

## Regulation of Benzoate Degradation in *Acinetobacter* sp. Strain ADP1 by BenM, a LysR-Type Transcriptional Activator

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In *Acinetobacter* sp. strain ADP1, benzoate degradation requires the *ben* genes for converting benzoate to catechol and the *cat* genes for degrading catechol. Here we describe a novel transcriptional activator, BenM, that regulates the chromosomal *ben* and *cat* genes. BenM is homologous to CatM, a LysR-type transcriptional activator of the *cat* genes. Unusual regulatory features of this system include the abilities of both BenM and CatM to recognize the same inducer, *cis,cis*-muconate, and to regulate some of the same genes, such as *catA* and *catB*. Unlike CatM, BenM responded to benzoate. Benzoate together with *cis,cis*-muconate increased the BenM-dependent expression of the *benABCDE* operon synergistically. CatM was not required for this synergism, nor did CatM regulate the expression of a chromosomal *benA::lacZ* transcriptional fusion. BenM-mediated regulation differs significantly from that of the TOL plasmid-encoded conversion of benzoate to catechol in pseudomonads. The *benM* gene is immediately upstream of, and divergently transcribed from, *benA*, and a possible DNA binding site for BenM was identified between the two coding regions. Two mutations in the predicted operator/promoter region rendered *ben* gene expression either constitutive or inducible by *cis,cis*-muconate but not benzoate. Mutants lacking BenM, CatM, or both of these regulators degraded aromatic compounds at different rates, and the levels of intermediary metabolites that accumulated depended on the genetic background. These studies indicated that BenM is necessary for *ben* gene expression but not for expression of the *cat* genes, which can be regulated by CatM. In a *catM*-disrupted strain, BenM was able to induce higher levels of *catA* expression than *catB* expression.

In the soil bacterium *Acinetobacter* sp. strain ADP1 (formerly *Acinetobacter calcoaceticus*), the enzymes needed for the conversion of benzoate to tricarboxylic acid cycle intermediates via the  $\beta$ -ketoacid pathway are encoded by the *ben* and *cat* genes (Fig. 1 and 2) (11). Although *cat* gene expression has been characterized, little is known about the regulation of the adjacent *ben* genes. The *ben*-encoded enzymes convert benzoate to catechol, a compound subsequently degraded by *cat*-encoded enzymes (26). During growth with benzoate, tight regulation of metabolic flow prevents the accumulation of most intermediary compounds (7). One metabolite whose transient production from benzoate can be detected, however, is *cis,cis*-muconate, the product of catechol ring cleavage and the inducer of all of the *cat*-encoded enzymes.

*cis,cis*-Muconate regulates its own formation and degradation. Acting on the LysR-type transcriptional regulator CatM, this compound activates transcription of *catA* and the *catBCIJFD* genes (30). Moreover, *cis,cis*-muconate derived from benzoate can prevent the degradation of an alternative carbon source, *p*-hydroxybenzoate, by an as yet undefined regulatory mechanism (7). The accumulation of *cis,cis*-muconate during growth with benzoate most likely reflects differential expression of *catA* and the *catBCIJFD* genes, which are physically separated on the ADP1 chromosome (Fig. 2). Unlike the other *cat* genes, *catA* is expressed in response to benzoate as well as *cis,cis*-muconate (23). Benzoate in the absence of its metabolism, however, yields only partial induction of CatA. In a similar fashion, partial induction of the *ben*-encoded enzymes can

be mediated by *cis,cis*-muconate (24). Full induction of these enzymes, however, requires growth with benzoate.

The ability of both *cis,cis*-muconate and benzoate to induce CatA and the Ben enzymes, albeit to different levels, suggests interconnected regulation of the corresponding genes. The goal of the current investigation was to determine the mechanisms and inducers of *ben* gene expression and to clarify the basis of benzoate-mediated expression of *catA*. The first step in this process was to characterize a genetic region adjacent to *benA* that appeared to control *cat* gene expression in a *catM* mutant strain (30). As described in this report, the region contains a gene immediately upstream of and divergently transcribed from *benA*, designated *benM*, that encodes a LysR-type transcriptional regulator with striking similarity to CatM. The ability of BenM to regulate the *ben* and *cat* genes in response to different inducers was tested. In addition, the effect of *benM* disruption on metabolic flow was examined by using nuclear magnetic resonance (NMR) techniques to monitor benzoate degradation (7).

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *Acinetobacter* strains and plasmids are described in Table 1 and Fig. 2. All *Acinetobacter* strains were derived from BD413 (14), also designated ADP1. Confusion about the taxonomic classification of ADP1 has prompted the use of *Acinetobacter* sp. until further characterization is complete (9). *Escherichia coli* DH5 $\alpha$  (Gibco BRL) and S17-1 (35) were used as plasmid hosts. Bacteria were cultured in Luria-Bertani (LB) broth and minimal medium (MM) at 37°C as previously described (32, 34). Carbon sources added to MM, at the final concentrations specified, were 10 mM succinate, 3 mM benzoate, 3 mM *cis,cis*-muconate, 2.5 mM anthranilate, and 2 mM catechol. Antibiotics were added as needed at the following final concentrations: tetracycline, 6  $\mu$ g/ml; kanamycin, 25  $\mu$ g/ml; streptomycin, 25  $\mu$ g/ml; spectinomycin, 25  $\mu$ g/ml; and ampicillin, 150  $\mu$ g/ml for *Acinetobacter* strains and 50  $\mu$ g/ml for *E. coli*.

**DNA manipulations and plasmid constructions.** Standard methods were used for chromosomal and plasmid DNA purifications, restriction enzyme digestions, electrophoresis, ligations, and *E. coli* transformations (32). Plasmids carrying

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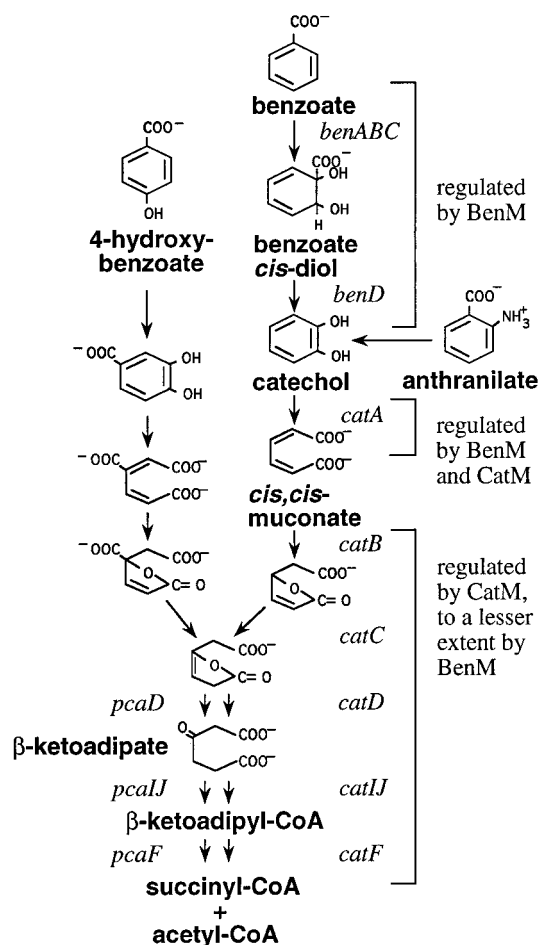


FIG. 1. The  $\beta$ -ketoadipate pathway of *Acinetobacter* sp. strain ADP1. Compounds and genes relevant to these studies are indicated by boldfaced and italicized text, respectively.

DNA fragments upstream of *benA* were generated from pIB1351 and pIB1352 (24). The sole *Sall* ADP1 DNA fragment of the pIB1351 or pIB1352 was deleted to form pIGG5 or pIGG26, respectively. The two *KpnI* ADP1 DNA fragments of pIB1351 were deleted to form pIGG23. The sole *HindIII* ADP1 DNA fragment of pIB1351 was deleted to form pIGG24. The DNA fragment between the *EcoRV* and *KpnI* sites of pIGG23 was ligated to pUC19 between the vector's *HincII* and *KpnI* sites to form pBAC64.

Plasmids pIGG27 and pBAC12, used for interposon mutagenesis, were made by inserting an omega ( $\Omega$ ) cassette conferring resistance to spectinomycin (*Sp*<sup>r</sup>) and streptomycin (*Sm*<sup>r</sup>) ( $\Omega$ S) or kanamycin (*Km*<sup>r</sup>) ( $\Omega$ K), respectively, in the sole *Sall* site of pIGG24. Plasmids pHP45 (29) and pUI1637 (6) were the sources of the  $\Omega$ S and  $\Omega$ K fragments, respectively. Each of these fragments carries transcriptional and translational stop signals following the drug resistance determinant. To generate the *benA::lacZ* fusion of pBAC54, the promoterless *lacZ*-*Km*<sup>r</sup> cartridge of pKOK6 (18) was isolated as a *PstI* fragment and inserted into the compatible *NsiI* site in *benA* of plasmid pIB1354 (24). To generate pBAC14, carrying *benM*, we made an intermediate plasmid in which the *EcoRI*-*PvuII* fragment (E, to P in Fig. 2) was ligated into pUC19 digested with *EcoRI* and *HincII*. The *benM* fragment was excised from this plasmid by digestion with *EcoRI* and *PstI*, and it was ligated into similarly digested pRK415 (16) to form pBAC14.

**Strain construction by interposon mutagenesis and allelic replacement.** A linear DNA fragment carrying either the *benM:: $\Omega$ S4036* or the *benM:: $\Omega$ K5008* allele was generated by restriction endonuclease digestion of plasmid pIGG27 with *PvuII* or plasmid pBAC12 with *BglII*, respectively. Each DNA fragment with an  $\Omega$  cassette was electrophoretically separated and purified from an agarose gel with the GeneClean purification kit (Bio101). As described previously (20), the DNA fragments were used to transform and to replace the corresponding chromosomal regions of recipient *Acinetobacter* strains ADP1 and ISA13, generating ISA36 and ACN9, respectively. Similarly, a linear DNA fragment carrying the

*benA::lacZ* fusion was generated by digestion of pBAC54 with restriction endonuclease *Asp718* and used to transform ADP1, thereby generating strain ACN32.

A crude cell lysate was made from strain ACN32 and used as previously described (20) to introduce the *benA::lacZ* fusion into the chromosomes of strains with different genetic backgrounds. In this way, new strains ACN39, ACN41, and ACN47 were generated from recipient strains ISA25, ISA29, and ISA36, respectively. DNA in a crude cell lysate made from strain ACN47 was used to transform ADP205 and generate strain ACN42. As previously described, the chromosomal configurations of all strains were confirmed by Southern hybridization analyses, and use of probes made from vector sequences confirmed that allelic replacement rather than plasmid integration had introduced modified DNA into the chromosomes of the recipient strains (9).

**Isolation of plasmids with the *benA* operator/promoter regions of mutants.** The gap repair method of Gregg-Jolly and Ornston (9a) was used to isolate the chromosomal DNA of selected mutants corresponding to the region between *K*<sub>1</sub> and *K*<sub>2</sub> in Fig. 2. Plasmid pIGG16 was constructed by ligating ADP1 DNA fragments E<sub>1</sub> to *K*<sub>1</sub> and *K*<sub>2</sub> to RV<sub>2</sub> (Fig. 2) into the vector pRK415 (16). Recipient strains were transformed with pIGG16, linearized at the sole *KpnI* site, and homologous recombination in vivo generated plasmids carrying the *benM*-*benA* intergenic regions of the mutants. The intergenic regions between *benM* and *benA* were sequenced as described below from plasmids isolated by the gap repair method, using two oligonucleotide primers, 5'-CAAGATTTTGAATTTGTCGGC-3' and 5'-GCTAGTATTAATGACGGGAAT-3', purchased from National Biosciences Inc.

**DNA sequence analysis.** The DNA sequence of plasmids pIGG5, -23, -24, and -26 and pBAC64 were determined with double-stranded templates and sequencing primers (Promega) which recognize the pUC19 cloning vector. An automated DNA sequencer (ABI373A; Applied Biosystems, Inc.) was used in the University of Georgia Molecular Genetics Instruments Facility. In addition, two oligonucleotide primers, 5'-GCTCTACTGGAATGGTT-3' and 5'-GCTCAGTAAAACCTTCAT-3' (Genosys Biotechnologies), were used with the pIGG5 DNA template. DNA sequences were analyzed with the Wisconsin Genetics Computer Group programs (5).

**CatA and  $\beta$ -galactosidase (*LacZ*) assays.** Catechol 1,2-dioxygenase (*CatA*) activity was measured in cell extracts of cultures grown in LB medium with or without 2 mM benzoate or 2 mM *cis,cis*-muconate as described previously (30). *CatA* activity was assayed spectrophotometrically by the increase in *A*<sub>260</sub>, indicative of *cis,cis*-muconate formation (25). For *LacZ* assays, cultures were grown overnight in 5 ml of LB with or without 3 mM benzoate, 3 mM *cis,cis*-muconate, 2.5 mM anthranilate, or 2 mM catechol. Cells were lysed with chloroform and sodium dodecyl sulfate, and  $\beta$ -galactosidase activities were determined as described by Miller (19).

**Metabolite monitoring by HPLC.** To monitor benzoate metabolism, 1-ml culture samples were centrifuged to pellet cells. Any cells remaining in the supernatant fractions were removed by passage through a low-protein-binding, 0.22- $\mu$ m-pore-size syringe filter (MSI). A 10- $\mu$ l sample of the filtrate was analyzed on a C<sub>18</sub> reversed-phase high-performance liquid chromatography (HPLC) column (Bio-Rad Laboratories). Elution at a rate of 0.8 ml/min was carried out with 30% acetonitrile and 0.1% phosphoric acid, and the eluant was monitored by UV detection at 254 nm to detect anthranilate, benzoate, and *cis,cis*-muconate and at 282 nm to detect catechol. Under these conditions, the retention times for benzoate, anthranilate, catechol, and *cis,cis*-muconate were 7.7, 6.0, 4.6, and 3.5 min, respectively. Peak areas corresponding to standards and experimental samples were integrated by using the ValuChrom software package (Bio-Rad). To assess the metabolism of benzoate by mutants blocked in its degradation, cultures were grown in LB broth with 2.5 to 3 mM benzoate added. Culture samples were analyzed by HPLC before and after 24 h of growth to determine the benzoate concentration. The concentration determinations of duplicate samples varied no more than 100  $\mu$ M.

**Metabolic observation.** Metabolic observation-NMR (MO-NMR) was carried out as described previously (7). Briefly, *Acinetobacter* strains were deuterated by overnight growth in a complex <sup>2</sup>H-IG medium prepared (by Isogenetics, Inc.) from algae grown photoautotrophically in deuterated water (7). The *Acinetobacter* cells were used to inoculate a tube containing 6 ml of a defined deuterated minimal medium, <sup>2</sup>H-MM (7), and 600  $\mu$ l of <sup>2</sup>H-IG medium. The addition of benzoate or a mixture of benzoate and *p*-hydroxybenzoate, dissolved in <sup>2</sup>H<sub>2</sub>O, marked the start of each experiment (time zero). The culture tubes were incubated at 34°C with aeration. Culture samples were taken periodically and analyzed by NMR spectroscopy. Concentrations of compounds were determined by integrating the appropriate spectral peaks.

**Northern hybridization analysis.** Total RNA was isolated from 20-ml *Acinetobacter* cultures by using a Snap-O-Sol kit (Biotex Laboratories). RNA species were electrophoretically separated on agarose-formaldehyde gels with 2 to 8  $\mu$ g of RNA per lane (32). A rapid downward transfer system was used to transfer RNA to Nytran nylon membranes (TurboBlotter; Schleicher & Schuell). RNA was cross-linked to the membranes by exposure to a total dose of UV light (254 nm) of 120 mJ cm<sup>-2</sup>. Sizes of the mRNAs were estimated by using RNA standards of sizes 9.4, 6.2, 3.9, 2.8, 1.9, 0.9, 0.6, and 0.4 kb (Promega) that were stained with methylene blue dye following transfer to the nylon membranes (32).

Nonradioactive probes, made by random-primed labeling of gel-purified DNA fragments with digoxigenin, were used in hybridizations as directed by the Genius system (Boehringer Mannheim Corp.). In the description of fragments, each

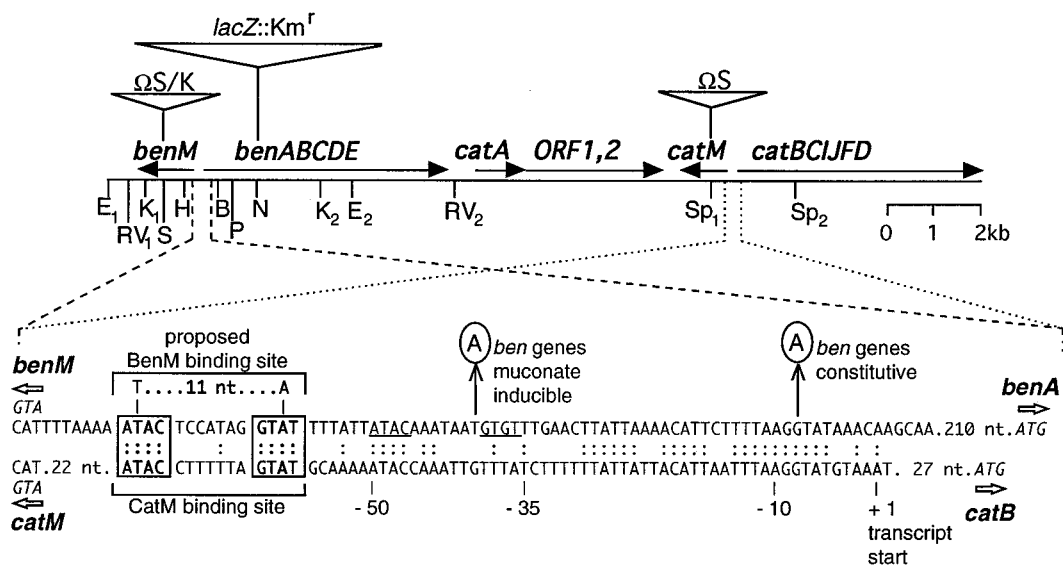


FIG. 2. Restriction map of the chromosomal *ben-cat* region. The locations of genes, and their transcriptional directions, are shown relative to some of the known restriction endonuclease sites: *EcoRI* (E), *EcoRV* (RV), *KpnI* (K), *Sall* (S), *HindIII* (H), *BsaAI* (B), *PvuII* (P), *NsiI* (N), and *SphI* (Sp). Triangles mark the insertion sites of  $\Omega$ S (29),  $\Omega$ K (6), and the *lacZ::Km<sup>r</sup>* cassette (18) of strains listed in Table 1. Below the map, the DNA sequence of the *benM-benA* intergenic region was aligned with the corresponding *catM-catB* intergenic region, with identical nucleotides indicated (:). The known CatM binding site (30) was used to predict a possible BenM binding site that has the consensus sequence (T-11 nt-A) and dyad symmetry (ATAC, GTAT) involved in binding LysR-type activators. An adjacent region with similar sequences is underlined. Circled nucleotides indicate mutations identified in strains ACN147 and ACN149 (G→A) and in strain ACN146 (T→A).

purified and used in labeling reactions, the numbers in parentheses correspond to positions in the 15,923-nucleotide (*nt*) *ben-cat* region sequence in GenBank, accession no. M76991. The *benA* probe was derived from DNA between *BsaAI* (351) and *NsiI* (1446) sites. A *benBC* probe was derived from DNA between *ClaI* sites (2143 and 2988). DNA between *EcoRI* sites (3348 and 3803) was used for a *benD* probe. A *benE* probe was made from DNA between the ATG start (4173) and TAA termination (5357) codons.

**Nucleotide sequence accession number.** The nucleotide sequence of *benM* was deposited in the GenBank database (accession no. AF009224).

**RESULTS**

**Identification of the *benM* gene.** In previous studies, a deletion encompassing the *benABC* genes and a region upstream of *benA* was shown to eliminate inducible *catA* and *catB* expression in a *catM* mutant (30). As presented here, the DNA sequence upstream of *benA* revealed a divergently transcribed 912-bp open reading frame, designated *benM*. The deduced 304-amino-acid sequence of BenM is homologous to sequences of LysR-type transcriptional activators including CatM, to which it is 59% identical and 75% similar. Similarities are greatest within a subset of this family containing proteins that regulate the degradation of catechol, such as CatM and CatR, or that regulate the degradation of chlorocatechols, such as ClcR, TcbR, and TfdR (Fig. 3). In pairwise sequence comparisons of BenM with *Pseudomonas* regulators CatR (12, 31), ClcR (3), and TcbR (36) or TfdR from *Ralstonia eutropha* (15), identity between aligned residues ranges from 29 to 41% and similarity ranges from 50 to 61%.

**Disruption of *benM* and isolation of suppressor mutations.** Strain ISA36 was created by interposon mutagenesis (29) of *benM* with the  $\Omega$ S cassette (Fig. 2 and Table 1). The *benM* disruption did not prevent the catabolism of *cis,cis*-muconate, catechol, or anthranilate, and with each of these as the sole carbon source, ADP1 and ISA36 grew at comparable rates (data not shown). The degradation of these substrates requires the gene products of *catA* and *catBCIJFD* but not those of *benABCD*. In contrast, the *benM* disruption prevented benzo-

ate degradation. Growth of ISA36 on catechol but not benzoate suggested that BenM regulates *ben* gene expression.

After 2 to 3 days of incubation on solid medium with benzoate as the sole carbon source, spontaneous mutants of ISA36 appeared at a rate of approximately  $10^{-5}$ . Crude DNA lysates made from six independently isolated mutants transformed an ISA36 recipient to be able to grow rapidly on benzoate, indicating that mutations distinct from the  $\Omega$ S disruption conferred growth with benzoate. These transformants remained *Sm<sup>r</sup> Sp<sup>r</sup>*, consistent with their retention of the  $\Omega$ S insertion in *benM*. When wild-type DNA was used to transform ISA36, benzoate-grown transformants were sensitive to streptomycin (*Sm<sup>s</sup>*) and spectinomycin (*Sp<sup>s</sup>*), as would be expected if the wild-type allele had replaced the  $\Omega$ S-disrupted *benM*.

DNA upstream of *benA* was similar in sequence to the characterized *catB* operator/promoter (Fig. 2) (30). Because mutations in the *ben* gene operator/promoter region might allow *ben* gene expression in the absence of BenM, a chromosomal region including *benA* and *benM* was isolated from the six ISA36-derived mutants by gap repair methods (9a). DNA sequencing revealed that two of the independently isolated strains, ACN147 and ACN149, had a G-to-A transition in the same position upstream of the *benA* coding region (Fig. 2). Another strain, ACN146, had a T-to-A transversion (Fig. 2). No mutations were identified in the *benA-benM* intergenic region of the remaining three strains. To determine how the identified mutations affect gene expression, a better understanding of wild-type *ben* gene expression was needed.

**Regulation of *benA* expression.** A *benA::lacZ* transcriptional fusion, on plasmid pBAC54 (Table 1), was introduced into the wild-type chromosome by allelic replacement, forming ACN32. In this strain, the promoterless *lacZ* cassette (18) lies 1.1 kbp downstream of the ATG initiation codon of *benA*, and  $\beta$ -galactosidase (LacZ) activity reflects *benA* expression. HPLC confirmed that the *benA* disruption had blocked further degradation of benzoate (data not shown). The addition of ben-

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid(s)	Relevant characteristic(s) <sup>a</sup>	Reference or source
<i>Acinetobacter</i> strains		
ADP1	Wild type (BD413)	14
ADP205	$\Delta(\text{catM-catB3205})$	20
ISA13	<i>catM::</i> ΩS4013	30
ISA25	$\Delta(\text{catBCIJF4025})$	7
ISA29	$\Delta(\text{benE-catA4029})$	7
ISA35	$\Delta(\text{benMABC4025})\text{catM}::\Omega\text{S4013}$	30
ISA36	<i>benM::</i> ΩS4036	This study
ACN9	<i>benM::</i> ΩK5008 <i>catM::</i> ΩS4013	This study
ACN32	<i>benA::lacZ-Km<sup>r</sup>5032</i>	This study
ACN39	<i>benA::lacZ-Km<sup>r</sup>5032</i> $\Delta(\text{catBCIJF4025})$	This study
ACN41	<i>benA::lacZ-Km<sup>r</sup>5032</i> $\Delta(\text{benE-catA4029})$	This study
ACN42	<i>benA::lacZ-Km<sup>r</sup>5032 benM::</i> ΩS4036 $\Delta(\text{catM-catB3205})$	This study
ACN47	<i>benA::lacZ-Km<sup>r</sup>5032 benM::</i> ΩS4036	This study
ACN146	<i>benM::</i> ΩS4036, point mutation T→A (Fig. 2)	This study
ACN147	<i>benM::</i> ΩS4036, point mutation G→A (Fig. 2)	This study
ACN149	<i>benM::</i> ΩS4036, point mutation G→A (Fig. 2)	This study
ACN157	<i>benA::lacZ-Km<sup>r</sup>5032 benM::</i> ΩS4036, point mutation T→A (Fig. 2)	This study
ACN158	<i>benA::lacZ-Km<sup>r</sup>5032 benM::</i> ΩS4036, point mutation G→A (Fig. 2)	This study
ACN160	<i>benA::lacZ-Km<sup>r</sup>5032 benM::</i> ΩS4036, point mutation G→A (Fig. 2)	This study
Plasmids		
pUC19	Ap <sup>r</sup> , cloning vector	37
pRK415	Tc <sup>r</sup> , cloning vector	16
pKOK6	Source of promoterless <i>lacZ-Km<sup>r</sup></i> cassette	18
pHP45	Source of ΩS	29
pUI1637	Source of ΩK	6
pIB1351, pIB1352	<i>benM</i> to -D (E <sub>1</sub> to E <sub>2</sub> ; Fig. 2) in pUC19	24
pIB1354	<i>benA</i> to -D (H to E <sub>2</sub> ; Fig. 2) in pUC19	24
pIGG5	Part of <i>benM</i> (E <sub>1</sub> to S; Fig. 2) in pUC19	This study
pIGG16	Part of <i>benK</i> , <i>benCDE</i> (E <sub>1</sub> to K <sub>1</sub> , K <sub>2</sub> to RV <sub>2</sub> ; Fig. 2) in pRK415	This study
pIGG23	Part of <i>benM</i> (E <sub>1</sub> to K <sub>1</sub> ; Fig. 2) in pUC19	This study
pIGG24	Part of <i>benM</i> (E <sub>1</sub> to H; Fig. 2) in pUC19	This study
pIGG26	Part of <i>benM</i> (S to E <sub>2</sub> ; Fig. 2) in pUC19	This study
pIGG27	ΩS in <i>benM</i> <i>Sa</i> I site (S; Fig. 2) of pIGG24	This study
pBAC12	ΩK in <i>benM</i> <i>Sa</i> I site (S; Fig. 2) of pIGG24	This study
pBAC14	All of <i>benM</i> (E <sub>1</sub> to P; Fig. 2) in pRK415	This study
pBAC54	<i>lacZ-Km<sup>r</sup></i> in <i>benA</i> <i>Nsi</i> I site (N; Fig. 2) of pIB1354	This study
pBAC64	Part of <i>benM</i> (RV <sub>1</sub> to K <sub>1</sub> ; Fig. 2) in pUC19	This study

<sup>a</sup> Km<sup>r</sup>, kanamycin resistant; Ap<sup>r</sup>, ampicillin resistant; Tc<sup>r</sup>, tetracycline resistant.

zoate to the growth medium increased β-galactosidase activity in ACN32 approximately 10-fold compared to additive-free medium (Fig. 4), establishing benzoate as an inducer of *benA* expression.

Since a role for *cis,cis*-muconate in regulating benzoate degradation had previously been suggested (4, 24), the effect of this compound on expression of the *benA::lacZ* fusion was tested. The addition of *cis,cis*-muconate led to even higher β-galactosidase levels than did benzoate, approximately 25-fold greater than without an added inducer (Fig. 4). To ensure that the true inducer was *cis,cis*-muconate and not a subsequent metabolite, strain ACN39 was constructed to carry the chromosomal *benA::lacZ* fusion (Table 1) and a chromosomal deletion of genes *catB* to *catF* which prevents *cis,cis*-muconate degradation. In ACN39, the pattern of β-galactosidase induction was similar to that of ACN32 (Fig. 4).

The ability of *cis,cis*-muconate to induce higher *ben* gene expression than benzoate was surprising, since benzoate-grown cells oxidize benzoate faster and have higher Ben enzyme activities than *cis,cis*-muconate-grown cells (1a, 4, 24). One possible cause for higher *ben* gene expression during growth on benzoate than on *cis,cis*-muconate is the ability of both inducers together to cause higher levels of *ben* gene expression than either alone. Consistent with this possibility, the addition of both *cis,cis*-muconate and benzoate to all strains with a *benA::lacZ* fusion induced the highest β-galactosidase levels in these studies, up to 80-fold higher than in uninduced cells (Fig. 4). Catechol, another metabolite of benzoate, also induced *benA* expression. In strain ACN41 (Table 1), which carries the chromosomal *benA::lacZ* fusion, the *catA* gene was deleted from the chromosome to block catechol degradation. In this strain, induction by benzoate and/or *cis,cis*-muconate was similar to that in ACN32. Catechol-induced expression was comparable to that of benzoate, with β-galactosidase activities sevenfold higher than in uninduced cells (Fig. 4).

***benM* is required for *benA* expression.** ACN47 carries a chromosomal *benM* disruption as well as the chromosomal *benA::lacZ* fusion (Table 1). In ACN47, expression of the *benA::lacZ* fusion was not inducible (Fig. 4). The wild-type pattern of induction was restored by *benM* in *trans* on pBAC14, although the induced levels of β-galactosidase were all approximately 40% lower than in ACN32 (Fig. 4). This same pattern of expression was observed with *benM* in *trans* in strain ACN42. Since ACN42 additionally carries a chromosomal deletion of the *catMBC* genes (Fig. 4), CatM is not required for the synergistic effect of benzoate and *cis,cis*-muconate on *benA* expression.

Introduction of the chromosomal *benA::lacZ* fusion into the *benM*-disrupted ACN146, ACN147, and ACN149 (Table 1) generated ACN157, ACN158, and ACN160, respectively. These strains each carry a mutation upstream of *benA* allowing benzoate degradation (selected as described above). Grown in the presence or absence of benzoate, ACN158 and ACN160, each with the same mutation (depicted in Fig. 2), constitutively expressed the *benA::lacZ* fusion, yielding LacZ levels of approximately 3,000 Miller units. In contrast, ACN157 grown with or without benzoate had low LacZ levels of 150 Miller units. The presence of *cis,cis*-muconate or both *cis,cis*-muconate and benzoate in the growth medium increased this activity in ACN157 to approximately 3,000 Miller units. The ACN157 mutation (depicted in Fig. 2) caused expression of the *benA::lacZ* fusion to be inducible by *cis,cis*-muconate but not benzoate.

**BenM-mediated regulation of *catA* expression.** CatM has been shown to activate *catA* expression in response to *cis,cis*-muconate but not benzoate (30). Since benzoate induces catechol 1,2-dioxygenase (CatA) independently of *cis,cis*-muconate (23) and CatM, the possibility that BenM regulates *catA* expression was tested. CatA activity was measured in the wild-type and *benM* mutants grown on LB to which no inducer,

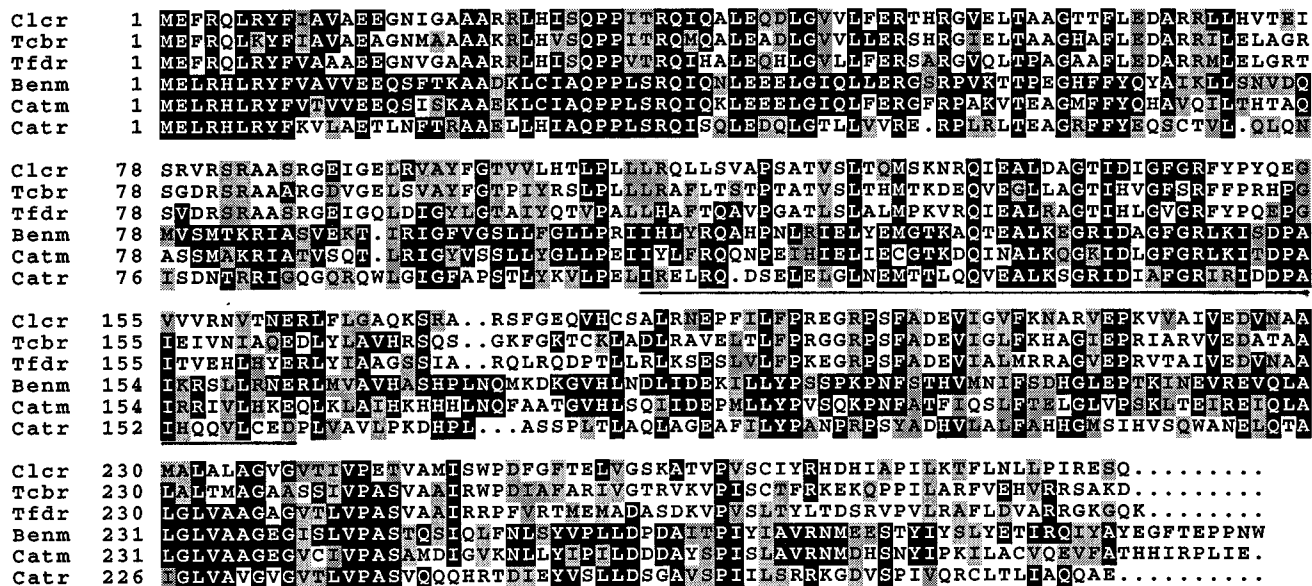


FIG. 3. Protein sequence alignment of homologous LysR-type transcriptional activators: ClcR (3), TcbR (36), TfdR (15), BenM, CatM (30), and CatR (31). Aligned residues identical or similar to those of BenM are in black or gray boxes, respectively. The underlined region, BenM residues 109 to 162, may be involved in inducer recognition.

*cis,cis*-muconate, or benzoate was added. In the *benM* mutant ISA36, *cis,cis*-muconate, but not benzoate, induced CatA to wild-type levels (Fig. 5). These results, consistent with CatM regulating *catA* expression in ISA36, suggested that the loss of benzoate induction was due to the absence of BenM-mediated *catA* expression.

The ability of *benM* in *trans* to regulate *catA* expression was tested in ISA35 (Table 1), a strain lacking functional chromosomal *catM* and *benM* alleles. The roles of benzoate and *cis,cis*-

muconate as effectors in ISA35 can be distinguished, since a chromosomal deletion encompassing the *benABC* genes prevents the conversion of benzoate to *cis,cis*-muconate. HPLC monitoring confirmed that no degradation of benzoate was detected during a 24-h period. Whereas ISA35 had no benzoate- or *cis,cis*-muconate-induced CatA activity, *benM* in *trans* restored induction in response to either inducer (Fig. 5). The response to benzoate was lower in ISA35(pBAC14) than that of the wild type (Fig. 5). In the latter case, however, benzoate can be degraded so that CatA levels stem from induction by both benzoate and *cis,cis*-muconate. CatA activity in ISA35 resulting from the presence of *benM* in *trans* was comparable to the level of benzoate-mediated CatA induction in a mutant unable to degrade benzoate (23). These results indicate that BenM is required for benzoate-mediated expression of *catA*

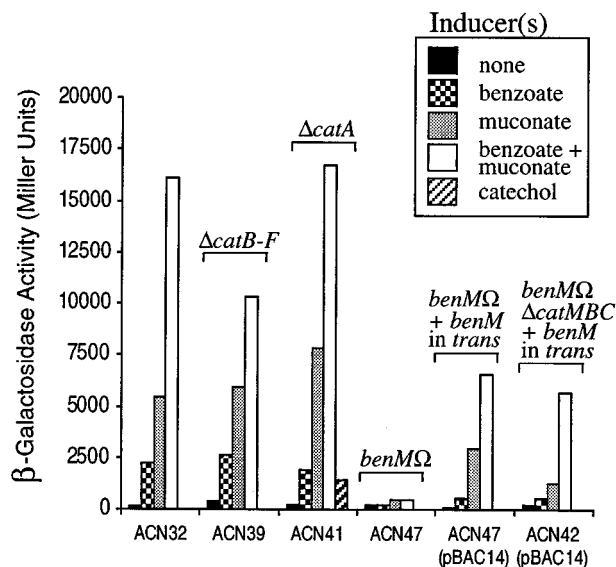


FIG. 4.  $\beta$ -Galactosidase (LacZ) activity resulting from expression of a chromosomal *benA::lacZ* fusion in strains ACN32, ACN39, ACN41, ACN47, ACN47(pBAC14), and ACN42(pBAC14) (Table 1). Genotypes are noted above bars. Strains were cultured in LB with the inducers indicated. Activities are averages of at least three independent repetitions, and the corresponding standard deviations were <20% of the average value.

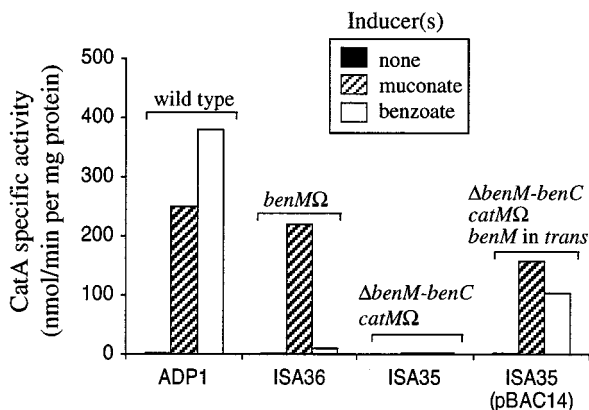


FIG. 5. CatA (catechol 1,2-dioxygenase) activity in strains ADP1, ISA36, ISA35, and ISA35(pBAC14) (Table 1). Genotypes are noted above bars. Strains were cultured in LB with the inducers indicated. Activities are the averages of at least three independent repetitions, and the corresponding standard deviations were <20% of the average value. In ADP1, but not the other strains, benzoate can be metabolized to *cis,cis*-muconate.

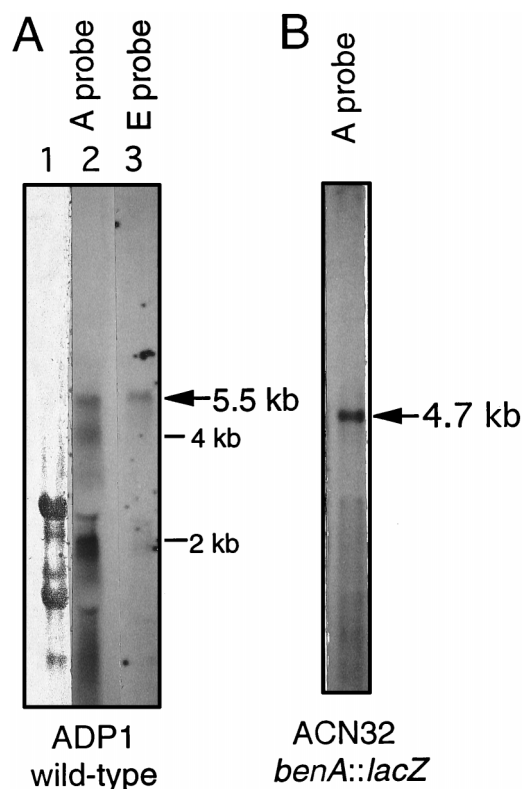


FIG. 6. Northern hybridization analysis of *ben* gene transcripts. Regions of *benA* and *benE* were labeled to make probes A and E, respectively, as described in Materials and Methods. Arrows indicate transcripts detected in RNA from the wild-type ADP1 grown with benzoate (A) or from ACN32, with a transcriptional terminator following the *benA::lacZ* fusion, grown with benzoate and *cis,cis*-muconate (B). Total wild-type RNA in one sample (A, lane 1) was stained with methylene blue to demonstrate the positions of the rRNA species.

whereas either CatM or BenM can regulate *catA* in response to *cis,cis*-muconate.

**Coexpression of the *benABCDE* genes.** Northern hybridization studies assessed coexpression of the *benABCDE* genes, which form an approximately 5-kbp region with no more than 50 nt separating each coding region (GenBank accession no. M76990; Fig. 2). Labeled probes were hybridized to RNA from cells grown with or without benzoate. Probe A, from the 5' region of *benA*, between *Bsa*AI and *Nsi*I restriction sites (Fig. 2), detected a 5.5-kb transcript in RNA from the wild type grown with benzoate (Fig. 6A, lane 2) but not in those grown without benzoate (data not shown). Hybridization signals, of variable relative intensities, corresponding to RNA sizes of approximately 4 and 2 kb were typically observed (Fig. 6A, lane 2).

The same *benA* probe detected a single transcript, 4.7 kb in size, in RNA from ACN32 grown with benzoate and *cis,cis*-muconate (Fig. 6B) but not from cells grown without inducers (data not shown). In ACN32, the known position of a transcriptional terminator that follows the *lacZ*-Km<sup>r</sup> cassette in *benA* (Fig. 2) (18) enables the size of the transcript to predict the position of a single initiation site upstream of the *benA* coding region (discussed later). Additional *ben* region probes (described in Materials and Methods) detected a 5.5-kb transcript in wild-type RNA from benzoate-grown cells but not in RNA from cells grown without benzoate (results shown only for the *benE* probe [Fig. 6A, lane 3]). The *benE* gene encodes a membrane protein of unknown function.

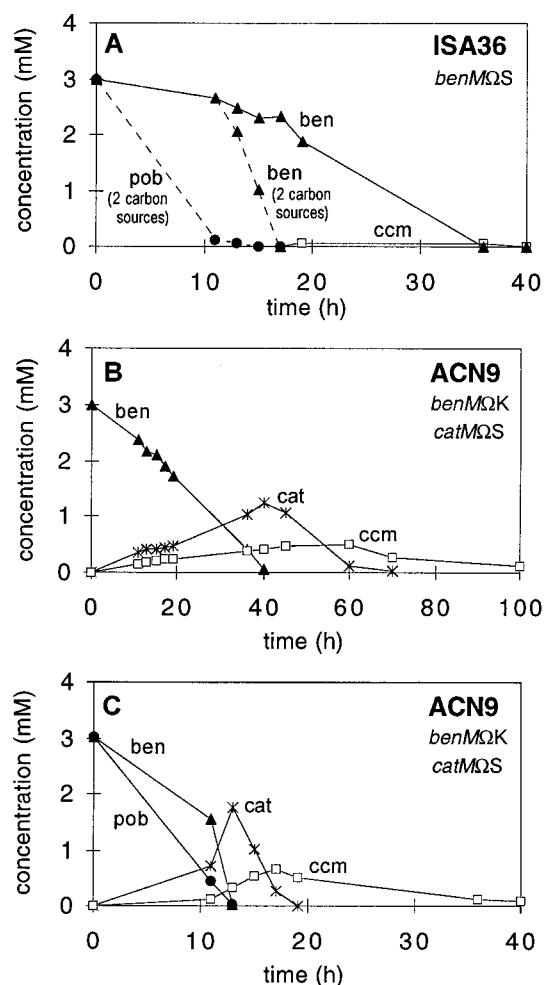


FIG. 7. Metabolites detected by MO-NMR following the addition, at time zero, of 3 mM benzoate (A and B) or 3 mM benzoate and 3 mM *p*-hydroxybenzoate (A and C) to deuterated cultures of ISA36 or ACN9. Concentrations of benzoate (ben), *p*-hydroxybenzoate (pob), catechol (cat), and *cis,cis*-muconate (ccm) were calculated by integration of NMR spectral peaks.

**Metabolic flow in regulatory mutants.** To assess the physiological significance of the individual CatM and BenM activators in *ben* and *cat* gene expression, metabolite flow was analyzed in a strain lacking a functional *benM* (ISA36) and a strain lacking both *benM* and *catM* (ACN9) (Table 1). Comparisons could then be made with results of previous studies in which identical methods were used to analyze the wild type and a *catM* mutant (ISA13) (7). Using MO-NMR techniques (7), either 3 mM [<sup>1</sup>H]benzoate or a mixture of 3 mM [<sup>1</sup>H]benzoate and 3 mM [<sup>1</sup>H]*p*-hydroxybenzoate was provided to deuterated bacterial cultures. <sup>1</sup>H compounds were detected by NMR analyses of samples taken during growth of the batch culture.

Although ISA36 cannot use benzoate as the sole carbon source, MO-NMR experimental conditions allowed this *benM::Ω* strain to degrade benzoate completely within 36 h of the addition of only this compound to the culture (Fig. 7A). During this time, ISA36 cells were most likely sustained by the small amount of the algae hydrolysate in the <sup>2</sup>H-medium. Consistent with the inability of CatM to substitute for BenM in *ben* gene expression, the complete consumption of benzoate by ISA36 was slower than that by the wild type or the *catM*-disrupted ISA13 under the same conditions, 8 and 18 h, re-

spectively (7). The accumulation of *cis,cis*-muconate by ISA36, approximately 100  $\mu$ M (Fig. 7A), was similar to that of the wild type (7). The *benM* disruption did not cause catechol to accumulate by lowering *catA* expression. In the absence of BenM, CatM-regulated *catA* expression allows rapid catechol degradation.

In contrast, catechol accumulated during the metabolism of benzoate by the *catM*- and *benM*-disrupted strain ACN9 (Fig. 7B). Although ACN9 did not grow with benzoate, *cis,cis*-muconate, anthranilate, or catechol as the sole carbon source, the growth medium used in the MO-NMR studies sustained the cells and allowed the very slow catabolism of benzoate, requiring more than 100 h for completion, to be monitored. The background levels of transcription occurring in the absence of regulated activation probably account for *ben* and *cat* gene expression. The high level of catechol accumulation by ACN9, as much as 1.3 mM, does not occur in either ISA36, ISA13, or ADP1 (7), suggesting that either CatM or BenM is sufficient to activate *catA* expression and allow the rapid degradation of catechol. In ISA13 (7), compared to ACN9, the relative decrease in catechol accumulation and increase in *cis,cis*-muconate accumulation from benzoate suggest that BenM activates *catA* expression to a greater extent than it does *catB*, since ISA13 differs from ACN9 only in having the wild-type *benM* allele.

The patterns of metabolites accumulating from benzoate were similar when ACN9 and ISA36 were provided with both [ $^1$ H]benzoate and [ $^1$ H]*p*-hydroxybenzoate (Fig. 7A and C). In each case, however, the time needed for complete benzoate consumption was much shorter in the presence of *p*-hydroxybenzoate (compare Fig. 7C with Fig. 7B). In the absence of a carbon source, such as *p*-hydroxybenzoate, low rates of benzoate catabolism may reflect carbon-limited growth, consistent with the inability of *benM*-disrupted strains to grow at the expense of benzoate as the sole carbon source.

## DISCUSSION

**BenM activates *ben* gene expression.** The dependence on *benM* for inducible expression of a *benA::lacZ* fusion identified BenM as a transcriptional activator of the *benABCDE* operon needed for the conversion of benzoate to catechol (Fig. 4). This metabolic conversion has been studied primarily in pseudomonads, where transcriptional control can be mediated by the XylS regulator encoded on the TOL plasmid pWWO. XylS, an AraC-type (8) rather than a LysR-type (33) activator, controls expression of the *xylXYZ* genes encoding broad-substrate-specific homologs of the BenABCD enzymes (10, 28). The *benR* locus of *Pseudomonas putida* has been suggested to encode a XylS-like transcriptional regulator of chromosomal *ben* genes (13). The lack of *benR-xylS* DNA hybridization, however, and differences between BenR- and XylS-mediated regulation (17) raise the possibility that BenR is not a XylS homolog and may be a BenM homolog.

The homology of BenM to LysR-type activators is not surprising, since several members of this large family regulate aromatic compound degradation (Fig. 3). The surprising observation was the relative effectiveness of benzoate and *cis,cis*-muconate in modulating *benM*-dependent transcription. Benzoate, or an intermediate in its conversion to *cis,cis*-muconate, had been the expected inducer of *ben* gene expression, since the initial rate of [ $^{14}$ C]benzoate uptake and metabolism by *cis,cis*-muconate-grown ADP1 is only 65% of that of benzoate-grown cells (4). Here we found that *cis,cis*-muconate was more than twice as effective as benzoate in increasing *benA* expression (Fig. 4). Nevertheless, benzoate itself did increase BenM-

regulated gene expression. Although HPLC analyses could not preclude the possibility that small amounts of benzoate were metabolized by strains with *ben* gene mutations, such mutations prevented the metabolism of benzoate from causing the expression of a *cis,cis*-muconate-regulated transcriptional fusion (4). Similarly, catechol itself, and not a subsequent metabolite, most likely causes BenM to activate *benA* expression in the *catA*-deleted strain ACN41 (Fig. 4).

Although *cis,cis*-muconate was more effective than benzoate as an individual inducer, both compounds together worked synergistically to induce higher levels of *benA* expression than either alone (Fig. 4). CatM is not essential for this synergism, since it occurred in the *catM*-deleted strain ACN42(pBAC14) (Fig. 4). In this strain, the relatively low response to all inducers may result from the expression of *benM* in *trans*, since a low response was also observed in a *catM*<sup>+</sup> strain with *benM* in *trans*, ACN47(pBAC14) (Fig. 4). Low levels might be caused by gene copy number effects or differences in expression of *benM* from the plasmid and the chromosome. Moreover, the chromosomal *benM*- $\Omega$  insertion could have *cis*-acting effects on *benA* expression.

**Interaction of BenM with effectors.** The ability of BenM to respond to multiple effectors raises questions about ligand binding. BenM probably binds *cis,cis*-muconate since its putative inducer binding region is approximately 60% identical in sequence to that of CatR and CatM, activators that also respond to this compound (Fig. 3, positions 109 to 162) (2, 33). The inducer binding regions of LysR-type proteins that recognize dissimilar ligands usually differ significantly from each other (33). For example, identity in sequence alignments of this region is only 15% between BenM and the *N*-acetylserine-responsive CysB, the only LysR-type protein for which the structure of the effector binding domain is known (35a). Therefore, the approximately 30% sequence identity in pairwise comparisons of this region of BenM with those of the chloromuconate-responsive ClcR, TcbR, and TfdR is significant. As expected, the central regions of ClcR, TcbR, and TfdR, are more similar to each other than each is to BenM.

If BenM binds to benzoate, it is not clear whether this occurs in the same region as that proposed for recognition of the structurally dissimilar *cis,cis*-muconate. NahR is a LysR-type transcriptional regulator that recognizes 2-hydroxybenzoate (salicylate), a compound similar in structure to benzoate (2). The effector binding region of NahR, however, is only 19% identical to the corresponding region of BenM. Mutations that allow NahR to recognize benzoate have been isolated (2) but are not sufficient to suggest amino acids in BenM specific for benzoate binding.

**Transcription initiation of the *ben* operon.** Amino acids in BenM likely to be involved in DNA recognition are nearly identical to those in CatM, in the helix-turn-helix regions of the N-terminal domains (20). Therefore, CatM and BenM operator sequences would be expected to be similar, leading to the prediction of a BenM binding site (Fig. 2) (30). Although protein binding to the proposed BenM operator depicted in Fig. 2 remains to be demonstrated, the ATACN<sub>7</sub>GTAT sequence is not only identical to that involved in CatM recognition and binding but also identical or nearly so to sequences that bind its most closely related homologs (discussed in reference 30). Binding of BenM to this site could have a negative autoregulatory function characteristic of LysR-type regulators (33). The spacing of this BenM binding site relative to a region in which two mutations affected *benA* expression corresponds to that between the operator and promoter regions upstream of *catB* (Fig. 2). The mutation causing constitutive *benA* expression, a G-to-A transition, may affect RNA polymerase

interactions in the  $-10$  promoter region, although confirmation requires mapping the transcription initiation site.

The mutation depicted in Fig. 2 that renders *ben* gene expression inducible by *cis,cis*-muconate but not benzoate might allow CatM to activate *benA* expression. In the mutant, CatM bound to the wild-type BenM operator could interact with RNA polymerase whose binding was altered by a promoter mutation. Alternatively, the mutation might create or alter an operator site to which CatM binds. As shown by the underlined sequences in Fig. 2, the mutation is adjacent to a motif that resembles, but does not exactly match, the dyad elements of the CatM operator. The inability of CatM to regulate *benA* expression in the wild type most likely does not reflect its inability to recognize benzoate, since effector binding to LysR-type regulators usually has a minimal effect on the affinity of the activators for their target DNA sequences (27). The ISA36 suppressor mutants that had wild-type *ben* region sequence might carry *catM* mutations.

The location of the *benA* regulatory region shown in Fig. 2 is consistent with the transcription initiation site predicted by the 4.7-kb truncated *ben* transcript in ACN32 (Fig. 6B), approximately 200 nt upstream of the ATG start codon in *benA*. The large size of the transcript, however, limits the accuracy of this prediction. The detection of only a single ACN32 transcript with the *benA* probe suggests that all signals detected by this probe in ADP1 RNA originated from the same transcriptional start site (Fig. 6A, lane 2). The small bands in ADP1 RNA, all variable in size and relative intensity, were detected with the *benA* but not the *benE* probe (Fig. 6A). These signals may result from different termination sites, degradation, and/or processing. The aberrant banding and exclusion patterns in regions corresponding in size to rRNA are not unusual.

**Benzoate catabolism by *benM*-disrupted mutants.** As would be expected from the coexpression of *benD* with *benABC* that was indicated by Northern hybridization studies (Fig. 6), benzoate *cis*-diol was never detected by MO-NMR (Fig. 7). In strains lacking BenM, it was most likely basal levels of *ben* gene expression that enabled the slow degradation of benzoate (Fig. 7). These basal expression levels, as much as 80-fold lower than in fully induced cultures, were not negligible: LacZ levels were approximately 200 to 400 Miller units in strains with a *benA::lacZ* transcriptional fusion that lacked BenM (Fig. 4). Similarly, in the absence of BenM and CatM, basal levels of *cat* gene expression probably enabled catechol degradation. A comparison of metabolite accumulations in strains with different regulatory mutations helped to assess the individual roles of BenM and CatM in *ben* and *cat* gene expression in vivo.

Whereas a critical role for BenM in *ben* gene expression was indicated by the inability of the *benM*-disrupted mutant ISA36 to utilize benzoate as the sole carbon source, the relative contribution of BenM and CatM to *cat* gene expression was less clear. CatM with its effector *cis,cis*-muconate is known to activate expression of *catA* and the *catBCIJFD* genes (30). In this report, we demonstrated that BenM is responsible for the previously identified benzoate-specific induction of CatA (23). The benzoate inducibility is lost in strain ISA35 (lacking CatM and BenM) and restored by *benM* in *trans* (Fig. 5) but not by *catM* in *trans* (30). Although this *benM*-mediated restoration results in low CatA activity relative to that of the wild type induced with benzoate (Fig. 5), it is comparable to that of strains unable to convert benzoate to *cis,cis*-muconate (23). With *catA* expression, as well as with *benA* expression, *cis,cis*-muconate was more effective than benzoate in eliciting a BenM-mediated response (Fig. 5). Collectively, these results confirm that both CatM and BenM activate *catA* expression.

The MO-NMR experiments indicated that in vivo, either

CatM or BenM alone allows the rapid degradation of catechol, presumably by activating *catA* expression (7) (Fig. 7). Strains in which either *catM* or *benM* was disrupted did not accumulate catechol, and it was only in the absence of both functional alleles in ACN9 that *catA* expression decreased to levels at which catechol was formed more rapidly than it was degraded. In contrast, the individual disruption of *catM* and *benM* had different effects on *cis,cis*-muconate accumulation, presumably reflecting differences in the ability of CatM and BenM to activate *catB* expression. In comparison to ACN9, the presence of a functional *catM* in ISA36 or a functional *benM* in ISA13 decreased or increased, respectively, the accumulation of *cis,cis*-muconate (7) (Fig. 7). These results indicate that in vivo, in a *catM*-disrupted background, BenM is better able to activate the transcription of *catA* than *catB*. Previous studies suggest that BenM is responsible for the CatM-independent regulation of the *catBCIJFD* genes (30), and recent gel shift mobility assays demonstrated the binding of BenM to the *catB* operator region in vitro (1).

BenM may also play a role in regulating *p*-hydroxybenzoate degradation. In ADP1, benzoate prevents *p*-hydroxybenzoate degradation, even in mutants unable to derive energy from benzoate degradation (7). The *benM* mutation in ISA36 and ACN9, however, alleviated this inhibition of *p*-hydroxybenzoate utilization, allowing *p*-hydroxybenzoate to be rapidly metabolized in the presence of benzoate (Fig. 7A and C). Since the inhibition appears to depend on the formation of *cis,cis*-muconate from benzoate (7), BenM may alleviate the repression either by delaying *cis,cis*-muconate formation or by a more direct mechanism.

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