Unraveling the Specific Regulation of the Central Pathway for Anaerobic Degradation of 3-Methylbenzoate*

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Background: The specific transcriptional regulation of the mbd pathway for anaerobic 3-methylbenzoate degradation is unknown.

Results: The MbdR/3-methylbenzoyl-CoA couple controls the induction of the mbd genes.

Conclusion: MbdR is the regulator of the mbd pathway in Azoarcus sp. CIB.

Significance: This work highlights the importance of the regulatory systems in the evolution and adaptation of bacteria to the anaerobic degradation of aromatic compounds.

The mbd cluster encodes the anaerobic degradation of 3-methylbenzoate in the β-proteobacterium Azoarcus sp. CIB. The specific transcriptional regulation circuit that controls the expression of the mbd genes was investigated. The P ΩP , P ΩV , and P sR promoters responsible for the expression of the mbd genes, their cognate MbdR transcriptional repressor, as well as the MbdR operator regions (ATACGTN5GTAT) have been characterized. The three-dimensional structure of MbdR has been solved revealing a conformation similar to that of other TetR family transcriptional regulators. The first intermediate of the catabolic pathway, i.e. 3-methylbenzoyl-CoA, was shown to act as the inducer molecule. An additional MbdR-dependent promoter, P A, which contributes to the expression of the CoA ligase that activates 3-methylbenzoate to 3-methylbenzoyl-CoA, was shown to be necessary for an efficient induction of the mbd genes. Our results suggest that the mbd cluster recruited a regulatory system based on the MbdR regulator and its target promoters to evolve a distinct central catabolic pathway that is only expressed for the anaerobic degradation of aromatic compounds that generate 3-methylbenzoyl-CoA as the central metabolite. All these results highlight the importance of the regulatory systems in the evolution and adaptation of bacteria to the anaerobic degradation of aromatic compounds.

Aromatic compounds are included among the most widespread organic compounds in nature, and some of them are man-made environmental pollutants (1–4). Microorganisms play a fundamental role in the degradation of these aromatic compounds in diverse ecological niches (3, 5–8). Many habitats containing large amounts of aromatic compounds are often anoxic. In the last decades, biochemical studies concerning the anaerobic degradation of aromatic compounds have been steadily accumulating, with benzoyl-CoA representing the intermediate to which most monocyclic aromatic compounds are converted (3–5, 9–12). On the contrary, the study on the specific regulatory systems controlling the expression of the gene clusters involved in the anaerobic degradation of aromatic compounds has been mainly restricted to the characterization of a few transcriptional regulators.

Anaerobic benzoate degradation via benzoyl-CoA was shown to be controlled by the two-component BamVW regulatory system (13) or the BgeR regulator (14) in the obligate anaerobes Geobacter strains, and by the BadR/BadM (15, 16) and BzdR/BoxR (17–20) regulators in the facultative anaerobes Rhodopseudomonas palustris and Azoarcus strains, respectively. Moreover, a few global regulators, e.g. AadR, AcpR, and AccR, that influence the anaerobic expression of the benzoyl-CoA central pathway have been reported (15, 21, 22). A TdiSR (TutC1B1) two-component regulatory system was described for the regulation of the bss/bbs genes encoding the peripheral pathway that converts toluene into benzoyl-CoA in denitrifying bacteria (4, 23–25). It was also reported that the regulation of the peripheral routes that funnel 4-hydroxybenzoate and p-coumarate into the benzoyl-CoA central pathway in the phototrophic R. palustris strain is accomplished by the HbaR and CouR proteins, respectively (26, 27). However, no specific-tran-
MbdR Regulator from Azoarcus sp. CIB

criptional regulators that control anaerobic degradation pathways, other than that of benzoyl-CoA and some peripheral routes that converge to the latter, have been described so far. Azoarcus sp. CIB is a denitrifying β-proteobacterium able to anaerobically degrade different aromatic compounds, including some hydrocarbons such as toluene, via benzoyl-CoA, and m-xylene, via 3-methylbenzoyl-CoA (28). The Azoarcus sp. CIB bzd genes responsible for the anaerobic degradation of benzoate are clustered and consist of the P₆ promoter-driven bzd-NOPQMOPTUVWXYZ catabolic operon and the bzdR regulatory gene (29). BzdR-mediated repression of P₆ is alleviated by the inducer molecule benzoyl-CoA, the first intermediate of the catabolic pathway (17, 18). In addition, the P₆ promoter is also subject to control by the benzoyl-CoA-dependent BoxR repressor, a BzdR paralog that regulates the expression of the box genes responsible for the aerobic degradation of benzoate in Azoarcus sp. CIB (20). The mbd cluster of Azoarcus sp. CIB encodes the central pathway responsible for the degradation of the 3-methylbenzoyl-CoA formed during the anaerobic degradation of m-xylene and 3-methylbenzoate (Fig. 1) (28). The mbd cluster is organized in at least three operons, i.e., the mbdO-orf9, mbdB1-mbdA, and mbdR operons (Fig. 1A). The mbdB1-mbdA operon is driven by the P₆ promoter and encodes a putative 3-methylbenzoate ABC transporter (MbdB1B2B3B4B5) and the 3-methylbenzoate-CoA ligase (MbdA) that activates 3-methylbenzoate to 3-methylbenzoyl-CoA (peripheral pathway) (Fig. 1B). The mbdO-orf9 operon is regulated by the P₇ promoter and encodes the enzymes for the anaerobic conversion of 3-methylbenzoyl-CoA to a hydroxymethylpimelyl-CoA (MbdMNOPQWXYZ) (upper central pathway) and the further degradation of the latter to the central metabolism (Orf1–9) (lower central pathway) (Fig. 1) (28). The mbdR gene was proposed to encode a transcriptional regulator of the TetR family that might regulate the inducible expression of the catabolic mbd genes (28). The efficient expression of the bzd and mbd genes required the oxygen-dependent AcpR activator, and it was under the control of AccR-mediated carbon catabolic repression by some organic acids and amino acids (22, 28).

In this work we have characterized the promoters of the mbd cluster and demonstrated the 3-methylbenzoyl-CoA/MbdR-dependent transcriptional control of the mbd genes in Azoarcus sp. CIB. The studies on the structural-functional relationships of the MbdR protein expand our current view on the transcriptional regulation of anaerobic pathways, and highlight the importance of the regulatory systems in the evolution and adaptation of bacteria to the anaerobic degradation of aromatic compounds.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Bacterial strains and plasmids used are listed in Table 1. Escherichia coli strains were grown in lysogeny broth (LB) medium (31) at 37 °C. When required, E. coli cells were grown anaerobically in M63 minimal medium (40) at 30 °C using the corresponding necessary nutritional supplements, 20 mM glycerol, as carbon source, and 10 mM nitrate, as terminal electron acceptor. Azoarcus sp. CIB strains were grown anaerobically in MC medium at 30 °C, using the indicated carbon source(s) and 10 mM nitrate as the terminal electron acceptor, as described previously (29).

For aerobic cultivation of Azoarcus strains, the same MC medium was used but without nitrate. When appropriate, antibiotics were added at the following concentrations: ampicillin (100 μg ml⁻¹), gentamicin (7.5 μg ml⁻¹), and kanamycin (50 μg ml⁻¹).

Molecular Biology Techniques—Standard molecular biology techniques were performed as described previously (31). Plasmid DNA was prepared with a High Pure plasmid isolation kit (Roche Applied Science). DNA fragments were purified with Gene-Clean Turbo (Q-biogene). Oligonucleotides were supplied by Sigma. The oligonucleotides employed for PCR amplification of the cloned fragments and other molecular biology techniques are summarized in Table 2. All cloned inserts and DNA fragments were confirmed by DNA sequencing with fluorescently labeled dideoxynucleotide terminators (41) and AmpliTaq FS DNA polymerase (Applied Biosystems) in an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Transformation of E. coli cells was carried out by using the RbCl method or by electroporation (Gene Pulser; Bio-Rad) (31). The proteins were analyzed by SDS-PAGE and Coomassie-stained as described previously (31). The protein concentration was determined by the method of Bradford (42) using bovine serum albumin as the standard. Nucleotide sequence analyses were done at the National Center for Biotechnology Information (NCBI) server (www.ncbi.nlm.nih.gov). Pairwise and multiple protein sequence alignments were made with the ClustalW program (43) at the EMBL-EBI server.

Synthesis and Purification of 3-Methylbenzoyl-CoA—The 3-methylbenzoyl-CoA was synthesized from the corresponding carboxylic acid via its succinimide ester as described (44). The CoA ester was purified by preparative reversed phase HPLC on a 1525 Binary HPLC Pump system (Waters) equipped with a NUCLEOSIL®100–7 C18 column (Macherey-Nagel, 50 ml total volume) using acetonitrile in 50 mM potassium phosphate buffer, pH 6.8, at a flow rate of 8 ml min⁻¹. The column was equilibrated with 5% acetonitrile; elution was at 25% acetonitrile in buffer. For removal of phosphate, the freeze-dried CoA ester was suspended in 2% aqueous acetonitrile; elution was with 25% aqueous acetonitrile. The purity was checked by reversed phase HPLC as described above and by the UV-visible spectrum. 3-Methylbenzoyl-CoA was stored at −20 °C as freeze-dried powder.

Construction of Azoarcus sp. CIBdmbdR and Azoarcus sp. CIBdmbdB1 Mutant Strains—For insertional disruption of mbdR and mbdB1 through single homologous recombination, an internal region of each gene was PCR-amplified with the primer pairs 5’mbdB1mutEcoRI5’/mbdB1mutXbaI3’ (Table 2). The obtained fragments were double-digested with the appropriate restriction enzymes and cloned into double-digested pk18mob vector, generating the pK18mbdRnew and pK18mbdB1 recombinant plasmids (Table 1). These plasmids were transferred from E. coli S17-1pir (donor strain) to Azoarcus sp. CIB (recipient strain) by biparental filter mating (32), and exconjugant strains Azoarcus sp. CIBdmbdR and Azoarcus sp. CIBdmbdB1 were isolated aerobi-
**MbdR Regulator from Azoarcus sp. CIB**

TABLE 1

| Strain or plasmid | Description* | Ref. or source |
|-------------------|---------------|---------------|
| **E. coli strains** |               |               |
| BL21 (DE3)        | F−, ompT, hsdS (r− m−), gal, dcm, met, ADE3 | 30 |
| S17-1Apur          | F−, ompT, hsdS (r− m−), gal, dcm, ADE3 | 31 |
| MC4100             | araD139 (argF-lacU169 rpsL150 (Smr) relA1 fliB5301 deoC1 ptsF25 rbsB | 33 |
| **Azoarcus sp. strains** |               |               |
| CIB               | Wild-type strain | 29 |
| CIBmmbdr          | Km7, CIB mutant strain with a disruption of the mbdR gene | This work |
| CIBmmbdb1         | Km7, CIB mutant strain with a disruption of the mbdB1 gene | This work |
| CIBΔP4s          | CIB mutant strain with a deletion of the P4 promoter | This work |

**Plasmids**

| Plasmid | Description* | Ref. or source |
|---------|---------------|---------------|
| pK18mob | Km7, oriColE1, Mob^−, lacZ^−, used for directed insertional disruption | 34 |
| pK18mmbDnew | Km7, pK18mob containing a 524-bp HindIII/EcoRI mbdR internal fragment | This work |
| pK18mmbBD1 | Km7, pK18mob containing a 728-bp EcoRI/XbaI mbdB1 internal fragment | This work |
| pK18mmbacsB | Km7, oriColE1, Mob^−, lacZ^−. Vector with a sacB selection marker for gene replacement by double site homologous recombination | 34 |
| pK18mmbacsBΔP4s | Km7, pK18mmbacsB containing a chimeric 2.6-kb XbaI/HindIII fragment carrying the ΔP4 | This work |
| pUC19    | Ap^R, oriColE1, lacZ^− | 31 |
| pUCmbdA  | Ap^R, pUC19 derivative expressing mbdA gene under Plac control | 28 |
| pIZ1016  | Gm^r, oripBB1, Mob^−, lacZ^−, Ptas/lacI^+, broad host range cloning and expression vector | 35 |
| pIZ1016P | Gm^r, pIZ1016 derivative expressing the P_{E. coli}::lacZ fusion | This work |
| pIZ253P  | Gm^r, pIZ1016 derivative expressing the P_{E. coli}::lacZ fusion | This work |
| pIZ253A  | Gm^r, pIZ1016 derivative expressing the P_{E. coli}::lacZ fusion | This work |
| pIZ253D  | Gm^r, pIZ1016 derivative expressing the P_{E. coli}::lacZ fusion | This work |
| pC0K1    | Cm^r, oripSC101, low copy number cloning vector | 36 |
| pC0mbdA  | Cm^r, pC0K1 derivative expressing mbdR gene under the control of Plac promoter | This work |
| pET-29a (+) | Km7, oriColE1, P_{ET-29a (+)} cloning and overexpression vector | Novagen |
| pETmbdR  | Km7, pET-29a (+) expressing mbdR-His^+ under P_{ET-29a (+)} | This work |
| pEHSTEV  | Km7, oriColE1, P_{EHSTEV} coding 6His, TEV, cloning, and overexpression vector | 37 |
| pEHSTEVmmbdr | Km7, pEHSTEV derivative expressing TEV protease-cleavable His^+_6 mbdR under P_{ET-29a} | This work |
| pSJ3     | Ap^R, oriColE1, lacZ^−, lacZ^− promoter probe vector | 38 |
| pSJ3P     | Ap^R, pSJ3 derivative carrying the P_{S. J. 3}::lacZ fusion | This work |
| pSJ3PST   | Ap^R, pSJ3 derivative carrying the P_{S. J. 3}::lacZ fusion | This work |
| pSJ3PSTT  | Ap^R, pSJ3 derivative carrying the P_{S. J. 3}::lacZ fusion | This work |
| pSJ3PSTT  | Ap^R, pSJ3 derivative carrying the P_{S. J. 3}::lacZ fusion | This work |
| pCD01    | Ap^R, pCD01 derivative harboring a 711-bp Scal/EcoRI fragment that includes the P_{0} promoter | 39 |
| pCD01P   | Ap^R, pCD01 derivative harboring a 251-bp Scal/EcoRI fragment that includes the P_{0} promoter | This work |

* The abbreviations used are as follows: Ap^R, ampicillin-resistant; Cm^r, chloramphenicol-resistant; Gm^r, gentamicin-resistant; Km^7, kanamycin-resistant; Smr^+, streptomycin-resistant; TEV, tobacco etch virus.

Finally, on kanamycin-containing MC medium harboring 10 mM glutarate as the sole carbon source for counterselection of donor cells. The mutant strains were analyzed by PCR to confirm the disruption of the target genes.

**Construction of Azoarcus sp. CIBΔP4s Mutant Strain**—The PA promoter was deleted by allelic exchange through homologous recombination using the mobilizable plasmid pK18mmbacsB, which allows positive selections of double-site recombinants using the sacB gene of Bacillus subtilis (34). In summary, two primer pairs (Table 2) were used to PCR-amplify the 1191-bp Z1 fragment and 1451-bp Z2 fragment flanking regions of the P4 promoter. Both fragments were digested with restriction endonuclease KpnI and ligated, and the chimeric DNA harboring a deleted PA promoter was PCR-amplified, double-digested, and cloned into the pK18mmbacsB plasmid. The resulting pK18mmbacsBΔP4s plasmid was transformed into the E. coli S17-1Apur strain (donor strain) and then transferred to Azoarcus sp. CIB (recipient strain) by biparental filter mating (32).

Exconjugants containing first site recombination were selected on kanamycin-containing MC medium harboring 10 mM glutarate as the sole carbon source for counterselection of donor cells. Second site recombination was selected by growth on the same medium supplemented with 5% sucrose and by plating on glutarate-containing MC plates supplemented with 5% sucrose. Correct allelic exchange in sucrose-resistant and kanamycin-sensitive Azoarcus sp. CIBΔP4s was verified by PCR with the appropriate primers (Table 2).

**Construction of a P_{A}::lacZ Translational Fusion**—The intergenic region between mbdB5 and mbdA genes that includes the P_{A} promoter was PCR-amplified using the primers Inter.mbdB5-A5' and Inter.mbdB5-A3'.2 (Table 2). The resulting 238-bp fragment was KpnI/XbaI double-digested and cloned upstream of the lacZ gene into the double-digested pSJ3 promoter probe vector, generating plasmid pSJ3P4s (Table 1). The recombinant pSJ3P4s plasmid was KpnI/HindIII double-digested and the 3.3-kb fragment containing the P_{A}::lacZ translational fusion was then cloned into the broad host-range plZ1016 cloning vector (Table 1). To this end, plZ1016 was KpnI/HindIII double-digested and its Ptac promoter and polylinker region were replaced by the P_{A}::lacZ translational fusion, generating plasmid pZP4s (Table 1).

**Construction of a P_{3}::lacZ Translational Fusion**—The intergenic region between tdrIR and mbdR genes that includes the P_{3R} promoter was PCR-amplified using the primers PmbdRKpnl5' and PmbdRXba13' (Table 2). The resulting 451-bp fragment was KpnI/XbaI double-digested and cloned upstream of a lacZ gene into the double-digested pSJ3 promoter probe vector, generating plasmid pSJ3P3R (Table 1). The recombinant pSJ3P3R plasmid was KpnI/HindIII double-digested, and the 3.5-kb fragment containing the P_{3R}::lacZ translational fusion...
TABLE 2
Oligonucleotides used in this study

| Primers          | Sequence (5’ → 3’)                  | Use                                                                 |
|------------------|--------------------------------------|----------------------------------------------------------------------|
| CIB+1P_mbdRmut2  | CATTTCAGTCCTTTCTTCTCTACCATCTG       | Primer extension P_{mbdR} promoter                                   |
| CIB+1P_mbdHmut2  | CATTTCAGTCCTTTCTTCTCTACCATCTG       | Primer extension P_{mbdH} promoter                                   |
| PmbdOF1          | GCCCTAGTATTCTTGGAGCTTCCTG           | Amplification of 203-bp fragment for RT-PCR assays                   |
| PmbdOR1          | GCCCTAGTATTCTTGGAGCTTCCTG           | Amplification of 278-bp fragment for RT-PCR assays                   |
| bctB1            | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{bctB} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdEcoRI3       | GGGAAGTGGGGCTTCTCCTCTCCTCTCTG       | Primer extension P_{mbdEcoRI} promoter                               |
| PmbdEcoRI13      | GGGAAGTGGGGCTTCTCCTCTCCTCTCTG       | Primer extension P_{mbdEcoRI} promoter                               |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| PmbdBF1          | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdB} promoter                                   |
| 5’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |
| 3’ mbdRmut2      | GGGGCTCTCTTCTCTGGAGCTTCCTG          | Primer extension P_{mbdR} promoter                                   |

Engineered restriction sites are underlined, and the corresponding restriction enzyme is shown in parentheses.
was then cloned into the broad host range pIZ1016 cloning vector (Table 1). To this end, pIZ1016 was KpnI/HindIII double-digested and its Ptac promoter and polylinker region were replaced by the Plac::lacZ translational fusion, generating plasmid pZIP3a (Table 1).

**Construction of the plZmbdA and pCKmbdR Plasmids**—The plZmbdA plasmid is a broad host range plasmid that expresses the mbdA gene under the control of the Ptac promoter (Table 1). For the construction of plZmbdA, the 1.7-kb HindIII/XbaI fragment containing the mbdA gene from pUCmbdA (28) was cloned into HindIII/XbaI double-digested plZ1016 plasmid. The pCKmbdR plasmid (Table 1) expresses the mbdr gene under control of the Plac promoter in the pCK01 cloning vector. To this end, the mbdr gene was PCR-amplified as a 676-bp fragment using mbdRSalI5’ and mbdRPstI3’ oligonucleotides (Table 2). The SalI/PstI double-digested PCR fragment was then cloned into double-digested pCK01 plasmid to generate pCKmbdR.

**Overproduction and Purification of MbdR**—The recombinant pETmbdR plasmid (Table 1) carries the mbdr gene, which was PCR-amplified (651-bp) with primers mbdRNdel1’ and mbdRXhoI3’ (Table 2), with a His6 tag coding sequence at its 3’-end, under control of the Ptac promoter that is recognized by the T7 phage RNA polymerase. The gene encoding T7 phase RNA polymerase is present in monocopy in *E. coli* BL21 (DE3), and its transcription is controlled by the Plac promoter and the LacI repressor, making the system inducible by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG). E. coli BL21 (DE3) (pETmbdR) cells were grown at 37 °C in 100 ml of kanamycin-containing LB medium until the culture reached an A600 of 0.5. Overexpression of the His-tagged protein was then induced during 5 h by the addition of 0.5 mM IPTG. Cells were harvested at 4 °C, resuspended in 10 ml of 20 mM imidazole-containing working buffer (50 mM NaH2PO4, pH 8, 300 mM KCl), and disrupted by passage through a French press operated at a pressure of 20,000 p.s.i. Cell debris was removed by centrifugation at 16,000 × g for 20 min at 4 °C, and the resulting supernatant was used as crude cell extract. The MbdR-His6 protein was purified from the crude cell extract by a single-step nickel-chelating chromatography (nickel-nitrilotriacetic acid spin columns, Qiagen). The column was equilibrated with resuspension buffer, loaded with the crude extract, and washed four times with working buffer plus increasing concentrations of imidazole (20, 75, and 100 mM). The MbdR-His6 protein was eluted in three steps adding to the column working buffer plus increasing concentrations of imidazole (250 and 500 mM and 1 M). The purity of MbdR-His6 protein was analyzed by SDS-12.5% PAGE. When necessary, the protein solutions were dialyzed against working buffer plus 20 mM imidazole, concentrated using Vivaspin 500 columns (Sartorius, 10,000 molecular weight cutoff membrane), and stored at 4 °C where they maintained their activity for at least 6 months.

**Analytical Ultracentrifugation Methods**—Sedimentation velocity and equilibrium were performed to determine the state of association of MbdR-His6. The analytical ultracentrifugation analysis was performed using several protein concentrations (from 11 to 46 μM). All samples were equilibrated in buffer containing 50 mM NaH2PO4, 300 mM KCl, 20 mM imidazole, pH 8. The sedimentation velocity experiments were carried out at 48,000 rpm and 20 °C in an Optima XL-A analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with UV-visible optic detection system, using an An50Ti rotor and 12-mm double sector centerpieces. Sedimentation profiles were registered every 1–5 min at 260 and 275 nm. The sedimentation coefficient distributions were calculated by least squares boundary modeling of sedimentation velocity data using the c(s) method (45), as implemented in the SEDFIT program. These s values were corrected to standard conditions (water at 20 °C and infinite dilution) using the SEDNTERP program (46) to get the corresponding standard s values (s20,w). Sedimentation equilibrium assays were carried out at speeds ranging from 5000 to 15,000 rpm (depending upon the samples analyzed) and at several wavelengths (260, 280, and 290 nm) with short columns (85–95 μl), using the same experimental conditions and instrument as in the sedimentation velocity experiments. After the equilibrium scans, a high speed centrifugation run (40,000 rpm) was done to estimate the corresponding baseline offsets. The measured low speed equilibrium concentration (signal) gradients of MbdR-His6 were fitted using an equation that characterizes the equilibrium gradient of an ideally sedimenting solute (using a MATLAB program, kindly provided by Dr. Allen Minton, National Institutes of Health) to obtain the corresponding buoyant signal average molecular weight.

**Crystallization and X-ray Crystal Structure Determination of MbdR**—To determine the three-dimensional structure of MbdR, the mbdr gene from *Azoarcus* sp. CIB was cloned into pEHISTEVM vector (37). To this end, the mbdr gene was PCR-amplified with primers mbdRBspHI5’ and mbdRBamHI3’ (Table 2) by using genomic DNA of *Azoarcus* sp. CIB as template, digested with BspHI and BamHI, and then ligated into the NcoI/BamHI double-digested pEHISTEVM vector, giving rise to plasmid pEHISTEVMbdR. Protein expression of the selenomethionine (SeMet)-substituted recombinant MbdR protein was carried out in *E. coli* B834(DE3) strain (Table 1) transformed with pEHISTEVMbdR, and purification was carried out essentially as described previously (47). The purified SeMet MbdR protein has an extra glycine and alanine at the N terminus resulting from cleavage of the engineered hexa-histidine tag. Crystallization of SeMet MbdR was carried out as described previously (47), and the MbdR crystals were finally grown in the optimized condition of 0.1 M MOPS, pH 7.0, 28% PEG3550, and 0.08% (NH4)2PO4. Structure was determined using SeMet MAD data and refined using CCP4 package (48). The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB) under accession number 4uds. Crystallization of MbdR-inducer complex was tried out using the purified MbdR protein with 3-methylbenzoyl-CoA either by co-crystallization or crystal soaking, but in both cases the production of crystals failed.

**RNA Extraction and RT-PCR Assays**—*Azoarcus* cells grown in MC medium harboring the appropriate carbon source were harvested at the mid-exponential phase of growth and stored at...
−80 °C. Pellets were thawed, and cells were lysed in TE buffer (10 Tris-HCl, pH 7.5, 1 mM EDTA) containing 50 mg ml^{-1} lysozyme. Total RNA was extracted using the RNeasy mini kit (Qiagen), including a DNase treatment according to the manufacturer’s instructions (Ambion), precipitated with ethanol, washed, and resuspended in RNase-free water. The concentration and purity of the RNA samples were measured by using a ND1000 spectrophotometer (Nanodrop Technologies) according to the manufacturer’s protocols. Synthesis of total cDNA was carried out with 20 µl of reverse transcription reactions containing 400 ng of RNA, 0.5 mM concentrations of each dNTP, 200 units of SuperScript II reverse transcriptase (Invitrogen), and 5 µM concentrations of random hexamers as primers in the buffer recommended by the manufacturer. Samples were initially heated at 65 °C for 5 min then incubated at 42 °C for 2 h, and the reactions were terminated by incubation at 70 °C for 15 min. In standard RT-PCRs, the cDNA was amplified with 1 unit of AmpliTaq DNA polymerase (Biotools) and 0.5 µM concentrations of the corresponding primer pairs (Table 2). Control reactions in which reverse transcriptase was omitted from the reaction mixture ensured that DNA products resulted from the amplification of cDNA rather than from DNA contamination. The dnaE gene encoding the α-subunit of DNA polymerase III was used to provide an internal control cDNA that was amplified with oligonucleotides 5′ POLIIIHK/3′ POLIIIHK (Table 2). The expression of the internal control was shown to be constant across all samples analyzed. For real time RT-PCR assays, the cDNA was purified using the GENE CLEAN® Turbo kit (MP Biomedicals), and the concentration was measured using an ND1000 spectrophotometer (Nanodrop Technologies). The IQ5 Multicolor Real Time PCR Detection System (Bio-Rad) was used for real time PCR in a 25-µl reaction containing 10 µl of diluted cDNA (5 ng in each reaction), 0.2 µM primer 5′, 0.2 µM primer 3′, and 12.5 µl of SYBR Green Mix (Applied Biosystems). The oligonucleotides used to amplify a fragment of mbda4 were mbdAQ-RT-PCRF3 and mbdAQ-RT-PCRS5 (Table 2). PCR amplifications were carried out as follows: 1 initial cycle of denaturation (95 °C for 4 min) followed by 30 cycles of amplification (95 °C, 1 min; test annealing temperature, 60 °C, 1 min; elongation and signal acquisition, 72 °C, 30 s). Each reaction was performed in triplicate. After the PCR, a melting curve was generated to confirm the amplification of a single product. For relative quantification of the fluorescence values, a calibration curve was constructed by 5-fold serial dilutions of an Azoarcus sp. CIB genomic DNA sample ranging from 0.5 to 0.5 × 10^{-4} ng. This curve was then used as a reference standard for extrapolating the relative abundance of the cDNA target within the linear range of the curve. Results were normalized relative to those obtained for the dnaE internal control.

**Results**

**DNA probes containing P_{O}, P_{B1}, P_{A}, and P_{AR}** probes were purified using GENE CLEAN® Turbo (Qiagen). The retardation reaction mixtures contained 20 mM Tris-HCl, pH 7.5, 10% glycerol, 50 mM KCl, 0.05 µM DNA probe, 250 µg/ml bovine serum albumin, 50 µg/ml unspecific salmon sperm DNA, and purified MbdR-His_{6} protein in a 9-µl final volume. After incubation of the retardation mixtures for 20 min at 30 °C, mixtures were fractionated by electrophoresis in 5% polyacrylamide gels buffered with 0.5× TBE (45 mM Tris borate, 1 mM EDTA). The gels were dried onto Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences) accompanied by amplifier screens (Cronex Lightning Plus, DuPont). The radioactivity present in the retardation complexes and free probes was quantified by using a densitometer with the Quantity One software (Bio-Rad).

**DNase I Footprinting Assays**—The DNA 32P-probes used for these experiments were labeled as indicated for the gel retardation assays. The reaction mixture contained 2 µM DNA probe (P_{O}, P_{B1}, or P_{A}), 500 µg/ml bovine serum albumin, and purified MbdR-His_{6} protein in 15 µl of buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 50 mM KCl). This mixture was incubated for 20 min at 30 °C, after which 3 µl (0.05 units) of DNase I (Roche Applied Science) (prepared in 10 mM CaCl_{2}, 10 mM MgCl_{2}, 125 mM KCl, and 10 mM Tris-HCl, pH 7.5) was added, and the incubation was continued at 37 °C for 20 s. The reaction was stopped by the addition of 180 µl of a solution containing 0.4 M sodium acetate, 2.5 mM EDTA, 50 µg/ml salmon sperm DNA, and 0.3 µl/ml glycerol. After phenol extraction, DNA fragments were precipitated with absolute ethanol, washed with 70% ethanol, dried, and directly resuspended in 90% (v/v) formamide-loading gel buffer (10 mM Tris-HCl, pH 8, 20 mM EDTA, pH 8, 0.05% w/v bromphenol blue, 0.05% w/v xylene cyanol). Samples were denatured at 95 °C for 3 min and fractionated in a 6% polyacrylamide-urea gel. A+G Maxam and Gilbert reactions (49) were carried out with the same fragments and loaded in the gels along with the footprinting samples. The gels were dried onto Whatman 3MM paper and visualized by autoradiography as described previously.

**Primer Extension Analyses**—Azoarcus sp. CIB cells were grown anaerobically on MC medium plus 3-methylbenzoate (inducing conditions) or benzoate (control condition) until mid-exponential phase. For the primer extension analysis of P_{O} and P_{B1} promoters, total RNA was isolated by using RNeasy mini kit (Qiagen) according to the instructions of the supplier. In the case of P_{A} and P_{AR} promoters, the procedure was the same but Azoarcus sp. CIB strains harboring pIZP_{A} or pIZP_{AR} plasmids were used instead of the parental strain due to the weaker nature of these promoters. Primer extension reactions were carried out with the avian myeloblastosis virus reverse transcriptase (Promega) and 15 µg of total RNA as described previously (17), using oligonucleotides CIB + 1P_{mbdC}3′, CIB + 1P_{mbdC}3′, PmbdREcoRI3′, and PmbdAEcoRI3′ (Table 2), which hybridize with the coding strand of the mbdO, mbdB1, mbdR, and mbda genes, respectively. These oligonucleotides were labeled at their 5′-end with phage T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences). To determine the length of the primer extension products, sequencing reactions of plasmids pSJ3PO, pSJ3PB1, pIZP_{A}, and pIZP_{AR} (Table 1) were carried out with oligonucleotide...
MbdR Regulator from Azoarcus sp. CIB

FIGURE 1. 3-Methylbenzoate anaerobic degradation pathway in Azoarcus sp. CIB. A, scheme of the mbd gene cluster of Azoarcus sp. CIB. Genes are represented by thick arrows, and their predicted function is annotated as follows: gray, regulatory gene; horizontal stripes, genes encoding a 3-methylbenzoate ABC-type transport system; stippling, gene encoding the 3-methylbenzoate-CoA ligase; black, genes encoding the 3-methylbenzoyl-CoA upper central pathway; white, genes involved in the 3-methylbenzoyl-CoA lower pathway (and some genes of unknown function). Bent arrows represent the promoters driving the expression of the mbd genes. The mbdO-orf9 operon and the mbd11-mbdA operon are indicated by broken arrows. B, scheme of 3-methylbenzoate activation and 3-methylbenzoyl-CoA anaerobic degradation pathway. The enzymes involved are indicated following the same code of A. The Bss-Bbs peripheral pathway that converts m-xylene into 3-methylbenzoyl-CoA is indicated by a dashed arrow. The compounds are as follows: 1, 3-methylbenzoate; 2, 3-methylbenzoyl-CoA; 3a, 3-hydroxy-6-methyl-pimelyl-CoA; 3b, 3-hydroxy-4-methyl-pimelyl-CoA; and 4, m-xylene.

otides CIB + 1P_mbdO3’, CIB + 1P_mbdB13’, PmdbAEcoRI3’, and PmbdREcoRI3’, respectively, using the T7 sequencing kit and [α-32P]dATP (PerkinElmer Life Sciences) as indicated by the supplier. Products were analyzed on 6% polyacrylamide-urea gels. The gels were dried on Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences).

In Vitro Transcription Experiments—Multiple-round in vitro transcription assays were performed as published previously (50). Plasmids pJCDPO and pJCDPB1 (Table 1) were used as supercoiled PO and PB1 templates. Reactions (50 μl mixtures) were performed in a buffer consisting of 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2, 0.1 mM bovine serum albumin, 10 mM dithiothreitol (DTT), and 1 mM EDTA. Each DNA template (0.25 nM) of supercoiled plasmids pJCDPO or pJCDPB1 was premixed with 30 nM probes CIB13, CIB13/H9251, and CIB13/H9262, respectively, using the T7 sequencing kit and [α-32P]dATP (PerkinElmer Life Sciences) as indicated by the supplier. Products were analyzed on 6% polyacrylamide-urea gels. The gels were dried on Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences).

RESULTS

mbdR Gene Encodes a Specific Repressor of the PO and PB1 Promoters in Azoarcus sp. CIB—In silico analysis at the 3′-end of the mbd cluster revealed a gene, mbdR, that encodes a putative specific transcriptional regulator (Fig. 1) (28). To analyze the role of the mbdR gene in the expression of the catabolic and transport mbd genes, an mbdR disruptional insertion mutant (Azoarcus sp. CIBmbdR strain; Table 1) was constructed. Because Azoarcus sp. CIBmbdR mutant strain grew normally on minimal medium containing 3-methylbenzoate as the only carbon source, the mbdR gene does not seem to function as a transcriptional activator of the mbd genes. Wild-type Azoarcus sp. CIB strain and Azoarcus sp. CIBmbdR mutant strain were grown anaerobically on minimal medium containing benzoate (control condition) or 3-methylbenzoate (inducing condition) as the only carbon sources, and the expression from PO and PB1 promoters was analyzed by RT-PCR experiments. Whereas the wild-type strain showed a clear induction of the PO and PB1 promoters when grown in 3-methylbenzoate, the MbdR mutant exhibited expression from the PO and PB1 promoters when growing both in benzoate or 3-methylbenzoate (Fig. 2, A and B). Hence, these results support the idea that MbdR acts as a specific transcriptional repressor of the PO and PB1 promoters.

MbdR Is a New Member of the TetR Family of Transcriptional Regulators—Analysis of the primary structure of MbdR shows an overall low amino acid sequence similarity to members of the TetR family of transcriptional regulators (Fig. 3) (51, 52). To
MbdR Regulator from Azoarcus sp. CIB

MbdR protein controls the P₀ and P₁₁ promoters. A and B, activity of the P₀ and P₁₁ promoters in wild-type Azoarcus sp. CIB and the Azoarcus sp. CIBmbdR mutant strain. Agarose gel electrophoresis of RT-PCR products obtained from the divergent promoters P₀ (A) and P₁₁ (B). Total RNA was extracted from Azoarcus sp. CIB (wt) and Azoarcus sp. CIBmbdR (mbdR) cells grown under denitrifying conditions using 3 mM benzoate (lane B) or 3 mM 3-methylbenzoate (lane 3M) as sole carbon sources. The primer pairs used to amplify the mbdO (P₀) and mbdB1 (P₁₁) gene fragments were as described in “Experimental Procedures.” C shows the interaction between increasing concentrations of purified MbdR-His₆ protein and a DNA probe (271-bp) containing the mbdO promoter. The DNA fragments used in gel retardation assays were prepared as indicated under “Experimental Procedures.”

FIGURE 2. MbdR Regulator from Azoarcus sp. CIB

MbdR protein interacts with the mbdO and mbdB1 promoters. A and B, activity of the P₀ and P₁₁ promoters in wild-type Azoarcus sp. CIB and the Azoarcus sp. CIBmbdR mutant strain. Agarose gel electrophoresis of RT-PCR products obtained from the divergent promoters P₀ (A) and P₁₁ (B). Total RNA was extracted from Azoarcus sp. CIB (wt) and Azoarcus sp. CIBmbdR (mbdR) cells grown under denitrifying conditions using 3 mM benzoate (lane B) or 3 mM 3-methylbenzoate (lane 3M) as sole carbon sources. The primer pairs used to amplify the mbdO (P₀) and mbdB1 (P₁₁) gene fragments were as described in “Experimental Procedures.” C shows the interaction between increasing concentrations of purified MbdR-His₆ protein and a DNA probe (271-bp) containing the P₀ promoter. D shows the interaction between increasing concentrations of purified MbdR-His₆ protein and a DNA probe (251-bp) containing the P₁₁ promoter. Lane numbers refer to the MbdR-His₆ protein concentration (nanomolar) used for each reaction. P₀ and P₁₁ probes as well as the major P₀/MbdR and P₁₁/MbdR complexes are marked with arrows.

The crystal structure of MbdR was determined using multi-wavelength anomalous diffraction data, and it was refined to 1.76 Å resolution. A summary of the crystallographic statistics is shown in Table 3. The crystal structure reveals that the crystallographic asymmetric unit contains a monomer of the protein (Fig. 4A). The N-terminal 14 amino acids, residues Thr-46 and Lys-47, and the C-terminal 10 residues in the structure are disordered. Helices α₁ to α₃ (Ala-13 to Phe-54) make up the N-terminal DNA binding domain and contain the helix-turn-helix motif (Fig. 3). The larger C-terminal ligand binding domain of MbdR (Fig. 3) consists of helices α₄ to α₉ (Lys-57 to Val-204) (Fig. 4A). The long axis of helices α₄, α₅, α₇, α₈, and α₉ are approximately parallel and at right angles to α₁. The short helix α₆ lies approximately parallel to α₁ and bisects the C-terminal domain with α₄ and α₇ on the one side and α₅, α₈, and α₉ on the other side (Fig. 4A). A 2-fold crystallographic symmetry operator (arises in space group P2₁2₁2₁) sits parallel to α₄ and generates a dimeric arrangement. The dimer interface is formed mainly by helices α₈ and α₉ with small contributions from helices α₆ and α₇. In total, the dimer buries 1759 Å²/monomer of surface area with mostly hydrophobic residues (Fig. 4B).

Taken together, all these results indicate that the MbdR homodimer shows the characteristic structure of the TetR family regulators. The members of the TetR family are mostly repressors (51, 52), and MbdR behaves also as a transcriptional repressor of the mbd genes responsible for the anaerobic catabolism of 3-methylbenzoate.

MbdR Binds to Palindrome Operator Sites within P₀ and P₁₁ Promoters—To confirm in vitro that the MbdR regulator directly interacts with the P₀ and P₁₁ promoters, gel retardation experiments were carried out with purified MbdR and a 271-bp DNA harboring P₀ or a 251-bp DNA containing P₁₁ as probes. The MbdR protein was able to retard the migration of both DNA probes in a protein concentration-dependent manner (Fig. 2, C and D). The affinity of MbdR for both P₀ and P₁₁ probes was very similar, showing a relative Kₐ of 1.71 ± 0.18 and 3.72 ± 0.03 nM, respectively. To further study the interaction of the MbdR protein with the P₀ and P₁₁ promoters, we mapped the transcription start sites of both promoters. Primer extension analyses were performed with total RNA isolated from Azoarcus sp. CIB cells grown exponentially in benzoate (control condition) or 3-methylbenzoate (inducing condition).
Whereas no transcript was observed from cells growing in benzoate, a transcript band was visible from cells growing in 3-methylbenzoate (Fig. 5, A and B), confirming a 3-methylbenzoate-dependent activation of the PO and PB1 promoters. The transcription start site at the PO and PB1 promoters was mapped at a guanine located 137 and 138 bp upstream of the ATG translation initiation codon of the mbdO and mbdB1 genes, respectively.

To characterize the DNA-binding sites of MbdR within the PO and PB1 promoters, we performed DNase I footprinting assays. As shown in Fig. 5, C and D, MbdR protected DNA regions spanning from positions 18 to 16 and from 4 to 34 with respect to the transcription start sites of the PO and PB1 promoters, respectively. The protected regions contained a conserved palindromic sequence (ATAC N10GTAT) that is suggested to be the operator sequence recognized by MbdR. The MbdR operator in PO and PB1 promoters spans the transcription initiation sites as well as the 10 and 35 (only in PB1) sequences for recognition of the σ70-dependent RNA polymerase (Fig. 5, C and D). Therefore, the characterization of the MbdR operator supports the observed repressor role of MbdR at the PO and PB1 promoters (Fig. 2, A and B).

3-Methylbenzoyl-CoA Is the Inducer That Alleviates the MbdR-dependent Repression of the mbd Genes—To identify the inducer molecule that alleviates the specific repression exerted by MbdR on the expression of the mbd genes, we first accomplished an in vivo approach. Thus, the activity of a PB1::lacZ translational fusion in plasmid pIZPB1 (Table 1) was measured...
in E. coli cells harboring also the pCKmbdR plasmid that expresses the mbdR gene under the IPTG-controlled Plac promoter (Table 1). As shown in Fig. 6A, the β-galactosidase activity levels of recombinant E. coli cells expressing the mbdR gene and grown anaerobically in minimal medium with glycerol as sole carbon source were significantly lower than those obtained in E. coli control cells lacking the mbdR gene. This result confirms in a heterologous host the role of MbdR as a transcriptional repressor of the mbd genes. Interestingly, the addition of 3-methylbenzoate to the culture medium of recombinant E. coli cells unable to metabolize this aromatic acid did not alleviate the repression exerted by MbdR (Fig. 6A), suggesting that

FIGURE 4. Three-dimensional structure of MbdR. A, ribbon diagram of the three-dimensional structure of the MbdR monomer, which belongs to space group I222. The refined structure has Rwork of 0.185 and Rfree of 0.242 with completeness of 99.3. B, ribbon diagram of the MbdR dimer generated using two neighboring monomers, showing the interface and the buried residues. C, molecular surface representation of the MbdR dimer with rotation of 90° backward to show the MbdR-DNA interaction surface. Red and blue surfaces represent negative and positive electrostatic potentials. D, similarity (superposition) of MbdR (red) to the structures of other TetR-like regulators such as AcrR (3LHQ, gold), EthR (3G1O, tan), HapR (2PBX, yellow), IcaR (2ZCN, green), QacR (3BTL, blue), and TetR (3LWJ, cyan). E, superimposition of the MbdR apo-structure (gold) and the QacR-4,4‘-[1,6-hexanediylbis(oxy)]bisbenzenecarboximidamide (red) complex structure (blue) (3BTJ) to show the proposed internal cavity of MbdR induced by 3-methylbenzoyl-CoA binding. F, putative key residues comprising the ligand-binding pocket of MbdR are shown as sticks. Figures were drawn using the PyMOL program.
3-methylbenzoate, the substrate of the mbd pathway, is not the specific inducer of the PB1 promoter. It has been described previously that the transcriptional activation of benzoate degradation operons in Azoarcus sp. CIB requires benzoyl-CoA, the first intermediate of the anaerobic/aerobic degradation pathways, as inducer molecule (17, 20). Thus, we checked whether 3-methylbenzoyl-CoA, the first CoA-derived intermediate of the mbd pathway, could be the specific inducer molecule of the mbd genes. To this end, we expressed the mbdA gene encoding the 3-methylbenzoate-CoA ligase (MbdA) that catalyzes the transformation of 3-methylbenzoate to 3-methylbenzoyl-CoA (28), in the reporter E. coli strain containing plasmids pIZPB1 and pCKmbdR. As shown in Fig. 6A, the activity of the PB1 promoter increased after the addition of 3-methylbenzoate to

**FIGURE 5. MbdR protein interacts with the PO and PB1 promoter regions.** A and B, determination of the transcription start site in the PO and PB1 promoters. Total RNA was isolated from Azoarcus sp. CIB cells growing on 3-methylbenzoate (inducing condition) or benzoate (control condition) as sole carbon sources as described under “Experimental Procedures.” The size of the extended products under inducing conditions (lane 3M) or noninducing conditions (lane B) was determined by comparison with the DNA sequencing ladder (lanes A, T, C, and G) of the PO (A) and PB1 (B) promoter regions. Primer extension and sequencing reactions of the PO and PB1 promoters were performed with primers CIB/H11001 and CIB/H11032, respectively, as described under “Experimental Procedures.” An expanded view of the nucleotides surrounding the transcription initiation site (circled) in the noncoding strand is shown. The longest extension product is shown by an arrow. C and D, DNase I footprinting analyses of the interaction of MbdR with the PO and PB1 promoter regions. The DNase I footprinting experiments were carried out using the PB1 (C) and PO (D) probes labeled as indicated under “Experimental Procedures.” Lanes AG, show the A+G Maxam and Gilbert sequencing reaction. Lanes A–G show footprinting assays containing increasing concentrations of MbdR-His6. Lanes F (C) and G (D) show footprinting assays containing MbdR-His6 plus 250 μM 3-methylbenzoyl-CoA (3MCoA). Phosphodiester bonds hypersensitive to DNase I cleavage are indicated by asterisks. On the left side of each panel, an expanded view of the promoter region is shown. Protected regions are shaded in gray over the promoter sequences. The −10/−35 regions are boxed, and the transcription initiation sites (+1) are underlined. The predicted MbdR operators are flanked by palindrome sequences indicated by convergent dotted arrows.
the culture medium, suggesting that 3-methylbenzoyl-CoA is the specific inducer of the MbdR repressor.

In vitro experiments were then performed to confirm the direct role of 3-methylbenzoyl-CoA as the inducer molecule of the mbd cluster. First, gel retardation experiments showed that the presence of 3-methylbenzoyl-CoA inhibited the interaction of MbdR with the PO and PB1 probes (Fig. 6B). On the contrary, 3-methylbenzoate or some 3-methylbenzoyl-CoA analogs, such as benzoyl-CoA or phenylacetyl-CoA, did not avoid the interaction of MbdR with its target promoters (Fig. 6C), suggesting that MbdR recognizes 3-methylbenzoyl-CoA specifically. The inducing effect of 3-methylbenzoyl-CoA was also observed in footprinting assays where the addition of 3-methylbenzoyl-CoA reverted the protection of MbdR against the DNase I digestion on the PO and PB1 promoters (Fig. 5, C and D).

The role of MbdR as a specific transcriptional repressor of the PO and PB1 promoters and 3-methylbenzoyl-CoA as the cognate inducer was also demonstrated by in vitro transcription assays using supercoiled DNA templates bearing each of the two promoters. Thus, Fig. 6D shows the MbdR-dependent repression of the PO and PB1 promoters, and it also reveals how the addition of increasing amounts of 3-methylbenzoyl-CoA leads to formation of the expected transcripts from both promoters.

Identification of Additional MbdR-dependent Promoters in the mbd Cluster, the P3R and PA Promoters—Nucleotide sequence analysis of the intergenic regions of the mbd cluster revealed putative MbdR binding regions that contain the conserved (ATACN14GTAT) palindromic sequence in the PA promoter (Fig. 1) (28) and upstream of the mbdA gene encoding the 3-methylbenzoate-CoA ligase (putative PA promoter). To experimentally validate that P3R and PA are functional promoters of the mbd cluster, the upstream region of mbdR and the mbdB5-mbdA intergenic region were cloned into the promoter probe vector pSJ3 and pSJ3P3R that contain the P3R::lacZ and PA::lacZ translational fusions, respectively (Table 1). Both
translational fusions were then subcloned into the broad host range vector pIZ1016 giving rise to plasmids pIZP3R (P3R::lacZ) and pIZPA (PA::lacZ) (Table 1). E. coli cells containing plasmids pIZP3R or pIZPA were grown in M63 minimal medium, and they showed 75 and 50 Miller units of β-galactosidase activity, respectively, suggesting that P3R and PA are functional but weak promoters. Primer extension experiments revealed that the transcription initiation sites (1) of P3R and PA promoters are located 120 bp (data not shown) and 117 bp (Fig. 7A) upstream of the mbdR and mbdA start codons, respectively.

To demonstrate the direct interaction of MbdR with the P3R and PA promoters, gel retardation assays were performed. To this end, purified MbdR was incubated either with a 352-bp DNA probe carrying the P3R promoter or with a 225-bp DNA fragment containing the PA promoter. Fig. 8, A and C, shows that MbdR was able to retard the migration of both DNA probes in a protein concentration-dependent manner. The binding was specific, because the addition of unlabeled heterologous DNA did not affect the protein-DNA binding, but the addition of unlabeled specific DNA inhibited the retardation of the probes (data not shown). Several P3R-MbdR retardation bands were observed (Fig. 8C), which agrees with the fact that several MbdR operator regions were suggested in P3R (Fig. 8E).

As observed previously with the PO and PB1 promoters, 3-methylbenzoyl-CoA behaved as the inducer of MbdR because binding of this protein to the PA and P3R promoters was significantly diminished in the presence of this aromatic CoA ester (Fig. 8, B and D).

FIGURE 7. MbdR protein interacts with the PA promoter region. A, determination of the transcription start site at the PA promoter. Total RNA was isolated from Azoarcus sp. CIB cells growing on 3-methylbenzoate (lane 3M) as sole carbon source as described under “Experimental Procedures.” The size of the extended product was determined by comparison with the DNA sequencing ladder (lanes A, T, C, and G) of the PA promoter region. Primer extension and sequencing reactions of the PA promoter were performed with primer PmbdAEcoRI3 (Table 2), as described under “Experimental Procedures.” An expanded view of the nucleotides surrounding the transcription initiation site (circled) in the noncoding strand is shown. The longest extension product is pointed by an arrow. B, DNase I footprinting analyses of the interaction of purified MbdR protein and the PA promoter region. The DNase I footprinting experiments were carried out using the PA probe labeled as indicated under “Experimental Procedures.” Lane A+G shows the A+G Maxam and Gilbert sequencing reaction. Lanes A–D show footprinting assays containing increasing concentrations of MbdR-His6. Lane E shows a footprinting assay containing MbdR-His6 (25 nM) in the presence of 250 μM 3-methylbenzoyl-CoA. Left side, an expanded view of the PA promoter region is shown. The protected region is shaded in gray over the promoter sequence. The −10/−35 regions are boxed, and the transcription initiation site (+1) is underlined. The predicted MbdR operator is flanked by palindrome sequences indicated by convergent arrows.
**MbdR Regulator from Azoarcus sp. CIB**

Although the role of $P_{3R}$ driving the expression of the mbdR regulatory gene is obvious, the role of the $P_A$ promoter located within the $P_{B1}$-driven operon (Fig. 1) is puzzling, and therefore, it was further investigated.

$P_A$ and $P_{B1}$ Promoters Are Essential for Growth of Azoarcus sp. CIB on 3-Methylbenzoate—As described previously, the $P_{B1}$ promoter drives the expression of the $mbdB1B2B3B4B5mbdA$ operon (Fig. 1) (28). We have shown above (Fig. 8A) that a new MbdR-dependent promoter, the $P_A$ promoter, is located upstream of $mbdA$ within the $P_{B1}$-driven operon (Fig. 1). To explore whether both promoters share a similar MbdR-dependent regulation, the sequence of the $P_A$ promoter recognized by MbdR was experimentally determined by DNase I footprinting assays. Fig. 7B shows that the region of $P_A$ protected by MbdR against the DNase I digestion includes the predicted (ATACN$_{10}$GTAT) operator region (Fig. 8E), and it spans the $–35$ sequence for recognition of the $σ^{70}$-dependent RNA polymerase. Moreover, the addition of 3-methylbenzoyl-CoA released the MbdR-dependent protection (Fig. 7B), confirming the role of this molecule as inducer. All these data support the hypothesis that MbdR behaves also as a transcriptional repressor for the $P_A$ promoter. To confirm in vivo the repressor role of MbdR on the $P_A$ promoter, the activity of a $P_A$-::lacZ translational fusion in plasmid pZIP$_A$ (Table 1) was measured in *E. coli* MC4100 cells harboring also the pCKmbdR and pUCmbdA plasmids that express the mbdR and $mbdA$ genes under the IPTG-controlled $Plac$ promoter, respectively (Table 1). The $β$-galactosidase activity levels (5 Miller units) of recombinant *E. coli* cells expressing the $mbdR$/$mbdA$ genes and grown anaerobically were significantly lower than those obtained in *E. coli* control cells expressing the $P_A$-::lacZ translational fusion but lacking the $mbdR$/$mbdA$ genes (50 Miller units). However, the addition of 3-methylbenzoate to the culture medium, which is transformed to 3-methylbenzoyl-CoA by the MbdA activity, alleviated the repression exerted by MbdR, and values of $β$-galactosidase activity of about 40 Miller units were obtained. Therefore, these results show that MbdR behaves as a functional repressor of the $P_A$ promoter, and 3-methylbenzoyl-CoA acts as the inducer molecule.

As suggested above by comparing the $β$-galactosidase values in *E. coli* cells expressing $P_A$-::lacZ (50 Miller units) and $P_{B1}$-::lacZ (4000 Miller units) fusions, the $P_A$ promoter appears to be significantly weaker than $P_{B1}$. To confirm the major role of $P_{B1}$ in the expression of the $mbdA$ gene in the homologous system, we checked by real time RT-PCR the expression of $mbdA$ in the wild-type *Azoarcus* sp. CIB strain and in *Azoarcus* sp. CIBdmdbB1, a mutant strain that contains an insertion within the $mbdB1$ gene that should block transcription from the $P_{B1}$ promoter but maintains a functional $P_A$ promoter (Table 1). The expression levels of the $mbdA$ gene in *Azoarcus* sp. CIBdmdbB1 grown in the presence of 3-methylbenzoate were similar to the basal levels observed with the wild-type CIB strain grown in the absence of 3-methylbenzoate, and they were more than 47 times lower than those observed in the wild-type CIB strain grown in 3-methylbenzoate (data not shown). These data suggested that $P_{B1}$, but not $P_A$, has indeed a major contribution to the $mbdA$ expression in *Azoarcus* sp. CIB. In agreement with this observation, the *Azoarcus* sp. CIBdmdbB1 mutant strain was unable to use 3-methylbenzoate as sole carbon source (Fig. 9A), and growth was restored when the $mbdA$
gene was provided in trans in plasmid pIZmbdA (Fig. 9A). In contrast, Azoarcus sp. CIBdmbdB1 mutant strain was still able to use m-xylene as a sole carbon source (data not shown), which is in agreement with the fact that the Bss-Bbs peripheral pathway for the anaerobic degradation of m-xylene generates 3-methylbenzoyl-CoA without the need of a specific CoA ligase activity (Fig. 1) (53–56). Taken together, all of these results indicate that \( P_{A} \) is essential for the anaerobic degradation of 3-methylbenzoate, an Azoarcus sp. CIB\( \Delta P_{A} \) mutant strain harboring a deletion of the \( P_{A} \) promoter but maintaining a complete \( mbdA \) gene and the native \( P_{B1} \) promoter was constructed (Table 1). Interestingly, Azoarcus sp. CIB\( \Delta P_{A} \) was not able to grow anaerobically in 3-methylbenzoate (Fig. 9A), suggesting that \( P_{A} \) is also necessary for an efficient expression of the \( mbdA \) gene, which in turn supports the presence of \( P_{A} \) within the \( P_{B1} \)-driven operon.

Because \( P_{B1} \) accounts for most of the \( mbdA \) expression, the role of the weak \( P_{A} \) promoter might be related to the initial induction of the \( mbdA \) expression when the cells start to grow in 3-methylbenzoate. To check this hypothesis, the activity of the \( P_{B1} \) and \( P_{A} \) promoters was analyzed by \( \beta \)-galactosidase assays along the growth curve of Azoarcus sp. CIB harboring pIZP\( B_{1} \) (\( P_{B1}::\text{lacZ} \)) and Azoarcus sp. CIB harboring pIZP\( A \) (\( P_{A}::\text{lacZ} \)) grown in the presence of 3-methylbenzoate. The activity of the weak \( P_{A} \) promoter was always higher than that of \( P_{B1} \) up to 6 h after the addition of 3-methylbenzoate, and then \( P_{B1} \) showed a significant induction and reached values about 20-fold higher than those of \( P_{A} \) (Fig. 9B). Therefore, these results suggest that the fast and modest induction of the \( P_{A} \) promoter will be critical to provide the required amount of the inducer molecule 3-methylbenzoyl-CoA for triggering the induction of the \( P_{B1} \) promoter and to allow growth on 3-methylbenzoate.

**DISCUSSION**

Bacterial metabolism of some compounds that usually are nonpreferred carbon sources, e.g. aromatic compounds, is generally strictly regulated at the transcriptional level (8). In this work, we have characterized the specific regulation of the \( mbd \) central cluster, which is responsible for anaerobic 3-methylbenzoate degradation in Azoarcus sp. CIB, by the MbdR transcriptional repressor. MbdR is an efficient repressor of the \( mbd \) genes whose expression can only be switched on when the Azoarcus sp. CIB cells grow anaerobically on 3-methylbenzoate (28) but not on benzoate (Fig. 2, A and B). This finding provides an explanation to the fact that Azoarcus sp. CIBd\( bzdN \), a strain lacking a functional benzoate degradation (\( bzd \)) pathway, cannot use benzoate anaerobically despite the Mbd enzymes that can activate benzoate to benzoyl-CoA and further metabolize this CoA-derived compound (28). On the other hand, it is worth noting that the \( bzd \) genes are not induced when Azoarcus sp. CIB grows anaerobically in 3-methylbenzoate (data not shown). Therefore, these results reveal that there is no cross-induction between the \( bzd \) and \( mbd \) pathways, supporting the existence of devoted BzdR- and MbdR-dependent regulatory systems that control, respectively, each of these two central catabolic pathways in Azoarcus sp. CIB.

Analytical ultracentrifugation and crystallographic data indicate that MbdR is a homodimer in solution, a common feature among other members of the TetR family, e.g. TetR (57), QacR (58), ActR (59), FadR (60, 61), PfmR (62), and the MbdR monomeric structure includes two domains with nine helices (\( \alpha1 \) to \( \alpha9 \)) linked by loops (Fig. 4A). The N-terminal DNA binding domain (helices \( \alpha1 \) to \( \alpha3 \)) contains the helix-turn-helix motif whose...
The mbd catabolic pathway, is the cognate inducer molecule been shown that in some members of the TetR family, for the cognate promoter (52). To date, all ligands bind in the same change in the protein that leads to changes in DNA recognition (51, 52, 57). It is able, with its specific surfaces required for the dimerization of the protein and for the interaction with the inducer (51, 52, 57).

TetR family and controls the anaerobic catabolism of aromatic degradation pathways and recognizes aromatic CoA thioesters as inducers. Thus, FerR/FerC recognize feruloyl-CoA; and PaaX/PaaR recognize phenylacetyl-CoA as inducer (63), shows two MbdR-specific hydrophobic clusters, Gln-107 to Gly-123 within α6 and the α6/α7 linkage loop, and Ser-165 to Ile-176 within α8. Some residues within these two clusters could be involved in discriminating between the 3-methylbenzoyl group of 3-methylbenzoyl-CoA and the phenylacetyl group of phenylacetyl-CoA (Fig. 6F).

Nevertheless, further experiments are needed to determine the structure of the MbdR3-3-methylbenzoyl-CoA complex for understanding the inducer specificity determinants and the molecular mechanism of transcriptional de-repression at the target promoters.

P3 and P3R are two additional promoters within the mbd cluster whose activity levels are lower than those of P1, but that share with the latter the 3-methylbenzoyl-CoA/MbdR-dependent control (Fig. 8). The P3R promoter drives the expression of the regulatory mbdR gene (Fig. 1). Interestingly, the amount of MbdR needed for the retardation of 50% of the P3R probe was at least 1 order of magnitude higher than that needed for the retardation of the P1 (Fig. 8A), P2 (Fig. 8C), and P3 (Fig. 2D) promoters. The fact that the activity from the P3R promoter is under auto-repression by MbdR at high protein concentrations underlines the importance of a negative feedback loop that would restrict the intracellular concentration of the transcriptional repressor when it reaches a given concentration.

The P1 promoter is located within the P3R-driven operon (Fig. 1). The predicted MbdR operator region (ATACNG6GTAT) was suggested to be the operator region recognition of the regulatory gene (Fig. 1), which might contact the phosphate backbone of the DNA. The sequence for recognition of the operator region as in the cases of other TetR family members, which might contact the phosphate backbone of the DNA to form a positively charged patch at the N-terminal domain of both monomers (Fig. 4C), which might contact the phosphate backbone of the target operator region as in the cases of other TetR family members (52).

Although the length of the MbdR operator region is similar to that described as the “induced fit” mechanism of QacR (58), do not have such a cavity (Fig. 4, B and C), which might contact the phosphate backbone of the DNA to form a positively charged patch at the N-terminal domain of both monomers (Fig. 4C), which might contact the phosphate backbone of the target operator region as in the cases of other TetR family members (52).

In vivo (Fig. 6A) and in vitro (Fig. 6, B and D) experiments revealed that 3-methylbenzoyl-CoA, the first intermediate of the mbd catabolic pathway, is the cognate inducer molecule that interacts with the MbdR repressor allowing transcription from the P1 and P3R promoters. There is an increasing number of regulators, i.e. PaaR (63) (TetR family), CouR, FerC, HcaR, Fer, and GenR (MarR family) (27, 64–67), PaaX (GntR family) (38), and BzdR and BoxR (XRE family) (17, 20), that control aromatic degradation pathways and recognize aromatic CoA thioesters as inducers. Thus, FerR/FerC recognize feruloyl-CoA; CouR/HcaR recognize p-coumaroyl-CoA; BzdR/BoxR/GenR recognize benzoyl-CoA; and PaaX/PaaR recognize phenylacetyl-CoA (Fig. 6D). In this work, we show that MbdR regulates the first member of this group of regulators that belongs to the TetR family and controls the anaerobic catabolism of aromatic compounds.

The C-terminal domain of TetR-like regulators is highly variable, with its specific surfaces required for the dimerization of the protein and for the interaction with the inducer (51, 52, 57). Based on the previously published studies of other TetR-like regulators, ligand binding usually induces a conformational change in the protein that leads to changes in DNA recognition and interaction, causing the dissociation of the repressor from the cognate promoter (52). To date, all ligands bind in the same general location at or near the dimer interface. However, it has been shown that in some members of the TetR family, for example AcrR (68), the ligand binds in a large internal cavity in the C-terminal region, surrounded by helices α4 through α8 of each monomer. In contrast, MbdR and other members of TetR family, such as QacR (58), do not have such a cavity (Fig. 4, A and C). By superimposing the apo-MbdR structure with the structure of the QacR-diamidine hexamidine complex (69), we could suggest the binding site of 3-methylbenzoyl-CoA in MbdR and a model of the MbdR-3-methylbenzoyl-CoA interaction (Fig. 4E).

Binding of 3-methylbenzoyl-CoA would require the movements of helices α5, α6, α8, and α9 in MbdR, similar to that described as the “induced fit” mechanism of QacR bound to its ligand (69, 70). Similar to what has been observed in the QacR-ligand complex structure, the movement of α6 after 3-methylbenzoyl-CoA binding to MbdR would induce a rotation of the helix-turn-helix domain (Fig. 4E), and as a consequence, this DNA binding domain would lose its DNA binding ability. Sequence comparison of MbdR and PaaR (Fig. 3), another member of the TetR family which uses phenylacetyl-CoA as inducer (63), shows two MbdR-specific hydrophobic clusters, Gln-107 to Gly-123 within α6 and the α6/α7 linkage loop, and Ser-165 to Ile-176 within α8. Some residues within these two clusters could be involved in discriminating between the 3-methylbenzoyl group of 3-methylbenzoyl-CoA and the phenylacetyl group of phenylacetyl-CoA (Fig. 4F). Nevertheless, further experiments are needed to determine the structure of the MbdR3-3-methylbenzoyl-CoA complex for understanding the inducer specificity determinants and the molecular mechanism of transcriptional de-repression at the target promoters.

P1 and P3R are two additional promoters within the mbd cluster whose activity levels are lower than those of P1, but that share with the latter the 3-methylbenzoyl-CoA/MbdR-dependent control (Fig. 8). The P3R promoter drives the expression of the regulatory mbdR gene (Fig. 1). Interestingly, the amount of MbdR needed for the retardation of 50% of the P3R probe was at least 1 order of magnitude higher than that needed for the retardation of the P1 (Fig. 8A), P2 (Fig. 8C), and P3 (Fig. 2D) promoters. The fact that the activity from the P3R promoter is under auto-repression by MbdR at high protein concentrations underlines the importance of a negative feedback loop that would restrict the intracellular concentration of the transcriptional repressor when it reaches a given concentration. The P1 promoter is located within the P3R-driven operon (Fig. 1). The predicted MbdR operator region (ATACNG6GTAT) (Fig. 8E) spans the −35 sequence for recognition of the σ70-dependent RNA polymerase in the P1 promoter (Fig. 7B), thus supporting the observed repressor role of MbdR on this promoter. Whereas the role of P3R driving the expression of the mbdR regulatory gene is obvious, the role of the P1 promoter was puzzling, and therefore, it was further investigated.

Inactivation of either the strong (P3B) or the weak (P3A) promoters in Azoarcus sp. CIBΔmbdB1 and Azoarcus sp. CIBΔPA mutant strains, respectively, revealed that both promoters are essential for the anaerobic growth of strain CIB in 3-methylbenzoate and thus will allow growth on this aromatic compound. In summary, these studies highlight the main role of some minor regulatory loops that control the expression of CoA ligases for triggering the efficient expression of aromatic catabolic pathways that use aryl-CoA compounds as central intermediates.
Mbd enzymes are able to activate benzoate and further convert benzoyl-CoA in vitro (28). We have shown here that MbdR is an efficient repressor of the mbd genes, and it recognizes 3-methylbenzoyl-CoA, but not benzoyl-CoA, as inducer. These results suggest that the broad substrate range mbd catalytic genes have recruited a regulatory system based on the MbdR regulator and its target promoters to evolve to a distinct central aromatic catabolic pathway that is only expressed for the anaerobic degradation of aromatic compounds that generate 3-methylbenzoyl-CoA as central metabolite. Thus, the existence in Azoarcus sp. CIB of two different central pathways, i.e., the bzd pathway, for the anaerobic degradation of aromatic compounds that generate benzoyl-CoA as central intermediate, and the mbd pathway, for the anaerobic degradation of aromatic compounds that generate 3-methylbenzoyl-CoA as central intermediate, could be mainly driven by the high specificity of the corresponding repressors, i.e., BzdR and MbdR, for their cognate inducers, i.e., benzoyl-CoA and 3-methylbenzoyl-CoA, respectively. If correct, this highlights the importance of the regulatory systems in the evolution and adaptation of bacteria to the anaerobic degradation of aromatic compounds.

The studies presented in this work expand our knowledge on the specific regulation of anaerobic pathways for the catabolism of aromatic compounds (4, 9, 14, 17, 20, 27, 28). Moreover, it worth noting that 3-methylbenzoyl-CoA is an uncommon intermediate in living cells, and MbdR-responsive promoters are likely to be also very infrequent in nature. Therefore, the P_{B1} promoter, mbdR regulator, and mbdA genes become potential BioBricks for creating new conditional expression systems that respond to 3-methylbenzoate in a fashion minimally influenced by the host and that has no impact on the host physiology (biological orthogonality), two major desirable traits in current synthetic biology approaches (71).

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