Transcriptional Activation of the Chlorocatechol Degradative Genes of *Ralstonia eutropha* NH9

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Received 21 May 1999/Accepted 21 August 1999

Ralstonia eutropha (formerly *Alcaligenes eutrophus*) NH9 degrades 3-chlorobenzoate via the modified *ortho*-cleavage pathway. A ca. 5.7-kb six-gene cluster is responsible for chlorocatechol degradation: the *cbnABCD* operon encoding the degradative enzymes (including *orfX* of unknown function) and the divergently transcribed *cbnR* gene encoding the LysR-type transcriptional regulator of the *cbn* operon. The *cbnRAB orfXCD* gene cluster is nearly identical to the chlorocatechol genes (*tcbRCD orfXEF*) of the 1,2,4-trichlorobenzene-degrading bacterium *Pseudomonas* sp. strain P51. Transcriptional fusion studies demonstrated that *cbnR* regulates the expression of *cbnABCD* positively in the presence of either 3-chlorobenzoate or benzoate, which are catabolized via 3-chlorocatechol and catechol, respectively. In vitro transcription assays confirmed that 2-chloro-*cis,cis*-muconate (2-CM) and *cis,cis*-muconate (CCM), intermediate products from 3-chlorocatechol and catechol, respectively, were inducers of this operon. This inducer-recognizing specificity is different from those of the homologous catechol (*catBCA*) and chlorocatechol (*clcABD*) operons of *Pseudomonas putida*, in which only the intermediates of the regulated pathway, CCM for *catBCA* and 2-CM for *clcABD*, act as significant inducers. Specific binding of CbnR protein to the *cbnA* promoter region was demonstrated by gel shift and DNase I footprinting analysis. In the absence of inducer, a region of ca. 60 bp from position –20 to position –80 upstream of the *cbnA* transcriptional start point was protected from DNase I cleavage by CbnR, with a region of hypersensitivity to DNase I cleavage clustered at position –50. Circular permutation gel shift assays demonstrated that CbnR bent the *cbnA* promoter region to an angle of 78° and that this angle was relaxed to 54° upon the addition of inducer. While a similar relaxation of bending angles upon the addition of inducer molecules observed with the *catBCA* and *clcABD* promoters may indicate a conserved transcriptional activation mechanism of *ortho*-cleavage pathway genes, CbnR is unique in having a different specificity of inducer recognition and the extended footprint as opposed to the restricted footprint of CatR without CCM.

The modified *ortho*-cleavage pathway plays a significant role in the aerobic bacterial degradation of chlorocatechols produced through converging pathways from various chlorinated aromatics (reviewed in references 12, 24, 47, 51, 55, and 56). Well-characterized examples include 3-chlorobenzoate (3-CB), 2,4-dichlorophenoxyacetic acid (2,4-D), and 1,2,4-trichlorobenzene, which are converted to the corresponding chlorocatechols by peripheral pathways and then catabolized to tricarboxylic acid cycle intermediates by the enzymes of the modified *ortho*-cleavage pathway encoded by the *clcABDE* genes from plasmid pAC27 of *Pseudomonas putida* (3, 20, 27) and the *clc* element (46) (or plasmid pB13 [4]) of *Pseudomonas* sp. strain B13, the *tfdCDEF* genes from plasmid pJP4 of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134 (14, 45), and the *tcbCDEF* genes from plasmid pP51 of *Pseudomonas* sp. strain P51 (57, 59), respectively. Sequence homology and overall structural similarity among the modified *ortho*-cleavage pathway operons and the catechol *ortho*-cleavage pathway operon *catBCA* (1, 26) indicate an evolutionary relationship (51, 56). Each operon has a *lysR*-type regulatory gene (50)

which is located upstream of and is divergently transcribed from the degradative gene clusters (9, 30, 48, 58). For the *tfdCDEF* operon of plasmid pJP4, however, *tfdT*, the *lysR*-type regulatory gene originally located upstream of the operon, is inactivated by an insertion sequence element, and its function has been taken over by distantly located *tfdR* (30, 61).

The regulatory mechanisms of the operons *catRBCA* and *clcRABD* have been studied both in vivo and in vitro (6–9, 26, 32–35, 41–44, 48; reviewed in reference 33). The regulators CatR and ClcR bind specifically to the *catB* and *clcA* promoter regions, respectively, and activate the expression of the degradative genes upon recognition of inducer. The inducers of the *catBCA* and *clcABD* operons have been identified as intermediates of their respective pathways, *cis,cis*-muconate (CCM) (43) and 2-chloro-*cis,cis*-muconate (2-CM) (35). In the 2,4-D degradation process, 2,4-dichloromuconate, the intermediate produced by TfdC, has been identified as an inducer of the *tfdCDEF* operon in vivo (18). DNA binding of the regulator TcbR to the *tcbC* promoter has been described (29), and initial in vivo characterization of the role of *tcbR* has been performed (30, 58). *P. putida* KT2442 harboring a plasmid containing the *tcbRCD orfXEF* genes was found to grow on 3-CB, while KT2442 containing the *tcbCD orfXEF* genes with an inactivated *tcbR* gene grew on 3-CB at a much lower rate (58). *P. putida* and *R. eutropha* strains harboring plasmids containing the *tcbRCD orfXEF* genes showed elevated (chloro)catechol 1,2-dioxygenase activity towards 3-chlorocatechol after cultivation on 3-CB (30, 58). This activity was not observed with cells of *R. eutropha* containing the *tcbCD orfXEF* genes with *tcbR*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
Strains		
<i>E. coli</i>		
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB⁺ lacI^a lacZΔM15]</i>	49
DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	23
BL21(DE3)pLysS	<i>hsdS gal (λcIts857 ind1 Sam7 nin5lacUV5-T7 gene 1) pLysS Cm^r</i>	Novagen
S17-1	<i>C600::RP4 2-(Tc::Mu)(Km::Tn7) thi pro hsdR hsdM^r recA</i>	53
<i>P. putida</i> PRS4020	<i>catR::Gm^r Ben⁻ derivative of PRS2000</i>	39
<i>R. eutropha</i> NH9D	3-CB ⁻ derivative of NH9	37
Plasmids		
pBluescript KS(-)	<i>lacZα⁺ Ap^r</i>	Stratagene
pUC18	<i>lacZα⁺ Ap^r</i>	60
pEKC1	9.2-kb <i>SacI cbnRAB orfXCD</i> insert from pENH91 in pKT230; Km ^r	38
pBLcbln1	2.8-kb <i>HindIII cbnRAB'</i> insert from pEKC1 in pBluescript KS(-); vector mcs <i>EcoRI</i> is located downstream of <i>cbnR</i> ; <i>cbnB</i> is truncated at 270 out of 370; Ap ^r	This study
pBLcbln1r	pBLcbln1 insert in reverse orientation; vector mcs <i>XhoI</i> and <i>SalI</i> are located downstream of <i>cbnR</i> ; Ap ^r	This study
pBLcbln2	5.6-kb <i>EcoRI-XhoI cbnAB orfXCD</i> insert from pEKC1 in pBluescript KS(-); Ap ^r	This study
pBLcbln3	6.7-kb <i>EcoRI-XhoI cbnRAB orfXCD</i> insert in pBluescript KS(-); Ap ^r	This study
pBLcbln3H	6.6-kb <i>XbaI-XhoI cbnRAB orfXCD</i> insert with 6 His codons on C terminus of <i>cbnR</i> in pBluescript KS(-); Ap ^r	This study
pT7-7	T7 promoter expression vector; Ap ^r	54
pT7cblnR	956-bp <i>NdeI-BamHI</i> PCR-generated fragment containing <i>cbnR</i> in pT7-7 under T7 promoter; Ap ^r	This study
pT7cblnRHis	911-bp <i>NdeI-HindIII</i> fragment of <i>cbnR</i> with 6 His codons on C terminus generated by PCR in pT7-7 under T7 promoter; Ap ^r	This study
pET16b	T7 promoter expression vector for constructing His fusion proteins; Ap ^r	Novagen
pEHiscblnR	956-bp <i>NdeI-BamHI cbnR</i> insert from pT7cblnR in pET16b; Ap ^r	This study
pCP13	Broad-host-range cosmid vector; <i>mob⁺ tra</i> ; Km ^r Tc ^r	11
pCbln13ABCD	5.6-kb <i>XbaI-XhoI cbnAB orfXCD</i> insert from pBLcbln2 in pCP13; Tc ^r	This study
pCbln13RABCD	6.7-kb <i>XbaI-XhoI cbnRAB orfXCD</i> insert from pBLcbln3 in pCP13; Tc ^r	This study
pCbln13RHABCD	6.6-kb <i>XbaI-XhoI cbnRHisAB orfXCD</i> insert from pBLcbln3H in pCP13; Tc ^r	This study
pQF50	Broad-host-range <i>lacZ</i> promoter probe vector; Ap ^r	17
pNO50RAB'	2.8-kb <i>HindIII cbnRAB'</i> insert from pBLcbln1 in pQF50; <i>cbnB</i> is truncated at aa 270 out of 370; Ap ^r	This study
pNO50AB'	1.7-kb <i>NcoI-HindIII cbnAB'</i> insert in pQF50; nearly complete <i>cbnA</i> promoter region is included; <i>cbnB</i> is truncated at aa 270; Ap ^r	This study
pNO50RA'	1.4-kb <i>SalI cbnRA'</i> insert from pBLcbln1r in pQF50; <i>cbnA</i> is truncated at aa 60 out of 251; Ap ^r	This study
pNO50RHAB'	2.6-kb <i>HindIII cbnRHisAB'</i> insert from pBLcbln3H in pQF50; <i>cbnB</i> is truncated at aa 270; Ap ^r	This study
pMP7	In vitro transcription vector; Ap ^r	25
pMPcbln1	417-bp <i>PstI-BamHI</i> insert containing the <i>cbnRA</i> divergent promoter region from pBLcbln1 in pMP7; Ap ^r	This study
pKS100	4.2-kb <i>PstI-BamHI</i> fragment from pUS1028 in pUC119; Ap ^r	13
pJET41	Supercoiled vector used for estimation of transcript size; Ap ^r	16

^a Ap^r, ampicillin resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; aa, amino acid; mcs, multicloning site.

inactivated. These results indicated that *tcbR* was required for the efficient expression of *tcbC*, which encodes chlorocatechol dioxygenase (30, 58). Further analysis of this operon including the identification of inducer is yet to be done.

The *cbnRAB orfXCD* operon on plasmid pENH91, found in a 3-CB degradative bacterium *R. eutropha* NH9, is highly homologous to the *tcbRCD orfXEF* operon (95.6 to 100% identity at the amino acid level and identical 150-bp divergent promoter regions) and is responsible for the degradation of 3-chlorocatechol (38). In this paper, we report the transcriptional regulation of the *cbnRAB orfXCD* operon, which includes the identification of inducers and a change in the bending angle of the promoter region upon recognition of an inducer. These observations provide a view of a conserved transcriptional mechanism of regulation plus the independent

evolution of inducer-recognizing specificity and DNA-binding property among the regulators of the *ortho*-cleavage pathway.

MATERIALS AND METHODS

Bacteria, plasmids, media, chemicals, and enzyme assays. The strains and plasmids used in this study are listed in Table 1. Preparation of media was performed as described previously (35, 37). As a selective medium for the transconjugants of *R. eutropha* NH9D, basal salts medium for NH9 (37) was supplemented with 0.2% sodium citrate. 2-Chloromuconate was produced by the enzymatic conversion of 3-chlorocatechol with chlorocatechol 1,2-dioxygenase (C1cA) as described previously (34). Quantitative determination of β-galactosidase activity in the reporter assay was performed by the method of Miller (35, 36). Each experiment was performed in triplicate.

DNA manipulations and construction of plasmids. Recombinant DNA techniques were performed according to standard procedures (49). Transformation of the plasmid pQF50 and its derivatives into *P. putida* PRS4020 was performed as described previously (35). Mobilization of the plasmid pCP13 and its deriva-

tives into *P. putida* PRS4020 and *R. eutropha* NH9D was conducted based on the method of Franklin (19). Plasmids to test growth complementation were constructed as follows: a plasmid, pBLcbln2, containing the *cbnAB orfXCD* genes with the *cbnA* promoter region in pBluescript KS(-), was constructed by using a 5.6-kb insert from pEKC1 which was cut out by using a unique *EcoRI* site 4 bp within the N terminus of *cbnR* (*EcoRI**) in the *cbnRAB orfXCD* operon and an *XhoI* site located downstream of the *cbnD* gene (Table 1). A 1.1-kb *EcoRI* fragment containing *cbnR* truncated at the *EcoRI** site was cut out from pBLcbln1 and inserted into the *EcoRI* site of pBLcbln2 to restore the original structure of the *cbnRAB orfXCD* operon, yielding pBLcbln3. From pBLcbln2 and pBLcbln3, the relevant fragments were excised and inserted into the cloning site of pCP13, yielding pCbln13ABCD and pCbln13RABCD, respectively. The broad-host-range vector pQF50 was used to produce the transcriptional fusion constructs (Table 1 and see Fig. 2).

To make constructs for expression of *cbnR* in *E. coli*, DNA fragments containing *cbnR* with *NdeI* and *BamHI* restriction sites were synthesized by PCR with plasmid pBLcbln1 as a template. A PCR-generated fragment using primers CBNR1 (5'-TTT TCA TAT GGA ATT CCG GCA GCT-3') and CBNR2 (5'-TTT TGG ATC CCT GTC CAG CGT GA-3') was digested and inserted into the cloning sites of pT7-7, yielding plasmid pT7cblnR. To make a construct of *cbnR* with six His codons on its carboxyl terminus (*cbnRHIS*), a PCR-generated fragment using primers CBNR1 and CBNRHIS1 (5'-TTT TAA GCT TCA ATG ATG ATG ATG ATG ATG GTC CTT CGC GGA TCG CCG CAC GTG TTC CAC GAA CC-3') was digested with *NdeI* and *HindIII* and was inserted into pT7-7, yielding pT7cblnRHIS. Nucleotide sequences of the PCR-derived inserts of the two plasmids were verified by sequencing analysis. In these two constructs, the initiation codons of *cbnR* or *cbnRHIS* were ligated with the *NdeI* site of the vector and thus were located 8 bp downstream of a vector-derived ribosomal binding site, and transcription was initiated from the T7 promoter of the vector. The insert *cbnR* was excised from pT7cblnR by digestion with *NdeI* and *BamHI* and cloned into pET16b, yielding pEHiscblnR. In this construct, *cbnR* was fused downstream of 10 His codons and a factor Xa site.

To demonstrate that CbnRHIS has the same function as wild-type CbnR, plasmids were constructed as follows. A 0.95-kb *EcoRI**-*XbaI* fragment containing *cbnRHIS* truncated near the amino terminus was excised from a pUC18-based construct, which was made by using the insert from pT7cblnRHIS, and was inserted into the *XbaI*-*EcoRI** site of pBLcbln2. The resulting plasmid, pBLcbln3H, contained a reconstructed *cbnRAB orfXCD* operon with six His codons attached to the C terminus of *cbnR*. Relevant fragments from pBLcbln3H were used (Table 1) to construct pCbln13RHABCD for the growth complementation test and pNO50RHAB' for the reporter assay in which the *cbnB'* gene was connected to a promoterless *lacZ* gene. A supercoiled template for the in vitro transcription assay, designated pMPcbln1, was constructed with the vector pMP7. The inserted fragment spanned positions -163 to +254 with respect to the *cbnA* transcriptional start site.

Purification of protein. Induction and extraction of protein from *Escherichia coli* BL21(DE3)pLysS containing a construct of pT7-7 or pET16b and further purification of the protein with the histidine tag were conducted according to the *His⁺ Bind Resin Manual* (36a). In order to partially purify CbnR and also to eliminate a contaminating protein of 27 kDa from crude lysate of cells with pT7cblnRHIS, a heparin-agarose column was used as described previously (8). The fractions that showed specific binding to the *cbnA* promoter fragment by gel retardation assay were pooled and concentrated by Centricon 10 (Amicon, Mass.). To prepare the vector (pT7-7) control for pT7cblnR, the fractions corresponding to the ones containing activity with pT7cblnR were used. CbnRHIS was purified further with His⁺ Bind resin.

Gel retardation assay. DNA binding reactions were performed in 20- μ l volumes consisting of 10 mM HEPES (pH 7.9), 10% glycerol, 100 mM KCl, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM dithiothreitol, 1.5 μ g of bovine serum albumin, 5 μ g of heparin (Sigma, H-3125), approximately 0.1 ng of a DNA fragment and the tested protein. The DNA fragments were synthesized by PCR and labeled internally with [α -³²P]dCTP, followed by a purification procedure (40). The binding reactions were initiated by the addition of CbnR and incubated for 20 min at room temperature. The binding reaction mixtures were then electrophoresed through 5% native polyacrylamide gel in 0.5 $\times$ Tris-borate-EDTA buffer for 2 h at 130 V with circulation of cooling water in Bio-Rad Protean apparatus. The DNA probe containing the 252-bp *cbnRA* promoter region was made by PCR with primers CBNFT1 (5'-TTG GCT GCT GCA GCC ATG TTC CC-3') and CBNFT2 (5'-AAT GCG GAC GCA ACC TGC TTC ACT CG-3') and with pBLcbln1 as a template. A 336-bp fragment containing a part of the hydroxyquinol 1,2-dioxygenase gene from *Burkholderia cepacia* AC1100 was used as a nonspecific DNA probe. The fragment was synthesized by PCR with primers ORF4P1 (5'-GCC TGC AGC GGC CCC TTC CAT GTG-3') and ORF4P2 (5'-GCC TGC AGC CTC AAG CAT TTG ACC-3') and with pKS100 as a template (13).

S1 nuclease analysis. Bacterial RNA was isolated via the RNeasy total RNA isolation kit (Qiagen, Chatsworth, Calif.) from a 9-ml culture of *P. putida* PRS4020 containing the plasmid pNO50RAB' cultivated in Luria broth supplemented with 5 mM 3-CB. To prepare the DNA probe, primer CBNFT2 was end labeled with T4 kinase (Gibco BRL, Gaithersburg, Md.) and [γ -³²P]ATP followed by PCR synthesis of a 252-bp fragment containing the promoter region with a second primer, CBNFT1, and pBLcbln1 as a template. The PCR product

was purified by QIA quick PCR purification kit (Qiagen). This double-stranded fragment was denatured by the addition of 1/10 volume of 2 N NaOH-2 mM EDTA (pH 8.0) and incubated at room temperature for 5 min. The denatured DNA was ethanol precipitated, washed with 70% ethanol, and resuspended in water. The labeled DNA fragment was hybridized with the RNA at 45°C overnight and incubated with S1 nuclease with the S1-Assay kit (Ambion, Austin, Tex.) according to the manufacturer's instructions. Sequencing reactions to juxtapose the protected DNA fragment resulting from the S1 reaction were performed with the SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, Wis.) with CBNFT2 as the primer.

DNase I protection assay. DNase I footprinting experiments were conducted based on the method described previously (40), except that the binding reaction was performed in 20 μ l of solution consisting of 100 mM Tris-HCl (pH 7.9), 1 mM EDTA, 4% glycerol, 100 mM KCl, 1 μ g of bovine serum albumin, 1 μ g of poly(dI-dC), approximately 10 ng of a DNA fragment plus the purified protein indicated. PCR was used to generate a 252-bp fragment spanning the *cbnA* promoter region using pBLcbln1 as a template. In each reaction, one of the primers, CBNFT1 or CBNFT2, was end labeled with T4 kinase (Gibco BRL) and [γ -³²P]ATP as described previously (40). To locate the footprint, sequencing reactions were performed as described above with either of the end-labeled primers, CBNFT1 or CBNFT2, and pBLcbln1 as the template.

In vitro transcription assays. In vitro transcription assays were performed as described previously (25, 35). The supercoiled template, pMPcbln1, and *E. coli* holo RNA polymerase (Epicentre Technologies) were used. For each reaction, 100 ng of purified CbnRHIS and a 1 mM concentration of each of the chemicals tested as effector molecules were used.

DNA bending by circular permutation gel shift assay. Circular permutation gel shift assays were conducted by the same method as the gel retardation assay with the following modifications: the binding reaction mixtures contained 0.2 μ g of purified CbnRHIS, approximately 0.3 ng of labeled DNA fragments and 2 μ g of heparin. In the inducer-containing samples, CCM was added at 1 mM in the binding reaction mixtures and at 0.5 mM in the running buffer. Electrophoresis was performed at 130 V for 5 h. Five DNA fragments of 257 bp containing the CbnR-binding sites at different positions from the ends to the middle were generated and labelled internally by PCR with [α -³²P]dCTP by using the following primer pairs: MCB1 (5'-GTT CGA TCC CGC GGT GGC TTC GCT CCA GAA GC-3') and MCB2 (5'-GCG CCG GCC ATG CCG TCC AAT ACC-3'), MCB3 (5'-TCC AGG GCT TGC ATC TGC CGC GTG ATGG-3') and MCB4 (5'-TTG TCG GTT TCG CCG GTCC-3'), MCB5 (5'-GGC GCT TGG CTG CTG CAG CCA TGT TCCC-3') and CBNFT2 (above), MCB6 (5'-GAA ATA CTT GAG CTG CCGG-3') and MCB7 (5'-TTC CGT CAC GCG TTG CTC CGT GAG GG-3'), and MCB8 (5'-GTG CCT ATA TTA CGC AAA CC-3') and MCB9 (5'-TTG GCC TCG GCC AGT TTC ATC ATG TAG CC-3'). The DNA fragments were purified with the QIA quick PCR purification kit (Qiagen). The bending angles were calculated as described previously (34).

RESULTS

Complementation by CbnR of growth on 3-CB of bacteria harboring *cbnABCD* genes. The *cbnABCD* genes encode enzymes of the modified *ortho*-cleavage pathway which degrade 3-chlorocatechol converted from 3-CB (Fig. 1) (38). To examine if *cbnR* was required for growth of NH9 on 3-CB, a complementation test was conducted. Either pCbln13RABCD or pCbln13ABCD, which retained the *cbnA* promoter region, was mobilized into *R. eutropha* NH9D, a derivative of NH9 which is cured of the *cbnRAB orfXCD* genes, or *P. putida* PRS4020, a *catR* knockout strain. The growth rates of the resultant four strains on 3-CB were compared together with those of strains containing the vector control, pCP13. Strains NH9D and PRS4020 with pCbln13RABCD showed growth on the 3-CB plate, while the strains with pCbln13ABCD or pCP13 did not. This result indicated that *cbnR* was necessary for the growth of these bacteria on 3-CB.

Activation of the *cbnA* promoter by CbnR during growth in the presence of Ben or 3-CB. It was presumed that CbnR may activate the *cbnA* promoter based upon the regulatory systems of other *ortho*-cleavage operons (29, 33, 58). To analyze the function of *cbnR* for the expression of the degradative genes in vivo, we used *P. putida* PRS4020 (*catR* knockout strain) as a host for reporter plasmids (Fig. 2). The cells were grown in

FIG. 1. Pathway for 3-CB degradation by *R. eutropha* NH9 and the genetic organization of the *cbnRAB orfXCD* operon. TCA, tricarboxylic acid.

basal synthetic medium (BSM) (1) supplemented with 10 mM glucose, 10 mM glucose, and 5 mM benzoate (Ben), or 10 mM glucose and 5 mM 3-CB for 18 h at 30°C. When the cells containing pNO50RAB' were grown on glucose with either Ben or 3-CB, the transcription from the *cbnA* promoter was activated 17- and 6.8-fold, respectively, compared to that in the cells grown on glucose alone (Table 2). The activation in the presence of Ben or 3-CB was not seen for the cells with pNO50AB', which did not contain *cbnR*. These results indicated that *cbnR* was a positive regulator of the transcription from the *cbnA* promoter.

Cells containing pNO50RA' did not induce substantially in the presence of 3-CB. When the same cells were grown in the presence of Ben, the transcription was activated 12-fold. The intact *cbnA* gene was necessary for the activation upon addition of 3-CB and also for the higher activation upon addition of Ben observed with pNO50RAB'. 3-CB and Ben are converted to 3-chlorocatechol and catechol, respectively, by enzymes encoded on the host chromosome, and CbnA (chlorocatechol dioxygenase) can further convert these (chloro)catechols to 2-CM and CCM, respectively. The above results suggested that 2-CM and CCM were the inducers of the *cbnA* promoter.

Purification of CbnRHis protein. Specific binding of CbnR to the *cbnA* promoter was demonstrated by gel retardation assay using the crude protein from *E. coli* BL21(DE3)pLysS containing pT7cbnR. In order to simplify protein purification, a plasmid expressing *cbnR* with six His codons on its C termi-

nus, pT7cbnRHis, was constructed. The crude soluble protein from *E. coli* BL21(DE3)pLysS containing pT7cbnRHis showed binding activity to the *cbnA* promoter, although a considerable portion of the protein was sequestered in inclusion bodies. When the crude protein was directly purified with the nickel affinity column, two major bands appeared in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile. One of the bands apparently corresponded to the calculated molecular mass of CbnRHis (32.9 kDa), and the other was a polypeptide of about 27 kDa. This 27-kDa protein was also produced from the cells of the vector control. To remove this contaminating protein, a heparin-agarose column was employed followed by the nickel affinity column, and the 32.9-kDa protein was recovered at more than 90% purity (Fig. 3, lane 3). The amino acid sequence of the N terminus of this protein was found to be identical to that of the deduced sequence of CbnRHis. The function of CbnRHis was evaluated with respect to partially purified wild-type CbnR in vivo and in vitro in the following sections.

Confirmation of the function of CbnRHis. Partially purified CbnR and purified CbnRHis were subjected to gel retardation assays with a 252-bp fragment containing the *cbnA* promoter region. The two proteins showed the same retardation mobility of the *cbnA* promoter fragment (Fig. 4, lanes 3 to 5 and 6 to 8). To test if CbnRHis had the same function as CbnR in vivo, growth complementation tests and reporter analysis were conducted. In growth complementation, strains NH9D and PRS4020 harboring pCbn13RHABCD grew on 3-CB, as did strains with pCbn13RABCD. In reporter analysis, PRS4020 containing pNO50RHAB' exhibited activation of transcription

FIG. 2. Diagram of the inserts of the constructs used for the *cbnA* promoter activity assay.

TABLE 2. The effect of Ben and 3-CB on transcriptional activation at the *cbnA* promoter

<i>P. putida</i> PRS4020 plasmid construct	β -Galactosidase activity (nmol/min/mg of extract) ^a		
	Glucose (10 mM)	Glucose (10 mM) + Ben (5 mM)	Glucose (10 mM) + 3-CB (5 mM)
Vector control (pQF50)	3.5 $\pm$ 0.2	1.8 $\pm$ 0.2	2.0 $\pm$ 0.2
pNO50RAB'	61.8 $\pm$ 2.3	1,038.1 $\pm$ 46.8	424.9 $\pm$ 22.4
pNO50AB'	116.1 $\pm$ 3.2	56.5 $\pm$ 3.9	71.1 $\pm$ 5.5
pNO50RA'	21.6 $\pm$ 0.7	261.9 $\pm$ 27.0	56.4 $\pm$ 8.7

^a Values are means $\pm$ standard deviation.

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of purification of CbnRHis. Lanes: 1, total protein from induced cells of BL21(DE3)pLysS containing pT7cbnRHis (100 μ g); 2, protein purified by heparin-agarose column (50 μ g); 3, CbnRHis purified by heparin-agarose column and nickel column (5 μ g); M, protein molecular mass markers. Sizes are shown in kilodaltons.

in the presence of either Ben or 3-CB to the same levels as PRS4020 containing pNO50RAB' did (data not shown). These results indicated that CbnRHis had the same function as CbnR in these *in vitro* and *in vivo* experiments.

Features of the *cbnA* promoter region bound by CbnR. The transcriptional start site of the *cbnA* promoter was determined by S1 nuclease analysis; the A on the template strand is marked by an arrow in Fig. 5 and was located 47 bp upstream from the translational start codon of the *cbnA* gene. The precise site of CbnR binding on the *cbnA* promoter was determined by the DNase I protection assay (Fig. 6). In the absence of inducer, approximately 60 bp from -76 to -19 (relative to the +1 of transcription) were protected from DNase I cleavage by CbnR. Both partially purified CbnR and CbnRHis exhibited the same protected patterns on the sense and antisense strands. These footprints were very similar to those of TcbR bound to the *tcbC* promoter (29). The addition of CCM or 2-CM up to 1 mM in

FIG. 4. Gel retardation assay demonstrating specific binding of CbnR and CbnRHis to the *cbnA* promoter region. A 252-bp fragment containing the *cbnA* promoter region was used as probe in lanes 1 to 8. A 336-bp fragment containing part of the hydroxyquinol 1,2-dioxygenase gene of *B. cepacia* AC1100 was used in lanes 9 to 12. Lanes: 1 and 9, no protein; 2 to 8, partially purified protein from BL21(DE3)pLysS/pT7-7 at 0.5 μ g; 3, 4, 5, and 11, partially purified CbnR at 0.05, 0.1, 0.5, and 0.5 μ g, respectively; 6, 7, 8, and 12, CbnRHis at 0.05, 0.1, 0.5, and 0.5 μ g, respectively.

FIG. 5. S1 nuclease assay to determine the transcriptional start site of *cbnA*. Sequencing reactions of the bottom strand of the *cbnA* promoter region are shown as A, T, G, and C. The products of S1 nuclease assay are shown in lanes 1 and 2 (marked by arrow). In lanes 1 and 2, 20 and 10 μ g of *in vivo*-derived RNA were used, respectively.

the binding reaction mixture did not change the footprinting pattern of CbnR to the *cbnA* promoter. Besides the inverted repeat containing T-N₁₁-A located in the recognition binding site (RBS) (50), there was a second inverted repeat (Fig. 6c) whose location is similar to those described recently by the study of BlaA, a LysR type regulator for β -lactamase genes of *Streptomyces cacaoi* (31).

Identification of the inducers by *in vitro* transcription assay. The results of the LacZ assays suggested that CCM and 2-CM act as the inducers of the *cbnA* promoter. Considerable activity, however, was also shown when the cells containing a truncated CbnA gene (pNO50RA') were grown in glucose with Ben (Table 2). The possibility that either Ben or catechol may also serve as an inducer could not be excluded. To examine the effect of these compounds on the transcriptional activation by CbnR, *in vitro* transcription assays were performed with purified CbnRHis and potential inducer compounds. Figure 7 shows that *cbnA* transcripts were produced only when either CCM or 2-CM was added to the reaction mixture (lanes 12 and 14). No transcript was produced with other compounds, including Ben (lane 4) or catechol (lane 8). Therefore Ben or catechol does not serve as an inducer for the *cbnA* promoter. The transcriptional start sites of the products with CCM or 2-CM were determined by S1 nuclease assay and were confirmed to be the same as that derived by *in vivo* assay (data not shown).

Bending of the *cbnA* promoter region. The presence of hypersensitive sites in the center of the footprint region of the *cbnA* promoter suggested that changes in DNA conformation might occur upon binding of CbnRHis. To examine this potential change with or without inducer, circular permutation gel shift assays were performed. Lanes 1 to 5 of Fig. 8 indicate that binding of CbnRHis caused bending of the promoter fragment; the bending angle was estimated to be 78°. When CbnRHis was bound to the promoter in the presence of CCM, relaxation of the bending angle to 54° was observed (lanes 6 to 10). The bending angles of three independent experiments varied by no more than 7.7%. Partially purified CbnR gave the same bending angles as CbnRHis (data not shown).

DISCUSSION

Transcriptional activation of the *cbnA* promoter by *cbnR* has been characterized both *in vivo* and *in vitro*. In growth comple-

FIG. 6. DNase I footprint of CbnRHis and CbnR on the *cbnA* promoter region. (a and b) Top strand (a) and bottom strand (b) of the *cbnA* promoter region. Lanes: 1, no protein; 2, CbnRHis (0.5 µg); 3, partially purified CbnR (1 µg); 4, partially purified protein from BL21(DE3)pLysS/pT7-7 (1 µg). (c) Schematic diagram of the *cbnA* promoter region protected from DNase I digestion by CbnRHis. The protected nucleotides are shown by the brackets. The vertical arrows indicate sites of hypersensitivity to DNase I digestion. The thick horizontal arrows show the inverted repeats containing T-N₁₁-A, regarded as a motif of LysR regulatory systems (50). The horizontal dotted arrows above the bottom strand indicate the second imperfect inverted repeat similar to those described recently (31). The horizontal solid lines under the top strand and above the bottom strand indicate the -35 and -10 regions of the *cbnA* promoter and the divergently transcribed *cbnR* promoter. The regions from nucleotide -76 to -49 and from -44 to -19 are suggested to be the RBS and the ABS, respectively (33). The numbering is relative to the transcriptional start site of *cbnA*.

mentation studies, *cbnR* was demonstrated to be necessary for growth on 3-CB for *R. eutropha* NH9D (3-CB⁻ derivative of NH9) and *P. putida* PRS4020 (*catR* knockout strain), when they harbor the *cbnABCD* degradative genes. CbnR has also been demonstrated to be a positive regulator by transcriptional reporter and in vitro transcription assays.

Both CCM and 2-CM have been identified as inducer molecules. In reporter analysis, the PRS4020 cells containing a plasmid with a truncated *cbnA* gene, pNO50RA', exhibited some elevated transcription in the presence of Ben. Therefore, it remained possible that the *cbnA* promoter could be induced by Ben or catechol. However, neither Ben nor catechol induces transcriptional activation by purified CbnRHis in vitro. The reason for the in vivo activation of the pNO50RA' construct is unclear. It is possible that the activity observed was induced by CCM supplied by the host-encoded catechol dioxygenase (CatA). Although *catR*, the activator of the host *catA* gene (26), is disrupted in PRS4020, there may be some low-level constitutive expression of *catA* which results in the conversion

FIG. 7. In vitro transcription assay demonstrating the requirement of both CbnR and inducer. In lanes 1 to 14, 0.1 µg of CbnRHis was used in the lanes with even numbers, while it was not added to the lanes with odd numbers. Lanes: 1 and 2, no chemicals; 3 and 4, Ben; 5 and 6, 3-CB; 7 and 8, catechol; 9 and 10, 3-chlorocatechol; 11 and 12, CCM; 13 and 14, 2-CM; 15, marker (a transcript of 428 bases derived from plasmid template pJET41) (16, 35) is shown as a faint band near the arrow marked *cbnA*. All of the chemicals were used at 1 mM concentrations. RNA-1 is the transcript from the ColE1 ori of the supercoiled plasmid pMP7.

of Ben to CCM, allowing the activation of the *cbnA* promoter. It is also possible that there are additional CbnR binding sites in the *cbnA* gene which add to the complexity of the *cbnA* promoter regulation. Both repressor and activator internal binding sites have been observed with other LysR family members, including CatR (6, 10). Although the activity observed with construct pNO50RA' in the presence of Ben cannot be explained, the in vitro transcription assays clearly demon-

FIG. 8. Circular permutation gel shift assay demonstrating CbnRHis bending of the *cbnA* promoter region in the absence (lanes 1 to 5) and presence (lanes 6 to 10) of 1 mM CCM. The ca. 60-bp region that showed a footprint by CbnR was distributed in different positions from the ends (lanes 1 and 6, left ends; lanes 5 and 10, right ends) to the center (lanes 3 and 8) of 257-bp fragments. The calculated bending angles were 78° and 54° in the absence and presence of CCM, respectively.

strated that CCM and 2-CM function as inducers for *cbnA* promoter expression.

It is puzzling that CbnR recognizes CCM as well as 2-CM as an inducing molecule. The enzymes of the *cbn* operon can catabolize catechol to a certain extent. Chlorocatechol 1,2-dioxygenase (CbnA) can utilize catechol as a substrate (15, 47), and chloromuconate cycloisomerase (CbnB) has maintained some activity against nonchlorinated CCM (47, 52). However, 3-oxoadipate enol-lactone, the product from CCM by CbnB, is presumed not to be catabolized further by the enzymes encoded by *cbnCD* (51). CatR and ClcR activate their regulated promoters significantly only in the presence of the intermediates of their respective pathways: CCM for CatR (43) and 2-CM for ClcR (35). The *clcABDE* operon is expressed when cells are grown in Ben because CatR binds to and activates the *clcA* promoter (34, 35, 42). However, CatR cannot bind to or activate the *cbnABCD* promoter (unpublished observations). It is possible that in addition to utilizing chlorocatechols as carbon substrates, the chlorocatechol dioxygenase enzymes act as important detoxifying agents. Catechol could be toxic to pseudomonads as chlorocatechols are (21). Because CatR cannot activate the expression of CbnA to detoxify catechol as it would ClcA, CbnA expression by CbnR upon recognition of CCM could be beneficial for the cell to decompose catechol rapidly.

The *clcABDE* operon was isolated from 3-CB-degrading bacteria (5) and thus functions to degrade 3-chlorocatechol in the original isolate. The high homology between the *cbnAB orfXCD* and the *tcbCD orfXEF* genes (38) suggests that the *cbnAB orfXCD* genes may have evolved to degrade more highly chlorinated catechols compared to the *clcABDE* genes. The recognition of more highly substituted catechols or corresponding muconates by the regulators of the *ortho*-cleavage pathway is mostly unknown. It has been demonstrated that 2,4-dichloromuconate acts as an inducer of *tfdCDEF* expression (18). However, the range of inducing molecules for these operons has not been explored. Since the products from the *tcbCD orfXEF* operon degrade dichlorocatechols and 3,4,6-trichlorocatechol, TcbR (CbnR) probably recognizes corresponding chloromuconates as inducers. Nevertheless, CbnR also has the ability to recognize CCM as an inducer, which is characteristic of CatR.

The specific binding of CbnRHis and CbnR to the *cbnA* promoter has been shown by gel retardation and DNase I protection assays. CbnRHis had the same function as CbnR in growth complementation, the reporter assay, and the bending assay of the *cbnA* promoter with and without inducer. Therefore, functional equality of CbnRHis with CbnR has been verified *in vivo* and *in vitro*. A similar observation about a His tag on the C terminus has been reported for NhaR, a LysR-type regulator of Na^+/H^+ antiporter gene of *E. coli* (2), suggesting the unaltered function of a C-terminally His-tagged LysR-type protein. On the other hand, the crude soluble protein from *E. coli* BL21(DE3)pLysS containing pEHiscbnR did not show binding activity to the *cbnA* promoter (data not shown). Although the N-terminal part of LysR family proteins functions as the DNA binding domain, addition of amino acids on the N terminus does not necessarily abolish binding activity. The addition of a polypeptide to the N terminus of SpvR, a LysR-type regulator of virulence genes of *Salmonella dublin*, showed little effect on the specific binding to the regulated promoters and activation of the promoters (22). It is not known whether the protein produced from the construct pEHiscbnR does not have the binding activity to the *cbnA* promoter or is exclusively retained in the inclusion bodies.

Under the established conditions for gel retardation assays

(40, 58), crude soluble protein from BL21(DE3)pLysS containing either pT7cbnR or pT7cbnRHis always formed aggregates with the *cbnA* promoter fragment in the well of the electrophoresis gel. The addition of heparin in the binding reaction dissolved the aggregate, and the protein-DNA complex was able to migrate in the gel. It was presumed that CbnR formed these aggregates because of its highly basic nature (calculated $\text{pI} = 10.3$ by Genetyx Software [Software Development Co., Tokyo, Japan]). Heparin has been used successfully in the study of the formation of splicing complexes on mRNAs, where it was presumed to quench the nonspecific binding of components in the nuclear extract with the highly negatively charged RNA (28). In our study, it is possible that the negatively charged heparin was able to cancel the electrostatic force among the complexes of CbnR(His)-DNA, but not the specific binding between CbnR(His) and the DNA fragment.

The length and the location of the footprinting pattern of CbnR to the *cbnA* promoter, with hypersensitive sites localized in the center, resemble those of CatR to the *catB* promoter (in the presence of the inducer CCM) (43) and of ClcR to the *clcA* promoter (8, 35). Thus, it is suggested that the region from nucleotide -76 to -49 is the RBS and the region from -44 to -19 is the activation binding site (ABS) (33), as shown in Fig. 6c. The RBS contains the conserved T-N₁₁-A motif critical for binding by the LysR-type regulators (33, 50). Further binding of the regulator to ABS helps RNA polymerase bind to the promoter region, and thus to activate transcription (33). Although both CbnR and CatR recognize CCM as an inducer, the extended footprinting pattern of CbnR in the absence of the inducer, encompassing both the RBS and ABS of the *cbnA* promoter, resembles that of ClcR to the *clcA* promoter (8, 35). This is in contrast to the restricted footprinting pattern of CatR to the RBS of the *catB* promoter in the absence of CCM (43). The DNA binding domain of the LysR-type regulators is believed to be located in the N terminus, and the regions responsible for inducer recognition are believed to be located in the middle of the protein (50). The difference in extent of the footprinting pattern without CCM between *cbn* and *cat* suggests that the evolution of the DNA binding domain and the inducer recognition region(s) could be discrete.

The ABS binding pattern of CbnR without inducer seemed similar to that of ClcR. However, the addition of an inducer, CCM or 2-CM, to the binding reaction mixture did not cause a change in the footprinting pattern of CbnR to the *cbnA* promoter, while addition of 2-CM caused a shift in the footprinting pattern of ClcR to the ABS of the *clcA* promoter region (35). It remains to be elucidated if the apparent lack of change in the footprinting pattern of CbnR is an intrinsic characteristic of the CbnR-*cbnA* promoter system or is due to experimental conditions.

The relaxation of the bending angles of *cbnA* promoter region bound by CbnR(His) upon the addition of inducer was similar to those shown by CatR (41) and ClcR (34). The footprinting patterns and changes in DNA bending upon addition of inducers of the *cat*, *clc*, and *cbn* promoters suggest that the outline of the transcriptional mechanism of these regulatory systems is conserved. Our present study further indicates independent evolution of the regulatory genes in terms of their DNA binding and recognition of inducer molecules.

ACKNOWLEDGMENTS

We are grateful to C. Douglas Hershberger for the preparation of the protein sample for N-terminal sequencing. We also thank the members of the Chakrabarty laboratory for aid and helpful discussions.

This work was supported by Public Health Service grant ES 04050-13 from the National Institute of Environmental Health Sci-

ences and by the Program for Promotion of Basic Research Activities for Innovative Biosciences of Japan.

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