The regulatory mechanism of 2,4,6-trichlorophenol catabolic operon expression by HadR in *Ralstonia pickettii* DTP0602

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*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 *hadD*. Purified HadR binds to the *hadX* promoter and HadR–DNA complex formation was induced in the presence of 16 types of substituted phenols, including chloro- and nitro-phenols and tribromo-phenol. In contrast with observations of other well-characterized LTTRs, the tested phenols showed no diversity of the bending angle of the 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 formation of the HadR–DNA complex, did not induce the transcription of *hadX*, *hadA* or *hadB* in vivo.

## 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 isolated a *Ralstonia pickettii* strain DTP0602 that utilizes 2,4,6-TCP as its sole source of carbon and energy (Kiyohara et al., 1989, 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*, *hadA*, *hadB*, *hadC*, *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 senses aromatic compounds (Cai & Xun, 2002; Hatta et al., 2012; Lessner et al., 2003; Miyachi et al., 2002; Sánchez & González, 2007). Orf2 and Orf3 exhibit homology with a hypothetical protein (accession no. EGE60039.1) in Rhizobium etli CNPAF512, and with 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 behaviour of HadR in the transcription of 2,4,6-TCP catabolic genes.

**METHODS**

**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) 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 SI7-1 μpir 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: ampicillin, 100 μg ml⁻¹; kanamycin, 25 μg ml⁻¹; and chloramphenicol, 25 μg ml⁻¹. For the DTP0602 derivative strain 100 μg kanamycin ml⁻¹ was used. Growth was determined by measuring the optical density at 600 nm (OD₆₀₀).

The substituted phenols (coinducers) used in this study were purchased from Tokyo Kasei Kogyo, Wako Pure Chemical Industry, Lancaster Synthesis, Cambridge Isotope Laboratories, Nacalai Tesque, Aldrich Chemical and Acros Organics. 6-Chlorohydroxyquinol was purchased from Tokyo Kasei Kogyo, Wako Pure Chemical Industry, and the substituted phenols (coinducers) used in this study were simultaneously ligated into the pK18mobscB (Schäfer et al., 1994). The resulting plasmids were named pK18mobscBHadA and pK18mobscBhadR.

**Recombinant DNA work.** To disrupt hadA and hadR, the flanking region of each gene was amplified using *R. pickettii* DTP0602 chromosomal DNA as template. The upstream and downstream flanking regions of hadA were amplified with primers hadA-EcoRI-F/hadR-HindIII-R. These fragments were digested with EcoRI and HindIII, respectively. After digestion, the two DNA fragments were simultaneously ligated into the pK18mobscB (Schäfer et al., 1994). The resulting plasmids were named pK18mobscBHadA and pK18mobscBhadR.

These suicide plasmids were independently transferred into *R. pickettii* DTP0602 by biparental filter mating using *E. coli* SI7-1. 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 DTP62A and DTP62dR, respectively.

To construct an hadA::lacZ reporter fusion in DTP0602, the PCR fragment containing the 5’ end of hadA was amplified by PCR with the primer hadX-Z-f hadA-Z-sall-R and was digested with *SalI*. This fragment was ligated into *SalI*-digested pVIK111 (Kalogeraki & Winans, 1997). The resulting plasmid (pVAl) was transferred into DTP0602 by biparental filter mating using *E. coli* SI7-1 μpir as a donor strain. The kanamycin-resistant colony with the hadA::lacZ reporter fusion was denoted as a colony arising from strain DTP62ZA.

Plasmid pTS1210 was used for the construction of hadR complementation vectors. The flanking region of hadR was amplified with primer phadr-Xhol-R/hadr-Xhol-R and the DNA fragment encoding HadR<sup>mal</sup> (described below) was amplified from pNC2 with primer phadr-Xhol-R/hadr-Xhol-R. Two DNA fragments were digested with *XhoI* and ligated into *SalI*-digested pTS1210, generating plasmids pTS1210 and pTS1210R. The plasmid was used for the construction of LacZ reporter plasmids (Blatny et al., 1997). DNA fragments encoding the hadR::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 EcoRI and *XbaI* and inserted into EcoRI/XbaI-digested pBl861, generating plBRY1 and plBEY1, respectively. These plasmids were transformed into DTP0602 or DTP62dR by electroporation.

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 RNA later solution (Ambion) and stored overnight at 4 °C. Total RNA was isolated using the PureLink RNA Mini kit (Invitrogen Life Technologies) according to the manufacturer’s instructions, and the RNA was subsequently treated with RNase-free DNase I (Takara Bio).

**DNA transformation of *R. pickettii*.** Electroporation was performed as described previously using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories) (Dennis & Sokol, 1995; Smith & Iglewski, 1989).
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). The hadR gene was amplified from pNC2 with the primer hadR-NcoI-F/hadR-SacI-R and digested with NcoI and SacI. The PCR fragment was inserted into NcoI/SacI-digested pET52b(+). The resulting plasmid was named pETHR1 and contained an additional 19 amino acids (ELALVPRGSSAHHHHHHH) at the C terminus including a thrombin cleavage site and an octahistidine tag. This plasmid was introduced into E. coli BL21(DE3) (pG-KJE8).

**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 random primers (Invitrogen Life Technologies) according to the manufacturer’s instructions. cDNA samples were amplified from pNC2 with the primer hadR-NcoI-F/hadR-SacI-R and digested with NcoI and SacI. The PCR fragment was inserted into NcoI/SacI-digested pET52b(+). The resulting plasmid was named pETHR1 and contained an additional 19 amino acids (ELALVPRGSSAHHHHHHH) at the C terminus including a thrombin cleavage site and an octahistidine tag. This plasmid was introduced into E. coli BL21(DE3) (pG-KJE8).
treated with 0.1 mg RNaseA ml\(^{-1}\) (Sigma Chemical) 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) 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. 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\(^{-6}\) to 3.0 × 10\(^{-10}\) copies µl\(^{-1}\). Specific primers were designed to amplify DNA segments of approximately 150 bp from hadR, hadX, hadA and hadB (Table S1).

β-Galactosidase assay was performed as described by 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.

**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-labelled primer, hadR-IRD-extension or instructions. From 3.0

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**Overexpression and purification of HadR and determination of oligomerization state of HadR.** _E. coli_ BL21(DE3) (pG-KER) (pETHR1) was grown at 30 °C with vigorous shaking. After OD\(_{600}\) 0.4, IPTG, l-arabinose and tetracycline were added to a final concentration of 0.05 mM, 10 mg ml\(^{-1}\) and 20 µg ml\(^{-1}\), respectively (Nishihara et al. 1998, 2000). Growth was continued for 3 h, and cells from 1000 ml of culture were centrifuged and resuspended in buffer A [50 mM K\(_2\)HPO\(_4\)–KHPO\(_4\) (pH 7.5), 300 mM NaCl, 2 mM mercaptoethanol and 10 % glycerol] at 10 volumes g\(^{-1}\) of wet weight and then disrupted by a French pressure cell press (Aminco). 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) 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 (cut-off 10 kDa; Vivascience). 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\(_{\text{mt}}\); 323 amino acids, 35.7 kDa) contained seven additional amino acid residues (ELALVPFR) from pET52b(+) at the C terminus.

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

**EMSA.** EMSAs for HadR were performed with the DIG gel shift kit (second generation) (Roche) 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 (see Fig. 3a, 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-labelled 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 labelled DNAs were electroblotted onto Bio Tyne PLUS nylon membranes (Pall Gelman Laboratory) and detected using the ImageQuant LAS-4000 UV 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) and expressed as arbitrary units.

For determination of the dissociation constants (\(K_D\)) of coinducers, EMSAs were performed using eight concentrations of coinducer (0–100 µM) and 1 ng of DIG-labelled probe E (see Fig. 3a). The intensities of the free probe and HadR\(_{\text{mt}}\)-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 labelled DNA was bound.

**Circular permutation analysis.** For circular permutation analysis, DNA fragments that contained the HadR\(_{\text{mt}}\) 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. S5a, probes 1–5). After three independent EMSAs were performed with these fragments, the mobilities of the HadR\(_{\text{mt}}\)-DNA complexes were determined by measuring the distance travelled from the well during electrophoresis. The bending angles were calculated as described by Thompson & Landy (1988).

**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 IRD-labelled probe, 2 µg herring sperm DNA, 5 µg BSA, 100 µM of substituted phenols and HadR\(_{\text{mt}}\) 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\(^{-3}\) units µl\(^{-1}\); 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 analysed using the Li-Cor 4300 DNA analyser as described above.

**Statistics and data analysis.** The Q-PCR analysis, β-galactosidase assay, circular permutations analysis and measurement of \(K_D\) values

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Fig. 2. Nucleotide sequence of the hadR-hadX divergent promoter and HadR<sup>mt</sup>-binding sites. The TSPs of the hadR and hadX-C operon are indicated by dotted arrows and bold type. 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 bold type. Bold arrows indicate T-N<sub>1</sub>-A sequences in the upstream region of the hadX promoter. The inverted repeat sequences and the A and T residues within the T-N<sub>1</sub>-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. The DNase I-hypersensitive sites in the presence of 2,4,6-TCP are indicated by filled arrowheads.

Fig. 3. EMSA of the binding of HadR<sup>mt</sup> 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 non-binding (−, 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<sup>mt</sup> and DIG-labelled fragments containing the hadX promoter region. 2,4,6-TCP at 100 μM used as coinducer in the reaction mixtures is indicated. Lane 1, EMSA with HadR<sup>mt</sup>; lane 2, EMSA with HadR<sup>mt</sup> and 2,4,6-TCP and no competitor DNA; lanes 3–5, EMSA with HadR<sup>mt</sup> and increasing concentrations (10, 100 and 200 ng, respectively) of unlabelled probe DNA fragment as specific unlabelled competitor; lane 6, EMSA with HadR<sup>mt</sup> and 200 ng of herring sperm DNA as non-specific competitor. Free DNA and the HadR<sup>mt</sup>–DNA complex are indicated by open arrowheads and filled arrowheads, respectively.
Table 2. $K_D$ values for substituted phenols and the absolute mRNA copy numbers of $hadR$, $hadX$, $hadA$ and $hadB$ in the presence or absence of these phenols

<table>
<thead>
<tr>
<th>Coinducer*</th>
<th>$K_D$ (µM)†</th>
<th>Absolute mRNA copy number‡,§ (fold induction!!) in DTP0602</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$hadR$</td>
</tr>
<tr>
<td>None</td>
<td>84.7 ± 21.9</td>
<td>4.98 ± 1.02</td>
</tr>
<tr>
<td>2,4,6-TBP</td>
<td>2.4 ± 0.4</td>
<td>74.6 ± 3.30</td>
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<tr>
<td>2,3,4,5-TeCP</td>
<td>3.8 ± 0.7</td>
<td>29.2 ± 13.9</td>
</tr>
<tr>
<td>2,4,6-TCP</td>
<td>6.2 ± 0.8</td>
<td>64.5 ± 9.62</td>
</tr>
<tr>
<td>Bromoxynil</td>
<td>6.6 ± 0.6</td>
<td>76.8 ± 7.24</td>
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<tr>
<td>2-C-4-7P</td>
<td>7.0 ± 1.5</td>
<td>38.0 ± 8.65</td>
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<tr>
<td>2,3,4,6-TeCP</td>
<td>8.1 ± 1.7</td>
<td>67.0 ± 9.93</td>
</tr>
<tr>
<td>2,3,5-TCP</td>
<td>10 ± 2.4</td>
<td>59.7 ± 22.8</td>
</tr>
<tr>
<td>PCP</td>
<td>16 ± 3.6</td>
<td>59.7 ± 6.32</td>
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<tr>
<td>2,3,4-TCP</td>
<td>18 ± 3.3</td>
<td>37.6 ± 14.2</td>
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<tr>
<td>2,4,6-TNP</td>
<td>30 ± 3.3</td>
<td>91.6 ± 25.8</td>
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<td>2,4-DNP</td>
<td>33 ± 1.0</td>
<td>51.5 ± 6.11</td>
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<tr>
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<td>36 ± 2.7</td>
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<tr>
<td>2,3,6-TCP</td>
<td>36 ± 3.3</td>
<td>76.2 ± 10.8</td>
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<tr>
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<td>40 ± 6.2</td>
<td>48.4 ± 13.1</td>
</tr>
<tr>
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<td>&gt;50</td>
<td>41.2 ± 5.97</td>
</tr>
<tr>
<td>2,6-DNP</td>
<td>&gt;50</td>
<td>45.3 ± 18.3</td>
</tr>
</tbody>
</table>

*TCP, TeCP, PCP, TBP, DNP, TN, 2-4-4-NP and bromoxynil indicate tri-, tetra- and penta-chlorophenol, tribromophenol, di- and tri-nitrophenol, 2-chloro-4-nitrophenol and 3,5-dibromo-4-hydroxybenzonitril, respectively.
†The dissociation constants ($K_D$s) for substituted phenols are the mean ± SD of three independent experiments.
‡Expressed as absolute mRNA copy number (× 10⁶)/total RNA (µg).
§Absolute mRNA copies of respective genes are the mean ± SD 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.

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.

RESULTS

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. 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 cathobic 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 an A nucleotide positioned 75 bp upstream from the ATG translation start codon of $hadX$. The putative $\sigma^{34}$ promoter sequence was found upstream from the TSPs of the $hadR$ and $hadX-C$ operon (Figs 2 and S1).

To ensure that the $hadX$ promoter controls the expression of the $hadX-C$ operon, the lacZ reporter plasmids pJBEY1 and pJBEY1, carrying an $hadR-hadX-hadA::lacZ-lacY$ (including the putative $\sigma^{34}$ promoter sequence of $hadX$) and an $hadX-hadA::lacZ-lacY$ (lacking the putative $\sigma^{34}$ promoter sequence of $hadX$), were constructed, respectively (Fig. 3a). The $\beta$-galactosidase activity in DTP0602-pJBEY1 and DTP0602-pJBEY1 cells was measured. After incubation with 2,4,6-TCP, the $\beta$-galactosidase activity increased significantly 3.8-fold in DTP0602-pJBEY1. The basal level of activity was significantly higher (approx. 6.0-fold) in DTP0602-pJBEY1 than in DTP0602-pJBEY1. The $\beta$-galactosidase
activity was repressed significantly 1.2-fold in the presence of 2,4,6-TCP in DTP0602-pIBEYI (Table S2). These results indicate that a single promoter of hadX is involved only in the transcription of the hadX-C operon.

Role of HadR for 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 an 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. S2).

To investigate the regulation of gene expression by HadR and whether 2,4,6-TCP acts as a coinducer of HadR, the transcript levels 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 increased significantly 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 (approx. 3- to 10-fold) than that of hadX in DTP0602, DTP62dA and DTP62dR (Tables 2 and S3). The transcription of hadR was significantly lower (approx. 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.

Determination of HadR<sup>mt</sup> protection sequence

For determination of the HadR-binding region, HadR<sup>mt</sup> (35.7 kDa) was purified to homogeneity. The molecular mass of HadR<sup>mt</sup> was estimated to be 137 ± 17.6 kDa by gel filtration chromatography, indicating that HadR<sup>mt</sup> exists as a tetramer in solution (Fig. S3). The oligomeric state of HadR<sup>mt</sup> is similar to that reported for other LTTRs (Bundy et al., 2002; Chang & Crawford, 1991; Kulik et al., 1995; Miller & Kredich, 1987; Muraoka et al., 2003; Schell et al., 1990). To confirm whether HadR<sup>mt</sup> altered the function of HadR, complementation studies using the plasmid pTSRm1 producing HadR<sup>mt</sup> were performed in DTP62dR. DTP62dR-pTSRm1 can normally grow on 2,4,6-TCP, suggesting that HadR<sup>mt</sup> showed functional complementarity to HadR (Fig. S2).

EMSAs were performed using HadR<sup>mt</sup> with DIG-labelled 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<sup>mt</sup> binding region (Fig. 3). 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<sup>mt</sup> to the hadX promoter specifically, we performed a competition test using herring sperm DNA as non-specific competing DNA and unlabelled DNA probes E, F and G as specific competitors for the binding assays. The band of HadR<sup>mt</sup>–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 unlabelled DNA. Therefore, HadR<sup>mt</sup> binds specifically to the hadX promoter and HadR<sup>mt</sup>–DNA complex formation was induced in the presence of 2,4,6-TCP (Fig. 3b).

DNase I footprinting analysis was performed in the presence or absence of 2,4,6-TCP (Figs 2, 3a and 4) to determine the HadR<sup>mt</sup>-binding site on the hadX promoter region. HadR<sup>mt</sup> 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-N11-A motif of the LTTR consensus binding sequences (AT-CGCCNNGGNG-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 hadX-C operon).
the TSP of the hadR). The footprint region of HadRmt 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.

**Effect of various substituted phenols on induction of the HadRmt–DNA complex formation and on the bending angle of the HadRmt binding region**

Because substituted phenols, including 2,4,6-TCP, were predicted to induce the HadRmt–DNA complex formation, EMSAs were performed in the presence of various substituted phenols and DIG-labelled probe E (Fig. 5). The band of HadRmt–DNA complex was not observed in the presence of mono- or di-chlorophenols (CPs, DCPs), mononitrophenols (NPs), hydroxyquinol, or 6-chlorohydroxyquinol, but it was observed in the presence of all tested tri- and 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). HadRmt showed binding to probe E without substituted phenols (Fig. 5, the first lane on the left in each gel). To determine whether HadRmt–DNA complex formation was specifically induced, competition tests were performed as described above. HadRmt–DNA complex formation occurred specifically by the tested phenols (Fig. 5b, c, lanes 3 and 4 in each gel). DNase I footprinting analysis was performed in the presence of 16 kinds of substituted phenols to confirm the diversity of HadRmt protection sequence in the presence of substituted phenols, including 2,4,6-TCP. As a result, HadRmt protection sequence showed no difference between 2,4,6-TCP and other substituted phenols (Fig. S4).

**Fig. 5.** Alternative coinducers of HadRmt to bind the upstream region of the hadX promoter. Free DNA and the HadRmt–DNA are indicated by open arrowheads and filled arrowheads, respectively. EMSA with HadRmt and DIG-labelled fragments (probe E) containing the hadX promoter region. (a) EMSAs were performed in the presence of 100 μM CPs and DCPs, NPs, hydroxyquinol and 6-chlorohydroxyquinol. The first lane on the left in each gel served as a control containing HadRmt. The second lane from the left in each gel contained HadRmt and the substituted phenol. (b, c) 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 HadRmt; lane 2, EMSA with HadRmt and substituted phenol and no competitor DNA; lane 3, EMSA with HadRmt and 200 ng of unlabelled probe E DNA fragment as specific unlabelled competitor; lane 4, EMSA with HadRmt and 200 ng of herring sperm DNA as non-specific competitor.
To test the hypothesis that HadRmt has a different affinity for each of the phenols, the $K_D$ values for the 16 types of substituted phenols were determined (see Methods for details). HadRmt showed a range of different affinities for the phenols (Table 2). The $K_D$ value for 2,4,6-TBP (2.4 ± 0.4 μM) was significantly lower than for 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 HadRmt also alters DNA bending when transcription is activated. The ability of HadRmt to bend DNA at its binding sites was examined by circular permutation analysis using 201 bp PCR fragments containing an HadRmt-protection sequence of the hadX promoter (Fig. S5a). As shown in Fig. S5(b–e), the estimated HadRmt-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 HadRmt-dependent DNA bending angle was not affected by the tested phenols.

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

For confirmation of the activation of the hadX-C operon transcription with substituted phenols, which induced HadRmt–DNA complex formation, Q-PCR analysis and β-galactosidase assays were performed to monitor the mRNA copy numbers of hadR, hadX, hadA and hadB, and the expression of hadA was monitored by using DTP0602 and DTP622ZA (hadA::lacZ) (Table 2 and Table S4). β-Galactosidase activity was increased significantly 47- to 540-fold in the presence of 2,4,6-TBP, 2,4,6-TCP, bromoxynil and 2-C-4-NP, which activated the transcription of hadX, hadA and hadB. However, HadRmt 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 operon transcription. In addition, HadRmt had a low affinity for 2,4-DNP and 2,3,6-TTCP, 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 2).

The HadRmt-dependent DNA bending angles were not significantly different in the presence or absence of coinducers (Fig. S5), indicating that HadR-dependent DNA bending was not correlated with the regulatory mechanism. This behaviour of HadR toward the hadX promoter was distinct from those of the LTTRs mentioned above, as 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 HadR. Lorenz & Stauffer (1995) suggested that MetR protected only a region of the glyA promoter (−155 to −109) without DNA bending relaxation in the presence or absence of homocystine. 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

**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-TCP deviated from established patterns for LTTRs. Transcription of the hadX-C operon in DTP0602 was induced in the presence of seven types of substituted phenols, but the HadRmt bound to the hadX promoter region in the presence of 16 kinds of substituted phenols (Table 2 and Fig. 5). The well-characterized LTTRs cause the coinducer-dependent shortening of the protected region and a relaxation of the DNA bending (Akakura & Winans, 2002a, b; 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 HadRmt-protection sequences, in the HadRmt-dependent bending angle or in the transcription level of hadR in the presence of seven types of substituted phenols. Induction of the hadX-C operon transcription was not affected by the difference of the autorepression levels of hadR, of HadRmt-protection sequences or of the HadRmt-dependent bending angles (Table 2, Figs S4 and S5). HadRmt 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, HadRmt 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 operon transcription. In addition, HadRmt 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 operon transcription. Overall, there was no clear pattern in the relationship between coinducer binding affinities and the hadX-C operon transcription (Table 2). The HadRmt-dependent DNA bending angles were not significantly different in the presence or absence of coinducers (Fig. S5), indicating that HadR-dependent DNA bending was not correlated with the regulatory mechanism. This behaviour of HadR toward the hadX promoter was distinct from those of the LTTRs mentioned above, as 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 HadR. Lorenz & Stauffer (1995) suggested that MetR protected only a region of the glyA promoter (−155 to −109) without DNA bending relaxation in the presence or absence of homocystine. 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. 4) 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. S6). 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. (2002) demonstrated that NahR-regulated promoter activation was induced by direct contact between NahR and the alpha subunit of RNA polymerase. The characterization using some of the NtdR, NahR and DntR variants suggested that LTTRs undergo the effector-binding domain (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 S6 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 was 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 2).

It is surprising that HadR<sup>mt</sup> 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 2), as 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 defence 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. (2008) suggested that there are a number of bromophenol-degrading bacteria in various ecological niches. 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 the 2,4,6-TBP catabolic gene in TB01 resembles that of HadR in DTP0602, these results perhaps indicate that the chlorophenol catabolic mechanism in DTP0602 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 2). 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 (DNA bending pattern, difference in the coinducer profile of hadX-C transcription and HadR binding) was distinct from the regulatory mechanism of other, previously characterized LTTRs.

**ACKNOWLEDGEMENTS**

This work was supported by the Program for Social Collaborative Research (Okayama University of Science) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank Dr Yuji Nagata, Tohoku University, and Dr Naoiumi Kamimura, Nagaoka University of Technology, for the helpful discussion and comments and Keiji Yoshimi for technical support.

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Edited by: E. L. Madsen