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CatM Regulation of the *benABCDE* Operon: Functional Divergence of Two LysR-Type Paralogs in *Acinetobacter baylyi* ADP1

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Two LysR-type transcriptional regulators, BenM and CatM, control benzoate consumption by the soil bacterium *Acinetobacter baylyi* ADP1. These homologs play overlapping roles in the expression of multiple genes. This study focuses on the *benABCDE* operon, which initiates benzoate catabolism. At this locus, BenM and CatM each activate transcription in response to the catabolite *cis*,*cis*-muconate. BenM, but not CatM, additionally responds to benzoate as an effector. Regulation by CatM alone is insufficient for growth on benzoate as the sole carbon source. However, three point mutations independently increased CatM-activated *benA* transcription and enabled growth on benzoate without BenM. Two mutations generate variants with one amino acid change in the 303-residue CatM, CatM(V158M) and CatM(R156H). These substitutions affected regulation of *benA* differently than that of *catB*, another CatM-regulated gene involved in benzoate catabolism. In relation to CatM, CatM(V158M) increased *cis*,*cis*-muconate-dependent transcription of *benA* but decreased that of *catB*. CatM(R156H) increased effector-independent expression of *catB* compared to CatM. In contrast, *cis*,*cis*-muconate was required with CatM(R156H) to activate unusually high *benA* expression. Thus, induction by *cis*,*cis*-muconate depends on both the sequence of CatM and the promoter. A point mutation at position −40 of the *benA* promoter enhanced CatM-activated gene expression and altered regulation by CatM(R156H). BenM and CatM bound to the same locations on *ben* region DNA. The frequency with which spontaneous mutations allow CatM to substitute for BenM might predict that one regulator would be sufficient for controlling benzoate consumption. This prediction is discussed in light of current and previous studies of the BenM-CatM regulon.

In the soil bacterium *Acinetobacter baylyi* ADP1 (formerly *Acinetobacter* sp. strain ADP1), BenM and CatM are homologous transcriptional regulators involved in aromatic compound degradation (Fig. 1) (8, 12, 29). These proteins are 59% identical in sequence, and both respond to a metabolite formed during benzoate consumption, *cis*,*cis*-muconate (referred to below as muconate) (Fig. 1A). However, unlike CatM, BenM also responds to benzoate as an effector. These two regulators jointly activate more than a dozen chromosomal *ben* and *cat* genes involved in benzoate catabolism (Fig. 1B). Furthermore, during growth with benzoate as a carbon and energy source, BenM and CatM repress genes needed to degrade alternative aromatic compounds (4).

In mutants lacking *catM* or *benM*, there is little effect on the expression of some genes such as *catA* or *benPK* (8, 12). At these loci, one regulator compensates for the loss of the other. This redundancy raises questions about the need for both regulators. To understand the evolution and retention of the *benM* and *catM* paralogs, these studies focused on the *benABCDE* operon. At this locus the regulators exert markedly different effects. BenM represses *benA* transcription in the absence of its effectors (6, 12). In response to muconate or benzoate, BenM activates *ben* operon transcription. Furthermore, both effectors together cause a synergistic increase in BenM-activated transcription (6).

CatM activates *benABCDE* transcription in response to muconate but not benzoate (11, 12). As assessed with a *benA*::*lacZ* fusion in a *benM* mutant, CatM with muconate activates expression at a level 7-fold below that of BenM with muconate and 21-fold below that of BenM with both effectors (11, 12). Regulation by CatM is insufficient to support the growth of mutants lacking BenM on benzoate. However, CatM alone activates high-level transcription from the other promoters involved in benzoate consumption (*benP*, *catA*, *catB*) (Fig. 1). Therefore, mutations that increase *ben* operon expression permit benzoate to be consumed without BenM. For example, a point mutation in the −10 region of the *benA* promoter enables a *benM* mutant to grow on benzoate (12). Without inducers, this mutation increases *benA* expression to a level fourfold higher than that induced from the wild-type promoter by CatM with muconate (12).

Here, mutations that increase CatM-activated transcription from the *benA* promoter were studied in order to identify constraints that normally prevent CatM from serving as the sole regulator of benzoate catabolism in ADP1. In the absence of CatM, BenM permits benzoate to be consumed very slowly (29). However, regulation by BenM in a *catM* mutant causes abnormally high levels of muconate to accumulate (12, 15). This observation suggests limitations on the ability of BenM to activate transcription from the *catB* promoter. BenM and CatM are members of the LysR-type family, a large group of homologous regulators that control diverse functions (31). The ADP1 regulators belong to a subclass of this family involved in the catabolism of aromatic compounds and pollutants in nu-

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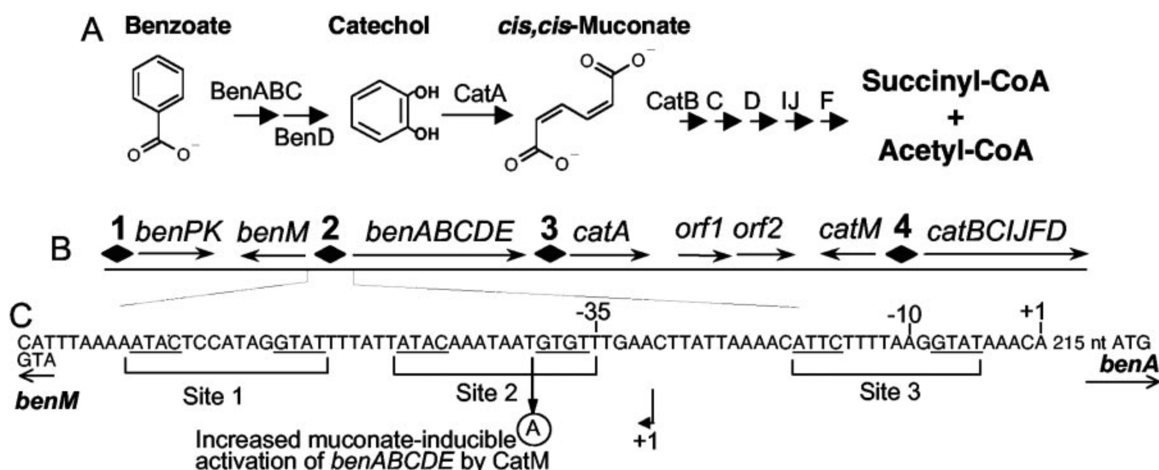

FIG. 1. BenM and CatM regulate benzoate degradation in ADP1. Catabolism depends on enzymes encoded by the *ben* and *cat* genes (A). These chromosomal genes are in an approximately 20 kbp cluster (B) (not drawn to scale). BenM and CatM regulate transcription initiation in four regions (diamonds 1 to 4). The functions of two open reading frames downstream of *catA* (*orf1* and *orf2*) are unknown, but they are not expressed during growth on benzoate. In the intergenic *benMA* region (C), there are three potential binding sites for BenM and CatM, as described in the text. Site 1 exactly matches the consensus sequence (underlined) of LysR-type regulators within a subclass to which BenM and CatM belong (10, 31). The sequences in sites 2 and 3 differ from the consensus by a single nucleotide that reduces the extent of dyad symmetry. Above the DNA sequence, the transcription initiation site (+1) and promoter regions (−10 and −35) are shown for *benA*. The initiation site for the divergently transcribed *benM* is also indicated (+1 below an arrow). A point mutation (circled A) increases the ability of CatM to activate transcription of the *benABCDE* operon (12).

merous bacterial genera (16, 32). Thus, the BenM-CatM regulon may serve as a good model for understanding complex regulatory circuits involved in biodegradation.

This report describes a variant, CatM(V158M), that enables growth on benzoate without BenM. CatM(V158M) was compared to CatM(R156H), a variant that activates *cat* operon expression without muconate (23). Additionally, the interactions of CatM with the *benA* promoter region were compared to those of BenM at the same region. The importance of the DNA sequence was explored in further investigations of a point mutation in the *benA* promoter that increases CatM activation of *benA*. These experiments help elucidate the threshold level of *benABCDE* operon expression needed for growth on benzoate. Furthermore, they highlight the importance of balanced expression from multiple promoters within the pathway. The accumulation of toxic metabolites may be a key factor in the evolution of this regulatory scheme.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Acinetobacter* strains (Table 1) were derived from ADP1, recently reclassified as an *A. baylyi* strain (33, 37). Bacteria were cultured in Luria-Bertani (LB) broth or minimal medium at 37°C (12). *Escherichia coli* DH5α (Invitrogen) was used as a plasmid host. *E. coli* BL21(DE3) (Stratagene) was used to express and purify BenM and CatM. Carbon sources were added at the following final concentrations: 3 mM benzoate, 3 mM muconate, or 10 mM succinate. Antibiotics were added as needed at the following final concentrations: ampicillin, 150 μg/ml for *A. baylyi* or 50 μg/ml for *E. coli*; kanamycin, 25 μg/ml; streptomycin, 13 μg/ml; spectinomycin, 13 μg/ml; tetracycline, 13 μg/ml. For growth curves, succinate-grown colonies were used to inoculate 5-ml cultures for overnight growth with succinate as the carbon source. In the morning, 500 μl of an overnight culture was used to inoculate 50 ml of minimal medium with benzoate or succinate as the sole carbon source. Cell growth was monitored turbidometrically with a Klett-Summerson colorimeter.

DNA sequencing and plasmid construction. Standard methods were used for DNA purification, digestion, ligation, electrophoresis, and bacterial transformation (30). Sequencing was carried out at the University of Georgia Integrated Biotech Laboratories. Plasmids are listed in Table 1. To purify CatM(V158M), pBAC383 was made in the same way as CatM-encoding pBAC381 (8) except that the *catM5153* allele was the template for PCR amplification. To generate a *catB*::*lacZ* fusion, the promoterless *lacZ*-Km^r cartridge of pKOK6 (20) was inserted into the SalI site in *catB* of pBAC673 to form pBAC674. To enable allelic exchange with the chromosome, *cat* region DNA was inserted downstream of the *lacZ*-Km^r cartridge as follows. A *catJFD* fragment was isolated as a BamHI-Asp718 fragment from pIGG14 and cloned into pBAC674 digested with BamHI and Asp718 to form pBAC675. To construct plasmid pBAC679, the *catM3102* allele was PCR amplified from the pIB17 template and cloned into pCR2.1-TOPO. At sites introduced by the amplification primers, the *catM3102* allele fragment was excised from this plasmid by digestion with NdeI and XhoI and ligated into similarly digested pET21b (Novagen) to create pBAC684. A plasmid with a *catM* disruption, pBAC6, was constructed by inserting an omega cassette encoding kanamycin resistance (14) into the HincII site of pIGG4.

BenM-independent Ben$^+$ mutants. Spontaneous mutants of *benM*-disrupted ISA36 (Table 1) that grow on benzoate (Ben$^+$) were selected after incubation on solid benzoate medium (12, 13). The *catM-catB* regions from Ben$^+$ mutants were isolated by gap repair (17). DNA segments were tested for the ability to transform ISA36 to a Ben$^+$ phenotype (12, 13). The *cat* region of ACN153 was isolated on pBAC234. The mutation conferring Ben$^+$ growth was localized to the *catM* segment on pBAC284, which was subjected to DNA sequence analysis.

Generation of *A. baylyi* strains by allelic exchange. Plasmid-borne alleles were introduced into the chromosome by methods that exploit the high efficiency of natural transformation and recombination in ADP1-derived strains (12, 23). Briefly, recipients were transformed with DNA, typically linearized plasmids, or crude cell-free lysates. Transformants in which homologous recombination had replaced the corresponding chromosomal region of the recipient with the donor DNA were initially identified by phenotypic changes. Strains generated for this study were tested for antibiotic resistance and carbon source utilization. Strains with the *catM3102* allele were tested for the characteristic high CatA enzyme activity in succinate-grown cultures (12, 23). Genotypes were confirmed by Southern hybridization, analysis of PCR-generated fragment sizes, and/or DNA sequencing of chromosomal regions.

With these methods, ACN153 was transformed with pBAC54 digested with Asp718. In a transformant, ACN164, homologous recombination had replaced the chromosomal *benA* locus with the fragment-borne *benA*::*lacZ* fusion. Similarly, a DNA fragment with *catB*::*lacZ* was made by digesting pBAC675 with Asp718. This fragment replaced the chromosomal *catB*-to-*catF* region of ISA36 and ACN153 to make ACN585 and ACN560, respectively. With the exception that it has the *catB*::*lacZ* fusion, ACN539 is isogenic to ACN293.

TABLE 1. *A. baylyi* strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
A. baylyi strains		
ADP1	Wild type (BD413)	18
ISA36	*benM*::ΩS*4036*	12
ADP102	*catM3102*[b]	23
ACN47	*benM*::ΩS*4036 benA*::*lacZ*-Km^r*5032*	12
ACN146	*benM*::ΩS*4036 benMA5146*	12
ACN153	*benM*::ΩS*4036 catM5153* [CatM(V158M)]	This study
ACN157	*benA*::*lacZ*-Km^r*5032 benM*::ΩS*4036 benMA5146*	12
ACN164	*benM*::ΩS*4036 benA*::*lacZ*-Km^r*5032 catM5153* [CatM(V158M)]	This study
ACN293	*benM*::ΩS*4036 benMA5147* Δ*catM5293*	27
ACN539	*benM*::ΩS*4036 benMA5147* Δ*catM5293 catB*::*lacZ*-Km^r*5534*	This study
ACN541	*benM*::ΩS*4036 benMA5146 catM*ΩK*5541*	This study
ACN547	*benM*::ΩS*4036 benMA5146 catM3102* [CatM(R156H)]	This study
ACN548	*benM*::ΩS*4036 benMA5146 benA*::*lacZ*-Km^r*5032 catM3102* [CatM(R156H)]	This study
ACN549	*catM3102* [CatM(R156H)]	This study
ACN558	*benM*::ΩS*4036 catM3102* [CatM(R156H)]	This study
ACN559	*benM*::ΩS*4036 benA*::*lacZ*-Km^r*5032 catM3102* [CatM(R156H)]	This study
ACN560	*benM*::ΩS*4036 catB*::*lacZ*-Km^r*5534 catM5153* [CatM(V158M)]	This study
ACN561	*benM*::ΩS*4036 benMA5146 catB*::*lacZ*-Km^r*5534 catM3102* [CatM(R156H)]	This study
ACN585	*benM*::ΩS*4036 catB*::*lacZ*-Km^r*5534*	This study
Plasmids		
PUC13, pUC19	Ap^r; cloning vector	36
pRK415	Tc^r; broad-host-range cloning vector	19
pCR2.1-TOPO	Ap^r; PCR cloning vector	Invitrogen
pET-21b	Ap^r; T7 expression vector	Novagen
pHP45	Ap^r Sm^r Sp^r; source of ΩS	25
pKOK6	Ap^r Km^r; source of promoterless *lacZ*-Km^r cassette	20
pUI1637	Ap^r Km^r; source of ΩK	14
pIB17	Ap^r; *catM3102* (11950–13205)[c] in pUC19	23
PIGG4	Ap^r; *catM* fragment (11950–12892)[c] in pUC19	This study
pIGG14	Ap^r Sm^r Sp^r; *catBCIJFD* (12892–18153)[c] in pUC13 with ΩS inserted in EcoRV site (15660)[c] of *catJ*	This study
pBAC6	Ap^r Km^r; fragment (11950–12892)[c] containing *catM* in pUC19 (ΩK in HincII site [12687][c] of *catM*)	This study
pBAC44	Ap^r Sm^r Sp^r; *benMABCDE* (563–7876)[c] in pUC19 (ΩS in multiple cloning site of vector)	This study
pBAC54	Ap^r Km^r; *lacZ*-Km^r cassette in NsiI site (3761)[c] in *benA* with adjacent *ben* region (2316–5663)[c] in pUC19	12
pBAC200	Tc^r; *cat* region DNA (9819–10649 and 15951–18153)[c] in pRK415; used to isolate the chromosomal *catMB* region	13
pBAC234	Tc^r; ACN153 *cat* region DNA (9819–18153)[c] in pRK415; isolated with pBAC200, *catM5153* mutation	This study
pBAC284	Ap^r; *catM* segment (11950–12892)[c] from pBAC234 cloned in pUC19, *catM5153* mutation	This study
pBAC364	Ap^r; *benMA5146* intergenic region (2293–2540)[c,d] in pUC19, DNase I footprinting, antisense strand labeling	This study
pBAC366	Ap^r; wild-type *benMA* intergenic region (2293–2540)[c,d] in pUC19, DNase I footprinting, antisense strand labeling	6
pBAC371	Ap^r; *benMA5146* region (2316–2540),[c,d] HindIII deletion of pBAC364, DNase I footprinting, sense strand labeling	This study
pBAC373	Ap^r; wild-type *benMA* region (2316–2540),[c,d] HindIII deletion of pBAC366, DNase I footprinting, sense strand labeling	6
pBAC383	Ap^r; PCR fragment with *catM5153* in pET-21b, for purification of CatM(V158M)	This study
pBAC669	Ap^r; *catBCIJ* (12892–15658)[c] in pUC13	This study
pBAC673	Ap^r; *catB* (13205–14225)[c] in pUC19	This study
pBAC674	Ap^r Km^r; *catB* (13205–14225)[c] upstream of *lacZ*-Km^r cassette in SalI site (14225)[c] of *catB* in pUC19	This study
pBAC675	Ap^r Km^r; *catB* (13205–14225)[c] *lacZ*-Km^r *catJFD* (15660–17347)[c] in pUC19	This study
pBAC679	Ap^r Km^r; PCR fragment with *catM3102* in pCR2.1-TOPO	This study
pBAC684	Ap^r; 0.91-kb *catM3102* NdeI-XhoI[d] fragment from pBAC679 in pET-21b, complete coding sequence	This study

[a] Ap^r, ampicillin resistant; Tc^r, tetracycline resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Km^r, kanamycin resistant; ΩS, omega cassette conferring Sm^r Sp^r; ΩK, omega cassette conferring Km^r.
[b] The original strain isolated with the *catM3102* allele has a mucoid colony morphology and is likely to carry additional uncharacterized mutations.
[c] Position in the *ben-cat* sequence in GenBank entry (accession number AF009224).
[d] Restriction sites added in primers used to generate PCR-amplified fragment for cloning.

Several strains were constructed in multiple steps. To facilitate the chromosomal introduction of the *catM3102* allele, a drug resistance marker was first inserted into *catM*. Exchange of the *catM* alleles could then be assessed by the acquisition of drug sensitivity. Plasmid pBAC6, digested with XmnI, was used to introduce the drug resistance marker into the chromosome of ACN146 to generate ACN541. A DNA fragment carrying the *catM3102* allele (pBAC684 digested with XhoI) was used to transform ACN541. In strain ACN547, the *catM3102* allele replaced the marker-disrupted *catM*ΩK*5541*of the recipient strain, ACN541. Strain ACN547 served as the recipient when transformed by a DNA fragment carrying the *catB*::*lacZ* fusion (pBAC675 digested with Asp718). Introduction of this *lacZ* reporter into the chromosome by allelic exchange yielded strain ACN561. Strain ACN547 was the recipient in a transformation with donor DNA from ACN157 (in the form of a cell-free lysate) used to introduce the *benA*::*lacZ* fusion into the chromosome. The resulting strain was ACN548.

ACN559 was also made in steps. ACN547 was transformed with wild-type *benM-benA* DNA (pBAC44 digested with XmnI). DNA sequencing confirmed that a transformant, ACN549, had wild-type *ben* region DNA. Next, the wild-type *benM* of ACN549 was replaced with the *benM*-disrupted allele of ISA36 by transforming the former strain with a cell-free lysate of the latter to generate ACN558. In the final step, a cell-free lysate of ACN47 was used to introduce the *benA*::*lacZ* fusion into the chromosome of ACN558 to generate ACN559.

β-Galactosidase (LacZ) assays. To assay the *benA*::*lacZ* transcriptional fusion, cultures were grown in minimal medium with muconate or succinate as the carbon source. To assay the *catB*::*lacZ* transcriptional fusion, cultures were grown in minimal medium on succinate. To assess induction, muconate (1 mM final concentration) was added to the growth medium for half the cultures. Growth was monitored by optical density at 600 nm (OD_{600}), and assays were done when cultures reached stationary phase. Culture samples (2 to 20 μl per assay) were lysed with sodium dodecyl sulfate and chloroform. Assay directions were followed for the FlourAce β-galactosidase reporter kit (Bio-Rad). The product of substrate hydrolysis, 4-methylumbelliferone (4MU), was detected with a TD-360 minifluorometer (Turner Designs). Relative fluorescence unit measurements enabled 4MU quantification by comparison with a standard curve.

Purification of CatM(V158M). A 50-ml culture of BL21(DE3)(pBAC383) was grown overnight in LB medium with ampicillin and used to inoculate 1 liter of the same medium. After a 4-h incubation at 37°C on a shaking platform, isopropyl-β-D-thiogalactopyranoside (1 mM final concentration) was added as an inducer. Following further incubation for 4 h, cells were harvested by centrifugation (6,000 × *g*). The cell pellet was stored at −70°C. The pellet was suspended in 30 ml buffer A (50 mM Tris [pH 8], 5 mM dithiothreitol, 10% [vol/vol] glycerol), and a crude extract was prepared by sonication. Following centrifugation (6,000 × *g*), the supernatant fraction was filtered through a 0.22-μm syringe filter, and subsequent purification was carried out with a fast protein liquid chromatography system (Pharmacia). The CatM(V158M) protein failed to bind to a 5-ml Hi-Trap heparin column (Pharmacia) and was recovered from the flowthrough fraction after 35% ammonium sulfate precipitation. The precipitate, which was collected by centrifugation (12,000 × *g*), was suspended in 7 ml buffer A and passed through a 5-ml Hi-Trap desalting column (Pharmacia). The protein sample was next loaded onto a 5-ml Hi-Trap heparin column that was then washed with 75 ml buffer A. Protein was eluted with 75 ml of a 0 to 0.6 M NaCl gradient in buffer A. Fractions containing CatM(V158M) were pooled after identification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. By using gels stained with Coomassie brilliant blue R-250 (Bio-Rad), the CatM(V158M) protein was estimated to be >90% pure. The Bradford method was used to assay protein concentrations with bovine serum albumin as the standard (3).

DNase I footprinting. As previously described, pBAC366 and pBAC373 were used to generate antisense and sense fragments of the wild-type *benMA* region end labeled with γ-^{32}P (6). Similarly, pBAC364 and pBAC371 were used to produce labeled fragments of the *benMA5146* region. For use in footprint reactions, the CatM and BenM proteins were purified by cation-exchange and heparin-agarose chromatography as described elsewhere (6, 8). Various concentrations of BenM, CatM, or CatM(V158M) were incubated with the radiolabeled DNA probe (ca. 300 pM; 200,000 cpm) at 30°C for 30 min with or without benzoate or muconate (0 to 5 mM). The binding reaction (20 μl total volume) was carried out in the following buffer: 80 mM Tris-acetate (pH 8.0), 100 mM potassium acetate, 25 mM ammonium acetate, 5 mM magnesium acetate, 0.1 mM EDTA, 1.0 mM dithiothreitol, 1 mM calcium chloride, 2 μg/ml calf thymus DNA, 50 μg/ml bovine serum albumin. DNase I cleavage reactions were carried out for 1 min at 30°C and analyzed by the methods used by Wang and Hoover (34).

RESULTS

A *catM* point mutation increases expression of *benA* without BenM. Sequence similarity between *benM* and *catM* suggested that CatM variants might substitute for BenM. To test this possibility, spontaneous mutants that grow on benzoate without BenM were isolated. Ten independent mutants derived from a *benM*-disrupted parent strain (ISA36) were selected on benzoate medium, as previously described (12, 13). From each mutant, chromosomal DNA in the *catM* region was isolated and tested for its ability to transform the parent strain to grow on benzoate. In one mutant (ACN153), the coding sequence of *catM* was responsible for conferring this Ben$^+$ phenotype. Sequence analysis of the mutant allele, designated *catM5153*, identified a single mutation, a G-to-A transition at position 474 of *catM* with respect to its translational start site. In the deduced sequence of the variant protein, CatM(V158M), a methionine residue substitutes for valine at position 158 of the 303-residue protein.

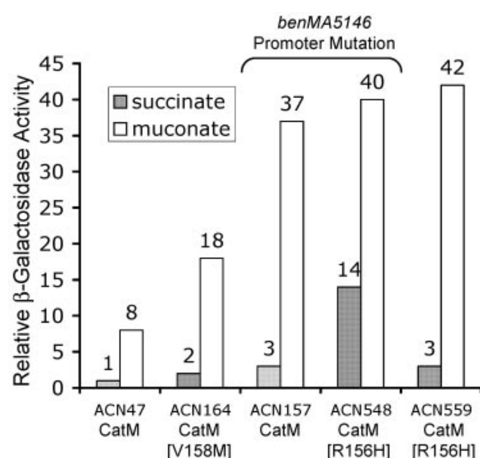

FIG. 2. Expression of a chromosomal *benA*::*lacZ* fusion in strains encoding CatM or variant regulators [CatM(V158M), encoded by *catM5153*, or CatM(R156H), encoded by *catM3102*]. All strains lack a functional *benM*, and two strains have a point mutation at position −40 relative to the *benA* transcription initiation site (*benMA5146*) (see Fig. 1). Cultures were grown on succinate or muconate as indicated. β-Galactosidase (LacZ) activity is shown relative to that measured for succinate-grown ACN47 (6.9 μmol/min/ml/OD_{600}). Activities are averages of at least four repetitions, and standard deviations were <20% of the average value.

Since the primary obstacle to BenM-independent growth on benzoate is low expression of the *benABCDE* operon (12, 13), the effect of the *catM* mutation on *ben* gene expression was assessed. A *benA*::*lacZ* transcriptional fusion was introduced into the chromosome of ACN153, which carries the *catM* mutation. In the resulting strain, ACN164, the replacement of the wild-type *benA* allele by the transcriptional fusion prevents consumption of benzoate as the sole carbon source. This strain was grown on muconate or succinate as the carbon source, and the activity of the *lacZ*-encoded enzyme, β-galactosidase, was evaluated in relation to that of the comparable strain with a wild-type *catM* allele (ACN47). The *benM* gene is disrupted in all strains tested for expression of the *benA*::*lacZ* fusion. With either the wild-type or mutant *catM* allele, *benA*::*lacZ* expression was inducible by growth on muconate (Fig. 2). However, the mutant allele caused an approximately twofold increase in expression relative to that in ACN47. To test whether the variant CatM might recognize benzoate as an effector, *lacZ* activity in ACN164 was measured with benzoate added to the growth medium alone or in combination with muconate. However, no increase in gene expression was observed in response to benzoate (data not shown) (11).

Effect of the *catM5153* allele on *catB* expression. It was not clear whether the twofold change in muconate-inducible *ben* gene expression was responsible for the ability of ACN153, which has *catM5153*-encoded CatM(V158M), to form colonies on benzoate medium within 3 days. The comparable strain with

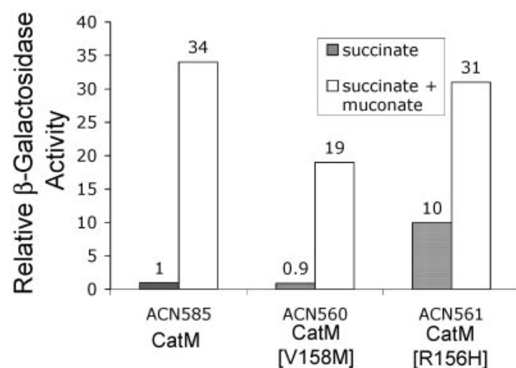

FIG. 3. Expression of a chromosomal *catB*::*lacZ* fusion in strains encoding CatM or variant regulators [CatM(V158M), encoded by *catM5153*, or CatM(R156H), encoded by *catM3102*]. All strains lack a functional *benM*. Cultures were grown on succinate with or without muconate as an inducer, as indicated. β-Galactosidase (LacZ) activity is shown relative to that measured for succinate-grown ACN585 (3.96 μmol/min/ml/OD_{600}). Activities are averages of at least three repetitions, and standard deviations were <20% of the average value.

wild-type *catM* does not form colonies on such a medium even after prolonged incubation. We tested whether *catM5153* might additionally affect *catB* expression. Regulation of this gene was studied because CatM-dependent *benA* expression is augmented by decreased CatB enzyme activity (13). This augmentation may result from the transient accumulation of muconate, the coactivator of CatM, when there are reduced levels of CatB, the enzyme that degrades muconate (Fig. 1) (13). Effects related to internal muconate concentration during growth on benzoate would not be observed under our assay conditions, since the chromosomal *benA*::*lacZ* fusion prevents the conversion of benzoate to muconate (Fig. 1 and 2). To test the effect of CatM(V158M) on *catB* expression, a *catB*::*lacZ* transcriptional fusion was used. The activity of LacZ was compared between ACN585, with the wild-type *catM*, and ACN560, with *catM5153*. In both strains *benM* is disrupted. The fusion replaces *catB* on the chromosome, thereby preventing growth on muconate as the sole carbon source. Cultures were grown with succinate as the carbon source in the presence or absence of muconate as an effector.

Expression of the *catB*::*lacZ* fusion was induced by muconate in both strains (Fig. 3). Deletion of *catM* in a *benM*-disrupted strain (ACN539) eliminated the inducible β-galactosidase (LacZ) activity (data not shown). This result supports the conclusion that CatM and CatM(V158M) are the sole regulators of *catB* expression in ACN585 and ACN560, respectively. Although *catB* expression in ACN560 increased in response to muconate, the maximum level was approximately half that of ACN585. As noted previously, lowered *catB* expression may increase the amount of muconate available to serve as the CatM coactivator (13). Thus, during growth on benzoate, the *catM5153* allele may increase *benA* expression to an extent greater than that measured by the *benA*::*lacZ* fusion (Fig. 2). Interestingly, the effect of *catM5153* on *catB* expression differed from that on *benA*, a locus where the *catM* mutation caused expression levels to increase rather than decrease relative to those with wild-type *catM* (Fig. 2). These differences indicate that the improved ability of CatM(V158M) to activate *benA* expression is not due to a general increase in responsiveness to muconate. Nevertheless, the amino acid substitution at residue 158 altered the response of CatM to muconate in the regulation of both *benA* and *catB*. The position of this substitution was near that at residue 156 in a previously characterized variant, CatM(R156H), that activates *cat* gene transcription in the absence of effectors (23).

Effect of CatM(R156H) on *benA* expression. To improve our understanding of CatM-regulated *benA* expression, we reexamined *catM3102*, an allele with a point mutation encoding histidine rather than arginine at position 156 of CatM. This mutation was selected by its ability to confer high-level expression of the *catIJF* genes in the absence of muconate as the coactivator for CatM (23). When this *catM* mutation was isolated approximately 25 years ago, the *benM* and *benA* genes had not yet been identified in any organism.

The effect of this CatM(R156H) variant on *benA* expression had not previously been addressed. For this purpose we constructed ACN559, a *benM*-disrupted strain in which the *catM3102* allele controls expression of the chromosomal *benA*::*lacZ* transcriptional fusion. As shown in Fig. 2, succinate-grown ACN559 expressed *benA*::*lacZ* at levels higher than those of ACN47, the comparable strain with wild-type *catM*. However, the *catM* mutation increased inducer-independent *benA* expression to a lesser extent than that observed for *catB*. Studies of the *catB*::*lacZ* fusion indicated a 10-fold increase in expression for succinate-grown strain ACN561 compared to ACN585 with wild-type *catM* (Fig. 3). Nevertheless, the CatM(R156H) variant significantly altered *benA* expression relative to that with wild-type CatM. In muconate-grown cells, the *benA*::*lacZ* expression level was approximately fivefold higher in ACN559 than in ACN47 (Fig. 2). This high level of *benA* expression in ACN559 raised the possibility that CatM(R156H) might substitute for BenM during growth on benzoate.

BenM-independent growth on benzoate. To test the effect of *catM3102* on benzoate catabolism, strain ACN558 was engineered to contain the wild-type *benA* region, the disrupted *benM* allele, and the *catM* mutation encoding CatM(R156H) (Table 1). ACN558 was able to grow with benzoate as the sole carbon source, although it grew more slowly than the wild type (ADP1), with both a longer generation time and a longer lag period (Table 2). This pattern of growth was nearly identical to that of ACN153, the strain with the CatM(V158M) variant (Table 2). These results demonstrate that a single CatM regulatory protein can control expression of both the *cat* and *ben* genes at levels sufficient to permit benzoate consumption without BenM.

To study BenM-independent regulation further, we used a previously isolated mutation that increases CatM-activated *benA* transcription (*benMA5146*, with a T-to-A transversion at −40 relative to the *benA* transcript start [Fig. 1C]) (12). The effect of this mutation on *benA*::*lacZ* expression was studied in succinate- and muconate-grown cultures (Fig. 2, strain ACN157). The mutation increased the level of muconate-inducible *benA* expression relative to that with the wild-type promoter (in ACN47). CatM-regulated *benA* expression patterns were similar to those of the CatM(R156H) variant at the wild-type *benA* promoter (Fig. 2, strain ACN559). Thus, a single mutation in either *catM* or the *benA* promoter is sufficient to increase

TABLE 2. Effects of *catM* and *benA* mutations on rates of BenM-independent growth on benzoate[a]

Strain	Relevant characteristic(s)[b]	Generation time (min)[c]	Lag time (h)[d]
ADP1	Wild type	60 ± 10	7 ± 0.5
ISA36	No BenM, wild-type CatM	No growth	No growth
ACN558	No BenM, CatM(R156H)	96 ± 8	9 ± 0.5
ACN153	No BenM, CatM(V158M)	93 ± 4	10 ± 0.5
ACN146	No BenM, −40 *benA* promoter mutation, wild-type CatM	50 ± 11	7 ± 0.5
ACN547	No BenM, −40 *benA* promoter mutation, CatM(R156H)	59 ± 8	4 ± 0.5

[a] Provided as the sole carbon source. When succinate was provided as the carbon source, the growth rates of all these strains were comparable (data not shown).
[b] See Table 1 for genotypes.
[c] Averages of three or more determinations.
[d] Time between inoculation and start of exponential growth.

CatM-activated *ben* gene expression. This *benMA5146* promoter mutation in ACN146 did not noticeably affect growth. With benzoate as the carbon source, the growth curves for ACN146 and the wild type were nearly the same (Table 2). Therefore, mutations that allow CatM to regulate BenM-independent growth on benzoate do not necessarily cause slower growth.

Combined effects of point mutations in the *benA* promoter and the *catM* gene. We tested the effect of the *benMA5146* promoter mutation on the ability of CatM(R156H) to activate *benA* expression. In ACN548, which lacks BenM and carries the *catM3102* allele, the *benA*::*lacZ* chromosomal fusion is under the control of the *benMA5146* promoter. Interestingly, the combination of the promoter mutation and the *catM* mutation resulted in significantly increased levels of *benA* expression in succinate-grown cells (Fig. 2, ACN548). Expression of the *benA*::*lacZ* fusion in this strain was further induced by growth on muconate. This combination of two mutations enabled a *benM* mutant, ACN547, to grow on benzoate with a generation time comparable to that of the wild type yet with a shorter lag time than those for the other strains in Table 2. Rapid initiation of growth may reflect increased muconate-independent expression of the *benABCDE* operon (as indicated by succinate-grown ACN548 [Fig. 2]). High levels of *ben* gene expression prior to the metabolic formation of muconate may allow benzoate consumption to commence without delay. Moreover, the ability of the *benMA5146* promoter mutation to alter CatM-regulated *benA* expression with both the wild-type and variant regulators raised questions about promoter-protein interactions.

CatM interactions with the *benA* promoter region. The N-terminal 58 amino acids of BenM are 85% identical to those of CatM. This resemblance predicts nearly identical DNA binding domains (22, 31, 32). To test whether BenM and CatM bind similarly to the *benA* promoter, DNase I footprinting was used. BenM, in the absence of effectors, binds to two regions upstream of the *benA* transcriptional start site (6). One region, designated site 3, overlaps the −10 region of the *benA* promoter. The second region, designated site 1, is adjacent to the −35 region of the *benA* promoter (Fig. 1C and 4E). Here, experiments with BenM (Fig. 4A and C, lanes 2) were used for comparison with experiments with CatM. Like BenM in the absence of effectors, the wild-type CatM (Fig. 4B, lane 2, and 4D, lane 3) or the CatM(V158M) variant (Fig. 4D, lane 2) protected the site 1 and 3 regions from DNase I cleavage compared to a reaction without any regulatory protein (Fig. 4, lane 1 in each panel). The binding of CatM or CatM(V158M) to the *benA* region is consistent with a previous model for BenM in which a regulatory tetramer, in the absence of effectors, represses *benA* transcription (Fig. 5, top).

With no effectors, BenM, CatM, or CatM(V158M) rendered some positions between the protected regions hypersensitive to DNase I cleavage (Fig. 4A and B, sites A to D, and Fig. 4E). These regularly spaced cleavage sites, on both the sense and antisense strands, are separated by the distance of one DNA helical turn (10 to 11 nucleotides). One site on the antisense strand was more sensitive to DNase I cleavage in the presence of CatM relative to BenM (position −20 [Fig. 4B and E, site D]). As interpreted for BenM, some sites may become more sensitive to DNase I cleavage than in the absence of protein via the formation of an exposed DNA loop when the tetrameric regulator binds. The spacing of the protein binding sites (sites 1 and 3) places them on the same side of the DNA helix (6).

Footprint changes in response to effectors. The addition of effectors resulted in a loss of protection from DNase I cleavage in the site 3 region (Fig. 4A to C, lanes 3 and 4, and 4D, lanes 4 to 5). Benzoate did not affect the DNase I cleavage patterns in reactions containing wild-type or variant CatM protein with or without muconate (data not shown), consistent with previous conclusions that CatM does not respond to benzoate (12). Loss of protection in the site 3 region should improve the ability of RNA polymerase to access the −10 promoter region. These inducing conditions are associated with the transcriptional activation of *benA*.

In the site 2 region, the effectors altered the cleavage patterns. For example, the BenM protein with both muconate and benzoate caused the continuous region of protection starting at site 1 to extend in size on both strands (Fig. 4A and C, lanes 4). Notably, the hypersensitive sites at −50 on the antisense strand and −46 on the sense strand became protected. The cleavage sites at positions −39 and −29 on the antisense strand became much less enhanced. Comparable changes occurred in the corresponding regions on the sense strand. Transcriptional activation may occur when a regulatory tetramer binds to the site 1 and site 2 regions (Fig. 5, bottom).

With BenM and muconate alone (Fig. 4A and C, lanes 3), the DNase I cleavage patterns were intermediate between those with no effectors and those with both effectors (Fig. 4A and C, lanes 2 and 4). Intermediate results also occur with benzoate as the sole effector (6). Similarly, this intermediate pattern resulted from wild-type CatM in the presence of muconate (Fig. 4B and D, lanes 4). These conditions correspond to low-level *benA* transcription and may reflect a mixture of DNA fragments with protein bound to sites 1 and 3 and others with protein bound to sites 1 and 2. Increasing the muconate concentration up to 5 mM with CatM also increased protection of bands at −50 or −46 on the antisense or sense strand, respectively (data not shown) (11). This site 2 position was also protected in the presence of muconate and CatM(V158M) (Fig. 4D, lane 5), an arrangement that correlates with increased *benA* transcription (Fig. 5, bottom). During *benA* tran-

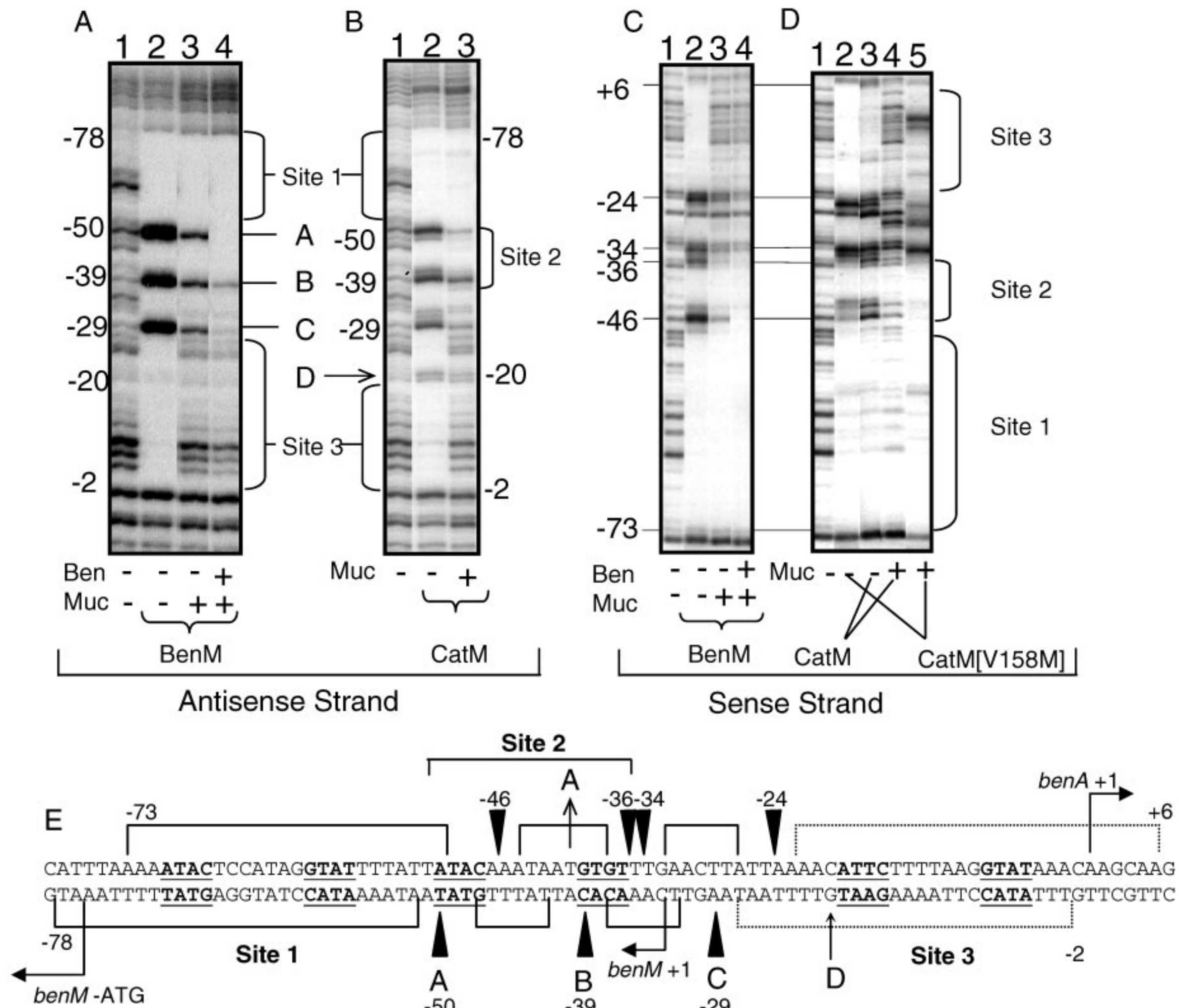

FIG. 4. DNase I footprinting of BenM, CatM, and CatM(V158M) at *benA*. DNase I-cleaved DNA was labeled on the antisense (A and B) or sense (C and D) strand of the *benA* promoter region. In lanes 2 to 5, a regulatory protein (0.15 μM) was present in the cleavage reaction, as indicated below panels A to D. The presence (+) or absence (−) of the effectors benzoate (Ben) and muconate (Muc) at a concentration of 1 mM in each reaction mixture is indicated. The binding sites labeled 1 to 3 are discussed in the text, and their positions are shown relative to the DNA sequence of the region (E). Nucleotides protected from DNase I digestion in both the absence and presence of inducers are indicated by solid brackets immediately above or below the *benA* sequence. Nucleotides protected from DNase I cleavage only in the absence of inducers are indicated by dotted brackets. Triangles and positions labeled A to D show sites that were hypersensitive to DNase I digestion. One hypersensitive site (site D) was evident in reactions with CatM but not BenM.

scription, the site 3 promoter region should interact with RNA polymerase and be unprotected in the absence of the polymerase. Thus, the anomalous site 3 banding pattern with CatM (V158M) was surprising (Fig. 4D, lane 5). Occasionally, a similar result was observed in footprints with wild-type BenM and CatM. Therefore, while the significance of this pattern is unclear, it may not depend solely on the variant CatM(V158M) protein.

The *benMA5146* DNA region was also used in DNase I footprints (data not shown) (11). No major differences occurred in the positions to which CatM binds the DNA. In the absence or presence of muconate, the cleavage patterns for CatM and the *benMA5146* region were similar to those for the wild-type *benA* promoter with CatM or the CatM(V158M) variant (Fig. 4).

DISCUSSION

CatM-*benA* DNA interactions. The overlapping DNA-binding functions of CatM and BenM reflect their sequence similarity, which is 85% overall and 98% in their N-terminal DNA binding domains. DNase I footprints suggested that without effectors, BenM, CatM, and CatM(V158M) recognized LysR-type binding sequences within site 1 and site 3 regions of *benA* DNA (Fig. 4A to C, lanes 2, and 4D, lanes 2 and 3). Such interactions should repress basal *benA* expression by blocking the −10 promoter region. Consistent with this interpretation, CatM-mediated repression occurs in vitro and in vivo in strains lacking BenM (6, 12).

Effector interactions with LysR-type proteins may cause global conformational changes in the tetramer (22). Effectors

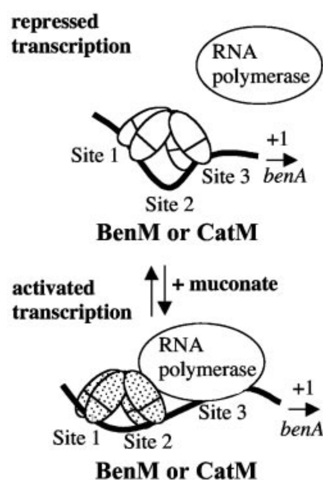

FIG. 5. Model of regulated *benA* expression. Footprint data (Fig. 4) suggest that CatM binds to the same regions of the *benA* promoter DNA as does BenM in the absence or presence of muconate. Models of *benA* regulation by BenM have been presented elsewhere (6, 9). BenM mediates higher levels of muconate-activated *benA* transcription than does CatM, as discussed in the text.

alter the number and/or the position of protein subunits bound to DNA (21, 31, 32). Effectors can also alter DNA bending and thereby impact transcription (1, 2). With *benA* DNA, muconate alleviated protection by BenM, CatM, or CatM(V158M) in the −10 (site 3) region of the promoter (Fig. 4A to C, lanes 3, and 4D, lane 4). With the variant protein in this region, alleviation of protection was accompanied by an altered DNase I cleavage pattern of unknown significance (Fig. 4D, lane 5 versus lane 1). Muconate also affected the site 2 region. With CatM, muconate reduced the extent of hypersensitivity in the cleavage of those positions indicated in Fig. 4E (Fig. 4B, lane 3, and 4D, lane 4). These patterns with CatM and muconate in the site 2 DNA were similar to those with BenM (Fig. 4A and C, lanes 3). The footprint patterns with muconate, which correlate with low-level *benA* transcription, may arise from a mixed population of DNA fragments in the active and repressed configurations (Fig. 5). Higher-level transcription correlates with protection of the cleavage sites at positions −50 on the antisense strand and −46 on the sense strand, as observed for BenM with muconate and benzoate (Fig. 4A and C, lanes 4) and for CatM(V158M) with muconate (Fig. 4D, lane 5).

Although there were some differences in the cleavage patterns, the overall *benA* regions to which the wild-type and variant CatM bound were similar to those of BenM. Therefore, the variations in *benA* expression levels could not be attributed to major shifts in the location of DNA-protein binding sites for BenM, CatM, CatM(V158M), and the mutant promoter *benMA5146* (data not shown). Moreover, the extent of BenM and CatM similarity raised questions concerning the evolutionary retention of both paralogs.

Functional divergence of BenM and CatM. CatM served as the sole *ben-cat* regulator when spontaneous mutations occurred in *catM* (*catM5153* and *catM3102*) or the *benA* promoter (*benMA5146*). Although the mutants rapidly consumed benzoate (Table 2), the relative fitness of the mutant and wild-type strains was not evaluated. Mutations that increased CatM-mediated *ben* gene transcription altered the balance of *ben* and *cat* gene expression. CatM(V158M) and CatM(R156H) affected *benA* and *catB* differently (Fig. 2 and 3). During benzoate consumption (Fig. 1), the altered regulation of these genes should affect metabolite flow and the accumulation of muconate, a key effector that is also toxic at high concentrations (15).

This toxicity prevents growth on alternative carbon sources when muconate accumulates endogenously during the metabolism of aromatic precursors in a strain lacking *catB* (5, 35). Interestingly, exogenous muconate does not inhibit growth in the same fashion. Perhaps the coupled uptake and degradation of muconate prevent its intracellular accumulation (15). Further evidence that endogenous muconate is harmful comes from the analysis of spontaneous mutants. Those selected mutants that grow without *catB* in the presence of an appropriate aromatic precursor, such as benzoate, anthranilate, benzyl alcohol, or benzaldehyde, invariably acquire secondary mutations that block muconate formation (5, 35). By this method, numerous *catA* mutations have been obtained in which catechol accumulation is evident but not lethal (5, 35). Thus, the endogenous accumulation of muconate appears to be more toxic than that of catechol.

The slow growth of ACN558 and ACN153, which encode CatM variants, may reflect the fact that these mutants are less adept than the wild type at balancing *ben* and *cat* gene expression to optimize muconate concentrations during growth on benzoate (Table 2). The key role of muconate as an effector allows this compound to control the genes needed for its own formation and degradation. The importance of the muconate concentration is also suggested by the short lag time of ACN547, a strain with high inducer-independent expression of the *ben* genes (Table 2). In this strain, muconate should not need to accumulate in order to initiate pathway induction.

In the wild type, pathway induction is initiated by benzoate in conjunction with BenM. As assessed by transcriptional fusions (12), muconate alone causes BenM to activate higher *benA* expression levels than do the CatM variants (Fig. 2). Thus, muconate alone should be sufficient to induce benzoate consumption under laboratory conditions. Nevertheless, the intricate synergistic response of BenM to two effectors allows benzoate consumption to initiate quickly, to reach high levels in the presence of the substrate (benzoate) and a catabolite (muconate), and to decrease upon substrate depletion (6). During evolution, selection may favor regulatory schemes that optimize the ability to adapt to changing conditions (28). Furthermore, the use of two regulators could help balance *ben* and *cat* gene regulation when muconate is derived from substrates other than benzoate. Some aromatic compounds, such as anthranilate, are degraded via catechol such that *cat* but not *ben* gene expression is required (5, 15, 37). The complexity of the regulatory circuit provides the potential for very rapid and large variations in gene expression.

Promoter dependence of regulation. An intriguing aspect of the complexity is that CatM and BenM function differently at multiple promoters. Studies of the CatM(R156H) variant emphasize the importance of specific promoter sequences. This variant activates high-level muconate-independent *catB* transcription (Fig. 3). Surprisingly, the main effect of the CatM(R156H) variant on *benA* transcription was to increase inducer-dependent

FIG. 6. Comparison of sequences in the *benA* and *catB* promoter regions. The sequences are aligned relative to the transcription initiation site (+1), and identity is indicated (:). The significance of the *ben* region sites 1, 2, and 3 is discussed in the text. Gray shading indicates a consensus sequence (ATAC-N_7-GTAT) used to bind CatM (29), BenM (6), and other related members in a subclass of LysR-type regulators.

gene expression (Fig. 2, ACN559 versus ACN47). The different effects on *catB* and *benA* (Fig. 2 and 3) suggest that protein-DNA interactions in the *benA* region do not properly situate CatM for optimal RNA polymerase contact. It may be that the affinity of the *benA* promoter for CatM is reduced relative to that for BenM and/or that the conformations of the two regulators in their activated states are sufficiently different to affect transcription.

The *benA* and *catB* promoters have greater sequence differences between their site 2 regions than between their site 1 and 3 regions (Fig. 6). Our regulatory model predicts that transcriptional activation results when a regulatory tetramer binds site 1 and site 2. The CatM(R156H) variant may have a higher proclivity for binding the *catB* site 2 region in the absence of muconate than it has for the comparable region of *benA*. Consistent with this possibility, a mutation of T to A at position −40 in the site 2 region of *benA* increased inducer-independent transcriptional activation by CatM(R156H) (Fig. 2, ACN548 versus ACN559). This mutation also increased CatM-activated inducible *benA* transcription (Fig. 2). In comparison to the wild-type sequence, the mutated sequence, AGTGT, more closely resembles the corresponding portion of the site 1 region of *catB*, AGTAT, to which CatM binding has been demonstrated (Fig. 6) (29).

It is not clear how the central portion of CatM affects protein binding to the *benA* site 2 region. Nevertheless, mutations at positions 156 and 158, in the effector-binding domain of CatM, increase transcriptional activation of *benA* (Fig. 2). In other LysR-type regulators, mutations have been identified that affect both inducer and DNA binding. For example, amino acid substitutions in a central position of the NahR protein affect both inducer response and DNA binding (24). The effector-binding domains of diverse LysR-type regulators may share a common Rossmann-fold topology characteristic of a family of periplasmic binding proteins (26). This topology involves two domains connected by a hinge that allows movement when an effector binds in the interdomain cavity. Residues 156 and 158 in CatM are predicted to be near this hinge-like region, and they could affect interdomain movement. Alternatively, these positions might affect the oligomerization of CatM. When mutations important to transcriptional activation by LysR regulators were mapped relative to the structure of CbnR, many corresponded to residues at the interface between subunits (22). It is also possible that the two CatM substitutions affect transcription by different mechanisms. More information about effector-induced conformational changes in CatM and its variants should be provided by current structural studies (7, 9). Structural investigations, which may also reveal the basis for the inability of CatM to respond to benzoate, will complement the physiological characterization of the regulatory mutations in this report.

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