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Roles of CatR and *cis,cis*-Muconate in Activation of the *catBC* Operon, Which Is Involved in Benzoate Degradation in *Pseudomonas putida*

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In *Pseudomonas putida*, the *catBC* operon encodes enzymes involved in benzoate degradation. Previous studies have determined that these enzymes are induced when *P. putida* is grown in the presence of benzoate. Induction of the enzymes of the *catBC* operon requires an intermediate of benzoate degradation, *cis,cis*-muconate, and a regulatory protein, CatR. It has been determined that CatR binds to a 27-bp region of the *catBC* promoter in the presence or absence of inducer. We have called this the repression binding site. In this study, we used a gel shift assay to demonstrate that the inducer, *cis,cis*-muconate, increases the affinity of CatR for the *catBC* promoter region by 20-fold. Furthermore, in the absence of *cis,cis*-muconate, CatR forms two complexes in the gel shift assay. The inducer *cis,cis*-muconate confers specificity primarily for the formation of complex 2. DNase I footprinting showed that an additional 27 bp of the *catBC* promoter region is protected by CatR in the presence of *cis,cis*-muconate. We have named this second binding site the activation binding site. Methylation interference footprinting determined that in the presence or absence of inducer, five G nucleotides of the *catBC* promoter region were necessary for CatR interaction with the repression binding site, while a single G residue was important for CatR interaction with the activation binding site in the presence of *cis,cis*-muconate. Using polymerase chain reaction-generated constructs, we found that the binding of CatR to the repression binding site is independent of the activation binding site. However, binding of CatR to the activation binding site required an intact repression binding site.

Pseudomonads are common soil bacteria which are capable of degrading many toxic compounds. These compounds are utilized as sources of nutrients after they are converted into intermediates common to conventional metabolic pathways. Understanding the regulation of genes which encode enzymes responsible for catabolism is of great interest to the field of biodegradation. Studies concerning the nature of substrate specificity for different catabolic enzymes may eventually lead to the engineering of novel catabolic pathways for compounds that currently cannot be degraded by nature. Since many of these recalcitrant compounds contain one or more aromatic rings (7, 8, 12), our laboratory has been studying the regulation of benzoate catabolism by *Pseudomonas putida* in order to better understand the regulation of the biodegradative processes.

The chromosomal genes of *P. putida* which code for the enzymes that catabolize benzoate to β-ketoadipate have been cloned and characterized (1, 2, 34, 35). Figure 1A shows that the *catBC* operon encodes the enzymes *cis,cis*-muconate lactonizing enzyme and muconolactone isomerase, respectively. Early studies demonstrated that growth on benzoate increased the levels of all β-ketoadipate enzymes (20, 22, 34, 35). Recently, our laboratory has shown that the *catR* gene is a positive transcriptional activator of the *catBC* operon when *P. putida* is grown in the presence of benzoate (25). The 32.2-kDa CatR protein encoded by this gene shows considerable homology to the LysR family of regulatory proteins (13, 24).

In an earlier study (25), our laboratory reported that *catR* and *catBC* are divergently transcribed with overlapping promoters (Fig. 1B). It was also reported that CatR binds to a single 27-bp site located in the region between the *catR* and *catBC* genes (Fig. 1C). In this study, we have examined the mechanism by which CatR activates the *catBC* operon. Gel shift studies have determined that *cis,cis*-muconate increases the binding affinity of CatR for the *catBC* promoter region. In addition, *cis,cis*-muconate allows CatR to bind to a second site of the *catBC* promoter region, thereby activating *catBC* transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *Escherichia coli* strain used for general cloning procedures was JM109 [Δ(*pro-lac*) *recA1 thi-1 supE endA gyrA96 hsdR relA1*(F' *traD36 proAB lacI*^{q} *lacZ*ΔM15)] (21). JM109 was grown at 37°C in Luria broth (LB) (19). Agar was added to 1.5% for plates. Antibiotics were added to sterile media in the following concentrations: ampicillin, 75 μg/ml; and kanamycin, 75 μg/ml.

Purification of CatR. The CatR protein was overexpressed in JM109 by using the expression vector pKR119ΔHf as described previously (25). Cells grown overnight in 500 ml of LB supplemented with ampicillin were added to 6 liters of the same medium and allowed to grow for 1 h at 37°C. At this time, isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 1 mM) was added to the culture, after which growth was allowed to continue for an additional 10 h. The

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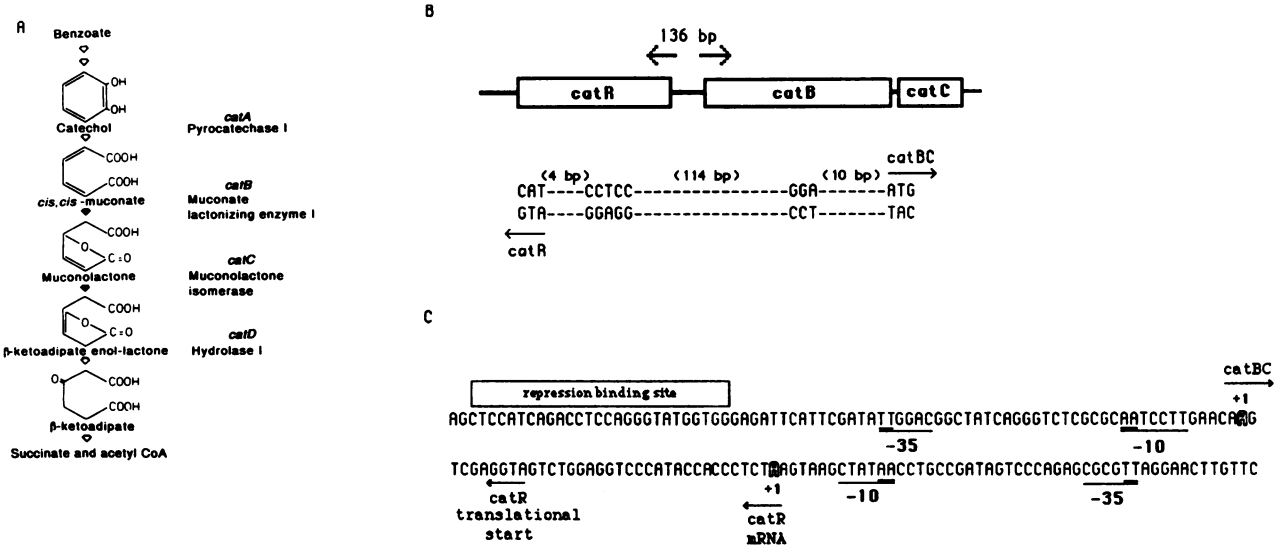

FIG. 1. (A) The catechol branch of the β-ketoadipate degradative pathway. The enzymes and metabolic intermediates of the pathway are shown. The filled arrows represent enzymatic steps in the pathway encoded by the *catB* and *catC* genes that are known to be positively regulated by CatR. (B) Structural arrangement of the *catR* gene and the *catBC* operon. Arrows show the direction of transcription of *catR* and the *catBC* operon. The translational starts of *catR* and *catBC* are separated by 136 bp of intervening DNA containing the Shine-Dalgarno sequences and promoter regions of these two genes. (C) The intervening region between the *catR* translational start and the *catBC* transcriptional start containing the overlapping promoters. The boxed region represents the *catBC/R* intergenic DNA that is bound by CatR, as determined previously by hydroxyl radical footprinting (25). Binding of this region by CatR results in repression of the *catR* gene.

cells were harvested by centrifugation at 7,000 × *g* for 10 min, resuspended in 1% NaCl, and pelleted once again. These cells were stored at −70°C.

Step I. Preparation of crude extract. The frozen cell pellet representing 6 liters of cells was resuspended in 25 ml of purification buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 250 mM NaCl, 0.05 mM EDTA, 5% glycerol). The cells were broken by two passages through a French pressure cell at 12,000 lb/in^2, and the resulting cell lysate was centrifuged for 30 min at 40,000 × *g*. The supernatant was removed and centrifuged at 100,000 × *g* for 1 h. The resulting supernatant represented the crude extract.

Step II. Heparin-agarose chromatography. The crude extract was passed over a 75-ml heparin-agarose column equilibrated with 200 ml of purification buffer. After washing of the column with 250 ml of the same buffer, bound protein was eluted with a 300-ml linear gradient of NaCl (0.25 to 1.0 M). Fractions containing CatR were identified by the gel shift assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (17). Protein concentration was determined by the Bradford assay (4). The appropriate fractions were pooled and dialyzed for 2 h in 4 liters of dialysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 0.05 mM EDTA).

Step III. S-Sepharose chromatography. The dialyzed protein was passed over a 30-ml S-Sepharose column equilibrated with 100 ml of dialysis buffer. The protein was eluted in a 60-ml linear gradient of NaCl (0.05 to 1.0 M), and fractions containing CatR were pooled and dialyzed for 2 h in 4 liters of Superose 12 running buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT, 200 mM NaCl, 0.05 mM EDTA).

Step IV. Gel filtration chromatography. CatR was further purified by gel filtration chromatography. The dialyzed protein collected from S-Sepharose chromatography was concentrated with a Centricon-10 ultrafiltration unit (Amicon, Danvers, Mass.) and resolved on a fast protein liquid chromatography (FPLC) Superose 12 column equilibrated in Superose 12 running buffer (see procedure described for S-Sepharose chromatography). The CatR collected after this final purification step was >95% pure. An equal volume of glycerol was added to the pooled Superose 12 fractions containing 1.5 mg of CatR protein per ml. The protein was stored at −70°C for later use in in vitro experiments.

DNA preparation and sequencing. Plasmid DNA was isolated from 40-ml cultures as described by Maniatis et al. (19). Single-stranded DNA was isolated as described elsewhere (30). Sanger dideoxy DNA sequencing was performed in order to confirm the fidelity of polymerase chain reaction (PCR) products (3, 21). The Maxam-Gilbert G+A reaction used as a reference ladder for the footprinting reactions was performed as described by Sambrook et al. (26).

Molecular cloning. Plasmids pMPH8, pMPF6, and pABS1 represent different regions of the *catBC* promoter segment cloned into pUC119. These plasmids were constructed by using PCR technology to amplify the appropriate DNA sequence. The template for the PCR reactions was plasmid pKRT6-1, which contains the entire *catBC* promoter region (24). The PCR protocol (23) utilized a 30-cycle run consisting of a 95°C denaturing step, 55°C annealing step, and 72°C extension step. To amplify the *catBC* promoter fragment used in the construction of pMPF6, two primers, P1 (5′-GGGAATTCCAGCAGCTCGGCGGCGCG-3′) and P2 (5′-GGGAGCTCAAGGATTGCGCGAGACCCT-3′), were selected to yield a 164-bp PCR product containing both of the CatR binding sites. To facilitate the subsequent cloning of the PCR product into pUC119, the two primers were synthesized (Operon Technologies, Inc., Alameda, Calif.) such that the final 164-bp PCR product contained an *Eco*RI site on one end and an *Sst*I site on the other end.

The 132-bp DNA fragment used to construct pMPH8 was

generated by using primers (P1 and P3 [5'-GGGAGCTCTC CCACCATACCCTGGA-3']) that allowed for the amplification of sequences containing only the first CatR binding site. Figure 1C depicts this first CatR binding site centered at −67 bp relative to the *catBC* transcriptional start site and is referred to as the repression binding site (RBS). The second CatR binding site, or activation binding site (ABS), was amplified by using primers (P2 and P4 [5'-GGGGATCCGAT TCATTCGATATTGG-3']) to create a 150-bp fragment which was cloned into pUC119 to create pABS1.

Restriction enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), and alkaline phosphatase was the product of Boehringer Mannheim Biochemicals (Indianapolis, Ind.). T4 DNA ligase was supplied by New England Biolabs (Beverly, Mass.).

Radioactive labeling of DNA. A 380-bp fragment containing the *catBC* promoter region was prepared as follows. Plasmid pKRT6-1 (5 μg) was digested with *Xho*I for 3 h, and the ends were filled in with Klenow fragment and [α-^{32}P]dCTP (19). After 30 min of incubation at 25°C, the sample was extracted with an equal volume of phenol-chloroform and precipitated with ethanol. The 380-bp promoter fragment was released after a 3-h *Pst*I digestion. The digest was loaded onto a 5% polyacrylamide gel and electrophoresed at 120 V for 3 h, using 1× running buffer (40 mM Tris acetate, 1 mM EDTA). Autoradiography of the gel allowed the radioactive promoter fragment to be excised and subsequently eluted from the gel, using an Elutrap electrophoresis chamber device (Schleicher & Schuell, Keene, N.H.). The eluted DNA was ethanol precipitated and resuspended in water.

The *catBC* promoter regions from pMPH8 and pMPF6 were released as *Eco*RI-*Sst*I fragments, while a *Hin*dIII-*Pvu*II digest was used to excise the pABS1 *catBC* promoter region. After digestion, these fragments were end labeled with [α-^{32}P]dATP at the *Eco*RI and *Hin*dIII ends, respectively. The labeled fragment was then isolated as described above. These radioactive promoter regions were used as a substrate for CatR binding and subsequent gel shift analysis.

Gel shift assays. The binding of CatR to the *catBC* promoter DNA was demonstrated by gel shift assays, using a modified Fried and Crothers procedure (9). The binding reaction mixture consisted of 2 μl of 10× binding buffer (100 mM Tris-HCl [pH 7.5], 100 mM β-mercaptoethanol, 10 mM EDTA, 1% Triton X-100, 40% glycerol, 500 mM KCl, 50 mM $MgCl_2$), 2 μl of bovine serum albumin (10 mg/ml), 2 μl of salmon sperm DNA (0.5 mg/ml), and 8 pM radioactive *catBC* promoter region DNA in a total volume of 20 μl. After 30 min of incubation at 30°C, the samples were electrophoresed in a 5% polyacrylamide gel for 3 h at 120 V, using Tris-EDTA as the running buffer. Some of the binding reaction mixtures and electrophoresis buffer contained 100 mM *cis,cis*-muconate, benzoate, or β-ketoadipate. The bands were visualized by autoradiography, and the radioactivity content was quantitated by using an AMBIS β scanner (AMBIS, San Diego, Calif.).

DNase I footprinting. For DNase I footprinting reactions, a modified Galas and Schmitz procedure (10, 16) was used. The 20-μl CatR binding reaction mixture described above was incubated for 30 min at 30°C and then shifted to 25°C for 5 min. DNase I digestion was initiated by adding 30 μl of DNase I reaction buffer (20 mM Tris-HCl [pH 7.9], 3 mM $MgCl_2$, 5 mM $CaCl_2$, 80 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 50 mg of bovine serum albumin per ml) and DNase I. After digestion of the CatR-*catBC* promoter complex for 1 min with various amounts of DNase I, the reactions were stopped by adding 50 μl of 500 mM EDTA. The digested DNA was prepared for electrophoresis by extraction twice with phenol-chloroform and precipitation with ethanol. The digested samples were resuspended in loading buffer (47% formamide, 10 mM EDTA, 0.05% bromophenol blue, 5% xylene cyanol) and applied to a standard sequencing gel for analysis.

Restriction protection assay. A 20-μl CatR binding reaction mixture was incubated in either the presence or absence of 100 μM *cis,cis*-muconate for 30 min at 30°C. The binding reaction mixtures were then digested with either *Mnl*I or *Taq*I for 90 min at 30°C and subjected to two phenol-chloroform extractions and an ethanol precipitation. The samples were resuspended in loading buffer and resolved on a 5% native polyacrylamide gel as described for the gel shift assays. Radioactive digestion products were visualized by autoradiography.

Methylation interference footprinting. The procedures of Sienbenlist and Gilbert (28) and Yang and Nash (36) were used, with the following modifications. A 164-bp *Eco*RI-*Sst*I fragment containing the *catBC* promoter region was isolated from pMPF6. After labeling of the *Eco*RI end with Klenow fragment and [α-^{32}P]dATP, the 164-bp promoter fragment was released with *Sst*I, phenol-chloroform extracted twice, and precipitated with ethanol. The labeled promoter fragment was resuspended in 200 μl of a solution containing 50 mM sodium cacodylate and 1 mM EDTA. The mixture was chilled on ice, and methylation was initiated by adding 5 μl of dimethyl sulfate stock (diluted 1:5 with water). The reaction mixture was immediately placed in a 20°C water bath for 3 min, and the reaction was terminated by the addition of 25 μl of 3 M sodium acetate and 600 μl of ethanol. The precipitated DNA was dried in vacuo, resuspended in water, and used as a substrate for CatR in the binding reaction described above. The binding reaction and gel shift electrophoresis assay were performed either in the presence or in the absence of *cis,cis*-muconate.

Autoradiography of the native 5% polyacrylamide gel allowed for the location and excision of radioactive *catBC* promoter fragments that migrated as either free or bound (to CatR) DNA. These bands were excised from the gel and subjected to electroelution as described above. The electroeluted protein-DNA complexes were extracted twice with phenol-chloroform, precipitated with ethanol, and dried in vacuo. The purified DNA samples were then resuspended in 100 μl of 0.5 M piperidine, incubated at 90°C for 30 min, and finally dried under reduced pressure. The DNA was resuspended in loading buffer and electrophoresed in a standard sequencing gel.

RESULTS

Purification of CatR. CatR was purified in order to biochemically characterize the protein in vitro. Figure 2 shows that the crude extract of IPTG-induced JM109/pKR119ΔHf consisted of approximately 20% CatR. Heparin-agarose chromatography provided a good first step in the purification process, with CatR eluting from the column at 0.6 M NaCl (data not shown). CatR was further purified by cation-exchange chromatography on an S-Sepharose column. Protein that eluted at approximately 0.9 M NaCl was analyzed by SDS-PAGE and judged to be about 80% pure (Fig. 2, lane 3). The final purification step required gel filtration chromatography on an FPLC Superose 12 column, yielding approximately 95% pure CatR. CatR was eluted from this column at the same volume as bovine serum albumin (66 kDa), suggesting that CatR is a dimer in solution. This purified CatR

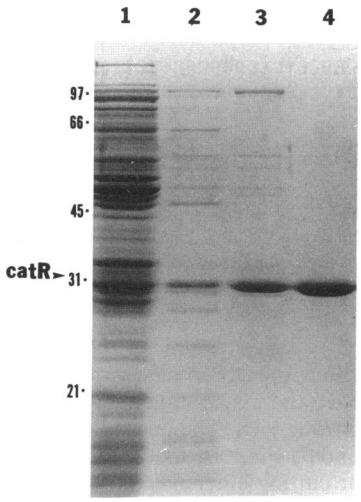

FIG. 2. SDS-PAGE analysis of CatR purification steps. Each lane was loaded with 20 μg of protein. Lanes: 1, crude extract (arrow indicates CatR); 2, the pooled fractions collected after heparin-agarose chromatography of the crude extract; 3, S-Sepharose chromatography of the pooled heparin-agarose fractions; 4, gel filtration chromatography of the S-Sepharose fractions containing CatR.

retained the ability to bind to the *catBC* promoter region throughout the entire purification process.

Effect of the inducer *cis,cis*-muconate on CatR binding to the *catBC* promoter. It was previously reported that partially purified CatR could specifically bind the *catBC* promoter region and that *cis,cis*-muconate did not affect the CatR-*catBC* interaction (25). Using pure CatR, we were able to demonstrate that *cis,cis*-muconate did affect CatR binding in vitro. Figure 3A shows that binding of CatR to its target

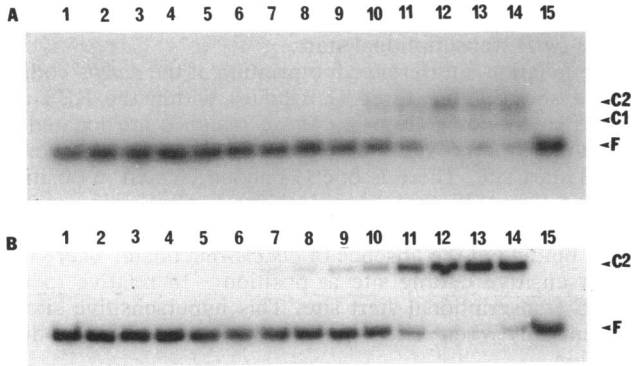

FIG. 3. (A) Gel shift analysis of purified CatR performed with and without *cis,cis*-muconate. Lanes 1 and 15 represent only ^{32}P-labeled *catBC* promoter DNA. Lanes 2 to 14 represent the following concentrations of purified CatR used in the binding assay: 2, 50 pg; 3, 100 pg; 4, 150 pg; 5, 200 pg; 6, 500 pg; 7, 1,000 pg; 8, 1,500 pg; 9, 2,000 pg; 10, 5 ng; 11, 10 ng; 12, 15 ng; 13, 20 ng; 14, 25 ng. The radiolabeled promoter fragment was present at a concentration of 8 pM. F, free DNA. (B) CatR binding to the *catBC* promoter region in the presence of 100 mM *cis,cis*-muconic acid. Lanes 1 and 15 represent only ^{32}P-labeled *catBC* promoter DNA. Lanes 2 to 14 represent the following concentrations of purified CatR used in the binding assay: 2, 5 pg; 3, 10 pg; 4, 15 pg; 5, 20 pg; 6, 50 pg; 7, 100 pg; 8, 150 pg; 9, 200 pg; 10, 500 pg; 11, 1,000 pg; 12, 1,500 pg; 13, 2,000 pg; 14, 2.5 ng.

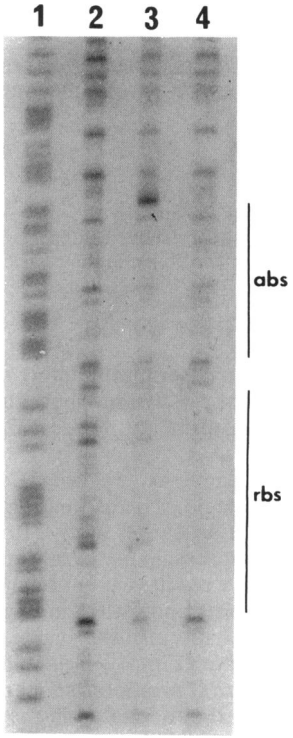

FIG. 4. DNase I footprinting. A 384-bp ^{32}P-labeled *XhoI-PstI* fragment containing the *catBC* promoter region was used for the footprinting analysis. Lanes: 1, Maxam-Gilbert G+A reaction performed on the same fragment; 2, DNase I cleavage of the promoter fragment performed in the absence of CatR; 3, the protection pattern observed in the presence of both CatR and 100 mM *cis,cis*-muconic acid; 4, the protection pattern observed when CatR is bound to the promoter region in the absence of *cis,cis*-muconate.

DNA results in the formation of two complexes, C1 and C2, as the protein concentration increases in the binding assay. The addition of 100 μM *cis,cis*-muconate to the binding reaction produced only the slower-migrating C2 (Fig. 3B), and the binding affinity of CatR for the promoter region was increased approximately 20-fold.

Since *cis,cis*-muconate favors the formation of C2, this complex may represent the interaction of CatR, inducer (*cis,cis*-muconate), and *catBC* promoter DNA necessary for activation of the *catBC* operon. This activation process would allow full expression of the *catB* and *catC* genes, enabling *cis,cis*-muconate to be further degraded by the enzymes *cis,cis*-muconate lactonizing enzyme and muconolactone isomerase, respectively. Other intermediates of the β-ketoadipate pathway such as benzoate, catechol, and β-ketoadipate did not alter the binding of CatR with the promoter region (data not shown), verifying that *cis,cis*-muconate has a specific in vitro effect on the binding of CatR to the *catBC* promoter region.

To examine the binding of pure CatR to the *catBC* promoter region, we used DNase I footprinting. Figure 4 shows that a 27-bp region was protected in the presence and absence of *cis,cis*-muconate. This region extended from −53 to −79 relative to the *catBC* transcriptional start site (Fig. 4 and 5). The presence of *cis,cis*-muconate in the CatR binding reaction resulted in CatR binding to a second site downstream of the first site. This second binding site protected an additional 27 bp spanning the region from −22 to −48

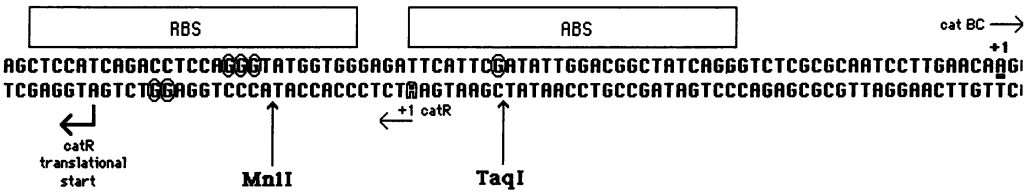

FIG. 5. Sequence of the promoter regions between the *catR* gene and the *catBC* operon. The boxed regions represent the sequence that is protected by CatR in the presence or absence of *cis,cis*-muconate (RBS) or only in the presence of *cis,cis*-muconate (ABS). The unique restriction sites *MnlI* and *TaqI* used in the restriction protection assay are depicted with arrows. The G residues critical for CatR binding are circled.

relative to the *catBC* transcriptional start site (Fig. 5). The lowest concentration of *cis,cis*-muconate that induced CatR to extend the DNA footprint to the second binding site was 100 μM.

Restriction protection assay. Figure 1C shows that the CatR RBS protects a region corresponding to +6 to +32 of the *catR* transcript. In an earlier study, Rothmel et al. (25) showed that CatR represses its own transcription when bound to this site. As a result, we have labeled this binding site RBS in Fig. 5. The second CatR binding site, revealed in this study by DNase I footprinting in the presence of the inducer *cis,cis*-muconate (Fig. 4), is labeled ABS (Fig. 5). Examination of the *catBC* promoter sequence revealed that there was a unique *MnlI* site located in the RBS. Likewise, a unique *TaqI* site was found in the ABS. Figure 6A shows that the *MnlI* site was protected from cleavage by *MnlI* when CatR was present in the binding reaction. Addition of *cis,cis*-muconate to the binding reaction did not significantly alter CatR protection of the *MnlI* site. However, the *TaqI* site located within the ABS was protected from cleavage only if both CatR and *cis,cis*-muconate were present in the binding reaction (Fig. 6B). The uncut DNA present (Fig. 6A, lanes 2, 5, 6, and 7; Fig. 6B, lanes 2 and 5) may have been due to the poor cutting efficiencies of these enzymes in the CatR binding buffer. This unique assay confirmed the results of the DNase I footprint, which showed that *cis,cis*-muconate induced CatR to bind to a second site located at positions −22 to −48 relative to the *catBC* transcriptional start site.

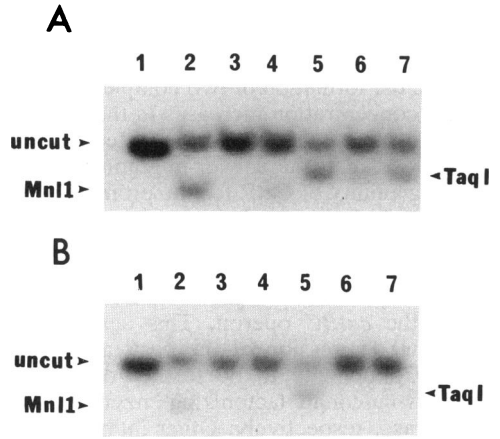

FIG. 6. Restriction protection assay. The RBS and ABS contain unique *MnlI* and *TaqI* sites, respectively. The assay determined whether CatR binding to the RBS or ABS protects these sites from restriction endonuclease cleavage by *MnlI* or *TaqI*. A binding reaction was performed by using pure CatR and a ^{32}P-labeled *catBC* promoter fragment, followed by the addition of the appropriate restriction enzyme. The reaction was then loaded onto a native 5% polyacrylamide gel and visualized by autoradiography. (A) Restriction protection assay performed in the absence of *cis,cis*-muconate. Lanes: 1, DNA only; 2, DNA and *MnlI*; 3, DNA, *MnlI*, and 34 ng of CatR; 4, DNA, *MnlI*, and 3.4 ng of CatR; 5, DNA and *TaqI*; 6, DNA, *TaqI*, and 34 ng of CatR; 7, DNA, *TaqI* and 3.4 ng of CatR. (B) Restriction protection assay performed in the presence of *cis,cis*-muconate. Each lane represents the same components as in panel A except that 100 mM *cis,cis*-muconate was present.

Methylation interference footprinting of CatR bound to the *catBC* promoter region. The methylation interference footprinting technique allowed us to examine the CatR-*catBC* promoter complex C2 observed in Fig. 2 and 3. Methylated *catBC* promoter DNA was incubated with CatR and electrophoresed to reveal C2 formed in the absence or presence of *cis,cis*-muconate. Isolation and subsequent footprinting of these complexes revealed that C2 formed in the presence of *cis,cis*-muconate contains CatR protected promoter DNA which has a methylation interference footprint pattern different from that of C2 formed in the absence of *cis,cis*-muconate. Methylation interference footprinting of the *catR* coding strand demonstrated that the G residues located at positions +21 and +22 relative to the *catR* transcriptional start interfere with CatR binding when methylated (Fig. 7). There is a hypersensitive site at positions +5 and +6 relative to the *catR* transcriptional start.

Methylation interference footprinting of the *catBC* coding strand showed that three G residues within the RBS are critical for binding (Fig. 8). These residues are located at positions −61 through −63 relative to the *catBC* transcriptional start site. There is one G residue located at position −41 of the ABS that is critical for CatR binding in the presence of *cis,cis*-muconate. This residue is not critical for CatR binding in the absence of *cis,cis*-muconate. There is a hypersensitive cutting site at position −15 relative to the *catBC* transcriptional start site. This hypersensitive site is present only when *cis,cis*-muconate is added to the binding reaction.

Cooperative binding of CatR to the *catBC* promoter. To evaluate the roles that the two CatR binding sites play in activating the *catBC* operon, PCR technology was used to generate specific *catBC* DNA fragments that replaced either the RBS or the ABS with pUC119 polylinker DNA. Replacing the ABS with pUC119 polylinker DNA had little effect on the CatR binding pattern in the absence of *cis,cis*-muconate in comparison with the binding pattern seen with the wild-type promoter (compare lanes 1 to 7 to lanes 8 to 14 in Fig. 9). With higher concentrations of CatR, the appearance of slower-migrating bands other than C1 and C2 becomes evident. We feel that this is a result of the nonspecific binding of additional dimers of CatR (see Discussion). The

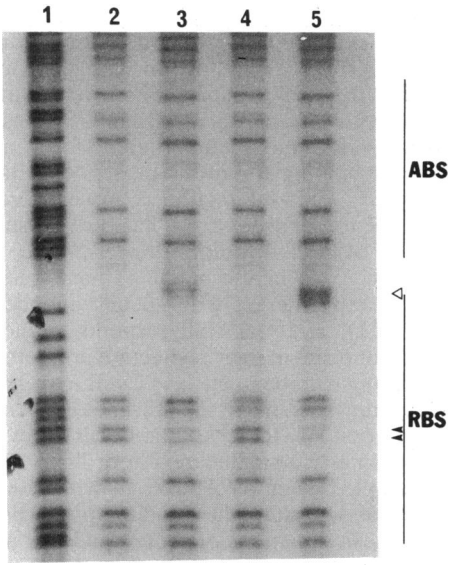

FIG. 7. Methylation interference footprinting of the ^{32}P-labeled 164-bp DNA fragment representing the *catR* coding strand. This footprint was performed with C2 which was isolated from the gel shift assay on SDS-PAGE. The G residues that are critical for CatR binding are marked with filled arrowheads. Nucleotides which represent hypersensitive cutting sites are marked with the open arrowhead. Lanes: 1, Maxam-Gilbert G+A reaction; 2, DNA and *cis,cis*-muconate; 3, DNA from C2 formed in the presence of *cis,cis*-muconate; 4, DNA in the absence of *cis,cis*-muconate; 5, DNA from C2 formed in the absence of *cis,cis*-muconate.

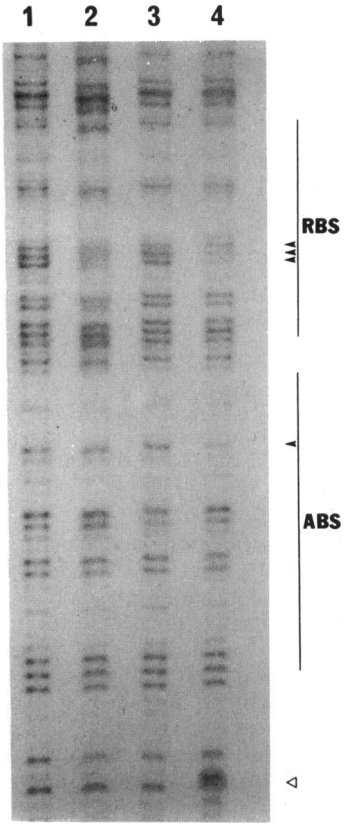

FIG. 8. Methylation interference footprinting of a 180-bp DNA fragment representing the *catBC* coding strand. Labeling is as in Fig. 7. Lanes: 1, DNA in the absence of *cis,cis*-muconate; 2, DNA from C2 formed in the absence of *cis,cis*-muconate; 3, DNA and *cis,cis*-muconate; 4, DNA from C2 formed in the presence of *cis,cis*-muconate.

affinity of CatR for the RBS was slightly decreased when polylinker DNA was substituted for the ABS (Fig. 9, lane 3 versus lane 10). When the RBS was replaced with polylinker DNA, leaving an intact ABS, CatR did not bind to this fragment in the absence of *cis,cis*-muconate (data not shown).

Addition of *cis,cis*-muconate to the binding reaction greatly affected the gel shift patterns of both the wild-type and ABS substitution DNA fragments. As expected, the wild-type *catBC* promoter region displayed a gel shift pattern in the presence of *cis,cis*-muconate which predominantly favors the formation of C2 along with slow-moving complexes at higher CatR concentrations (Fig. 10, lanes 9 to 11). However, Fig. 10 (lanes 1 to 7) shows that the ABS substitution DNA fragment produced a gel shift similar to that formed in the absence of *cis,cis*-muconate, including formation of traces of C1 (lane 4). The *cis,cis*-muconate-induced specificity for C2 formation and the increased binding affinity of CatR characteristic of the wild-type promoter are absent from the ABS deletion fragment. CatR did not bind to the RBS$^-$ ABS$^+$ fragment in the presence of *cis,cis*-muconate (data not shown).

DISCUSSION

The regulatory protein CatR has been shown to be necessary for transcriptional activation of the *catBC* operon (25). It was noted that *cis,cis*-muconate was not required for partially purified CatR to bind to and protect a 27-bp sequence located near the *catR* transcriptional start site (25). In this study, we have purified CatR and demonstrated the molecular interaction between this regulatory protein, the benzoate catabolism intermediate *cis,cis*-muconate, and the *catBC* operon.

Gel shift analysis of CatR binding to the *catBC* promoter region has shown that in the absence of the inducer *cis,cis*-muconate, CatR at relatively low concentrations forms two

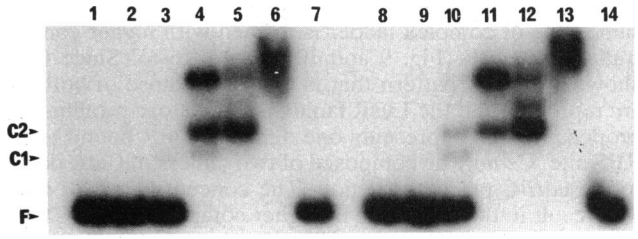

FIG. 9. Gel shift assay of CatR binding to the RBS and ABS deletion fragments of the *catBC* promoter. Purified CatR was incubated with ^{32}P-labeled DNA fragments representing either both binding sites (RBS$^+$ ABS$^+$) or only one binding site (RBS$^-$ ABS$^+$ and RBS$^+$ ABS$^-$). The binding reaction was performed in the absence of *cis,cis*-muconate. Lanes 1 to 7 represent the RBS$^+$ ABS$^-$ DNA; lanes 1 to 6 represent increasing amounts of purified CatR; lane 7 represents free DNA; lanes 8 to 14 represent the wild-type (RBS$^+$ ABS$^+$) promoter region (CatR concentration of lanes 8 to 13 correspond to those of lanes 1 to 6); lane 14 contains DNA only. C1 and C2 are indicated. F, free DNA.

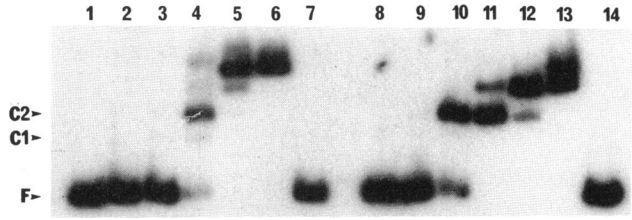

FIG. 10. Effect of *cis,cis*-muconate on CatR binding to the RBS and ABS deletion fragments. Gel shift assay conditions were those used for Fig. 9 except that 100 mM *cis,cis*-muconate was added to the binding reaction mixture and to the electrophoresis buffer. Lanes 1 to 7 represent the RBS$^+$ ABS$^-$ DNA fragment; lanes 1 to 6 represent the same concentrations of purified CatR used in lanes 1 to 6 of Fig. 9; lane 7 represents DNA only; lanes 8 to 14 represent the wild-type (RBS$^+$ ABS$^+$) promoter region (CatR concentrations of lanes 8 to 13 corresponds to those of lanes 1 to 6 in Fig. 9); lane 14 represents DNA only. C1 and C2 are indicated. F, free DNA.

complexes with the *catBC* promoter. When *cis,cis*-muconate is present in the binding reaction, the affinity of CatR for the promoter region is increased approximately 20-fold. In addition, C2 is predominantly formed in the presence of this inducer. It has been previously shown that other LysR family members produce altered gel shift patterns in response to their respective inducers. For example, the TrpI-*trpBA* interaction favors the formation of a second complex when incubated with the inducer indoleglycerol phosphate (5, 6). However, unlike the CatR-*catBC* interaction, the TrpI binding pattern has revealed that a significant amount of C1 remains despite the presence of inducer. CatR binding to the ABS may be the result of a conformational change that the protein undergoes in the presence of *cis,cis*-muconate that results in a form of CatR that is capable of interacting with the ABS. The CatR-DNA complex represented by the occupation of both the RBS and ABS appears to be more stable than the complex found when CatR interacts with the RBS alone. Therefore, the 20-fold affinity increase that CatR exhibits for the *catBC* promoter must be represented by CatR binding to the ABS, in the presence of its inducer.

Fried and Crothers (9) have previously shown that the *lac* repressor is capable of forming eight different complexes with its operator binding sites, depending on the amount of protein present in the binding reaction. Each of these successively slower migrating complexes represented the nonspecific binding of an additional *lac* repressor molecule. This same type of complex ladder is formed with higher concentrations of CatR (Fig. 9 and 10, lanes 4 to 6). Since CatR shows a binding pattern that is similar to those of both the *lac* repressor and the LysR family of regulatory proteins, we propose that C1 represents one dimer of CatR bound to the RBS site. C2 may be composed of two dimers of CatR bound to the *catBC* promoter region. The concentrations of CatR that result in the formation of higher complexes than C1 and C2 in the gel shift assay still give the same footprinting pattern in the presence or absence of *cis,cis*-muconate (data not shown). This finding suggests that the slower-migrating complexes do not represent the binding of CatR to functional binding sites.

DNase I footprinting and the restriction protection assay demonstrated that an additional binding site (the ABS) is occupied by CatR in the presence of *cis,cis*-muconate. This interaction with the ABS is very sensitive to the in vitro conditions used in this study. Unlike findings for the RBS, examination of the DNA sequence of the ABS failed to reveal any obvious inverted or direct repeats that may serve as a recognition sequence. In addition, methylation interference footprinting showed that only one G residue in the ABS site was critical for CatR binding. These observations suggest that the CatR-RBS and CatR-ABS interactions are quite different. The LysR family member NahR, like CatR, occupies an adjacent binding site in the presence of its inducer, salicylate (14, 27). This interaction is so sensitive that the binding of NahR to the second site has been observed only through the use of in vivo footprinting (14).

Several of the LysR family members have been demonstrated to indirectly interact with specific inducers. NahR (14), TrpI (5, 11), and CatR all respond to inducers by binding to a second site in their respective promoter regions. Some LysR family members, such as CysB and OxyR, show altered footprinting patterns in the presence of their inducers (15, 29). In the case of OccR, addition of inducer results in the protein binding to a smaller region of DNA (31). There are other examples, such as AmpR, in which the inducer apparently does not alter the binding properties of the protein (18). These variations of protein-inducer interaction suggest that there are several unique mechanisms of transcriptional activation within the LysR family of regulatory proteins.

Methylation interference footprinting revealed that the interactions between CatR and its two binding sites are quite different. The RBS contains five different G residues that are critical for CatR binding, and this finding is supported by the hydroxyl radical footprinting study of Rothmel et al. (25). In the current study, only one G residue of the ABS was necessary for CatR binding. We are currently examining the non-G residues of the two binding sites for their importance to the overall transcriptional activation of the *catBC* operon by CatR and *cis,cis*-muconate.

In a previous study, hydroxyl radical footprinting showed that the binding of CatR to the RBS produced a region of the *catBC* coding strand that was hypersensitive to cleavage (25). This hypersensitive area was located at −49 to −52 (AGAG) relative to the *catBC* transcriptional start and was proposed to result from a conformational change in the DNA which was mediated by CatR binding. In this study, we observed a hypersensitive region (two C residues) on the *catR* coding strand located at −52 to −53 relative to the *catBC* transcriptional start. This area is located directly between the RBS and ABS sites and may represent DNA that bends as a result of CatR binding. It is unclear why the hypersensitive site consists of two C residues instead of two G residues in the methylation interference assay. The role that this change in DNA topology may play in the transcriptional activation mechanism is uncertain, but the importance of DNA curvature in the recognition of promoters by RNA polymerase has been demonstrated (33).

It has been previously demonstrated with the LysR family members IlvY and TrpI that binding to their two respective operator sites is cooperative (32). The role of cooperativity in CatR binding was determined by deleting either the RBS or the ABS and testing the resulting DNA fragments for binding potential in a gel shift assay. The binding of CatR to an RBS$^+$ ABS$^-$ DNA fragment (minus *cis,cis*-muconate) was similar to the wild-type RBS$^+$ ABS$^+$ binding pattern, despite the substitution of polylinker DNA for the ABS. This finding indicates that CatR binding to the RBS is independent of the ABS.

With the wild-type *catBC* promoter, the addition of *cis,cis*-muconate to the binding reaction produces predominantly C2. However, the RBS$^+$ ABS$^-$ DNA fragment did

not produce C2 predominantly in the presence of *cis,cis*-muconate. Therefore, there must be some type of sequence specificity in the ABS which allows CatR to bind in the presence of *cis,cis*-muconate. Likewise, the binding of CatR to the ABS required an intact RBS. Replacement of the RBS with polylinker DNA (RBS^- ABS^+) prevented the formation of either C1 or C2, and *cis,cis*-muconate did not allow binding to occur. These experiments suggest that the binding of CatR to the RBS and ABS is cooperative in that both sites must be present in order for full *catBC* transcriptional activation to occur.

The exact mechanism of *catBC* operon transcriptional activation that occurs when *P. putida* is exposed to benzoate is not fully understood. This study shows that benzoate catabolism produces a specific inducer, *cis,cis*-muconate, which somehow interacts with the regulatory protein CatR to create a CatR-promoter complex which is recognized by RNA polymerase. Some investigators have proposed that a protein-protein interaction between RNA polymerase and the transcriptional activator stabilizes RNA polymerase binding to the promoter region (11). Others have suggested that changes in DNA topology at the −35 region brought about by the transcriptional activator-inducer complex allow proper recognition of the −35 region by RNA polymerase (32). The nature of the specific activation mechanism for the CatR-*catBC* system will be the focus for future experiments. Understanding the nature of inducer-activator interactions, and the role that they play in transcriptional activation, will help us to engineer biodegradative pathways for compounds that currently cannot be degraded by the indigenous environmental population of bacteria.

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