension (*hadX* coding strand) and hadR-IRD-footprint-F/hadX-footprint-R (*hadR* coding strand). The binding reactions were carried out at room temperature in a final volume of 20 μl containing 100 ng of

IRD-labeled probe, 2 μg of herring sperm DNA, 5 μg of bovine serum albumin, 100 μM of substituted phenols and $HadR^{mt}$ in binding buffer (20 mM Tris-HCl, 50 mM NaCl, 0.1 mM EDTA; pH 8.0) for 15 min. Subsequently, 20 μl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added along with 1 μl of a DNase I solution (1×10^{-3} unit/μl; Takara Bio) followed by incubation for 2 min at room temperature. Reactions were stopped by the addition of 10 μl of 0.1 M EDTA. The mixture was treated with phenol, precipitated with ethanol, and resuspended in 1 μl IR2 stop solution (Li-Cor). The dissolved sample was analyzed using the Li-Cor 4300 DNA analyzer as described above.

2-4-11. Statistics and data analysis

The Q-PCR analysis, β-galactosidase assay, circular permutations analysis and measurement of K_Ds were performed using a Student's *t*-test. A *p*-value of less than 0.05 was considered significant. Each measurement was carried out at least in triplicate, and the means and standard deviations were calculated.

2-5. RESULTS

2-5-1. Determination of the operon structure and promoter region of *had* genes

RT-PCR analysis was performed with total RNA isolated from DTP0602 cells grown on 2,4,6-TCP. The amplification products of the expected sizes were detected for the genes *hadX-hadA* (R3), *hadA-hadB* (R4), *hadB-hadC* (R5), *hadC-orf4* (R7, 8), *hadR* (R2), *hadC* (R6) and *orf4* (R9) (Fig. 1c). No RT-PCR products using a primer that spans the *orf1-hadR* (R1) and *hadC-orf4-orf5* (R10) were obtained. The presence of rho-independent terminator in the R1 and R10 regions was sought using FindTerm (Softberry), but not located. This implies that an alternative type of terminator may have stopped the transcription. We hypothesized that the promoters of the 2,4,6-TCP catabolic genes caused the read-through transcription of *hadC*, because the *orf4* gene lies just downstream from *hadC* in the opposite direction and amplification products that span the *hadC-orf4-orf5* gene were not obtained. The results demonstrate that the 2,4,6-TCP catabolic pathway genes in DTP0602 consist of two transcriptional units, the *hadX-hadA-hadB-hadC* operon and monocistronic *hadR*.

Transcriptional start points (TSPs) of the *hadR* and *hadX-C* operon were determined by primer extension analyses using total RNA isolated from DTP0602 cells or DTP0602-pJBECl, which carry *orf1*, *hadR*, *hadX*, *hadA* and *hadB*. The TSPs (+1) of the *hadR* and *hadX-C* operon were found to be a C nucleotide positioned 26 bp upstream from the ATG translation start codon of *hadR* and a A nucleotide positioned 75 bp upstream from the ATG translation start codon of *hadX*. The putative σ^{70} promoter sequence was found upstream from the TSPs of the *hadR* and *hadX-C* operon (Fig. 2 and 3).

To ensure that the *hadX* promoter controls the expression of the *hadX-C* operon, the *lacZ* reporter plasmids pJBRYl and pJBEYl, carrying a *hadR-hadX-hadA*::*lacZ-lacY* (including the putative σ^{70} promoter sequence of *hadX*) and a *hadX-hadA*::*lacZ-lacY* (lacking the putative σ^{70} promoter sequence of *hadX*) were constructed, respectively (Fig. 6a). The β-galactosidase activity in DTP0602-pJBRYl and -pJBEYl cells was measured. After incubation with 2,4,6-TCP, the β-galactosidase activity significantly increased 3.8-fold in DTP0602-pJBRYl. The basal level of activity was significantly higher (approximately 6.0-fold) in DTP0602-pJBEYl than in DTP0602-pJBRYl. The β-galactosidase activity was significantly repressed 1.2-fold in the presence of 2,4,6-TCP in DTP0602-pJBEYl (Table 3). These results indicate that a single promoter of *hadX* is involved only in the transcription of the *hadX-C* operon.

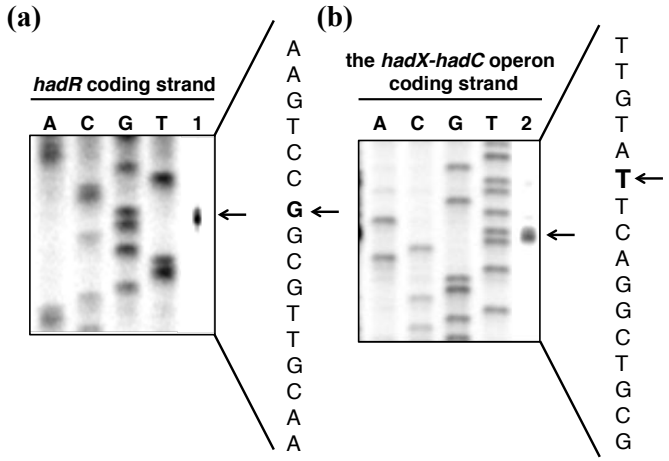

FIG. 2. Determination of the transcriptional start points (TSPs) of the *hadR* and *hadX-C* operons using primer extension analysis. Primer extension products were synthesized as described in the Materials and Methods section. DNA sequencing ladders were generated with the same primer as that used in the primer extension reactions. The nucleotide sequence of each promoter region is indicated to the right of each panel. The TSPs of the *hadR* and *hadX-C* operons are indicated by arrow and boldface, respectively. (a) Primer extension analysis of *hadR*. Lanes: A, C, G, and T, DNA sequencing ladders; 1, the primer extension product of the *hadR* transcripts from 200 ng total RNA isolated from strain DTP0602. (b) Primer extension analysis of the *hadX-C* operon. Lanes: A, C, G and T, DNA sequencing ladders; 2, the primer extension product of the *hadX-C* operon transcripts from 200 ng total RNA isolated from strain DTP0602 harboring pJBEC1.

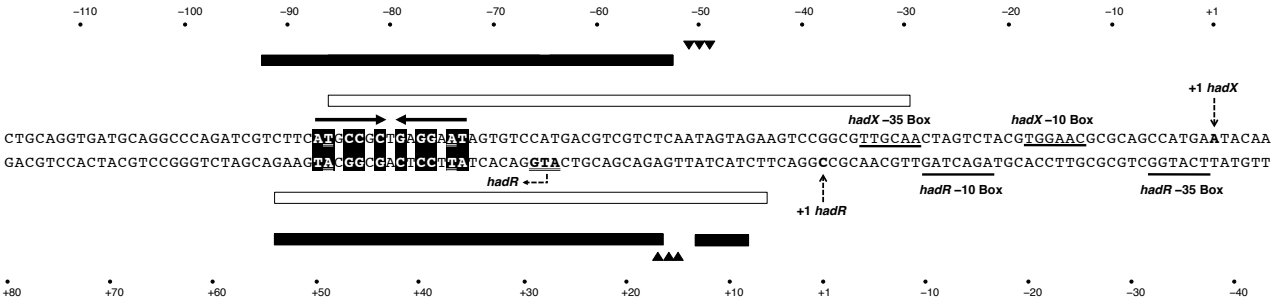

FIG. 3. Nucleotide sequence of the *hadR*-*hadX* divergent promoter and HadR^{mt}-binding sites. The TSPs of the *hadR* and *hadX*-*C* operon are indicated by dotted arrows and boldface. The putative −35 and −10 regions of the *hadR* and *hadX* promoters are indicated by solid single underlines. The translation start codon (ATG) of *hadR* is indicated by a solid double underline and boldface. Bold arrows indicate T-N_{11}-A sequences in the upstream region of the *hadX* promoter. The inverted repeat sequences and the A and T residues within the T-N_{11}-A sequences are highlighted and solid double underlined. The numbers indicate the distance in base pairs from the transcriptional initiation site with respect to the TSP of the *hadR* or *hadX*-*C* operon.

Table 3. Activity of the *hadX* promoter in DTP0602

Plasmid	β-galactosidase activity* (fold induction†)		
	none	2,4,6-TCP‡, §	
pJBRY (*hadR-hadX-hadA*::*lacZ*)	18.6 ± 0.695	73.2 ± 3.98	(3.8)
pJBEY (*hadX-hadA*::*lacZ*)	112 ± 1.74	94.9 ± 4.72	(0.86)

* Expressed as Miller units ± standard deviation.

† Fold induction = Miller units (2,4,6-TCP added)/Miller units (none).

‡ TCP indicate trichlorophenol.

§ $P < 0.05$, versus without coinducers, Student's t test.

2-5-2. Role of HadR for the transcription of the *hadX-C* operon

To demonstrate the role of HadR for the 2,4,6-TCP utilization in *R. pickettii* DTP0602, a null mutant of *hadR* (DTP62dR) was constructed. DTP62dR showed no growth on 2,4,6-TCP as a sole carbon source, whereas a *hadR* complement strain (DTP62dR-pTSR1) grew to the same extent as the wild type (DTP0602). This indicated that the HadR protein was necessary for 2,4,6-TCP utilization (Fig. 4).

To investigate the regulation of gene expression by HadR and whether 2,4,6-TCP acts as a coinducer of HadR, the transcript level of *hadR*, *hadX*, *hadA* and *hadB* in the presence or absence of 2,4,6-TCP in DTP0602, DTP62dR and the *hadA* null-mutant (DTP62dA), which lacks the ability to utilize 2,4,6-TCP, were examined by Q-PCR. By addition of 2,4,6-TCP, the transcription of *hadX*, *hadA* and *hadB* was significantly increased by 120-, 230- and 170-fold in DTP0602 and by 130-, 230- and 170-fold in DTP62dA, respectively, but the transcription was not induced in DTP62dR. Furthermore, the transcription level of *hadA* was significantly higher (approximately 3- to 10-fold) than that of *hadX* in DTP0602, DTP62dA and DTP62dR (Table 4 and 6). The transcription of *hadR* was significantly lower (approximately 2.4-fold) in DTP62dR than in DTP0602 without 2,4,6-TCP. These results indicated that HadR requires 2,4,6-TCP as a coinducer to positively regulate the transcription of the *hadX-C* operon.

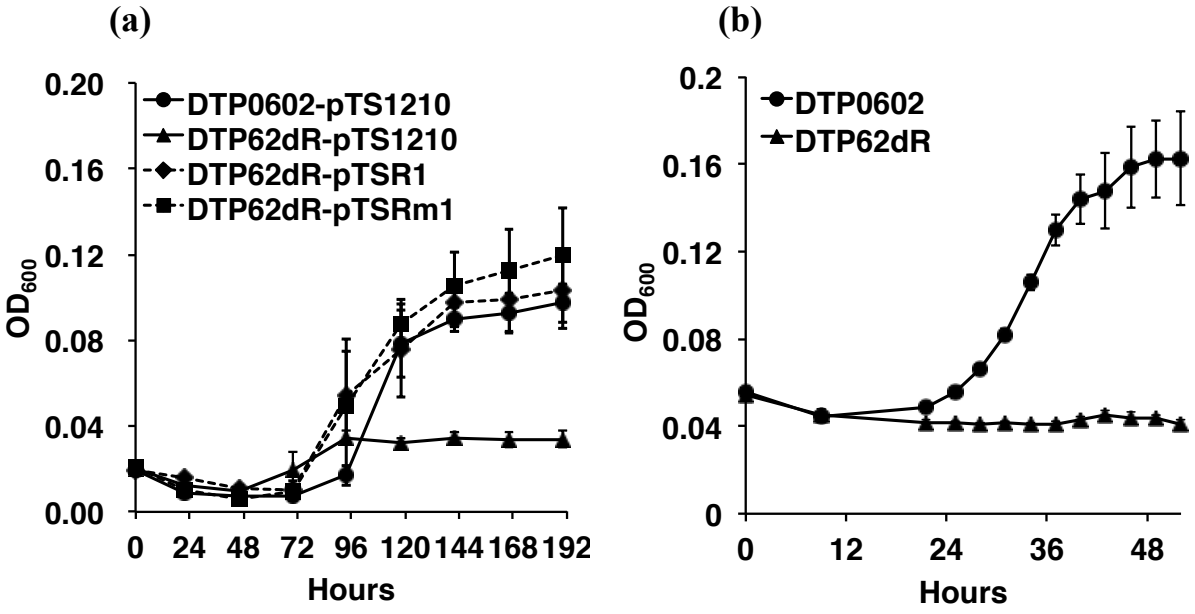

FIG. 4. Growth curves of *R. pickettii* DTP0602, *hadR* mutant strain, complementation with *hadR*, and complementation with *hadR*^{mt} on MM containing 500 μM 2,4,6-TCP. Growth conditions are described in the Materials and Methods section. Data points and error bars represent averages and standard deviations of triplicate cultures, respectively. (a) The growth curves of DTP0602-pTS1210, DTP62dR-pTS1210, DTP62dR-pTSRl (*hadR*$^{+}$), and DTP62dR-pTSRml (*hadR*^{mt}). (b) The growth curves of DTP0602 and DTP62dR.

Table 4. The absolute mRNA copy number of *hadR*, *hadX*, *hadA* and *hadB* in the presence or absence of those phenols in DTP62dR and DTP62dA

Strains and coinducers*	Absolute mRNA copy number†, ‡ (fold induction§) in DTP62dR			
	hadR	***hadX***	***hadA***	***hadB***
DTP62dR				
none	35.6 ± 6.51	2.78 ± 0.600	12.1 ± 2.18	20.3 ± 4.80
2,3,6-TCP	65.6 ± 2.69 (1.8)	3.21 ± 0.198 ‖ (1.1)	13.7 ± 0.448 ‖ (1.1)	14.9 ± 0.766 ‖ (0.73)
2,4,6-TCP	54.6 ± 12.9 (1.5)	2.74 ± 0.434 ‖ (0.99)	12.1 ± 2.34 ‖ (1.0)	13.0 ± 2.58 (0.64)
2,3,4,6-TeCP	56.9 ± 5.48 (1.6)	1.70 ± 0.116 (0.61)	9.16 ± 0.360 (0.76)	9.05 ± 1.04 ‖ (0.45)
2,4,6-TBP	65.9 ± 12.1 (1.9)	3.42 ± 0.329 ‖ (1.2)	17.2 ± 1.76 (1.4)	17.1 ± 1.75 ‖ (0.84)
Bromoxynil	40.1 ± 4.34 ‖ (1.1)	0.850 ± 0.053 (0.31)	4.98 ± 0.330 (0.41)	4.26 ± 0.359 (0.21)
2-C-4-NP	49.7 ± 2.71 ‖ (1.4)	2.02 ± 0.179 (0.73)	11.2 ± 0.842 (0.93)	9.81 ± 0.637 (0.48)
2,4-DNP	42.0 ± 6.06 ‖ (1.2)	1.37 ± 0.195 (0.49)	8.89 ± 0.673 (0.74)	6.64 ± 0.660 (0.33)
DTP62dA				
none	59.3 ± 4.47	2.60 ± 0.242	18.7 ± 2.48	13.3 ± 1.14
2,4,6-TCP	73.1 ± 0.696 (1.2)	327 ± 49.9 (130)	4200 ± 406 (230)	2290 ± 155 (170)

* Compounds abbrevations are as indicated in Table 2.

† Experessed as absolute mRNA copy number ($\times 10^6$)/total RNA (μg).

‡ Absolute mRNA copies of respective genes are the average ± standard deviation of three independent experiments.

§ Fold induction = absolute mRNA copy number (coinducer added)/absolute mRNA copy number (none).

‖ $P > 0.05$, versus without coinducers, Student's *t* test.

2-5-3. Determination of HadR^{mt} protection sequence

For the determination of HadR-binding region, HadR^{mt} (35.7 kDa) was purified to homogeneity. The molecular mass of HadR^{mt} was estimated to be 137 ± 17.6 kDa by gel filtration chromatography, indicating that HadR^{mt} exists as a tetramer in solution (Fig. 5). The oligomeric state of HadR^{mt} is similar to that reported for other LTTRs (Bundy *et al.*, 2002; Chang & Crawford, 1991; Kullik *et al.*, 1995; Miller & Kredich, 1987; Muraoka *et al.*, 2003; Schell *et al.*, 1990). To confirm whether HadR^{mt} altered the function of HadR, complementation studies using the plasmid pTSRml producing HadR^{mt} were performed in DTP62dR. DTP62dR-pTSRm1 can normally grow on 2,4,6-TCP, suggesting that HadR^{mt} showed functional complementarity to HadR (Fig. 5).

EMSAs were performed using HadR^{mt} with DIG-labeled DNA fragments encompassing the *hadR-hadX-hadA* region from positions −247 to +877 relative to the TSP of the *hadX* with or without 2,4,6-TCP to determine the HadR^{mt} binding region (Fig. 6). Irrespective of the presence or absence of 2,4,6-TCP, only a single retarded band was observed for probes E, F and G and no bands were observed for probes A, B, C, D and H. To evaluate the binding of HadR^{mt} to the *hadX* promoter specifically, we performed a competition test using herring sperm DNA as nonspecific competing DNA and unlabeled DNA probes E, F and G as specific competitors for the binding assays. The band of HadR^{mt}–DNA complex formation still occurred in the presence of a 200-fold excess of herring sperm DNA, whereas it was substantially reduced in the presence of a 100-fold excess of unlabeled DNA. Therefore, HadR^{mt} binds specifically to the *hadX* promoter and HadR^{mt}–DNA complex formation was induced in the presence of 2,4,6-TCP (Fig. 6b).

DNase I footprinting analysis was performed in the presence or absence of 2,4,6-TCP (Fig. 3, 6a and 7) to determine $HadR^{mt}$-binding site on the *hadX* promoter region. $HadR^{mt}$ protects a continuous region from positions −86 to −30 on the *hadX* coding strand and positions +54 to +7 on the *hadR* coding strand in the absence of 2,4,6-TCP and a continuous region from positions −92 to −53 on the *hadX* coding strand and two regions from positions +54 to +17 and +13 to +9 on the *hadR* coding strand in the presence of 2,4,6-TCP. These protection sequences contained the T-N_{11}-A motif of the LTTR consensus binding sequences (AT-NCCNCNGNGGN-AT; positions −87 to −73 relative to the TSP of the *hadX-C* operon) and portions of the structural genes of *hadR* (positions +54 to +27 relative to the TSP of the *hadR*). The footprint region of $HadR^{mt}$ was significantly shortened in the presence of 2,4,6-TCP, because positions downstream of −52 of the *hadX* coding strand and upstream of +18 of the *hadR* coding strand were no longer protected. In the presence of 2,4,6-TCP, a DNase I-hypersensitive site appeared at positions −49, −50 and −51 on the *hadX* coding strand and +15, +16 and +17 on the *hadR* coding strand.

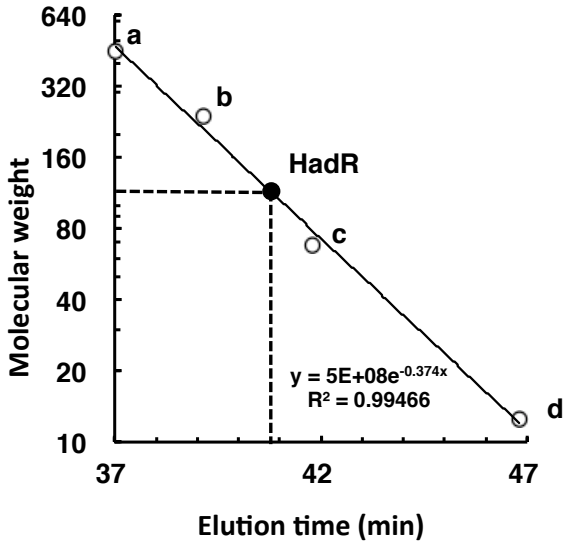

FIG. 5. Determination of the oligomerization state of $HadR^{mt}$ using gel filtration. The retention time and apparent molecular mass during gel filtration are presented on the X and Y axes, respectively. The open and closed circles indicate size standards and $HadR^{mt}$, respectively. Molecular masses of standard proteins are as follows: a, ferritin (450 kDa); b, catalase (250 kDa); c, bovine serum albumin (68 kDa); d, cytochrome C (12.5 kDa).

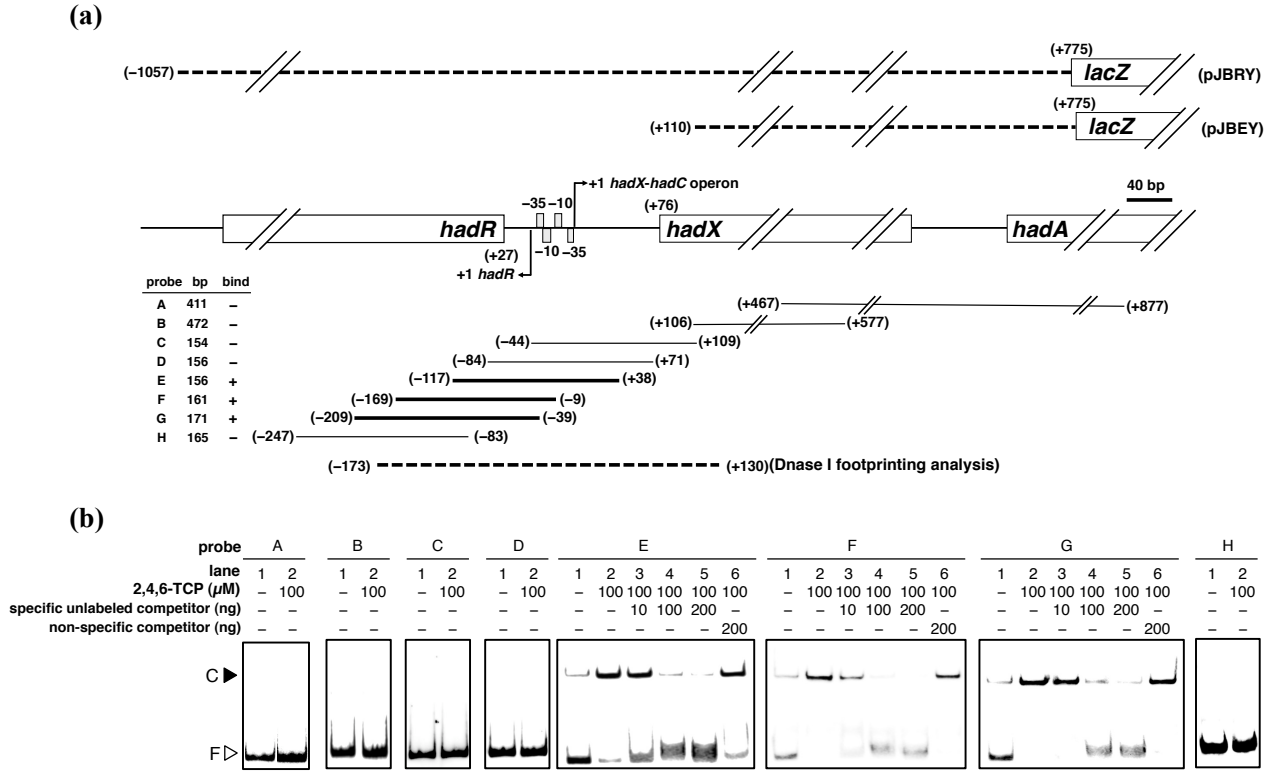

FIG. 6. EMSA of the binding of HadR^{mt} to the upstream region of the *hadX* promoter. (a) DNA fragments used for the promoter probe vector; pJBRY, pJBEY (dotted bars), the EMSA and DNase I footprinting analysis (dotted bars). Binding (+: thicker bars) and nonbinding (–: thinner bars) of the respective fragments are indicated. The numbers noted next to the fragments indicate the positions of the ends of the fragments relative to the TSP of the *hadX-C* operon. (b) EMSA with HadR^{mt} and DIG-labeled fragments containing the *hadX* promoter region. 100 μM 2,4,6-TCP used as coinducers in the reaction mixtures is indicated. Lane 1, EMSA with HadR^{mt}; lane 2, EMSA with HadR^{mt} and 2,4,6-TCP and no competitor DNA; lanes 3–5, EMSA with HadR^{mt} and increasing concentrations (10, 100 and 200 ng, respectively) of unlabeled probe DNA fragment as specific unlabeled competitor; lanes 6, EMSA with HadR^{mt} and 200 ng of herring sperm DNA as non-specific competitor. Free DNA and the HadR^{mt}–DNA complex are indicated by open arrowheads and closed arrowheads.

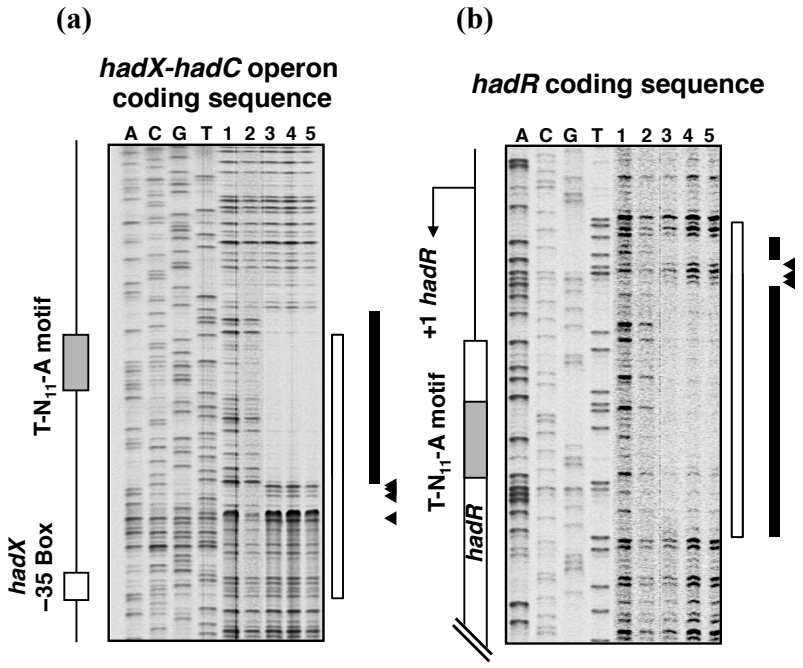

FIG. 7. DNase I footprinting of HadR^{mt} on the *hadR-hadX* divergent promoter region. (a and b) The patterns of fragments resulting from digestion with DNase I of the IR800-labeled fragments represent the *hadX-C* operon- (a) or *hadR*-coding (b) strand. HadR^{mt} concentrations were 0 (lane 1), 14.6 μM (lane 2), 1.4 μM (lane 3), 2.8 μM (lane 4) and 7.3 μM (lane 5). Reactions were performed in the absence (lanes 1 and 2) or presence (lanes 3–5) of 100 μM 2,4,6-TCP. Lanes A, C, G and T represent sequencing lanes. Open box indicates DNase I protection in the absence of 2,4,6-TCP and closed box (black) indicates DNase I protection in the presence of 2,4,6-TCP. The DNase I-hypersensitive sites are indicated by arrowheads. The closed arrowheads indicate the reactions performed in the presence of 2,4,6-TCP.

2-5-4. Effect of various substituted phenols on the induction of the HadR^{mt}–DNA complex formation and on the bending angle of the HadR^{mt} binding region

Because substituted phenols, including 2,4,6-TCP, were predicted to induce the HadR^{mt}–DNA complex formation, EMSAs were performed in the presence of various substituted phenols and DIG-labeled probe E (Fig. 8). The band of HadR^{mt}–DNA complex was not observed in the presence of mono-, di-chlorophenols (CPs, DCPs), mononitrophenols (NPs), hydroxyquinol, or 6-chlorohydroxyquinol, but it was observed in the presence of all tested tri-, tetra-chlorophenols (TCPs, TeCPs), PCP, 2,4,6-tribromophenol (2,4,6-TBP), 2,4-, 2,5-, 2,6-dinitrophenols (DNPs), 2,4,6-trinitrophenol (2,4,6-TNP), 2-chloro-4-nitrophenol (2-C-4-NP) and 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil). HadR^{mt} showed binding to probe E without substituted phenols (Fig. 8, the first lane on the left in each gel). To determine whether HadR^{mt}–DNA complex formation was specifically induced, competition tests were performed as described above. The HadR^{mt}–DNA complex formation occurred specifically by the tested phenols (Fig. 8b and 8c, lane 3 and 4 in each gel). DNase I footprinting analysis was performed in the presence of sixteen kinds of substituted phenols to confirm the diversity of HadR^{mt} protection sequence in the presence of substituted phenols, including 2,4,6-TCP. As a result, HadR^{mt} protection sequence showed no difference between 2,4,6-TCP and other substituted phenols (Fig. 9).

To test the hypothesis that HadR^{mt} has a different affinity for each of the phenols, the K_Ds for the sixteen types of substituted phenols were determined (see the Materials and Methods section for details). HadR^{mt} showed a range of different affinities for the phenols (Table6). The K_D value for 2,4,6-TBP (2.4 ± 0.4 μM) was significantly lower than the other

substrates tested, even that of 2,4,6-TCP (6.2 ± 0.8 μM). The K_D value for 2,4,6-TCP was not significantly different from those for bromoxynil (6.6 ± 0.6 μM) and 2-C-4-NP (7.0 ± 1.5 μM).

The appearance of DNase I-hypersensitive sites (−49, −50 and −51) on the *hadX* promoter region suggests that $HadR^{mt}$ also alters DNA bending when transcription is activated. The ability of $HadR^{mt}$ to bend DNA at its binding sites was examined by circular permutation analysis using 201-bp PCR fragments containing a $HadR^{mt}$-protection sequence of the *hadX* promoter (Fig. 10a). As shown in Figure 10b, 10c, 10d and 10e, the estimated the $HadR^{mt}$-dependent bending angles were 14 ± 1.4° in the absence of coinducers and 15 ± 1.7°, 15 ± 1.8° and 15 ± 2.2° in the presence of 5 μM 2,4,6-TCP, 10 μM 2,3,4,5-TeCP and 20 μM PCP, respectively. These bending angles were not significantly different. These results indicated that the $HadR^{mt}$-dependent DNA bending angle was not affected by the tested phenols.

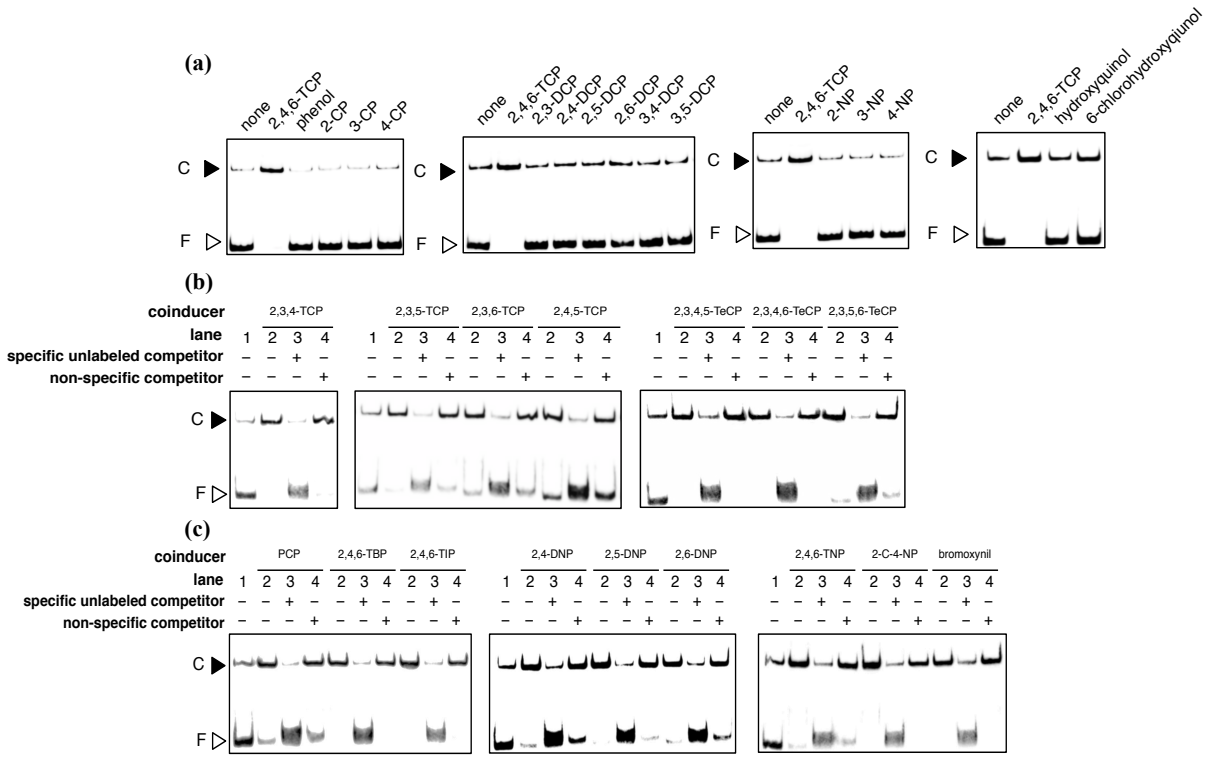

FIG. 8. Alternative coinducers of HadR^{mt} to bind the upstream region of the *hadX* promoter. Free DNA and the HadR^{mt}–DNA are indicated by open arrowheads and filled arrowheads, respectively. EMSA with HadR^{mt} and DIG-labeled fragments (probe E) containing the *hadX* promoter region. (a) EMSAs were performed in the presence of 100 μM of CPs and DCPs, NPs, hydroxyquinol and 6-chlorohydroxyquinol. The first lane on the left in each gel served as a control containing HadR^{mt}. The second lane from the left in each gel contained HadR^{mt} and the substituted phenol. (b) EMSAs were performed in the presence of 100 μM TCPs, TeCPs and PCP, TBP, DNPs and TNP, 2-C-4-NP and bromoxynil. Lane 1, EMSA with HadR^{mt}; lane 2, EMSA with HadR^{mt} and substituted phenol and no competitor DNA; lanes 3, EMSA with HadR^{mt} and 200 ng of unlabeled probe E DNA fragment as specific unlabeled competitor; lanes 4, EMSA with HadR^{mt} and 200 ng of herring sperm DNA as nonspecific competitor.

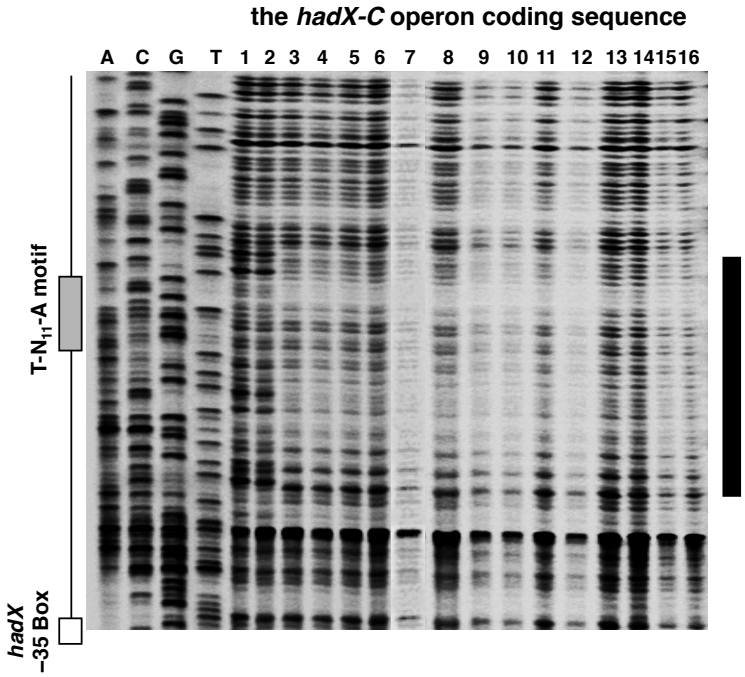

FIG. 9. DNase I footprinting of HadR^{mt} on the *hadX-C* promoter region. The patterns of fragments resulting from digestion with DNase I of the IR800-labeled fragments represent the *hadX-C* operon coding strand. HadR^{mt} concentrations were 0 (lane 1) and 14.6 μM (lanes 2–16). Reactions were performed in the absence (lanes 1 and 2) or presence (lanes 3–16) of 100 μM of substituted phenols. Lane 3, 2,4,6-TBP; lane 4, 2,4,6-TCP; lane 5, bromoxynil; lane 6, 2,3,4,6-TeCP; lane 7, 2-C-4-NP; lane 8, 2,4-DNP; lane 9, 2,3,4,5-TeCP; lane 10, PCP; lane 11, 2,4,6-TNP; lane 12, 2,3,5-TCP; lane 13, 2,3,6-TCP; lane 14, 2,3,5,6-TeCP; lane 15, 2,3,4-TCP; lane 16, 2,4,5-TCP. Lanes A, C, G and T represent sequencing lanes. Closed box (black) indicates DNase I protection in the presence of substituted phenols.

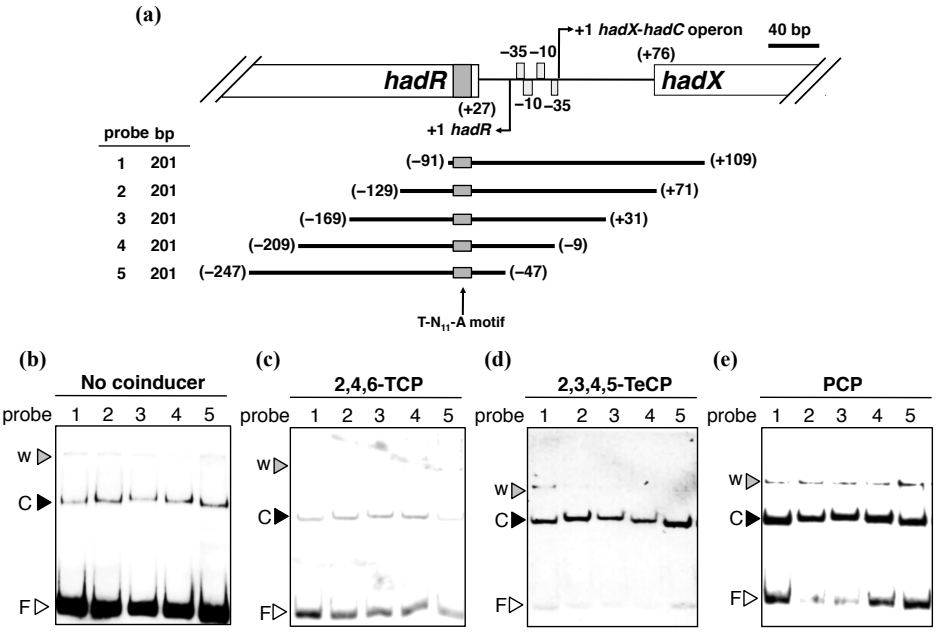

FIG. 10. Determination of HadR^{mt}-induced DNA bending by circular permutation analysis. (a) The DNA fragment used in circular permutation analysis. The T-N_{11}-A motif is indicated by the shaded bar. The 201-bp DNA fragments (1–5) contain the T-N_{11}-A motif in different positions relative to the end of the fragment. The numbers next to the probes indicate the positions of the ends of the probes relative to the TSP of the *hadX-C* operon. (b–e) EMSA demonstrating DNA bending by HadR^{mt}. Binding reactions were performed in the absence (b) or presence of 5 μM 2,4,6-TCP (c), 10 μM 2,3,4,5-TeCP (d), and 20 μM PCP (e). Free DNA is indicated by open arrowheads, the HadR^{mt}–DNA complex is indicated by closed arrowheads (black), and the wells are indicated by shaded arrowheads.

2-5-5. Variations in the transcription of *hadR*, *hadX*, *hadA* and *hadB* induced by substituted phenols

For the confirmation of the activation of the *hadX-C* operon transcription with substituted phenols, which induced the $HadR^{mt}$–DNA complex formation, Q-PCR analysis and β-galactosidase assay were performed to monitor the mRNA copy numbers of *hadR*, *hadX*, *hadA* and *hadB*, and the expression of *hadA* monitored by using DTP0602 and DTP62ZA (*hadA*::*lacZ*) (Table 5 and 6). β-galactosidase activity was significantly increased 47- to 540-fold in the presence of 2,4,6-TBP, 2,4,6-TCP, bromoxynil, 2-C-4-NP, 2,3,4,6-TeCP, 2,4-DNP and 2,3,6-TCP. The mRNA copy numbers of *hadX*, *hadA* and *hadB* was significantly induced 7.4- to 280-fold by 2,4,6-TBP, 2,4,6-TCP, bromoxynil, 2-C-4-NP, 2,3,4,6-TeCP, 2,4-DNP and 2,3,6-TCP. However, it was not significantly induced in DTP0602 by other substituted phenols. As compared with the phenols that were absent, the transcription level of *hadR* was significantly decreased by 50% with 2-C-4-NP, 2,3,4-TCP, 2,4,5-TCP, 2,4-DNP, 2,5-DNP and 2,6-DNP and by 70% with 2,3,4,5-TeCP; however, the transcription level of *hadR* did not change with other substituted phenols. To determine whether or not the activation of these genes is under the control of HadR, Q-PCR analysis was performed using DTP62dR cells in the presence of seven types of substituted phenols: 2,4,6-TBP, bromoxynil, 2,4,6-TCP, 2,3,6-TCP, 2,3,4,6-TeCP, 2-C-4-NP and 2,4-DNP. The transcription of these genes was not significantly induced in the presence of these phenols, and the basal level of *hadR* transcription was significantly lower in DTP62dR than in DTP0602 (Table 4).

Table 5. Activity of the *hadA-lacZ* fusion in DTP62ZA

Coinducers*	**(fold induction‡)**			
none	0.379	±	0.649	
2,3,4-TCP	1.51	±	1.35 §	(4.0)
2,3,5-TCP	3.12	±	3.48 §	(8.2)
2,3,6-TCP	42.3	±	0.991	(110)
2,4,5-TCP	3.13	±	1.32	(8.3)
2,4,6-TCP	153	±	1.77	(400)
2,4,6-TBP	113	±	12.6	(300)
2,3,4,5-TeCP	0.878	±	0.217 §	(2.3)
2,3,4,6-TeCP	17.8	±	0.215	(47)
2,3,5,6-TeCP	1.25	±	0.434 §	(3.3)
PCP	2.00	±	0.434	(5.3)
Bromoxynil	203	±	9.06	(540)
2-C-4-NP	57.9	±	6.08	(160)
2,4-DNP	101	±	13.1	(270)
2,5-DNP	0.00	±	0.942	(0.0)
2,6-DNP	0.00	±	0.432	(0.0)
2,4,6-TNP	0.00	±	0.779 §	(0.0)

* Compound abbrevations are as indicated in Table 2.

† Expressed as Miller units ± standard deviation.

‡ Fold induction = Miller units (coinducer added)/Miller units (none).

§ $P > 0.05$, versus without coinducers, Student's t test.

Table 5. The K_Ds values for substituted phenols and the absolute mRNA copy number of *hadR*, *hadX*, *hadA* and *hadB* in the presence or absence of those phenols

		Absolute mRNA copy number‡, § (fold induction‖) in DTP0602			
Coinducers*	**K_D (μM)†**	***hadR***	***hadX***	***hadA***	***hadB***
none		84.7 ± 21.9	4.98 ± 1.02	23.6 ± 10.1	23.1 ± 4.05
2,4,6-TBP	2.4 ± 0.4	74.6 ± 3.30 ¶ (0.88)	573 ± 122 (120)	5,510 ± 1,000 (230)	3,930 ± 850 (170)
2,3,4,5-TeCP	3.8 ± 0.7	29.2 ± 13.9 (0.35)	3.02 ± 2.91 ¶ (0.61)	9.28 ± 9.29 ¶ (0.39)	3.70 ± 2.39 (0.16)
2,4,6-TCP	6.2 ± 0.8	64.5 ± 9.62 ¶ (0.76)	604 ± 126 (120)	6,700 ± 1,900 (280)	4,130 ± 1,180 (180)
bromoxynil	6.6 ± 0.6	76.8 ± 7.24 ¶ (0.91)	467 ± 44.8 (94)	4,350 ± 527 (180)	2,660 ± 150 (120)
2-C-4-NP	7.0 ± 1.5	38.0 ± 8.65 (0.45)	134 ± 27.1 (27)	1,130 ± 151 (48)	847 ± 169 (37)
2,3,4,6-TeCP	8.1 ± 1.7	67.0 ± 9.93 ¶ (0.79)	42.0 ± 4.79 (8.5)	374 ± 34.0 (16)	249 ± 16.1 (11)
2,3,5-TCP	10 ± 2.4	59.7 ± 22.8 ¶ (0.71)	7.15 ± 5.53 ¶ (1.4)	6.7 ± 5.38 (0.28)	3.04 ± 1.94 (0.13)
PCP	16 ± 3.6	59.7 ± 6.32 ¶ (0.71)	3.19 ± 0.926 (0.64)	21.7 ± 2.14 ¶ (0.92)	16.7 ± 1.39 (0.72)
2,3,4-TCP	18 ± 3.1	37.6 ± 14.2 (0.44)	8.10 ± 6.42 ¶ (1.6)	12.7 ± 10.9 ¶ (0.54)	3.59 ± 2.69 (0.16)
2,4,6-TNP	30 ± 3.3	91.6 ± 25.8 ¶ (1.1)	12.5 ± 6.40 ¶ (2.5)	32.1 ± 11.3 ¶ (1.36)	24.7 ± 9.16 ¶ (1.1)
2,4-DNP	33 ± 1.0	51.5 ± 6.11 (0.61)	181 ± 32.4 (37)	1,660 ± 396 (70)	850 ± 69.6 (37)
2,3,5,6-TeCP	36 ± 2.7	77.5 ± 10.4 ¶ (0.92)	3.40 ± 1.11 ¶ (0.68)	17.3 ± 5.69 ¶ (0.73)	15.3 ± 4.77 (0.66)
2,3,6-TCP	36 ± 3.3	76.2 ± 10.8 ¶ (0.90)	36.8 ± 7.75 (7.4)	450 ± 133 (19)	277 ± 90.7 (12)
2,4,5-TCP	40 ± 6.2	48.4 ± 13.1 (0.57)	5.52 ± 4.15 (1.1)	13.6 ± 7.52 ¶ (0.57)	6.56 ± 6.42 (0.28)
2,5-DNP	>50	41.2 ± 5.97 (0.49)	7.52 ± 5.25 ¶ (1.5)	41.5 ± 32.4 ¶ (1.8)	19.3 ± 11.4 ¶ (0.83)
2,6-DNP	>50	45.3 ± 18.3 (0.54)	1.56 ± 0.292 (0.31)	8.55 ± 3.73 (0.36)	8.03 ± 2.85 (0.35)

* TCP, TeCP, PCP, TBP, DNP, TNP, 2-C-4-NP, and bromoxynil indicate tri-, tetra-, penta-chlorophenol, tribromophenol, di-, tri-nitrophenol, 2-chloro-4-nitrophenol, and 3,5-dibromo-4-hydroxybenzonitril, respectively.

† The dissociation constants (K_Ds) for substituted phenols is the average ± standard deviation of three independent experiments.

‡ Experessed as absolute mRNA copy number ($\times 10^6$)/total RNA (μg).

§ Absolute mRNA copies of respective genes are the average ± standard deviation of three independent experiments.

‖ Fold induction = absolute mRNA copy number (coinducer added)/absolute mRNA copy number (none).

¶ $P > 0.05$, versus without coinducers, Student's t test.

2-6. DISCUSSION

When a transcriptional activator induces target promoter activity, the affinity of the regulator for the promoter sequence increases. Established gene regulatory paradigms indicate that the degree of induction of the target-regulated promoter is generally proportionate to the conformational change exhibited by the regulator-DNA complex. Such patterns are expected for LTTR promoters, which are induced in the presence of several kinds of coinducers. We found that activation of the target-regulated promoter for 2,4,6-trichlorophenol deviated from established patterns for LTTRs.

The transcription of the *hadX-C* operon in DTP0602 was induced in the presence of seven types of substituted phenols, but the $HadR^{mt}$ bound to the *hadX* promoter region in the presence of sixteen kinds of substituted phenols (Table 6 and Fig. 8). The well-characterized LTTRs cause the coinducer-dependent shortening of the protected region and a relaxation of the DNA bending (Akakura & Winans, 2002a; Akakura & Winans, 2002b; Kullik *et al.*, 1995; Ogawa *et al.*, 1999; Porrúa *et al.*, 2007). We hypothesize that the induction of the *hadX-C* operon transcription was caused by a change in the $HadR^{mt}$-protection sequences, in the $HadR^{mt}$-dependent bending angle, or in the transcription level of *hadR* in the presence of seven types of substituted phenols. The induction of the *hadX-C* operon transcription was not affected by the difference of the autorepression levels of *hadR*, of $HadR^{mt}$-protection sequences or of the $HadR^{mt}$-dependent bending angles (Table6, Fig. 9 and 10). $HadR^{mt}$ had a high affinity for 2,4,6-TBP, 2,4,6-TCP, bromoxynil and 2-C-4-NP, which activated the transcription of *hadX*, *hadA* and *hadB*. However, $HadR^{mt}$ had a high affinity for 2,3,4,5-TeCP and 2,3,5-TCP, which did not affect the induction of the *hadX-C* transcription. In addition, HadR had a low affinity for

2,4-DNP and 2,3,6-TCP, which affected the induction of transcription. Overall, there was no clear pattern in the relationship between coinducer binding affinities and the *hadX-C* operon transcription (Table 6).

The $HadR^{mt}$-dependent DNA bending angles were not significantly different in the presence or absence of coinducers (Fig. 10), indicating that HadR-dependent DNA bending was not correlated with the regulatory mechanism. This behavior of HadR toward the *hadX* promoter was distinct from those of the LTTRs mentioned above, since the DNA bending in itself might be important for the interaction of LTTR with RNA polymerase. MetR and TrpI were reported as exceptions, because the DNA bending angle was not affected by the coinducer, which was also the case for as well as HadR. Lorenz *et al.* suggested that MetR protected only a region of the *glyA* promoter (−155 to −109) without DNA bending relaxation in the presence or absence of homocysteine (1995). The TrpI-dependent DNA bending angle was not affected with or without indole glycerol phosphate (InGP). However, the protection region of TrpI extended to −32 in the presence of InGP (Gao & Gussin, 1991; Piñeiro *et al.*, 1997). The protection region of MetR was different from that of HadR (Fig. 7) and no reduction in length of the MetR- or TrpI-protection region was observed. Furthermore, the conserved amino acids and distinctive domain were not conserved between HadR, MetR and TrpI, and HadR was not more closely related to MetR and TrpI (Fig. 11). Unlike in the regulatory mechanisms of MetR and TrpI, a conformational difference in the HadR-DNA complex other than DNA bending, such as an interaction with another protein or DNA looping, might be important in the regulatory mechanism of HadR.

Park *et al*. demonstrated that NahR-regulated promoter activation was induced by direct contact between NahR and the alpha subunit of RNA polymerase (2002). The characterization using some of the NtdR, NahR and DntR variants suggested that LTTRs

undergo the effector-binding domains (EBD) conformational change, when activation of the target-regulated promoter occurs (Cebolla *et al.*, 1997; Ju *et al.*, 2009; Lönneborg *et al.*, 2007). These observations support the hypothesis that in the presence of any of the seven types of substituted phenols, the EBD conformational change of HadR allows the *hadX* promoter region to contact the alpha subunit of RNA polymerase without DNA bending, resulting in the induction of the *hadX-C* operon. These hypotheses should be further investigated.

Figure 11 shows an unrooted phylogenetic tree of HadR and other previously characterized LTTR proteins. We observed that HadR was more closely related to the TcpR, DntR, NtdR, NagR, NahR, PcpR and LinR proteins, which might sense aromatic compounds. The *hadX-C* operon transcription induced in the presence of bromo-, chloro- and nitro-substituted phenol. The coinducer profile of *hadX-C* operon transcription was distinctive, because other LTTR-regulated operon transcription was induced in the presence of nitrophenol, chlorophenols, tribromophenol, chlorohydroxyquinol or salicylate (Table 6).

It is surprising that $HadR^{mt}$ had a greater affinity for 2,4,6-TBP than for 2,4,6-TCP and that the transcription of *hadX*, *hadA* and *hadB* was induced by 2,4,6-TCP and 2,4,6-TBP (Table 6), since DTP0602 was isolated for its ability to use 2,4,6-TCP. DTP0602 showed growth on 2,4,6-TBP as a sole carbon source, whereas DTP62dR did not show growth (unpublished data). This result suggested that the *hadX-C* operon played a key role in 2,4,6-TBP utilization. Previously, *Ochrobactrum* sp. TB01 also showed growth on 2,4,6-TBP as a sole carbon source, and 2,4,6-TBP and 2,4,6-TCP were degraded by TB01 cells. The NAD(P)H-dependent reductive dehalogenase is involved in 2,4,6-TBP degradation in TB01, but the detailed 2,4,6-TBP catabolic pathway and regulatory

mechanism in TB01 were not known until the present study (Yamada *et al.*, 2008). The bromophenols, including 2,4,6-TBP, are naturally occurring compounds excreted by a diverse range of marine organisms as a defense against predators (Ashworth & Cormier, 1967; Fielman *et al.*, 1999; King, 1986; Pedersen *et al.*, 1974). On the other hand, 2,4,6-TCP has been artificially produced. Yamada *et al.* suggested that there are a number of bromophenol-degrading bacteria in various ecological niches (2008). If the catabolic operon of 2,4,6-TCP is identical to that of 2,4,6-TBP in TB01, and the transcriptional regulatory mechanism of 2,4,6-TBP catabolic gene in TB01 resembles that of HadR in DTP0602, these results perhaps indicate that the trichlorophenol catabolic mechanism in DTP0602 was evolved from the bromophenol catabolic mechanism.

In addition, the interesting findings that the transcription level of *hadA* was significantly higher than that of *hadX* might provide clues about another regulatory mechanism of *hadX* and *hadA* transcription (Table 6). The characterization of this regulatory mechanism is in progress. Overall, our results revealed that the regulatory mechanism of 2,4,6-TCP catabolic operon expression in DTP0602 (the DNA bending pattern, the difference in the coinducer profile of *hadX-C* transcription and HadR binding) was distinct from the regulatory mechanism of other, previously characterized LTTRs.

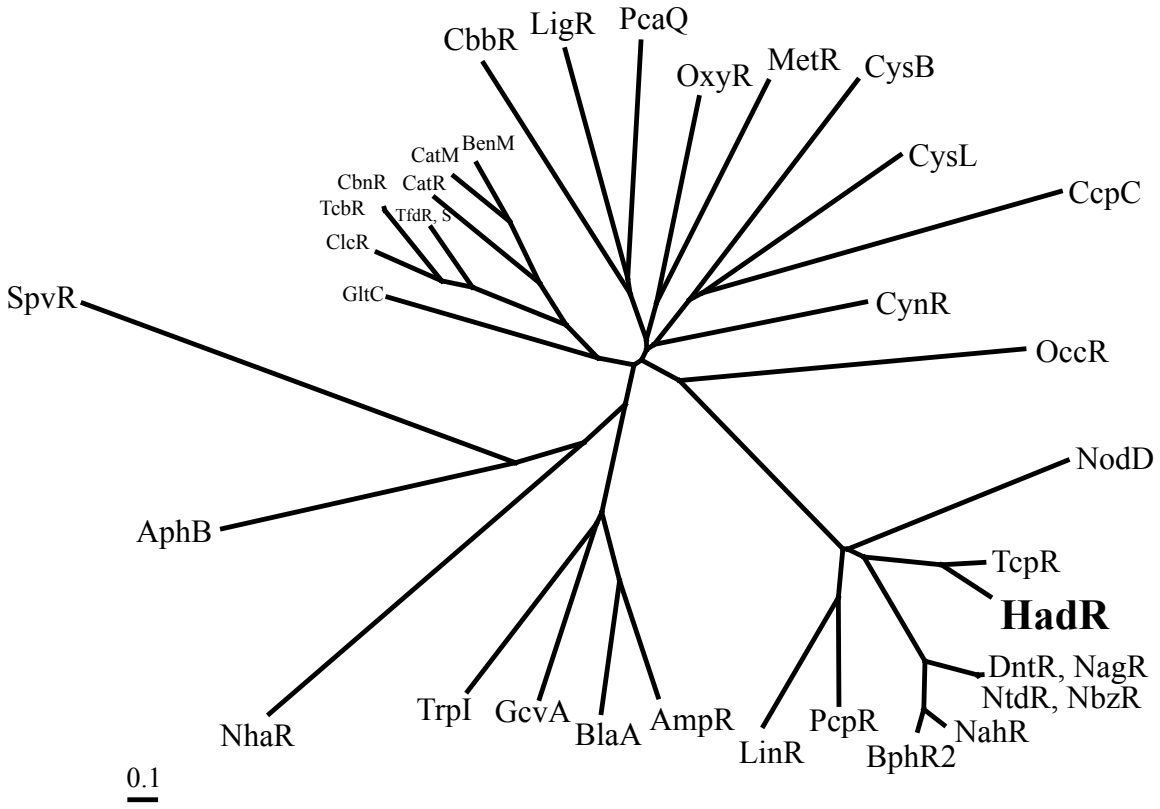

FIG. 11. Unrooted phylogenetic tree depicting distribution of HadR and LTTR proteins in different bacterial species. Multiple alignment of the sequences was done with ClustalX version 2.1, and the phylogenetic tree was constructed using the neighbor-joining method. The figure was generated with Tree View version 1.6.6. Sequences are as follows (organism name and accession number in parentheses): HadR (*Ralstonia pickettii* DTP0602, AB767276), DntR (*Burkholderia* sp. DNT, AAP70493.1), NagR (*Ralstonia* sp. U2, AAG13636.1), NtdR (*Acidovorax* sp. JS42, AAP70492.1), NbzR (*Comamonas* sp. JS765, AAP70491.1), NahR (*Pseudomonas putida* WCIB 9816-4, NP_863107.1), BphR2 (*Pseudomonas pseudoalcaligenes* KF707, AAZ08063.1), PcpR (*Sphingobium chlorophenolicum* ATCC 39723, AAM96667.1), LinR (*Sphingomonas paucimobilis* UT26, BAA36280.1), AmpR (*Citrobacter freundii* OS60, AAA64510.1), BlaA (*Proteus vulgaris* 5E78-1, BAA07083.1), GcvA (*Escherichia coli* 026:H11 str. 11368, YP_003230809.1), TrpI (*Pseudomonas aeruginosa* PAO1, NP_248727.1), NhaR (*Escherichia coli,* AAA24221.1), AphB (*Vibrio cholera* CG842, AAD45271.1), SpvR (*Salmonella enterica* subsp. enterica serovar Dublin str. CT_02021853, YP_002213864.1), GltC (*Bacillus subtilis* SMY, AAA16437.1), ClcR (*Pseudomonas putida* PRS2000, CAE92862.1), TcbR (*Pseudomonas aeruginosa* P51, ABP88941.1), CbnR (*Cupriavidus necator* NH-1,

BAA74529.1), TfdR (*Ralstonia eutropha* JMP134, AAB47014.2), TfdS (*Ralstonia eutropha* JMP134, AAR31051.1), CatR (*Pseudomonas putida* PRS2000, AAA66201.1), CatM (*Acinetobacter* sp. ADP1, AAC46429.1), BenM (*Acinetobacter* sp. ADP1, AAC46441.1), CbbR (*Xanthobacter flavus* H4-14, CAA80406.1), LigR (*Sphingobium* sp. SYK-6, YP_004834386.1), PcaQ (*Sinorhizobium meliloti* SM11, YP_005722466.1), OxyR (*Escherichia coli* str. K-12 substr. MG1655, NP_418396.1), MetR (*Escherichia coli* str. K-12 substr. MG1655, NP_418272.1), CysB (*Salmonella enterica* subsp. enterica serovar Typhi-murium str. LT2, NP_460672.1), CysL (*Bacillus subtilis* subsp. subtilis str. 168, NP_391645.1), CcpC (*Bacillus subtilis* subsp. subtilis str. 168, NP_389297.1), CynR (*Escherichia coli* K-12 substr. MG1655, NP_414872.3), OccR (*Agrobacterium tumefaciens,* AAA98378.1), NodD (*Rhizobium leguminosarum* bv. viciae, AA048452.1) and TcpR (*Cupriavidus necator* JMP134, AAZ60949.1).

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Chapter3. The Characterization of the HadP-HadQ system

3-1. SUMMARY

The two-component regulatory system, BphP-BphQ system of *Acidovorax* sp. KKS102 involved in catabolite repression (CR) of biphenyl catabolism. The genes highly similar to *bphQ* were found from several β-proteobacteria, such as *Burkholderia* and *Ralstonia*. But, the establishment of their roles, excepted for CR, in their original hosts did not know. In this study, we described the HadP-HadQ system of *Ralstonia pickettii* DTP0602 that showed homology with the BphP-BphQ system of KKS102. DTP0602 utilizes 2,4,6-trichlorophenol (2,4,6-TCP) as its sole source of carbon and the regulatory mechanism of catabolic pathway genes (*hadX-C* operon) for 2,4,6-TCP has been characterized. As queries to search the draft genome sequence of DTP0602, there were two open reading frames (ORF) that has 43, 64% similarity to BphP and BphQ. Two ORFs were named *hadP* and *hadQ*. With 2,4,6-TCP as a sole carbon source, the *hadP*, *hadQ* and *hadPQ* deletion mutant did not show the activation of *hadX-C* operon expression and dehalogenation of 2,4,6-TCP. The *hadP* and *hadQ* gene products showed homology with C_4-dicarboxylate sensor system (DctS and DctR) in *Rhodobacter capsulatus* and which are essential to utilization of C_4-dicarboxylate (succinate, fumarate and malate). However, the *hadPQ* deletion mutant showed the growth on C_4-dicarboxylate. With C_6-tricarboxylate (citrate) as a carbon source, the *hadPQ* deletion mutant showed no growth. Therefore, the HadP-HadQ that exhibited homology with C_4-dicarboxylate sensor system (DctS-DctR) system regulated the dehalogenation of 2,4,6-TCP, the activation of *hadX-C* operon expression and the utilization of citrate.

3-2. FOOTNOTE

Abbreviations: LTTR, LysR-type transcriptional regulator; 2,4,6-TCP, 2,4,6-trichlorophenol; MM, minimal medium; CR, catabolite repression; TCS, two-component regulatory system; Q-PCR, quantitative-PCR; EMSAs, Electrophoretic mobility shift assays

3-3. INTRODUCTION

There were the characterizations of regulatory mechanism including fermentation system and response system in carbon sources metabolism. The C_4-dicarboxylates (malate succinate and fumarate) and C_6-tricarboxylate (citrate) are effective carbon sources for the growth of many bacteria. In *Escherichia coli* and *Klebsiella pneumonia*, C_4-dicarboxylate and citrate-response and utilization system has been characterized. The two-component regulatory system (TCS), DcuS-DcuR and CitA-CitB system responded to C_4-dicarboxylates or citrate and regulated the C_4-dicarboxylates- or citrate-transporter genes (Bott *et al.*, 1995; Golby *et al.*, 1999). *Rhodobacter capsulatus* also contains a two-component regulatory system DctS-DctR. The *dctS-dctR* insertional mutants did not show growth on C_4-dicarboxylates. And, the *dctS* and *dctR* gene controlled expression of *dctPQM*, which were essential to the growth on the C_4-dicarboxylates (succinate, fumarate, malate) (Forward *et al.*, 1997; Hamblin *et al.*, 1993; Janausch *et al.*, 2002). Therefore, the DctS-DctR system was stimulated that DctS sensed the C_4-dicarboxylates and DctR regulated the C_4-dicarboxylate transporter.

Ralstonia pickettii strain DTP0602 utilizes 2,4,6-trichlorophenol (2,4,6-TCP) as its sole source of carbon and energy. A catabolic pathway for 2,4,6-TCP and the regulatory mechanism of 2,4,6-TCP degradation had been previously characterized (Fig. 1). The 2,4,6-TCP 4-monooxygenase gene (*hadA*), probable electron transfer protein gene (*hadB*), hydroxyquinol 1,2-dioxygenase gene (*hadC*) and maleylacetate reductase gene (*hadD*) were characterized, previously (Hatta *et al.*, 1999; Takizawa *et al.*, 1995). The *hadX* gene might be the 2,4,6-TCP monooxygenase component of a probable electron transfer gene. The *hadR* gene, which belongs to the LysR-type transcriptional regulator (LTTR), lies just

upstream from *hadX* in the opposite direction (accession no. AB767276). A single promoter of *hadX* is involved only in the transcription of the *hadX-C* operon, and HadR requires 2,4,6-TCP as a coinducer to positively regulate the transcription of the *hadX-C* operon. Furthermore, the *hadR* deletion mutant did not show growth on 2,4,6-TCP. These results supposed that HadR is only the regulator involved in the 2,4,6-TCP degradation (Torii *et al.*, 2013).

Ohtsubo *et al*. had been described the catabolite control in *Acidovorax* sp. KKS102 and observed that the two-component regulatory system (TCS), BphP-BphQ system involved in catabolite repression (CR) of biphenyl catabolism (2006). And, the genes highly similar to *bphQ* were found from several β-proteobacteria, such as *Burkholderia cenocepacia* J2315, *B*. *multivorans* ATCC17616, *B*. *xenovorans* LB400 and *Ralstonia solanacearum* RS1085. However, the establishment of their roles in their original hosts did not know in each β-proteobacteria. In this study, to perform the characterization the HadP-HadQ system (BphP-BphQ homologues of *Ralstonia pickettii* DTP0602), we constructed the *hadP-hadQ* deletion mutants and growth test on several carbon sources. The expression of *hadX-C* operon, the dehalogenation of 2,4,6-TCP and utilization of citrate (C_6-tricarboxylate) were regulated by the HadP-HadQ system. This study provides a new insight into citrate and aromatic compounds utilization.

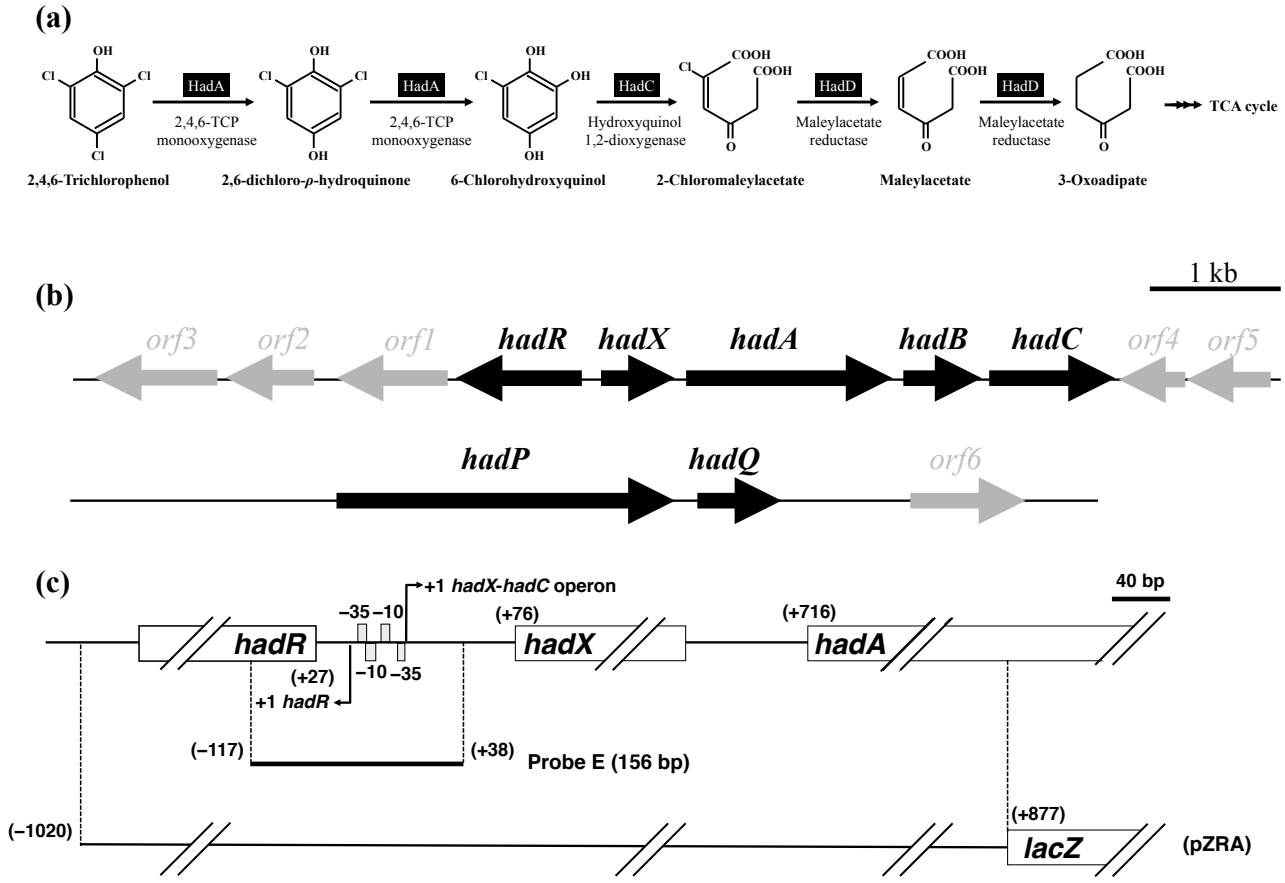

FIG. 1. 2,4,6-TCP catabolic pathway and gene cluster in *Ralstonia pickettii* DTP0602. (a) 2,4,6-TCP catabolic pathway. (b) Identical genetic organization of the *had* genes catalyzing 2,4,6-TCP degradation. Bar represents 1 kb. (c) DNA fragments used for pzRA (dotted bars), and the EMSA (probe E; thicker bar). The numbers noted next to the fragments indicate the positions of the ends of the fragments relative to the TSP of the *hadX-C* operon.

3-4 MATERIALS AND METHODS

3-4-1. Bacterial strains, chemicals and DNA manipulation

The strains and plasmids used in this study are listed in Table 1. For routine culture, *R. pickettii* DTP0602 and the DTP0602 derivative strain were grown in 1/5 Luria-Bertani (LB) medium or minimal medium (MM) containing 0.3% (w/v) succinate (Wako Pure Chemical Industry, Osaka, Japan) at 30°C, with shaking (140 r.p.m.) (Kiyohara *et al.*, 1992). *Pseudomonas putida* PpY101 and derivative strain were grown in 1/5 LB at 30°C with shaking (Fukuda & Yano, 1985). *Escherichia coli* SI7-1 or S17-l λpir were used for biparental filter mating and were grown in LB at 37°C with shaking (Miller & Mekalanos, 1988; Simon *et al.*, 1983). Antibiotics were used at the following concentrations for *E. coli* strains: 25 μg ml^{-1} of kanamycin, 25 μg ml^{-1} of chloramphenicol, 12.5 μg ml^{-1} of tetracycline and 100 μg ml^{-1} of ampicillin, for DTP0602 derivative strain: 100 μg ml^{-1} of kanamycin, 25 μg ml^{-1} of tetracycline, for PpY101 derivative strain: 50 μg ml^{-1} of kanamycin, 250 μg ml^{-1} of chloramphenicol.

The 2,4,6-TCP used in this study was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and was dissolved in DMSO and stored at −20°C.

The primers used in this study are listed in Table 2 in the supplemental material. Plasmid isolation, restriction enzyme digestion, DNA ligation and *E. coli* transformation were performed as described by Sambrook *et al.* (1989). PCR was performed with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan).

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Description*	Reference(s)
R. pickettii		
DTP0602	Wild type; 2,4,6-TCP^r	Kiyohara *et al.* (1989)
DTP62P	DTP0602 derivative; *hadP* null-mutant	This study
DTP62Q	DTP0602 derivative; *hadQ* null-mutant	This study
DTP62PQ	DTP0602 derivative; *hadPQ* null-mutant	This study
DTP0602-pJB866	DTP0602 harboring pJB866; Te^r	This study
DTP62P-pJB866	DTP62P harboring pJB866; Te^r	This study
DTP62Q-pJB866	DTP62Q harboring pJB866; Te^r	This study
DTP62PQ-pJB866	DTP62PQ harboring pJB866; Te^r	This study
DTP62P-pJTP6	DTP62P harboring pJTP6; Te^r	This study
DTP62Q-pJTQ1	DTP62Q harboring pJTQ1; Te^r	This study
DTP62PQ-pJTPQ1	DTP62PQ harboring pJTPQ1; Te^r	This study
DTP62ZA	DTP0602 derivative; *hadA*::*lacZ*; Km^r; *lacZ* was inserted 60-bp downstream from ATG translation codon of *hadA*	Torii *et al.* (2013)
DTP62ZAd	DTP0602 derivative; *hadA*::*lacZ*; Km^r; *lacZ* was inserted 300-bp downstream from ATG translation codon of *hadA*	This study
DTP62PZA	DTP62P derivative; *hadA*::*lacZ*; Km^r	This study
DTP62QZA	DTP62Q derivative; *hadA*::*lacZ*; Km^r	This study
DTP62PQZA	DTP62PQ derivative; *hadA*::*lacZ*; Km^r	This study
DTP62ZA-pJB866	DTP62ZA harboring pJB866; Te^r, Km^r	This study
DTP62PZA-pJB866	DTP62PZA harboring pJB866; Te^r, Km^r	This study
DTP62QZA-pJB866	DTP62QZA harboring pJB866; Te^r, Km^r	This study
DTP62PQZA-pJB866	DTP62PQZA harboring pJB866; Te^r, Km^r	This study
DTP62PZA-pJTP6	DTP62PZA harboring pJTP6; Te^r, Km^r	This study
DTP62QZA-pJTQ1	DTP62QZA harboring pJTQ1; Te^r, Km^r	This study
DTP62PQZA-pJTPQ1	DTP62PQZA harboring pJTPQ1; Te^r, Km^r	This study
E. coli		
S17-1	*thi pro hsdR hsdM*$^+$ *recA*; integrated plasmid RP4-Tc::Mu-Km::Tn7	Simon *et al.* (1983)
S17-1 λpir	*thi pro hsdR hsdM*$^+$ *recA*; integrated plasmid RP4-Tc::Mu-Km::Tn7 *λpir*	Kalogeraki *et al.* (1997)
P. putida		
PpY101	*met*; Nal^r	Fukuda *et al*. (1985)
PpY101-pZRA, pTS1210	PpY101 harboring pZRA and pTS1210; Cm^r, Km^r	This study
PpY101-pZRA, pTSPQ	PpY101 harboring pZRA and pTSPQ; Cm^r, Km^r	This study
Plasmid		
pK18*mobsacB*	Integration vector, *oriT*, *sacB*, Km^r	Simon *et al.* (1983)
pVIK111	Suicide vector for *lacZ* translational fusions	Kalogeraki & Winans (1997)
pJB866	Expression vector containing *xylS* and the Pm promoter, Te^r Tra$^-$ Mob$^+$ IncP	Blatny *et al.* (1997)
pPR9TZ	pPR9TT with a 3.6-kb *SmaI*-*ScaI* fragment containing *lacZ*	Kamimura *et al.* (2010)
pTS1210	Broad-host-range vector, pSa *ori* pBR *ori*, Km^r Ap^r	T. Nakazawa
pNC2	pUC19 with a 5.3-kb EcoRI-EcoRI fragment carrying *orf1*, *hadR*, *hadX*, *hadA*, and *hadB*	This study
pK18*mobsacB*Δ*hadP*	pK18*mobsacB* with a 3.0-kb PCR fragment carrying the flanking regions of *hadP* from DTP0602	This study
pK18*mobsacB*Δ*hadQ*	pK18*mobsacB* with a 3.0-kb PCR fragment carrying the flanking regions of *hadQ* from DTP0602	This study
pK18*mobsacB*Δ*hadPQ*	pK18*mobsacB* with a 3.0-kb PCR fragment carrying the flanking regions of *hadPQ* from DTP0602	This study
pVA1	pVIK111 with a 0.7-kb PCR fragment carrying 60-bp 5'end of *hadA*	Torii *et al.* (2013)
pVAd	pVIK111 with a 1.0-kb PCR fragment carrying 1,300-bp 5'end of *hadA*	This study
pJTP6	pJB866 with a 2.8-kb PCR fragment carrying *hadP* from DTP0602	This study
pJTQ1	pJB866 with a 0.9-kb PCR fragment carrying *hadQ* from DTP0602	This study
pJTPQ1	pJB866 with a 3.7-kb PCR fragment carrying *hadPQ* from DTP0602	This study
pZRA	pPR9TZ with a 2.0-kb PCR fragment carrying *hadR*, *hadX* and 50-bp 5'end of *hadA* from DTP0602	This study
pTSPQ	pTS1210 with a 3.7-kb PCR fragment carrying *hadPQ* from DTP0602	This study

* Cm^r, Km^r and Te^r resistance to chloramphenicol, kanamyci and tetracycline.

Table 2. Oligonucleotides used as PCR primers

Primer and fuction(s)	**Sequence (5'-3')***
For construction of the gene disruption strain and complementation vectors	
hadP-EcoRI-FF	AATTGAATTCCAGCGCAGCAACAAT
hadP-BamHI-FR	CACAGGATTCACAGCAGGAAGCAGG
hadP-BamHI-RF	GTGGGGATTCATATCGACGGCATCG
hadP-HindIII-RR	TGTCAAGCTTCTGGGGCACGGCTAT
hadQ-EcoRI-FF	TTCGGAATTCACATGCAGAAGTGGT
hadQ-XbaI-FR	CTTCTCTAGAGTCGACGATGAACAC
hadQ-XbaI-RF	CAACTCTAGAGAAAAGCTCAACGTC
hadQ-HindIII-RR	GGCAAAGCTTGACCTTGTTGCGCAC
hadQ-F	CCATGGCCGGTTACACTTCG
hadQ-R	TGGTCTACCGGCCACAATGG
For construction of pzRA	
hadR-EcoRI-F	AGCGGAATTCCCGGCATAGCCACCATAACC
For Q-PCR analysis	
qhadX-F	GCAGCCAGGACAGAGAAGTTCCCG
qhadA-F	CAGTACCTGGAATCCCTGAACGACG
qhadB-F	CACCATTTCCCTTGCTACCCTGTT
qhadX-R	CGGGTTGTCGCAGACCGAACAGAC
qhadA-R	TGCAGGTCCGGGCGATGGTGCA
qhadB-R	CTTGGCTTCCATGCTGCGGACGAA
For confirmation of the HadR binding siteE-F	CCAGGTTTCGCCACCGGCGCGATGCGCGCT
siteE-R	TGCAGGTGATGCAGGCCCAGATCGTCTTCA
For construction of DTP62ZAd (*hadA*::*lacZ* fusion) strain	
hadAz2-EcoRI-F	AAAGAATTCTGACCACCTACATCGACGACCC
hadAz2-XbaI-R	GGGTCTAGATCGGTGAGGAACAGGTCGCGGAT

* Primers include a restriction site which is underlined.

3-4-2. RNA isolation and Q-PCR analysis

DTP0602 were grown in MM containing 10 mM L-aspartate and harvested at an OD_{600} of 0.4–0.5 (mid-log phase). The cells were centrifuged and the pellet was washed twice with MM, the cell pellet was suspended to an OD_{600} of 1.0 in the MM. The cell suspension (15 ml) was placed in a 200-ml baffle flask. Subsequently, 200 μM 2,4,6-TCP or 4 mM organic compounds were added to the cell suspension and agitated. The mRNA copy number of *hadX-C* operon peaked at 4 h (data not shown). Therefore, incubation time was defined as the point at which the peak of mRNA copy number of *hadX-C* operon (4 h). To isolate total RNA, the cells that were incubated with the appropriate growth condition were centrifuged to pellet the cells. The isolation of total RNA and Q-PCR analysis were described previously (Torii *et al.*, 2013).

3-4-3. The measurement of the dechlorination of 2,4,6-TCP, the growth on organic compounds and β-galactosidase activity

DTP0602, derivative strain (DTP62ZA and DTP62ZAd) and *P.putida* derivative strain grown in 1/5 LB or MM containing 10 mM L-aspartate and were prepared and harvested at an OD_{600} of 0.75–0.80 (mid-log phase on 1/5 LB medium or on MM containing aspartate). The cells were centrifuged and the pellet was washed twice with MM.

For β-galactosidase assay, the cell pellet was suspended to an OD_{600} of 0.5 or 1.0 in the MM. The cell suspension (2 ml) was placed in a test tube. Subsequently, 200 μM 2,4,6-TCP or 4 mM organic compounds were added to the cell suspension and agitated. The β-galactosidase activity of DTP0602 derivative strain and *P. putida* derivative strain peaked at 2, 4 h and 10 h, respectively (unpublished data). Therefore, incubation time (2, 4 and 10 h) was defined as the point at which the peak of the rate of β-galactosidase translation.

After incubation, 100 µl of DTP0602 derivative cells was mixed with 900 µl of Z buffer and assayed for β-galactosidase activity. β-galactosidase assay was described previously (Torii *et al.*, 2013)

For the confirmation of the dechlorination of 2,4,6-TCP and the growth on organic compounds, the cell pellet was suspended to an OD_{600} of 0.05 in the MM. The cell suspension (40 ml) was placed in a 200-ml baffle flask. Subsequently, 500 µM 2,4,6-TCP or 10 mM organic compounds were added to the cell suspension and agitated. Growth was determined by measuring optical density at 600 nm (OD_{600}). The determination of the amount of chloride released by the mercuric thiocyanate method described by Iwasaki *et al.*(1952). The 0.6 ml of DTP0602 and derivative cells were centrifuged, and the supernatant was mixed with mercuric thiocyanate and ferric ammonium sulfate. After 10min, halide production was monitored spectrophotometrically at 460 nm.

3-4-4. EMSAs

The purification of HadR and EMSAs were described previously (Torii *et al.*, 2013). The DNA fragment for EMSAs was amplified by PCR using primer pair siteE-F/siteE-R with pNC2 as the template (Fig. 1a). The DNA–protein binding reactions were carried out at 25°C in a final volume of 10 µl containing 1 ng of DIG-labeled probe E, 100 µM 2,4,6-TCP or 5 mM organic compounds as coinducers and the purified HadR (0.35 µM) for 15 min.

3-4-5. DNA transformations of *R. pickettii* and *P. putida*

E. coli SI7-1 or S17-l λpir were used for biparental mating. *E. coli* strains (donor) and *R. pickettii* DTP0602 and *P.putida* PpY101 (recipients) were cultivated in LB or 1/5 LB. The biparental mating was described previously (Torii *et al.*, 2013).

3-4-6. Recombinant DNA work

To construct a *hadA::lacZ* reporter fusion in DTP0602, the PCR fragment containing the 5′ end of *hadA* (1300-bp) was amplified by PCR with the primer hadAz2-EcoRI-F/hadAz2-XbaI-R and was digested with *Eco*RI and *Xba*I. This fragment was ligated into *Eco*RI/*Xba*I -digested pVIK111 (Kalogeraki & Winans, 1997). The resulting plasmid (pVAd) was transferred into DTP0602 by biparental filter mating using *E. coli* SI7-1 λpir as a donor strain, resulting strain were named DTP62ZAd.

To disrupt *hadP* and *hadQ,* the flanking region of each gene was amplified using *R. pickettii* DTP0602 chromosomal DNA as the template. The upstream and downstream flanking regions of *hadP* and *hadQ* were PCR-amplified by using primers hadP-EcoRI-FF/hadP-BamHI-FR, hadP-BamHI-RF/hadP-HindIII-RR, hadQ-EcoRI-FF/hadQ-XbaI-FR and hadQ-XbaI-RF/hadQ-HindIII-RR. These PCR fragment was TA-cloned into pTA2 (Toyobo) and resulting plasmids were digested with *Eco*RI, *Xba*I or *Bam*HI, *Xba*I or *Bam*HI and *Hind*III, respectively. After digestion, the upstream and downstream flanking regions of *hadP* or *hadQ*, the upstream of *hadP* and the downstream of *hadQ* were simultaneously ligated into the pK18*mobsacB*. The resulting plasmids were named pK18*mobsacB*Δ*hadP*, pK18*mobsacB*Δ*hadQ* and pK18*mobsacB*Δ*hadPQ*. These suicide plasmids were independently transferred into *R. pickettii* DTP0602 by biparental filter mating using *E. coli* S17-1, resulting strain were named DTP62P, DTP62Q and DTP62PQ.

To construct a *hadA::lacZ* reporter fusion in DTP62P, DTP62Q and DTP62PQ, the pVA1 plasmid was transferred into these strains by biparental filter mating using *E. coli* SI7-1 λpir as a donor strain. The kanamycin-resistant colony with the *hadA::lacZ* reporter fusion was denoted as a colony arising from the strain DTP62PZA (*hadP*$^{-}$, *hadA*::*lacZ*), DTP62QZA (*hadQ*$^{-}$, *hadA*::*lacZ*) and DTP62PQZA (*hadPQ*$^{-}$, *hadA*::*lacZ*).

Plasmid pJB866 was used for the construction of *hadP* and *hadQ* complementation vectors. The flanking region of *hadP*, *hadQ* and *hadPQ* were amplified with primer hadP-EcoRI-FF/hadQ-XbaI-FR, hadQ-F/hadQ-R, and hadP-EcoRI-FF/hadQ-R. These PCR fragments was TA-cloned into pTA2 and resulting plasmids was digested *Eco*RI. This fragment was ligated into *Eco*RI-digested pJB866 to generate pJTP6, pJTQ1, and pJTPQ1. These plasmids were introduced into DTP0602, DTP62P, DTP62Q, DTP62PQ, DTP62ZA and DTP62PQZA by biparental filter mating, resulting strain were named DTP0602-pJB866, DTP62P-pJB866, -pJTP6, DTP62Q-pJB866, -pJTQ1, DTP62PQ-pJB866, -pJTPQ1, DTP62ZA-pJB866, DTP62PQZA-pJB866 and DTP62PQZA-pJTPQ1, respectively.

To perform β-galactosidase assay in *P. putida* PpY101, pPR9TZ and pTS1210 were used. The flanking region of *hadR*, *hadX* and *hadA* was amplified with primer hadR-EcoRI-F/qhadA-R. The PCR fragment was TA-cloned into pTA2 and resulting plasmids was digested *Bam*HI and *Xho*I. This fragment was ligated into *Bgl*II/*Xho*I-digested pJB866 to generate pZRA. The PCR-amplified *hadPQ* fragment (for complementation vector) was ligated into *Eco*RI-digested pTS1210 to generate pTSPQ. These plasmids were transferred into PpY101 by electroporation, resulting strain were named PpY101-pZRA, pTS1210 and PpY101-pZRA, pTSPQ.

3-4-7. Statistics and data analysis

The Q-PCR analysis and β-galactosidase assay were performed using a Student's *t*-test. A *p*-value of less than 0.05 was considered significant. Each measurement was carried out at least in triplicate, and the means and standard deviations were calculated.

3-5. RESULTS

3-5-1. The catabolite repression on the *hadX-C* operon transcription

Previously, kiyohara *et al*. suggested that due to catabolite repression, DTP0602 preferentially consumes succinate or glucose over 2,4,6-TCP (Kiyohara *et al.*, 1992). The expression of *hadX-C* operon was examined in the presence of 2,4,6-TCP and 2,4,6-TCP plus succinate by using the Q-PCR analysis and β-galactosidase assay in DTP0602, since the dehalogenation of 2,4,6-TCP was repressed by the presence of succinate.

First, we investigated the effects of several organic compounds on the *hadX* promoter using strain DTP62ZA, which carried in its chromosome a *lacZ* gene inserted at the 60-bp downstream from ATG translation codon of *hadA*. However the repression patterns were not measured exactly, because the β-galactosidase activity was high in the presence of 2,4,6-TCP plus succinate. Therefore, DTP62ZAd, which carried in its chromosome a *lacZ* gene inserted at the 1,300-bp downstream from ATG translation codon of *hadA*, was constructed and measured. The β-galactosidase activity was significantly repressed 0.22-, 0.62, 0.33-, 0.61-, 0.064- and 0.20-fold by six organic compounds – succinate, fumarate, malate, citrate, pyruvate and acetate in DTP62ZAd (Table 3). The mRNA copy number of *hadX*, *hadA* and *hadB* was significantly repressed 0.19-, 0.22- and 0.21-fold in the presence of 2,4,6-TCP plus succinate as compared to the amount of mRNA copy number in the presence of 2,4,6-TCP (Table 4). These results indicated that the decrease of the activation level of *hadX* promoter participate in catabolite repression of the dehalogenation of 2,4,6-TCP.

Here, we raise the possibility that HadR protein do not bind in the presence of 2,4,6-TCP plus carbon source, because HadR directly regulate the transcription of *hadX-C*

operon. To confirm this possibility, EMSAs were performed in the presence of 2,4,6-TCP plus succinate or glucose. A previous study demonstrated that $HadR^{mt}$ binds specifically to the probe E and $HadR^{mt}$–DNA complex formation was induced in the presence of 2,4,6-TCP (Fig. 1c). Irrespective of the presence or absence of carbon source, the $HadR^{mt}$–DNA complex formation was not changed (Fig. 2). Furthermore, β-galactosidase assay were performed using *P. putida* –pZRA, pTS1210. The β-galactosidase activity was not decreased in the presence of 2,4,6-TCP plus succinate with respected to this with 2,4,6-TCP (Table 6). These results showed that HadR protein does not play a role in catabolite control and suggested he involvement of an unidentified regulatory system that modulates the expression of the *hadX-C* operon in response to the preferred carbon sources.

Table 3. Activity of the *hadA*::*lacZ* fusion in DTP62ZAd in the presence of 2,4,6-TCP plus carbon sources

Coinducers*	**β-galactosidase activity† (fold induction‡)**				
2,4,6-TCP	2001	±	409		
2,4,6-TCP + succinate	431	±	28.3	(0.22)	§
2,4,6-TCP + fumarate	1238	±	250	(0.62)	§
2,4,6-TCP + malate	651	±	109	(0.33)	§
2,4,6-TCP + citrate	1215	±	125	(0.61)	§
2,4,6-TCP + pyruvate	129	±	7.34	(0.064)	§
2,4,6-TCP + acetate	404	±	15.4	(0.20)	§
2,4,6-TCP + ethanol	3137	±	261	(1.6)	§
2,4,6-TCP + lactose	2967	±	77.0	(1.5)	§

*TCP indicate trichlorophenol.

†Expressed as Miller units ± standard deviation.

‡Fold induction = Miller units (2,4,6-TCP plus carbon source)/Miller units (2,4,6-TCP).

§ $P < 0.05$, versus with none, Student's t test.

Table 4. The absolute mRNA copy numbers of *hadX*, *hadA* and *hadB* in the presence of coinducers in DTP0602

	Absolute mRNA copy number†, ‡ (fold induction§) in DTP0602		
Coinducers*	***hadX***	***hadA***	***hadB***
2,4,6-TCP	192 ± 58.8	6880 ± 2111	3016 ± 803
2,4,6-TCP plus succinate	37.4 ± 6.38 (0.19) ||	1510 ± 443 (0.22) ||	629 ± 193 (0.21) ||

* TCP indicate trichlorophenol.

† Experessed as absolute mRNA copy number ($\times 10^6$)/total RNA (μg).

‡ Absolute mRNA copies of respective genes are the average ± standard deviation of three independent experiments.

§ Fold induction = absolute mRNA copy number (2,4,6-TCP plus succinate)/absolute mRNA copy number (2,4,6-TCP).

\|\| $P < 0.05$, versus with 2,4,6-TCP, Student's *t* test.

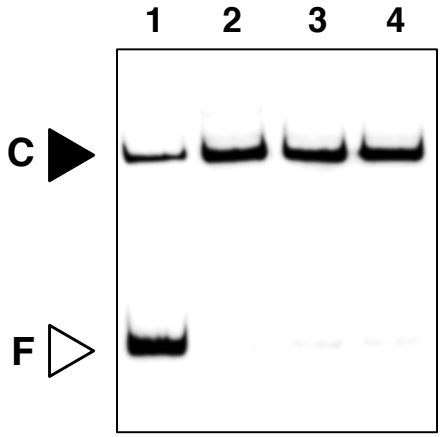

FIG. 2. EMSA of the binding of HadR^{mt} in the presence of 2,4,6-TCP and carbon source. EMSA with HadR^{mt} and DIG-labeled E. 100 μM 2,4,6-TCP and 4 mM glucose and 4 mM succinate used as coinducers in the reaction mixtures is indicated. Lane 1, EMSA with HadR^{mt}; lane 2, EMSA with HadR^{mt} and 2,4,6-TCP; lanes 3, EMSA with HadR^{mt}, 2,4,6-TCP and succinate; lanes 4, EMSA with HadR^{mt}, 2,4,6-TCP and glucose. Free DNA and the HadR^{mt}–DNA complex are indicated by open arrowheads and closed arrowheads.

3-5-2. Identification of the *hadP* and *hadQ* gene

Ohtsubo *et al*. suggested that *bphQ* of *Acidovorax* sp. KKS102 and *bphQ* homologues in several β-proteobacteria were the genes for catabolite control in this group of bacteria (Ohtsubo *et al.*, 2006). However, the establishment of their roles in catabolite control in their original hosts needs further investigations in each strain. Here, we arise the possibility that *bphQ* homologues in DTP0602 involved another mechanism of utilization.

As queries to search the draft genome sequence of DTP0602, there were two open reading frames (ORF) that has 43, 64% similarity to BphP and BphQ. Two ORFs were named *hadP* and *hadQ* (Fig. 1b). HadP had a histidine residue that is conserved and involved in phosphorylation in many sensor kinases. The hydropathy plot of HadP predicted that it contains one transmembrane domain at its N-terminus. The deduced protein product of the *hadQ* gene, which had a putative signal receiving domain at its N-terminus and a helix–turn–helix DNA-binding motif at its C-terminus, was similar to the response regulators of two-component regulatory systems. Orf6 is located downstream from *hadQ* and exhibit homology with 5,10-methylene-tetrahydrofolate dehydrogenase (accession no. YP_725874.1) in *Ralstonia eutropha* H16.

3-5-3. The dehalogenation of 2,4,6-TCP in *hadP* and *hadQ* deletion mutant

To demonstrate the role of HadP and HadQ for the 2,4,6-TCP utilization in *R. pickettii*, *hadP*, *hadQ* and *hadPQ* deletion mutants (DTP62P, DTP62Q and DTP62PQ) were constructed through homologous recombination. These mutants and DTP0602 were grown on 2,4,6-TCP and measured the amount of chloride released. DTP0602 released approximately 1.5 mM chloride ions, suggesting that 3 mol of chlorine atoms was liberated from 1mol of 500 μM 2,4,6-TCP (Fig. 3a). On the other hand, the DTP62P, DTP62Q, DTP62PQ, DTP62P-pJB866, DTP62Q-pJB866 and DTP62PQ-pJB866 did not release chloride ions. On the other hand, complementation strain (DTP62P-pJTP6, DTP62Q-pJTQ1 and DTP62PQ-pJTPQ1) released approximately 1.5 mM chloride ions (Fig.3b and 3c). Therefore, HadP and HadQ protein were necessary for dechlorination of 2,4,6-TCP that is the initial step in the 2,4,6-TCP degradation pathway.

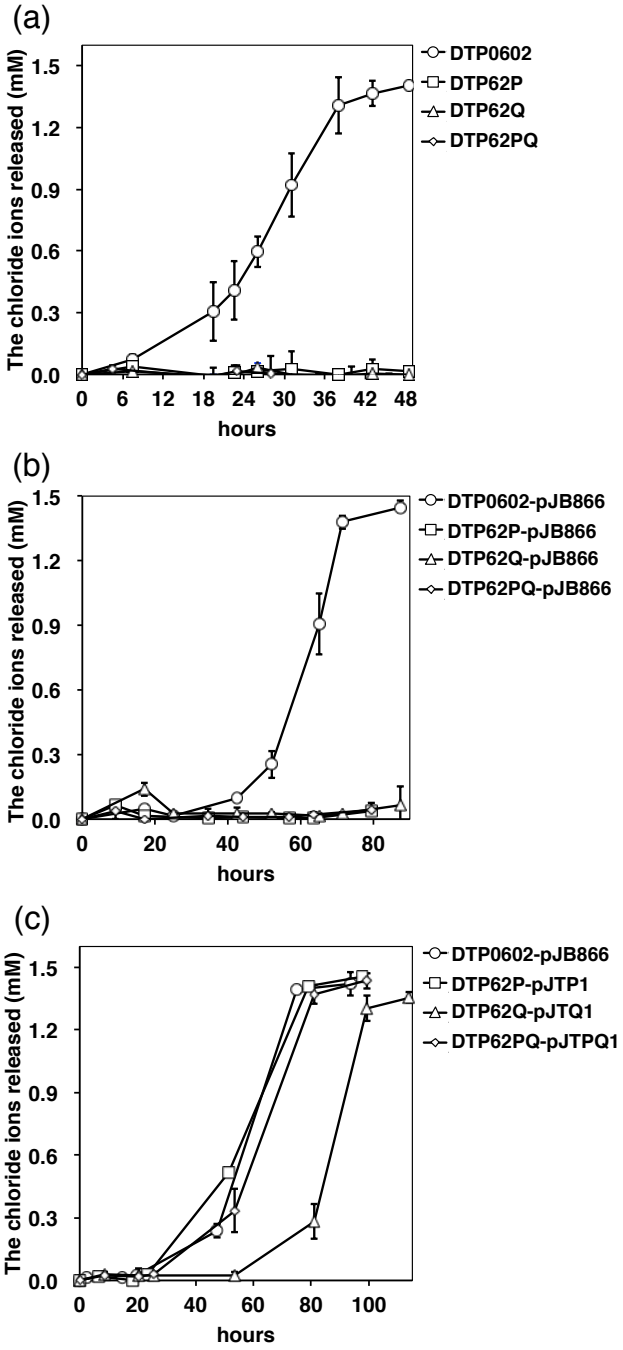

Fig. 3. The chloride ions released of *R. pickettii* DTP0602, mutant strain of *hadP, Q, PQ* and complementation with *hadP, Q, PQ*. (a) The chloride ions released of DTP0602, DTP62P, DTP62Q and DTP62PQ. (b) The chloride ions released of DTP0602-pJB866, DTP62P-pJB866, DTP62Q-pJB866 and DTP62PQ-pJB866. (c) The chloride ions released of DTP0602-pJB866, DTP62P-pJTP1, DTP62Q-pJTQ1 and DTP62PQ-pJTPQ1.

3-5-4. The activation of the *hadX-C* operon expression by HadP and HadQ

For the confirmation of the regulation of *hadX-C* operon transcription by HadP and HadQ, β-galactosidase assay were performed to monitor the *hadA* expression by using DTP62ZA (wild type, *hadA*::*lacZ*), DTP62PZA (Δ*hadP*, *hadA*::*lacZ*), DTP62QZA (Δ*hadQ*, *hadA*::*lacZ*) and DTP62PQZA (Δ*hadPQ*, *hadA*::*lacZ*). In DTP62ZA, β-galactosidase activity was induced 40-fold in the presence of 200 μM 2,4,6-TCP relative to that in the absence of coinducer. In the presence of 2,4,6-TCP, the *hadX* promoter activity was decreased by a deletion of *hadP*, *hadQ* and *hadPQ*, since β-galactosidase activity was induced by 0.51-, 03.6- and 0.45-fold in DTP62PZA, DTP62QZA and DTP62PQZA. The complementation vectors (pJTP6, pJTQ1 and pJTPQ1) and pJB866 were introduced into DTP0602, DTP62PZA, DTP62QZA and DTP62PQZA (DTP62ZA-pJB866, DTP62PZA-pJB866, DTP62QZA-pJB866, DTP62PQZA-pJB866, DTP62PZA-pJTP6, DTP62QZA-pJTQ1 and DTP62PQZA-pJTPQ1). With 2,4,6-TCP, the β-galactosidase activity was increased 2.0-, 4.2-, and 2.5-fold in DTP62PZA-pJB866 DTP62QZA-pJB866 and DTP62PQZA-pJB866. On the other hand, it was increased 150-, 84- and 16-fold in DTP62PZA-pJTP6, DTP62QZA-pJTQ1 and DTP62PQZA-pJTPQ1 (Table 5). These results indicated that the HadP and HadQ protein involved the activation of *hadX-C* operon expression.

To test whether or not the HadP and HadQ directly involves in the activation of the *hadX* promoter, β-galactosidase assay was performed by using *P.putida*-pZRA, pTS1210 (*hadPQ*$^{-}$) and -pZRA, pTSPQ (*hadPQ*$^{+}$) as surrogate host. The β-galactosidase levels with 2,4,6-TCP in *P.putida*-pZRA, pTSPQ were 1.6-fold higher, as compared to those in *P.putida*-pZRA, pTS1210 (Table 6).

These findings demonstrated that the activation of *hadX* promoter was regulated the HadP-HadQ system and might indicated that HadP protein sensed 2,4,6-TCP and HadQ activated the *hadX* promoter.

Table 5. Activity of the *hadA*::*lacZ* fusion in DTP0602, *hadP*, *hadQ* deletion mutant and complementation strains

Strain	β-galactosidase activity* (fold induction†)	
	none	2,4,6-TCP‡, §
DTP62ZA (wild type)	3.26 ± 0.122	130 ± 5.90 (40)
DTP62PZA (Δ*hadP*)	2.13 ± 0.00262	1.09 ± 0.00362 (0.51)
DTP62QZA (Δ*hadQ*)	2.15 ± 0.00268	7.75 ± 0.00991 (3.6)
DTP62PQZA (Δ*hadPQ*)	3.17 ± 0.00670	1.44 ± 0.628 (0.45)
DTP62ZA-pJB866 (negative control)	5.32 ± 0.627	266 ± 23.6 (50)
DTP62PZA-pJB866 (negative control)	0.911 ± 0.00115	1.84 ± 0.00234 (2.0)
DTP62QZA-pJB866 (negative control)	4.6 ± 0.00585	19.2 ± 0.52974 (4.2)
DTP62PQZA-pJB866 (negative control)	1.82 ± 0	4.55 ± 0.01147 (2.5)
DTP62PZA-pJTP6 (*hadP* complement strain)	2.81 ± 1.85	414 ± 17.6 (150)
DTP62QZA-pJTQ1 (*hadQ* complement strain)	7.23 ± 0.679	609 ± 2.74 (84)
DTP62PQZA-pJTPQ1 (*hadPQ* complement strain)	9.8 ± 0.628	161 ± 12.6 (16)

*Expressed as Miller units ± standard deviation.

†Fold induction = Miller units (2,4,6-TCP added)/Miller units (none).

‡TCP indicate trichlorophenol.

§ $P < 0.05$, versus without coinducer, Student's *t* test.

Table 6. Activity of the *hadA*::*lacZ* fusion in *P. putida*

Plasmids and coinducers*	**β-galactosidase activity† (fold induction)**
pzRA and pTS1210	
none	38.6 ± 1.66
succinate	31.1 ± 0.590
2,4,6-TCP	72.0 ± 5.89 (1.9) ‡, ‖
2,4,6-TCP + succinate	432 ± 13.7 (11) ‡, ‖
pzRA and pTSPQ	
none	38.9 ± 2.94
2,4,6-TCP	113 ± 11.0 (2.9) ‡, ‖

* TCP indicate trichlorophenol.

† Expressed as Miller units ± standard deviation.

‡ Fold induction = Miller units (2,4,6-TCP)/Miller units (none).

‖ $P < 0.05$, versus with none, Student's t test.

3-5-5. Growth properties of the *hadPQ* deletion mutant

HadP shared 97% and 35 % amino acids identity with C_4-dicarboxylate transport sensor protein DctS of *Cupriavidus necator* N-1 (YP_004685227.1) and in *Rhodobacter capsulatus* (YP_003579156.1). The HadQ protein shared with 99% and 38 % amino acids identity with NarL family response regulator of *Cupriavidus necator* N-1 (YP_004685226.1) and DctR of *R. capsulatus* (YP_003579157.1). HadP shared 14%, 10% and 16% amino acids identity with the citrate sensor kinase TctE of *Salmonella thphimurium*, CitA of *E. coli* and CitA of *Klebsiella pneumoniae* (Bott *et al.*, 1995; Liu *et al.*, 2008; Scheu *et al.*, 2012). The possibility that the regulatory defect of the *hadPQ* mutant leads to an associated growth deficiency was tested with a *hadPQ* deletion mutant DTP62PQ, because the DctS-DctR system was essential to C_4-dicarboxylate metabolism (Hamblin *et al.*, 1993; Janausch *et al.*, 2002).

The parental (DTP0602) and DTP62PQ strain grew identically on minimal medium agar plate containing 10 mM citrate, succinate, fumarate or malate as the sole carbon source (Fig. 4). The DTP62PQ strain grew on succinate, fumarate or malate as same extent as DTP0602. With citrate as a carbon source, the DTP62PQ showed no growth, whereas the DTP62PQ-pJTPQ1 (*hadPQ* complementation strain) showed the growth on citrate. These results demonstrated that the HadP-HadQ system did not involved in the utilization of C_4-dicarboxylates, but essential to the utilization citrate metabolism in DTP0602.

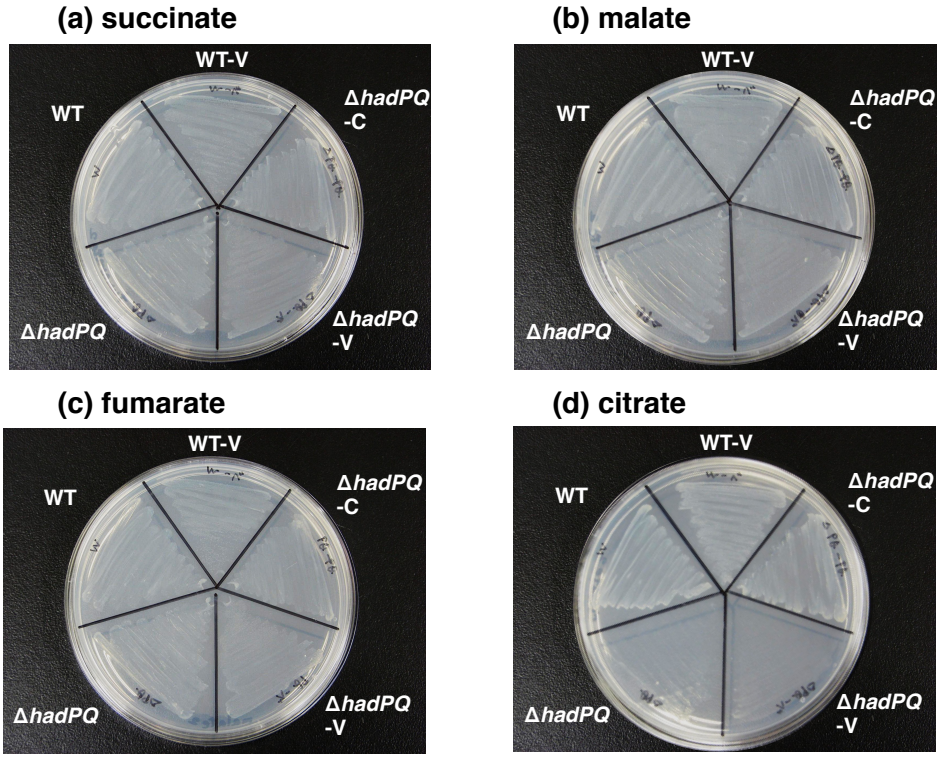

Fig. 4. Growth of the *hadPQ* deletion mutant and DTP0602 in MM containing 10 mM succinate (A), fumarate (B), malate (C) and citrate (D). WT, WT-V, Δ*hadPQ,* Δ*hadPQ*-V and Δ*hadPQ*-C were indicated DTP0602, DTP0602-pJB866, DTP62PQ, DTP62PQ-pJB866 and DTP62PQ-pJTPQ1.

3-6. DISCUSSION

As described in introduction, the response mechanisms of citrate and C_4-dicarboxylates have been clarified in several bacteria. And, a large variety of aromatic compounds metabolic pathways have been discovered in very different microorganisms. It was discovered that a large diversity of regulatory systems (LTTR family, IclR family, DntR family, MarR family and two-component family) existed for mediating the expression of catabolic pathways. Established gene regulatory paradigms indicate that the response system of TCA cycle compounds and aromatic compounds degradation system operate independently. The β-galactosidase assay and growth test by using wild type and the *hadPQ* deletion mutant indicated that *hadPQ* essential to the expression of 2,4,6-TCP catabolic operon and the growth of citrate. Therefore, these results indicated that the HadP-HadQ system of DTP0602 that has closely related to the BphP-BphQ system of KKS102 regulated citrate metabolism and the dehalogenation of 2,4,6-TCP. Furthermore, this is the first report that the regulator that involved in the utilization of TCA cycle compounds is essential to the first step in the aromatic compounds degradation. If HadP sense C_6-tricarboxylate and 2,4,6-TCP and activate the phosphorylation of HadQ, possible mechanisms of signal reception could suggest that the regulatory mechanism of citrate metabolism also serve as activator of chlorophenols degradation. The answer to this possibility awaits further investigation.

Our results indicated that the HadP and HadQ were essential to the 2,4,6-TCP utilization of DTP0602. In addition to LysR-type transcriptional regulator HadR, the two-component regulatory system HadP and HadQ were also essential to the 2,4,6-TCP degradation and the expression of *hadX*-C operon. Previously, we had been indicated that

HadR was more closely related to LTTR proteins that sense aromatic compounds and regulate the expression of aromatic compound catabolic operon (Cai & Xun, 2002; Cebolla *et al.*, 1997; Lessner *et al.*, 2003; Lönneborg *et al.*, 2007; Miyauchi *et al.*, 2002; Sánchez & González, 2007). These report demonstrated that the expression of aromatic compounds degradation operon was only activated by LTTR. The expression of *xpsR* promoter in *Ralstonia solanacearum* was regulated the LTTR PhcR and TCS VsrAD (Huang *et al.*, 1998). The XpsR regulated the *eps* operon that encoded biosynthetic enzymes for the exopolysaccharide, which it requires for efficient colonization, wilting, and killing of plants. However, the observation that the second activator involved in the aromatic compounds degradation was not known. This is the first reported that the regulatory mechanism in which both LTTR and TCS essential to the transcription of aromatic compound catabolic gene.

The phenotype of DTP62PQ with citrate as a sole carbon source is also interesting, since the HadP-HadQ system showed homology with the DctS-DctR system of *Rhodobacter capsulatus* (Hamblin et al., 1993; Janausch et al., 2002). There was no report that DctS-DctR system regulated citrate metabolism. The TctE-TctD system of *Salmonella thphimurium*, the DcuS-DcuR system of *E. coli*, the CitA-CitB system of *E. coli* and of *Klebsiella pneumoniae* were reported the TCS that involved the C_4-dicarboxylate or citrate metabolism and regulated the transporter gene or fermentation gene (Bott *et al.*, 1995; Golby *et al.*, 1999; Krämer *et al.*, 2007; Liu *et al.*, 2008; Scheu *et al.*, 2012; Zientz *et al.*, 1998). However, HadP-HadQ is not closely related to these TCS. The studies described here reveal that DTP0602 contains a DctS-DctR-like two component regulatory system that involved in citrate utilization.

Some interesting questions remain to be answered concerning the nature of the

HadP-HadQ system: what does the function of the HadP-HadQ system regulate for citrate utilization; does the HadP-HadQ system regulate expression jointly with other regulators; and what does the signal catalyze a phosphorylation of HadP (citrate, 2,4,6-TCP or other intermediates). These hypotheses should be further investigated.

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Chapter4. Discussion

The regulatory mechanism of 2,4,6-TCP degradation pathway genes has been not known, although the 2,4,6-TCP degradation bacteria and degradation pathway genes were reported. The studies described here reveal that the regulatory mechanism of 2,4,6-TCP catabolic operon (*hadX-C* operon) in *Ralstonia pickettii* DTP0602. This article suggested LTTR, HadR and TCS, HadP-HadQ system were essential to the expression of *hadX-C* operon in DTP0602.

The transcription of the *hadX-C* operon in DTP0602 was induced in the presence of seven types of substituted phenols, but the HadR bound to the *hadX* promoter region in the presence of sixteen kinds of substituted phenols. Established gene regulatory paradigms indicate that the degree of induction of the target-regulated promoter is generally proportionate to the conformational change exhibited by the regulator-DNA complex. Therefore, the activation of the target-regulated promoter for 2,4,6-trichlorophenol deviated from established patterns for LTTRs. Numerous LTTRs have been shown to induce DNA bending upon binding of the protein, which induces a conformational change and typically alters the binding region and DNA bending angle. However, HadR-dependent DNA bending angles were not significantly different in the presence or absence of coinducers. These results might indicate that conformational change of HadR allows the *hadX* promoter region to contact the alpha subunit of RNA polymerase without DNA bending.

To date, a number of regulators that regulate the expression of aromatic compounds catabolic gene have been identified and characterized. However, the regulator that involved in both utilization of TCA cycle intermediates and the aromatic compounds degradation has not been reported. The HadP-HadQ system was essential to citrate metabolism. Thus, this is the first report that the regulatory mechanism of citrate metabolism serves as activation of

chlorophenols degradation.

Our results revealed that the regulatory mechanism of 2,4,6-TCP catabolic operon expression in DTP0602 was distinct from the regulatory mechanism of characterized LTTRs. In this article, we provide new insight into the regulatory mechanism of aromatic compounds degradation pathway. Finally, these findings contribute to bioremediation of soil contaminated with 2,4,6-TCP by using microorganisms including *Ralstonia pickettii* DTP0602.

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岡山理科大学大学院

工学研究科　システム科学専攻

鳥　居　英　人

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