catM Encodes a LysR-Type Transcriptional Activator Regulating Catechol Degradation in Acinetobacter calcoaceticus

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Received 27 March 1995/Accepted 10 August 1995

On the basis of the constitutive phenotypes of two catM mutants of Acinetobacter calcoaceticus, the CatM protein was proposed to repress expression of two different loci involved in catechol degradation, catA and catBCIJFD (E. Neidle, C. Hartnett, and L. N. Ornston, J. Bacteriol. 171:5410–5421, 1989). In spite of its proposed negative role as a repressor, CatM is similar in amino acid sequence to positive transcriptional activators of the LysR family. Investigating this anomaly, we found that insertional inactivation of catM did not cause the phenotype expected for the disruption of a repressor-encoding gene: in an interposon-generated catM mutant, no cat genes were expressed constitutively, but rather catA and catB were still inducible by muconate. Moreover, this catM mutant grew poorly on benzoate, a process requiring the expression of all cat genes. The inducibility of the cat genes in this catM mutant was completely eliminated by a 3.5-kbp deletion 10 kbp upstream of catM. In this double mutant, catM in trans restored muconate inducibility to both catA and catB. These results suggested the presence of an additional regulatory locus controlling cat gene expression. The ability of CatM to function as an activator was also suggested by these results. In support of this hypothesis, in vivo methylation protection assays showed that CatM protects two guanines in a dyad 65 nucleotides upstream of catB transcriptional start site, in a location and pattern typical of LysR-type transcriptional activators. Gel mobility shift assays indicated that CatM also binds to a region upstream of catA. DNA sequence analysis revealed a nucleotide near the 3′ end of catM not present in the published sequence. Translation of the corrected sequence resulted in the deduced CatM protein being 52 residues longer than previously reported. The size, amino acid sequence, and mode of action of CatM now appear similar to, and typical of, what has been found for transcriptional activators in the LysR family. Analysis of one of the constitutive alleles of catM previously thought to encode a dysfunctional repressor indicated instead that it encodes an inducer-independent transcriptional activator.

In the soil bacterium Acinetobacter calcoaceticus, the catM gene regulates all of the cat genes needed for catechol degradation via the β-ketoadipate pathway (Fig. 1) (28). The cat genes include catA, encoding catechol 1,2-dioxygenase, which converts catechol to cis,cis-muconate (CCM), and the catBCIJFD genes, encoding enzymes for the formation of tricarboxylic acid cycle intermediates from CCM. These genes are clustered on the A. calcoaceticus chromosome, with catM and two open reading frames separating catA from the catBCIJFD genes (Fig. 2) (33). In the wild-type strain, cat gene expression is induced by CCM, which increases the levels of cat-encoded enzymes by approximately 300-fold (2). However, mutants in which some or all of the cat genes are constitutively expressed have been isolated. In one such mutant, ADP163, a point mutation that substitutes His for Arg-156 of CatM was found to cause constitutive expression of all of the cat genes. This observation and constitutive catA expression in the catM-catB deletion strain ADP205 contributed to the hypothesis that CatM was a transcriptional repressor of the cat genes (28).

In amino acid sequence alignments, the amino-terminal region of CatM aligns well with those of several members of the LysR family of transcriptional activators. In this region, which appears to be involved in the specific binding of the regulators to their target DNA sequences (41), approximately 30% of the CatM residues are identical to those of other LysR proteins (47). The carboxy-terminal region of CatM, however, did not align well with those of other LysR proteins, in part because CatM appeared to be significantly shorter than all other family members (12, 41). Additional analyses of LysR proteins identified a region likely to be involved in inducer-compound recognition and/or binding (41). Although an inducer is normally necessary for transcriptional activation, several mutations cause the production of inducer-independent LysR activators. Single amino acid substitutions near residue 150 of several LysR proteins lead to constitutive expression of the target genes (41). Because this position is close to that of the substitution which causes constitutive gene expression in the catM mutant ADP163, we reconsidered the roles of the wild-type and mutant CatM proteins in the regulation of cat gene expression.

Analysis of an A. calcoaceticus mutant in which we inactivated catM by interposon insertion clearly showed that CatM does not repress its regulated targets. Other experiments, including in vivo footprinting to localize the CatM binding sites within the regulated catB promoter, suggested that CatM, like most other LysR-type proteins, functions predominantly as a transcriptional activator. Consistent with this role of CatM, catM of ADP163 was found to encode an inducer-independent transcriptional activator. During these analyses, we obtained evidence for an additional cat gene regulator whose presence may have obscured the role of CatM in previous studies.

MATERIALS AND METHODS

Bacterial strains and plasmids. A. calcoaceticus strains and plasmids are described in Table 1 and Fig. 2. Bacteria were cultured in Luria broth (LB) and
minimal media at 37°C as previously described (39, 43). All A. calcoaceticus strains were derived from BD413 (15, 16), which we designate ADP1. The 5′ ends of the catB mRNA transcripts were mapped as described by Delic et al. (5). The oligonucleotide primer, 5′-TCCACTGATTTATACATTCCG-3′ (primer B), was complementary to the first 17 nucleotides of the catB coding sequence as well as 3 nucleotides upstream of the ATG translational start codon. This primer was labeled at its 5′ end with 32P-ATP and T4 polynucleotide kinase (39). The oligonucleotide (2 × 105 cpm) was annealed with 30 μg of RNA in 20 μl of 50 mM Tris-HCl (pH 8.0) and 100 mM KCl after the mixture was heated at 90°C for 1 min, 60°C for 2 min, and then 4°C for 15 min. The resultant hybrids served as templates in extension reactions at 42°C for 50 min with 10 U of avian myeloblastosis virus reverse transcriptase, 0.5 mM deoxynucleoside triphosphates, and 6 U of RNasin ribonuclease inhibitor (Promega Corp., Madison, Wis.) (39). The same labeled oligonucleotide was used in parallel dideoxy chain termination sequencing reactions with primer A to generate a size standard sequence ladder. DNA fragments were analyzed on 6% polyacrylamide–urea gels.

In vivo footprint analysis with DMS. Footprint analysis was carried out by the method of Gralla (11) as modified by Huang and Schell (14). Recombinant plasmids with the catB promoter of A. calcoaceticus were methylated with dimethylsulfate (DMS) in vivo in E. coli strain JM83 in the presence and absence of catM. Methylated plasmid DNA was purified and cleaved with piperidine, and the cleavage products were analyzed by primer extension with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio). A 32P-labeled oligonucleotide, 5′-ATCTGGGTTGTAGTACC-3′ (primer M), complementary to bases 22 to 22 of the catM coding sequence and a second labeled oligonucleotide (primer B, described above for transcript mapping) were used for analysis of the sense (bottom) strand and the antisense (top) strand, respectively.

Nucleotide sequence accession number. The corrected DNA sequence of catM has been deposited with GenBank under accession number M76991.

RESULTS

Construction and characterization of catM insertion mutants ISA13 and ISA35. Although the hypothesis that CatM represses cat gene expression was consistent with the constitu-
The possibility that CatM regulates by other mechanisms could not be eliminated by previous studies. Whereas catA is constitutively expressed in catM mutant strain ADP205, the deletion also encompasses the catB gene, making the specific role of catM in the resultant phenotype unclear. To further evaluate the function of CatM, we inactivated the genomic copy of catM by \Omega interposon mutagenesis (36), leaving the catBCIJFD gene cluster intact. First, we constructed pIGG9, which has a 2-kbp \OmegaS cartridge (a Sp' cassette with flanking transcriptional and translational stop signals) inserted in the SpI site of catM (Fig. 2). The pIGG9 DNA was then linearized with EcoRI and used to transform the wild-type strain ADP1. An Sp' transformant, designated ISA13, was isolated, and Southern hybridization analysis confirmed that in it, the catM:\OmegaS allele replaced the chromosomal wild-type catM.

In contrast to the previous catM mutants, ISA13 did not express the cat genes constitutively. The catM disruption only slightly reduced the maximal expression of catA and catB in response to CCM (Table 2). However, the ability of benzoate or its metabolites to induce catB expression was reduced fivefold by catM disruption (Table 2). This is consistent with the reduced ability of ISA13 to metabolize benzoate. Whereas both the wild-type strain and the constitutive mutant ADP163 utilize benzoate as the sole carbon source, each with a doubling time of 60 to 90 min, the catM disruption of ISA13 resulted in 3.5 times slower growth on benzoate. These results suggested that CatM does not negatively regulate expression of the cat genes. In ISA13, the ability to induce catA and catB expression appeared to be mediated by a regulator(s) other than CatM, since as described below, the presence of a 3.5-kbp deletion 10 kbp upstream of catM in a catM-disrupted mutant eliminated all inducible catA and catB expression.

Strains blocked in the metabolism of benzoate have been used to show that benzoate itself induces cat gene expression (29). In order to determine whether benzoate alone was responsible for CatA and CatB induction in ISA13 (Table 2), it was necessary to block its metabolism to CCM. The benABC genes, involved in the initial step of benzoate catabolism (30) (Fig. 1), were first deleted from a ben gene-carrying plasmid by the removal of a 3.5-kbp KpnI fragment, generating pIGG16. This ben deletion was transformed into the genome of ADP1 by linearizing pIGG16 with BamHI, mixing the DNA with wild-type ADP1 cells, and screening the transformants for the loss of the ability to utilize benzoate as the sole carbon source, thereby generating strain ISA21. ISA21 was further trans-

### Table 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristic(s) | Reference or source |
|-------------------|----------------------------|---------------------|
| A. CALCOACETICUS |                           |                     |
| ADP1              | Wild type (BD413)          | 15 and 16           |
| ADP163            | pcaE3125 catM3102          | 28                  |
| ADP205            | Δ(catM-catB3205)           | 28                  |
| ISA13             | catM::\OmegaS 4013         | This study          |
| ISA21             | Δ(benM-benC4021)           | This study          |
| ISA35             | Δ(benM-benC4021) catM::\OmegaS 4013 | This study |
| ACN10             | benM::\OmegaK 5008 Δ(catM-catB3205) | This study |
| Plasmids          |                            |                     |
| pUC13 and pUC19   | Ap'                         | 50                  |
| pTZ18U            | Ap'                         | 25                  |
| pRK415            | 2.5                         | 20                  |
| pIB15\(^b\) and pIB16\(^b\) | 1.3-kb HindIII catM fragment in pUC19 | 28                  |
| pIB25\(^\alpha\)  | 1.3-kb HindIII catM fragment in pRK415 | 28                  |
| pIB27\(^\alpha\)  | 1.3-kb HindIII catM3102 fragment in pRK415 | 28                  |
| pIGG9             | EcoRI-HindIII catM::\OmegaS fragment in pUC19 | This study |
| pIGG16            | EcoRI-EcoRV ben fragment with Δ(3.5-kbp KpnI) in pRK415 | This study |
| pIGG19            | StuI-HindIII catM fragment in pUC19 | This study |
| pRA316            | EcoRI-HindIII catM-catB region in pTZ18U | This study |
| pRA3030           | catM::Tn5lacZ B20 in pIB25 | This study          |
| pBAC12            | 1.9-kb EcoRI-HindIII ben::\OmegaK in pUC19 | This study |

\(^a\) catM allele transcriptionally controlled by the lac promoter of the vector.

\(^b\) catM allele transcribed in the opposite orientation relative to that of the lac promoter of the vector.

### Table 2. Inducible expression of catA and catB in the A. CALCOACETICUS wild type and catM mutants

| Strain | Relevant characteristic(s) | No inducer CatA | No induction CatB | Benzoate\(^a\) CatA | Benzoate\(^a\) CatB | CCM CatA | CCM CatB |
|--------|-----------------------------|------------------|-------------------|---------------------|---------------------|----------|----------|
| ADP1   | Wild type                   | <5               | <10               | 393                 | 190                 | 167      | 230      |
| ISA13  | catM::\OmegaS 4013          | <5               | <10               | 237                 | 43                  | 93       | 193      |
| ISA35  | Δ(ben-benC4025) catM::\OmegaS 4013 | <5               | <10               | <5                  | <10                 | <5       | <10      |
| ISA35(pIB25) | catM in trans | <5               | <10               | <5                  | <10                 | 80       | 150      |

\(^a\) Values are averages of at least three repetitions, with standard deviations of <20%. Cells were grown in LB with either 3 mM benzoate or 3 mM CCM as an inducer.

\(^b\) In ADP1 and ISA13, benzoate can be metabolized to CCM.
formed to Sp' by the same method with linearized pIGG9 which contains the catM::3 IS allele. Southern hybridization analysis confirmed that the genome of the resultant strain ISA35 carries both the IS insertion in catM and the ben deletion (including benABC and an additional 1-kbp region upstream of benA).

In addition to blocking the conversion of benzoate to CCM, the ben deletion in ISA35 unexpectedly eliminated all induction of catA and catB by either benzoate or CCM (Table 2; compare ISA35 to ISA13). This result suggests that a locus in the deleted ben region controls the expression of catA and catB in ISA13. In the absence of this regulatory locus, plasmid pIB25, carrying only wild-type catM, restored the CCM inducibility of both catA and catB but did not restore the induction of catA by benzoate in strain ISA35 (Table 2). CatM, therefore, appeared to be responsible for transcriptional activation of both catA and catB in response to CCM. These results further argue against a role for CatM as a repressor of catA and catB expression.

Reanalysis of the catM DNA sequence. Previous DNA sequence data (28) implied both that the CatM protein was much smaller than any other LysR-type regulator and that it lacked conserved carboxy-terminal domains likely to be involved in transcription activation (41). Therefore, the sequence of catM near the published termination codon of the catM open reading frame was again determined, with plasmid pIGG19 (Table 1) being used as the template. An additional C nucleotide was found 17 nucleotides upstream of the original stop codon. Starting at nucleotide 738, with position 1 being the first nucleotide of the catM coding region, the sequence was found to be GGCATGGrather than the published TGCAATGG. This indicates that the coding region was found to 17 nucleotides upstream of the original stop codon.

This sequence correction resulted in the extension of the deduced amino acid sequence by 52 residues. This new 303-residue CatM is very similar in size and sequence to other LysR-type activators. In sequence alignments of CatM with each of the LysR activators—CatR (37), TdR (24), Ccr (4), TcR (46), and BphR (21)—the identity of the aligned residues ranged from 28 to 41% (similarity between 50 and 60%). These proteins are involved in the regulation of aromatic compound degradation by diverse bacteria, and each controls the expression of genes that are either the same as or similar to those regulated by CatM of A. calcoaceticus. CatM was most similar to CatR of Pseudomonas species (13, 37), with the new 52 C-terminal CatM residues showing 45% similarity to those of the homologous CatR region.

CatM specifically binds to the catM-catB intercistronic region and to a region upstream of catA. To obtain additional evidence that CatM directly activates transcription from the catB promoter, we used gel retardation assays. A 313-bp EcoRI-HindIII fragment containing the catM-catB intercistronic region (Fig. 3A) was end labeled with 32P and incubated with cell extracts of E. coli JM83 either carrying plasmids with catM transcriptionally controlled by the vector's lac promoter (pIB15) or with catM in the opposite orientation relative to the lac promoter (pIB16) or carrying no plasmid. The electrophoretic mobility of the catM-catB intercistronic DNA fragment was retarded only when it was preincubated with crude extracts of catM-containing cells (Fig. 3B). Increasing concentrations of crude extracts from E. coli JM83 (pIB15) or E. coli JM83 (pIB16) caused an increasing amount of the fragment to show retarded mobility. Electrophoretic mobility shifts of the labeled fragment were not significantly affected by 0.1 to 10 mM inducer CCM added to the binding reactions prior to electrophoretic separations (data not shown). The lack of an obvious effect of the inducer on apparent DNA binding by CatM is consistent with behavior reported for other LysR proteins that are transcriptional activators (41).

To further localize DNA sequences that specifically interact with CatM, the 313-bp EcoRI-HindIII fragment was digested with AseI, and the mixture was 32P end labeled. The mixture of the two labeled fragments was then incubated with the same crude extracts described above. The 211-bp EcoRI-AseI fragment showed retarded migration when it was incubated with extracts from cells harboring plasmids with catM, indicating that CatM binds to this fragment (Fig. 3C). Extracts from cells carrying pIB15 show greater binding activity than those from cells carrying pIB16, most likely because the A. calcoaceticus DNA fragment is oriented so that the lac promoter of pIB15 drives catM expression. The lac promoter drives higher catM expression than does the catM promoter alone, which appears to be weakly expressed in E. coli. Protein binding to the 211-bp EcoRI-AseI fragment was sequence specific, because migration of the 102-bp AseI-HindIII fragment was unaffected by incubation with any of the crude extracts tested (Fig. 3C).

To more precisely locate the sequence binding CatM, the mixture of the labeled EcoRI-AseI and AseI-HindIII fragments
were carried out with a 32P-labeled, RNA from wild-type ADP1 grown in LB with either 5 mM benzoate (+, induced) or 10 mM succinate (−, noninduced). The same oligonucleotide was used to generate the sequencing ladder (lanes G, A, T, and C). The arrows show the major cDNA products and indicate catB transcript initiation sites 26 to 28 bp upstream of the catB ATG translation initiation codon on the coding strand.

was digested with DdeI and used as a binding substrate. Only the 86-bp DdeI-AseI fragment (Fig. 3A) showed a mobility shift dependent on preincubation with crude extracts containing CatM (data not shown), indicating that the CatM binding site in the catB-catM intercistronic region lies between the DdeI and AseI sites. Similarly, a labeled fragment carrying 388 bp between the SspI and SalI restriction sites and ending 52 bp upstream of the catA coding region was preincubated with cell extracts of E. coli JM83(pIB15), and this retarded its electrophoretic mobility (data not shown). Preincubation with extracts from E. coli JM83 had no effect, indicating that CatM also binds to a specific sequence upstream of catA.

**Localization of the transcription start site and promoter of catB.** The results outlined above showed that CatM binds to an 86-bp region located 48 bp upstream of the catB translation start site, a region also likely to contain the catB promoter. The position of the CatM binding site relative to that of the promoter elements should give insight into how it regulates catB expression. Thus, we localized the catB transcriptional start site by primer-extension techniques: total RNA was prepared either from wild-type A. calcoaceticus cells induced for CatB production by growth in LB with benzoate or from uninduced cells grown in LB with succinate. RNA samples annealed with a 32P-labeled, catB-specific primer served as templates for reverse transcriptase-mediated extension reactions. cDNAs were detected only in the presence of RNA isolated from induced cultures (Fig. 4), indicating that increased catB expression in response to benzoate and/or its metabolic descendants occurs at the transcriptional level. The majority of benzoate-induced catB transcripts started between 26 and 28 nucleotides upstream of the ATG-methionine translational initiation codon (Fig. 4).

**In vivo interactions between CatM and nucleotides of the catM-catB intercistronic region.** Next, we used in vivo methylation protection analysis to define specific nucleotides involved in CatM binding. DMS methylates the N-7 of guanines and, at a lower rate, the N-3 of adenines; this methylation can be dramatically inhibited or enhanced if the bases are interacting with a protein (11, 40). The strength and position of such interactions can be investigated by comparing the base cleavage products of target DNA treated with DMS in the presence and absence of a DNA-binding protein.

Cultures of E. coli cells containing a plasmid with either the catM-catB intercistronic region and the complete catM gene (pIB16) or with the catM-catB intercistronic region and only the first 135 bp of catM (pRA316) were treated with DMS. Plasmid DNA was isolated and cleaved at methylated sites with piperidine. Methylation patterns were determined by analyzing the cleavage fragments by means of primer extension reactions (Fig. 5). The presence of CatM specifically caused strong protection of a guanine at position −61 (relative to the +1 catB transcription initiation site) on the antisense (top) strand (G-61) (Fig. 5A). Similarly, a guanine and an adenine at −68 and −67, respectively, on the sense (bottom) strand were protected from methylation, again only in the presence of CatM (G-68 and A-67) (Fig. 5B). A guanine at position −56 (and possibly one at −57) as well as several adenines between −66 and −62 showed increased methylation caused by the presence of CatM.

These results indicated that CatM interacts with nucleotides centered around position −65 of the catB promoter. This CatM-binding region (−72 to −58) contains an interrupted dyad (ATAC-N7-GTAT) whose position and methylation protection pattern are similar to those that have been observed for other LysR-type transcriptional activators, including CatR and ClcR, which control the aromatic degradation genes with a similar function in Pseudomonas spp. (4, 34, 35, 41). Addition of the inducer CCM to the E. coli cultures prior to methylation with DMS did not consistently produce any new changes in the protection pattern. However, the permeability of E. coli to CCM is not known, and thus the effects of the inducer on CatM interactions with its binding site remain unclear. Finally, guanines at −10 and −9 on the antisense strand showed consistent protection from DMS methylation only in the presence of CatM.

**CatM negatively autoregulates.** The CatM binding site identified above is 23 bp upstream of the catM translational start codon and thus is likely to be near the catM promoter. On the basis of similarity to other LysR systems, we expected that the
binding of CatM at this site should reduce its own transcription. To evaluate this, we analyzed the expression of a plasmid-borne catM::lacZ fusion on pRA3030 in E. coli MC1061 both in the presence and in the absence of catM on pIB13. The β-galactosidase activity directed from the catM::lacZ fusion, 51 Miller units, was reduced by threefold to 15 Miller units when catM was placed in trans. Thus, like many other LysR transcriptional activators (41), CatM is negatively autoregulated.

Explanation of the constitutive phenotypes of mutants ADP163 and ADP205. All the data presented above are consistent with the hypothesis that CatM acts as a transcriptional activator. To determine how the point mutation in catM of ADP163 caused high CatA level, we cloned the 1.3-kb Hin fragment containing this allele into a broad-host-range plasmid, forming pIB27. The presence of pIB27 in trans in wild-type ADP1 caused constitutive catA and catB expression (Table 3) consistent with the idea that catM in ADP163 encodes a trans-dominant, inducer-independent CatM activator, not an inactivator.

High CatA levels in catM-catB deletion mutant ADP205 had been attributed to the deletion of catM (28), although here we found that specific disruption of catM did not cause the same effect. Because a regulatory locus in the ben region appeared to affect catA expression in ISA13, we tested the possibility that this region was involved in the constitutive expression of catA in ADP205. Interposon mutagenesis was used to disrupt the putative regulatory locus: plasmid pBAC12 (Table 1) carrying an ΩK cassette (7) immediately upstream of benA was linearized and used to transform ADP205 to Km. Southern hybridization confirmed the presence of the ADP205 catM-catB3205 deletion and the ben::ΩK insertion in the resultant strain ACN10 (Fig. 2). Constitutive expression of catA in ADP205 was eliminated by introduction of the ben::ΩK insertion; the specific activity of CatA (cathol pH oxidase) in LB-grown ACN10 was <5 nmol/min/mg of protein, compared with a value of 250 nmol/min/mg of protein determined for similarly grown ADP205 cultures. This suggests that CatM is not the sole regulator of catA, and therefore the constitutive phenotype associated with the catM-catB deletion results from events other than the repression of catA by CatM. The possible involvement of the catB deletion in causing the constitutive catA expression of ADP205 is discussed below.

### DISCUSSION

CatM activates cat gene expression. Three lines of evidence demonstrated that A. calcoaceticus CatM is a LysR-type regulator that activates, rather than represses, cat gene expression. First, in a ben-deleted background, inactivation of catM caused a loss of muconate-inducible catA and catB expression that was specifically restored by catM in trans. Second, analysis of the DNA-protein interactions of CatM with the catB promoter showed that the position and DNA sequence of regions which interact with CatM are characteristic of those involved in LysR-type transcriptional activation. Third, the trans-dominant nature of the ADP163 catM allele which causes constitutive cat gene expression indicated that it encodes a mutant CatM protein that is an inducer-independent activator, not a dysfunctional repressor.

Analysis of a revised catM DNA sequence showed that CatM is a 303-residue protein, very similar in size and amino acid sequence to many other LysR transcriptional activators. Strong homology (41% identity and 60% similarity) to CatR, a transcriptional activator of Pseudomonas sp. catB, a gene which is isofunctional to A. calcoaceticus catB (13, 37, 38), was detected. The DNA sequences of catM and catR are 45% identical, despite significant differences in G + C content; catM contains 44% G + C, in contrast to catR, which contains 65% G + C. This degree of homology strongly suggests a common ancestry for both genes. Both CatM and CatR appear to be members of a subfamily of LysR activators, including CtcR (3, 4), TcbR (46), TidR (18, 24), and TidS (19, 24). Members of this subfamily regulate genes encoding muconate- or chloromuconate-lactonizing enzymes and/or genes encoding oxygenases that act on catechol or chlorinated aromatic compounds from Acinetobacter, Pseudomonas, and Alcaligenes species.

Sequences involved in the binding of many of these LysR-type regulators to their target DNA regions have been identified (3). Characteristic of LysR-type binding sites is a T-N11-A motif and a small region of interrupted dyadic symmetry centered approximately 65 nucleotides upstream of the regulated transcriptional start sites (3, 10). Exactly such a sequence, ATAC-N7-GTAT, is present at the A. calcoaceticus catB promoter in the region identified by in vivo footprinting as the likely CatM recognition and binding site (Fig. 6). This sequence is nearly identical to those proposed as the binding sites of CatR, CtcR, TidR, and TcbR (3, 22, 35). Additionally, the two guanines of the dyad which we showed by in vivo footprinting to be involved in the binding of CatM to the A. calcoaceticus catB promoter are at the same locations as ones which have been shown by site-directed mutagenesis and methylation analysis to be essential for binding. The three lines of evidence suggest that the two guanines of the dyad are involved in the binding of CatM to the A. calcoaceticus catB promoter.
ylation interference to be important for binding and/or transcriptional activation of the Pseudomonas catB promoter by CatR (35). We did not observe any interactions between CatM and guanines downstream of the −65 binding site, even in the presence of its inducer. For many LysR-type activators, including CatR, inducer-dependent interactions with nucleotides downstream of the primary binding site, near position −40, have been reported (35, 38). The failure to observe such interactions may result from the low permeability of E. coli to the inducer CCM, the low number of available guanines in this region of the catB promoter, or different mechanisms of action. The sequences of the catB promoter downstream of the CatM binding site (−55 to +1) in A. calcoaceticus differ extensively from those downstream of the CatR binding site in Pseudomonas putida, suggesting fundamental differences in promoter structures and RNA polymerase binding sites. Moreover, we observed apparent CatM-dependent protection of guanines at positions −9 and −10, which, although atypical for LysR-type regulators, may suggest DNA bending by CatM, a possibility supported by the presence of A tracts in the promoter region. It is also possible that this CatM-dependent protection of guanines close to the catB transcriptional start site might reflect aberrant sequestration of RNA polymerase at the catB promoter (23). Nonetheless, conservation of the upstream sequences involved in the binding of CatM, CatR, and ClcR indicates a common ancestry not only for the genes encoding the homologous activators, all of which respond to similar inducer compounds, but also for the cognate regulatory sites.

A regulator distinct from CatM regulates catA in response to benzoate and CCM. Although we clearly showed that CatM can activate cat gene expression, simple disruption of catM in strain ISA13 had only a slight effect on the inducible expression of catA and catB. Moreover, inducible expression of catA in response to either benzoate or CCM is in a strain which lacks catM and which cannot further metabolize either inducer was observed (28). These results suggest the existence of a cat gene regulator(s) distinct from CatM. In the presence of such a regulator, the overlapping ability of catM to activate cat gene expression is obscured. The ben deletion in conjunction with the catM disruption in strain ISA35 eliminated all inducible expression of catA and catB, thereby allowing us to examine the role of catM by placing it in trans. The ability of catM to restore CCM-inducible but not benzoate-inducible cat gene expression in this strain indicated that CatM responds to CCM but not to benzoate.

The 3.5-kbp ben region deletion in ISA35 eliminated all of the catM-independent regulation of catA and catB, suggesting that the deletion contains a regulatory region affecting the expression of catA and catB. Consistent with this, insertion of an ΩS or ΩK cartridge in a region just upstream of benA also eliminated the inducibility of catA in response to benzoate (27). Finally, when the ΩK cartridge was introduced in the same ben region of the ADP205 chromosome, it eliminated the constitutive catA expression caused by the chromosomal catM-catB deletion. Although high catA expression in ADP205 was originally attributed to the loss of catM, it now seems more likely that the constitutive phenotype results from the loss of catB, since we have observed constitutive catA expression in a catB-deleted strain which has a wild-type catM (27). One possible explanation is that the deletion of catB causes CCM accumulation, perhaps from the metabolism of endogenous aromatic compounds such as anthranilate, and that CCM thus generated can cause the ben region regulator to activate catA expression.

Reanalysis of the constitutive mutant ADP163. Although here we found that the catM3102 allele carried on pIB27 was dominant with respect to that of the wild type and caused constitutive catA and catB expression in ADP1 (Table 3), previous experiments showed the opposite: wild-type catM in trans on pIB25, in ADP163 eliminated constitutive cat gene expression (28). Thus, it appears that in these merodiploids, cat gene expression depends on which catM allele is plasmid borne, implicating gene dosage in this effect. It is also possible that the ratio of the constitutive and the wild-type catM gene products affects the formation or the levels of functional CatM oligomers. The possibility that the phenotype of these strains results from the formation of aberrant CatM hetero-oligomers is consistent with the recent observation that the presence of the wild-type catM in trans in the constitutive ADP163 mutant not only eliminated the constitutive expression of catA and catB but also prevented high induced levels of catA and catB in response to CCM and benzoate (27). Although CatR appears to function as a dimer (35), the subunit structure of active CatM remains to be determined.

ACKNOWLEDGMENTS

This research was supported by NIH grants GM32255 (to M.A.S.) and SBR IR43ES06304 (to G.L.G.). Additional support was provided by National Science Foundation grant MCB-9507393 (to E.L.N.), by the University of Georgia Research Foundation, Inc. (to E.L.N.), and by the Administration for Economic Development of the government of Puerto Rico (to C.E.R.-A.). We gratefully acknowledge L. S. Collier for the construction and characterization of plasmid pBAC12 and strain ACN10. In addition, we thank J. Huang for assistance with sequencing and footprinting and L. N. Orrison for helpful discussions.

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VOL. 177, 1995

A. CALCOACETICUS

CatM ACTIVATOR

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