BzdR, a Repressor That Controls the Anaerobic Catabolism of Benzoate in Azoarcus sp. CIB, Is the First Member of a New Subfamily of Transcriptional Regulators*

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In this work, we have studied the transcriptional regulation of the bzd operon involved in the anaerobic catabolism of benzoate in the denitrifying Azoarcus sp. strain CIB. The transcription start site of the PN promoter, driving the expression of the bzd catabolic genes was identified. Gel retardation assays and PGLacZ translational fusion experiments performed both in Azoarcus sp. CIB and Escherichia coli cells have shown that bzdR encodes a specific repressor that controls the inducible expression of the adjacent bzd catabolic operon, being the first intermediate of the catabolic pathway (i.e. benzyol-CoA, the actual inducer molecule). This is the first report of a transcriptional repressor and a CoA-derived aromatic inducer controlling gene expression in the anaerobic catabolism of aromatic compounds. DNase I footprinting experiments revealed that BzdR protected three regions (operators) at the PN promoter. The three operators contain direct repetitions of a TGCA sequence that forms part of longer palindromic structures. In agreement with the repressor role of BzdR, operator region I spans the transcription initiation site as well as the −10 sequence for recognition of the RNA polymerase. Primary sequence analyses of BzdR showed an unusual modular organization with an N-terminal region homologous to members of the HTH-XRE family of transcriptional regulators and a C-terminal region similar to shikimate kinases. A three-dimensional model of the N-terminal and C-terminal regions of BzdR, generated by comparison with the crystal structures of the SinR regulator from Bacillus subtilis and the shikimate kinase I protein from E. coli, strongly suggests that they contain the helix-turn-helix DNA-binding motif and the benzyol-CoA binding groove, respectively. The BzdR protein constitutes, therefore, the prototype of a new subfamily of transcriptional regulators.

In the last 30 years, some insights have been added to the knowledge of the anaerobic catabolism of aromatic acids. Most of the studies have focused on the biochemical steps that lead to the mineralization of the aromatic ring in a few microorganisms such as certain strains of Rhodopseudomonas (photosynthetic α-Proteobacteria), Thauera, and Azoarcus (denitrifying β-Proteobacteria), in some Fe(III) and sulfate reducers, and in fermentative bacteria (1–4).

Benzoate is a good model compound by which to study the anaerobic catabolism of aromatic compounds (1). A general strategy used by microbes to degrade anaerobically benzoate is its activation to benzyol-CoA that is then degraded to central biosynthetic intermediates through a series of reactions that involve aromatic ring reduction, β-oxidation-like reactions, and ring cleavage as main steps. Biochemical and genetic insights on benzoate degradation have been reported in Rhodopseudomonas palustris, Magnetospirillum magneto tacticum MS-1, Thauera aromatica, Azoarcus evansii, and Azoarcus sp. CIB (1, 2, 5). Despite some progress on the enzymology of anaerobic catabolism of benzoate by these bacteria in recent years, very little is known about the regulation of the genes involved in this particular catabolic process. Although several genes have been suggested to be involved in the regulation of the anaerobic benzoate degradation, so far only bzdR and aadR have been shown to control the expression of a benzoate catabolic cluster in R. palustris (6, 7).

The bzd cluster of Azoarcus sp. CIB codes for the set of proteins involved in the anaerobic catabolism of benzoate (2) (Fig. 1A). The bzd catabolic genes are organized in a single operon (bdzNOPQSTUVWXYZA) encoding the enzymes that activate, deammatate, and cleave the aromatic ring of benzoate yielding CoA derivative molecules that are channeled into the central metabolism of the cell (2). The bzd catabolic operon is driven by the PN promoter (Fig. 1A). Upstream of the bzd operon is located the bzdR gene that is expressed from the PR promoter (Fig. 1A) (2). It has been suggested previously that the bzdR gene might encode the specific transcriptional regulator (BzdR) of the bzd cluster (2).

By using different genetic and biochemical approaches, we show in this work that bzdR codes for a specific regulator of the bzd catabolic genes, being the first example of a benzyol-CoA-dependent transcriptional repressor that is described in anaerobic catabolism of aromatic compounds. Moreover, primary sequence analyses and three-dimensional modeling of BzdR reveal an unusual modular structure that allows us to consider BzdR as the first member of a new subfamily of transcriptional regulators.
Regulation of Benzoyl-CoA Cluster in Azoarcus sp. CIB

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Growth Conditions—The E. coli and Azoarcus strains as well as the plasmids used in this work are listed in Table I. To construct plasmid pCK01BzdR, a 1.6-kb EcoRI/BamHI DNA fragment containing the bzdR gene was PCR-amplified from plasmid pECOR7 (Table I) by using oligonucleotides 5’-GGGAAGCTTTCAGCGTGCCAG-3’ (an engineered BamHI site is underlined) and 3’-GGGATCCTTCGACATACTCGGC-3’ (an engineered HindIII site is underlined). Unless indicated otherwise, E. coli cells were grown at 37 °C in Luria-Bertani (LB) medium (15). When required, E. coli cells were grown aerobically in M63 minimal medium (15) at 30 °C using the corresponding necessary nutritional supplements and 20 mM glycerol, as carbon source, and 10 mM nitrate, as terminal electron acceptor. Azoarcus strains were grown aerobically in MC medium as described previously (2). Where appropriate, antibiotics were added at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), gentamycin (7.5 μg/ml), kanamycin (50 μg/ml), and rifampicin (50 μg/ml).

Molecular Biology Techniques—Oligonucleotide primers were designed using the methods (6). 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 synthesized on an Oligo-1000 M nucleotide synthesizer (Beckman Instruments, Inc.). All cloned inserts and DNA fragments were confirmed by DNA sequencing through an ABI Prism 377 automated DNA sequencer (Applied Biosystems Inc.). Transformation of E. coli cells was carried out by using the BBI method or by electroporation (Gene Pulser; Bio-Rad) (8). Plasmids were transferred from E. coli to Azoarcus (donor strain) into Azoarcus sp. strain CIB by using conjugative transfer (2). Exconjugants containing the bzdR gene were selected on LB medium lacking nitrate and containing 0.4% citrate as the sole carbon source for counterselection of donor cells. The mutant strain was analyzed by PCR to confirm the disruption of the target gene.

Overproduction and Purification of His6::BzdR—The recombinant plasmid pCK01BzdR (Table I) carries the bzdR gene under the control of the ATG start codon and with a His6 tag coding sequence at its 5′-end, under control of the lac promoter and two lac operon boxes. The His tag added 13 amino acids (MGCGSHHHHHHGGIL) to the N-terminal end of His6::BzdR-protein (34.842 Da). The His-tagged protein was overproduced in E. coli M15 strain harboring plasmid pREFP4 (Table I) that produces the LacI repressor to strictly control gene expression from the lac promoter and two lac operon boxes (2). The His-tagged bzdR protein was eluted by using elution buffer (50 mM NaH2PO4, 300 mM NaCl, 750 mM imidazole, pH 8.0). The purified protein was dialyzed at 4 °C in FP buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM β-mercaptoethanol, and 50 mM KC1) and stored at −20 °C.

Gel Retardation Assays—A 268-bp DNA fragment spanning from position 174 to +79 with respect to the transcription start site of pY promoter (Fig. 1B) was PCR-amplified from plasmid p3SP3 (Table I) by using oligonucleotides 5’-GCTTAGAC CCATGCAATATCTCCTCTGATG-3’ (an engineered HindIII site is underlined) and 3’-AGCCGGCTGCTGCTGTGCTGTTGG-3’ (an engineered KpnI site is underlined) and 5’-GZN, The PCR-amplified fragments were digested with KpnI and XbaI restriction endonucleases and ligated to the KpnI/XbaI double-digested pSU3 promoter probe vector giving rise to plasmid pSU3-P (pSU3-lacZ) and pSU3-PbzdR (pSU3-P-bzdR-lacZ) (Table I, Fig. 1). The correct lacZ translational fusions were confirmed by nucleotide sequence analysis. Plasmids p5UTMINtSN and p5UTMINtSN5 were constructed by subcloning the NotI cassettes from p5JS3P and p5JS3P into the mini-Tn5 delivery plasmid p5UTMINtSN5km2 (Table I, Fig. 4), and they were transferred from E. coli S17-1lpI to E. coli AFMC cells (Table I) by biparental mating (22). Exconjugants containing the p5JS3P and p5JS3PbzdR translational fusions insertions into their chromosomal DNA sequences for the transposon marker, kanamycin, on rifampicin-containing LB medium, generating the E. coli AFMCPN and AFMCRPN strains, respectively (Table I).

Construction of Azoarcus sp. Strain CIBdbzdR—For disruption of the bzdR gene through single homologous recombination, a 209-bp internal fragment of bzdR was PCR-amplified by using primers BzdRm5’ (5’-CGGAGGCCGAGGGGGAATGCT-3’) and N-INV-III (5’-GCTTGGCACC CCAGGACTTC-3’), and it was cloned into the Smal-digested pKM18 (a mobilizable plasmid that does not replicate in Azoarcus) (Table I). The resulting construct, pKIMobbdZR (Table I), was transferred from E. coli S17-1lpI (donor strain) into Azoarcus sp. strain CIB (recipient strain) by using conjugative transfer (2). Exconjugants containing the disrupted bzdR gene by insertion of the suicide plasmid, namely Azoarcus sp. strain CIBdbzdR, were isolated aerobically on kanamycin-containing MC medium lacking nitrate and containing 0.4% citrate as the sole carbon source for counterselection of donor cells. The mutant strain was analyzed by PCR to confirm the disruption of the target gene.

Sequence Data Analyses—The amino acid sequences of open reading frames were compared with those present in finished and unfinished microbial genome data bases using the TBLAST algorithm (18) at the National Center for Biotechnology Information server (available on the World Wide Web at www.ncbi.nlm.nih.gov/blast/blast.cgi). Multiple protein sequence alignments were made with the ClustalW (19) program at the INFOBIGEN server (available on the World Wide Web at www.infobiogen.fr/services/menuuser.html). Phylogenetic analysis of the protein sequence alignments were made with the ClustalW (19) program. The sequence of BzdR was determined by Edman degradation with a 477A sequence of BzdR was determined by Edman degradation with a 477A automatic protein sequencer (Applied Biosystems Inc.). A crude extract of E. coli DH5α (pBzdR4) cells was loaded in a 12.5% SDS-polyacrylamide gel, and the BzdR (288 amino acids) protein encoded by plasmid pBzdR4 was directly electrophoresed from the gel onto a polyvinylidene difluoride membrane as described previously (17). β-Galactose-β-Galactoside Ligase Assays—β-Galactosidase activities were measured with permeabilized cells as described by Miller (15). Benzoate-CoA ligase activities were measured through a French press (Aminco Corp.) operated at a pressure of 20,000 p.s.i. The cell lysate was centrifuged at 26,000 × g for 20 min at 4 °C. The clear supernatant fluid was carefully decanted and applied to nickel-nitriolactaetric acid-agarose columns (Qiagen). Columns were then washed at 4 °C with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5 mM mercaptoethanol, and 50 mM β-mercaptoethanol, and 50 mM NaCl, 750 mM imidazole, pH 8.0. The purified protein was dialyzed at 4 °C in FP buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM β-mercaptoethanol, and 50 mM KC1) and stored at −20 °C.
footprinting assays was PCR-amplified from plasmid pECOR7 by using oligonucleotides 5IVTPN (5'-CGGAATTCCGATTACCATCAATGATCCG-GCAAG-3'), which hybridizes with the 3'-end of bzdR, and 3IVTPN (5'-CGGAATTCCATGAAACTACTCTCTTCTGATG-3'); an engineered EcoRI site is underlined, and the bzdN start codon is indicated in boldface type). The amplified DNA fragment was then digested with PvuII and EcoRI restriction enzymes, and the resulting 376-bp substitution was singly 3'-end-labeled by filling in the overhanging EcoRI-digested end with [α-32P]dATP and the Klenow fragment as indicated above. The labeled fragment was purified by using Gene-Clean Turbo (Q-BIOgene). For DNase I footprinting assays, the reaction mixture contained 2 nM DNA probe, 500 nM oligonucleotide (Q-BIOgene). The purified protein in 15 ml of TP buffer (see above). This mixture was incubated for 20 min at 30 °C, after which 3 ml of DNase I (Amersham Biosciences) (prepared in 10 ml CaCl2, 10 mM MgCl2, 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 calf thymus DNA, and 0.5 µg/ml glycogen. After phenol/ chloroform extraction, DNA fragments were precipitated with absolute ethanol, washed with 70% ethanol, dried, and directly resuspended in 5 µl of 90% (v/v) formamide-loading gel buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 0.05% (w/v) bromphenol blue, 0.05% (w/v) xylene cyanol). Samples were then denatured at 95 °C for 2 min and fractionated in a 6% polyacrylamide-urea gel. A + G Maxam and Gilbert reactions (23) 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 exposed to Hyperfilm MP (Amersham Biosciences). The labeled fragment was purified by using Gene-Clean Turbo (Q-BIOgene). For DNase I footprinting assays, the reaction mixture contained 2 nM DNA probe, 500 nM oligonucleotide (Q-BIOgene). The purified protein in 15 ml of TP buffer (see above). This mixture was incubated for 20 min at 30 °C, after which 3 ml of DNase I (Amersham Biosciences) (prepared in 10 ml CaCl2, 10 mM MgCl2, 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 calf thymus DNA, and 0.5 µg/ml glycogen. After phenol/ chloroform extraction, DNA fragments were precipitated with absolute ethanol, washed with 70% ethanol, dried, and directly resuspended in 5 µl of 90% (v/v) formamide-loading gel buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 0.05% (w/v) bromphenol blue, 0.05% (w/v) xylene cyanol). Samples were then denatured at 95 °C for 2 min and fractionated in a 6% polyacrylamide-urea gel. A + G Maxam and Gilbert reactions (23) 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 exposed to Hyperfilm MP. 

**RESULTS AND DISCUSSION**

BzdR Is the Prototype of a New Subfamily of Transcriptional Regulators—Determination of the N-terminal amino acid sequence (MSNDENSSRL) of the bzdR gene product (see “Experimental Procedures”), suggests that BzdR is a protein of 298 amino acids (33,488 Da). Primary sequence analyses and amino acid sequence comparisons revealed that the BzdR protein exhibits two distinct regions. The N-terminal region (residues 1–130) shows a significant similarity with members of the helix-turn-helix (HTH)1-XRE family of transcriptional reg-
identical residues in more than 80% of the BzdR-like sequences. The Walker A motif is
acids are indicated by their standard
residues of each sequence are
numbered
B. subtilis
(AAC76415), SinR of
subgroups, respectively. Secondary structure elements predicted from the BzdR three-dimensional model (Fig. 10) are drawn at the
bottom
shows a sequence identity of 23% with the
PF01202), enzymes that catalyze the conversion of shikimate
homologous to shikimate kinases (PFAM accession number
(38). The C-terminal region of BzdR (residues 131–298) is
pression (34%) was found with SinR (Fig.
AAN32624), A. evansii (ANN39374), M. magnetotacticum MS-1 (1) (ZP_00056533), shikimate kinase I (SK) of E. coli
AAC76415), SinR of B. subtilis (BAA12542). At the bottom is shown a suggested consensus sequence for BzdR-like proteins. The amino acid residues of each sequence are numbered on the right. Sequences were aligned using the multiple sequence alignment program Clustal (19). Amino acids are indicated by their standard one-letter code. Dark grey shows identical residues in all of the BzdR-like sequences. Light grey indicates identical residues in more than 80% of the BzdR-like sequences. The Walker A motif is boxed, α and β refer to α-Proteobacteria and β-Proteobacteria
subgroups, respectively. Secondary structure elements predicted from the BzdR three-dimensional model (Fig. 10) are drawn at the bottom of the alignment.

FIG. 2. Multiple amino acid sequence alignment of BzdR. The accession numbers of the sequences are as follows: R. palustris CGA009 (CAE30227), B. japonicum USDA110 (NP_767116), M. magnetotacticum MS-1 (2) (ZP_00052337), R. eutropha JMP134 (ZP_00170685), R. metallidurans CH34 (ZP_00274120), B. fungorum LB400 (1) (ZP_00279512), B. fungorum LB400 (2) (ZP_00283918), Azoarcus sp. CIB (AAQ08805), T. aromatica (ANN32624), A. evansii (ANN39374), M. magnetotacticum MS-1 (1) (ZP_00056533), shikimate kinase I (SK) of E. coli
AAC76415), SinR of B. subtilis (BAA12542). At the bottom is shown a suggested consensus sequence for BzdR-like proteins. The amino acid residues of each sequence are numbered on the right. Sequences were aligned using the multiple sequence alignment program Clustal (19). Amino acids are indicated by their standard one-letter code. Dark grey shows identical residues in all of the BzdR-like sequences. Light grey indicates identical residues in more than 80% of the BzdR-like sequences. The Walker A motif is boxed, α and β refer to α-Proteobacteria and β-Proteobacteria
subgroups, respectively. Secondary structure elements predicted from the BzdR three-dimensional model (Fig. 10) are drawn at the bottom of the alignment.

uloters (SMART release; available on the World Wide Web at
smart.embl-heidelberg.de/smart) that includes more than 1300 proteins from eukaryota, archaea, bacteriophages, and bacteria with an ITH DNA-binding motif similar to that of the well characterized Cro protein of λ phage (36, 37). The highest amino acid sequence identity (34%) was found with SinR (Fig. 2), a 14-kDa multifunctional protein that, besides repressing sporulation, regulates genes for motility, alkaline protease expression (aprE), and competence development in Bacillus subtilis (38). The C-terminal region of BzdR (residues 131–298) is homologous to shikimate kinases (PFAM accession number PF01202), enzymes that catalyze the conversion of shikimate to shikimate 3-phosphate using ATP as co-substrate, and it shows a sequence identity of 29% with the E. coli shikimate kinase I (SK gene product) (Fig. 2). BzdR-like proteins are found encoded in the genomes of other denitrifying bacteria such as T. aromatica (51% identity; AAN32624), A. evansii (50% identity; AAN39374), Burkholderia fungorum LB400 (two proteins with 51 and 48% identity; ZP_00279512 and ZP_00283918, respectively), Ralstonia metalidurans (53% identity; ZP_00274120), Ralstonia eutropha (53% identity; ZP_001700685), M. magnetotacticum MS-1 (two proteins with 47 and 44% identity; ZP_00056533 and ZP_00052337, respectively), Bradyrhizobium japonicum (47% identity; NP_767116), and the photosynthetic bacterium R. palustris (45% identity; CAE30227) (Fig. 2). With the exception of the BzdR-like protein of M. magnetotacticum
ZP_00056533), there is a good correlation between the taxonomical position of the organism and the level of identity among BzdR homologues (Fig. 2). Thus, BzdR-like proteins from α and β-Proteobacteria cluster as different branches of the phylogenetic tree (data not shown). Whereas in most of the bacteria, the bzdR-like genes are in the vicinity of or integrated into the aerobic benzoate degradation cluster (40), Azoarcus sp. CIB is the only case described so far with the bzdR gene located in the anaerobic benzoate degradation cluster.

All of these data strongly suggest that BzdR is the specific regulator that controls expression of the bzd catabolic operon in Azoarcus sp. CIB. Since the modular organization of BzdR has not been reported so far in other regulators of the ITH-XRE family, BzdR might constitute the prototype of a new subfamily of transcriptional regulators.

BzdR Is the Specific Repressor of the bzd Catabolic Operon—
To determine whether bzdR is indeed involved in the regulation of the anaerobic benzoate degradation, a bzdR mutant of Azoarcus sp. strain CIB was constructed by gene disruption as described under “Experimental Procedures.” The mutant, Azoarcus sp. CIBΔbzdR (Table I), grew as the wild-type Azoarcus sp. strain CIB in benzoate-containing minimal medium (data not shown), which suggests that the BzdR protein, if playing any regulatory role, is not an activator but a repressor of the bzd catabolic operon. When we measured expression of the last gene of the bzd operon (i.e. the bzdA gene encoding benzoate-CoA ligase) (Fig. 1A), we observed that whereas...
Azoarcus sp. CIB cells showed a benzoate-inducible expression of \( bzdA \), a high level of benzoate-CoA ligase activity was detected in Azoarcus sp. CIBd\( bzdR \) cells growing either in the presence of benzoate (inducing conditions) or pyruvate (noninducing conditions) (Fig. 3A). This result indicates a constitutive expression of \( bzdA \) in Azoarcus sp. CIB cells that lack the \( bzdR \) gene, which is in agreement with BzdR being a putative repressor of the \( bzdA \) catabolic operon. To confirm that BzdR controls the expression of the \( bzdA \) catabolic operon at the level of the \( P_{bzdA} \) promoter, plasmid pBBR5\( P_{bzdR} \), which harbors a \( P_{bzdA} \) translational fusion (Table 1), was introduced into wild-type Azoarcus sp. CIB cells and in Azoarcus sp. CIBd\( bzdR \) cells. Whereas a significant \( bgd \)-galactosidase activity was observed when Azoarcus sp. CIB (pBBR5\( P_{bzdR} \)) cells grew in benzoate-containing minimal medium but not when the cells used pyruvate as the sole carbon source (Fig. 3B), high levels of \( bgd \)-galactosidase were observed when Azoarcus sp. CIBd\( bzdR \) (pBBR5\( P_{bzdR} \)) cells grew either in benzoate or pyruvate as the sole carbon sources (Fig. 3B). These data indicate a benzoate-inducible expression of the \( P_{bzdA} \)lac\( Z \) reporter fusion when the cells contain the BzdR protein and a constitutive expression of the reporter fusion in the absence of BzdR. All of these results taken together suggest that BzdR negatively regulates the expression of the \( bzdA \) catabolic operon by repressing the \( P_{bzdA} \) promoter when Azoarcus sp. CIB cells do not use benzoate as a carbon source.

To further investigate the regulatory role of the BzdR protein on the expression of the \( P_{bzdA} \) promoter, \( E. coli \) AFMC cells were transformed with plasmids pS33\( P_{bzdA} \) (\( P_{bzdA} \)lac\( Z \)), pS33\( P_{bzdR} \) (\( P_{bzdA} \)lac\( Z \)), which harbors the \( bzdA \) gene under control of its own promoter located adjacent to the \( P_{bzdA} \)lac\( Z \) reporter fusion (Fig. 4), and pCK01BzdR (\( Plac:bzdR \)), which encodes

### Table 1

| Strain or plasmid | Relevant genotype and characteristic(s) | Reference or source |
|-------------------|-----------------------------------------|---------------------|
| **E. coli strains** |                                          |                     |
| DH5a              | endA1 hsdR17 supE44 thi-1 recA1 gyrA(Nal') relA1 ΔargF-lac) U169 depR | Ref. 8              |
| S17-1Apir         | Tp' Sm' recA1 hsdRM' R B 2-Cm tet M: Km Tn7 7Cm Ap' phage lysogen | Ref. 9              |
| MC4100            | F', araD319 Δ(argF-lac) U169 rpsL150(3'm') relA1 tbrB5301 deoC1 T1S(pT25) rbs | Ref. 10             |
| M15               | Strain for regulated high level expression with pQE vectors | Qiagen              |
| AFMC              | MC4100 spontaneous rifampicin-resistant mutant | Ref. 11             |
| AFMCRP\(_N\)      | AFMC harboring a chromosomal insertion of a \( P_{bzdA} \)translational fusion; Km' | This work           |
| AFMCP\(_N\)       | AFMC harboring a chromosomal insertion of a \( P_{bzdA} \)translational fusion; Km' | This work           |
| **Azoarcus strains** |                                          |                     |
| Azoarcus sp. strain CIB | Wild type strain | Ref. 2              |
| Azoarcus sp. strain CIB\( bzdR \) | Azoarcus sp. strain CIB with a disruption in the \( bzdR \) gene | This work           |
| pUC18             | Ap', oriColE1 high copy number cloning vector, lacZa | Ref. 8              |
| pBzdR4            | Ap', pUC18 containing the \( bzdA \) gene under the control of the \( Plac \) promoter | This work           |
| pUCBZDA           | Ap', pUC18 containing the \( bzdA \) gene under the control of the \( Plac \) promoter | This work           |
| pS3J3             | Ap', oriColE1 lacZ promoter probe vector, lacZ fusion flanked by NotI sites | Ref. 2              |
| pS3J3F\(_N\)      | Ap', 598-bp KpnI/XbaI \( bzdRN \) fragment (\( P_{bzdn} \) promoter) cloned into KpnI/XbaI double-digested pS3J3 vector | Ref. 2              |
| pS3J3RP\(_N\)     | Ap', 2070-bp KpnI/XbaI \( P_{bzdA} \)\( P_{bzdR} \)\( P_{bzdR} \) cloned into KpnI/XbaI double-digested pS3J3 vector | This work           |
| pUMiniTn5Km2      | Ap', Km', B6KorV RP4orT, miniTn5Km2 transposon delivery plasmid | Ref. 9              |
| pUMiniTn5F\(_N\)  | Ap', Km', pUMiniTn5Km2 derivative harboring a 4.5-kb \( NtI \) fragment that includes the \( P_{bzdA} \)lac\( Z \) translational fusion | This work           |
| pECOR7            | Ap', pUC19 harboring a 7.1-kb EcoRI DNA fragment containing the \( bzdRN \) genes | Ref. 2              |
| pBRR1MCS-2        | Km', oriPpBRR1MCS Mob+ lacZa, broad-host-range cloning and expression vector | Ref. 13             |
| pBRR1MCS-5        | Gm', oriPpBRR1MCS Mob+ lacZa, broad-host-range cloning and expression vector | Ref. 13             |
| pBRR2P\(_N\)      | Km', pBRR2MCS-2 derivative harboring the 4.2-kb EcoRI/HindIII fragment that contains the \( P_{bzdA} \)lac\( Z \) translational fusion from pS3J3P\(_N\) | This work           |
| pBRR5\(_N\)       | Gm', pBRR5MCS-5 derivative harboring the 4.2-kb EcoRI/HindIII fragment that contains the \( P_{bzdA} \)lac\( Z \) translational fusion from pS3J3P\(_N\) | This work           |
| pCK01             | Km', oriPpCS101, low copy number cloning vector polylinker flanked by NotI sites | Ref. 12             |
| pCK01BzdR         | Km', pCK01 derivative harboring a 1.6-kb DNA fragment from pECOR7 containing the \( bzdR \) gene under the control of \( Plac \) | This work           |
| pCK01BzdA         | Km', 1.9-kb fragment from pUCBZDA containing the \( bzdA \) gene cloned into EcoRI/XbaI double-digested pCK01 vector under the control of the \( Plac \) promoter | This work           |
| pK1Smob           | Km', oriColE1 Mob+ lacZa, used for directed insertional disruption | Ref. 14             |
| pK1SmobddzR       | Km', 499-bp blunt-ended \( bzdR \) internal fragment cloned into SmaI-digested pK1Smob | This work           |
| pREP4             | Km', plasmid that expresses the lac repressor | Qiagen              |
| pQE32             | Ap', oriColE1 T5promoter lac operator, \( \lambda t_1/E. coli \) rimB T1 terminators, \( NtI \)-terminal His\(_9\) | Qiagen              |
| pQE32-His\(_9\)BzdR| Ap', pQE32 derivative harboring the His\(_9\)bzdR gene | This work           |
| pAFK3             | Cm', pCK01 derivative expressing the \( E. coli \) paaK gene | Ref. 12             |
| pUC18FP4          | Ap', pUC18 derivative harboring the 268-bp XbaI/EcoRI \( P_{bzdR} \) fragment | This work           |
the bzdR gene under control of the heterologous Plac promoter (Table I). Whereas β-galactosidase assays of permeabilized E. coli AFMC (pSJ3P₆) cells grown anaerobically in glycerol-containing mineral medium revealed expression of P₆ (Fig. 5A), the presence of the BzdR protein both in cis (E. coli AFMC (pSJ3RP₆) cells) or in trans (E. coli AFMC (pSJ3P₆, pCK01BzdR) cells) repressed the expression of the P₆::lacZ translational fusion (Fig. 5A). As expected, β-galactosidase levels in E. coli AFMC cells harboring plasmid pSJ3P₆ and the control plasmid pCK01 were similar to those observed in E. coli AFMC (pSJ3P₆) cells (Fig. 5A). To analyze faithfully the bzd regulatory system at monocopy dosage, the P₆::lacZ and P₆::bzdR-P₆::lacZ reporter fusions were subcloned as NotI-DNA cassettes within mini-Tn₅ vectors, rendering plasmids...
Regulation of Benzoyl-CoA Cluster in Azoarcus sp. CIB

Control plasmid pCK01 (medium. PN the decrease in the genetic dosage, the repressor role of BzdR on 3-chlorobenzoate (PA)

Azoarcus pathway from pBadR, AadR, and HbaR controlling degradation of benzoate and (6, 7) and TutBC and TdiRS

afact that benzoate is not able to act as an inducer of the

As shown in Fig. 5C, only 2-fluorobenzoate was able to avoid significantly the repression of the P_N::lacZ fusion by the BzdR protein. Since Azoarcus sp. strain CIB is able to grow on benzoate and 2-fluorobenzoate as the sole carbon sources but, however, is unable to catabolize any of the other aromatic
compounds cited above, it is tempting to speculate that BzdR is only able to efficiently recognize aromatic CoA derivatives that can be catabolized through the bzd-encoded pathway, suggesting that this regulator has evolved to become efficiently adapted to such a particular catabolic system.

Although previous reports have suggested that the inducer of the genes involved in anaerobic catabolism of benzoate in *R. palustris* and *T. aromatica* might be benzoyl-CoA rather than benzoate (1, 43), no direct experimental evidence supporting this assumption has been provided. Here we present for the first time experimental evidence that benzoyl-CoA is the actual inducer of the pathway in *Azoarcus* sp. CIB. It should be noted that another aromatic CoA derivative, phenylacetyl-CoA, was shown to be the inducer of an aerobic hybrid pathway, the phenylacetic acid degradation pathway, whose intermediates are also CoA-derived compounds (11). Whether transcriptional regulators of both anaerobic and aerobic hybrid pathways for catabolism of aromatic acids have evolved to recognize CoA-derived aromatic compounds rather than the free acids is a hypothesis that requires further confirmation when additional regulatory systems of such type of degradative routes become characterized.

In Vitro Binding of BzdR to the *PNo* Promoter—To study in vitro the interaction of the BzdR protein with the *PNo* promoter, we first mapped the transcription start site of this promoter and overproduced the regulatory protein in recombinant *Escherichia coli* cells. Primer extension analysis was performed with total RNA isolated from *Azoarcus* sp. strain CIB cells containing plasmid pBBR2Pf (Table I). The transcription initiation site in the *PNo* promoter was mapped 75 nucleotides upstream of the ATG translation initiation codon of the *bzdN* gene (Fig. 6), showing putative −10 (TAACAT) and −35 (TCAACA) boxes typical of σ^70^-dependent promoters (Fig. 1B).

To overproduce BzdR, we have engineered plasmid pQE32-His6BzdR (Table I) that expresses a His-tagged BzdR protein (see “Experimental Procedures”). As expected, the His6-BzdR protein was a repressor of the *PNo* promoter in *E. coli* AFMCNP (pQE32-His6BzdR) cells growing anaerobically in glycerol-containing minimal medium (Fig. 7A). Induction by benzoyl-CoA of the His6-BzdR mediated repression of *PNo* was confirmed in *E. coli* AFMCNP (pQE32-His6BzdR, pCK01BzdA) cells growing anaerobically in glycerol-containing minimal medium in the presence (induction) or absence of benzoate (Fig. 7A). These results indicate that the functionality of His-tagged BzdR is similar to that of the wild-type protein, and, thus, His6-BzdR can be used to study the interaction of this regulator with the cognate *PNo* promoter. The purification of the His6-BzdR protein was carried out through affinity chromatography as described under “Experimental Procedures,” and it was checked by SDS-PAGE (Fig. 7B). To demonstrate the interaction of the BzdR regulatory protein with the *PNo* promoter, the purified His6-BzdR protein was subjected to gel retardation assays, using as probe a 268-bp DNA fragment that carries the *PNo* promoter region from position −174 to +79 (*PNo* probe) (Fig. 1B). The His6-BzdR protein was able to retard the migration of the *PNo* probe in a protein concentration-dependent manner (Fig. 8A). Moreover, binding of BzdR to the *PNo* promoter was highly specific, because whereas it was inhibited by adding an unlabeled *PNo* probe to the retardation assays, it was not affected by adding an unlabeled heterologous probe (data not shown). Depending on the amount of His6-BzdR protein used in the retardation assay, two distinct complexes, complex 1 (higher mobility) and complex 2 (lower mobility), were observed (Fig. 8A). Thus, whereas complex I appeared preferentially at low amounts (2.5–10 nM) of His6-BzdR protein, 5-fold higher amounts of His6-BzdR gave rise to complex II (Fig. 8A). These results might suggest that BzdR binds to different regions at the *PNo* promoter (see below). Gel retardation assays with His6-BzdR were also carried out in the presence of different concentrations of the benzoyl-CoA (Sigma) inducer molecule. As shown in Fig. 8B, increasing concentrations of benzoyl-CoA ranging from 250 μM to 2 mM significantly reduced the amount of bound *PNo* probe, suggesting that benzoyl-CoA inhibits binding of His6-BzdR to the *PNo* promoter. Although a percentage of *PNo* probe (about 20%) remained free when 2 mM benzoate was added to the retardation assay, this percentage increased to 95% when 2 mM benzoyl-CoA was used (Fig. 8B). All of these data taken together are in agreement with the lacZ-reporter fusion experiments reported above, and they confirm BzdR as the repressor of the *PNo* promoter and benzoyl-CoA as the actual inducer molecule. Nevertheless, the molecular mechanism of the benzoyl-CoA-mediated induction of the *PNo* promoter is still unknown, and it will be subject of future research.

**Identification of the BzdR Binding Site(s)—**The purified His6-BzdR protein was also used to determine the BzdR-binding site(s) (operator) in the *PNo* promoter region by DNase I footprinting. As shown in Fig. 9, the His6-BzdR protein protected three different regions: region I (63 bp) spanning from position −32 to +31, region II (21 bp) spanning from position −83 to −63, and region III (21 bp) spanning from position −146 to −126 (Fig. 1B). The three protected regions contain direct repetitions of a sequence, TGCA, that forms a part of longer palindromic structures (Fig. 1B). Whereas the TGCA sequences are separated by 6 nucleotides in regions II and III, the longer region I presents a pair of TGCA sequences separated by 1 nucleotide and another pair of TGCA sequences separated by 15 nucleotides (Fig. 1B). Other members of the HTH-XRE family of transcriptional regulators (see above) such as the SinR regulator from *B. subtilis* and the Cro and 434 repressors
from phages λ and 434, respectively, also bind to short repeated sequences that span the promoters (38, 44–46). Binding of such regulators to the multiple operators of their cognate promoters generates different protein-DNA complexes in gel retardation assays (38, 47), a pattern of binding that was also observed with BzdR at the PN promoter (Fig. 8). Moreover, binding of BzdR induces changes in the DNA structure of PN as revealed by several phosphodiester bonds that become hypersensitive to DNase I cleavage (Fig. 9). The fact that BzdR-binding region I spans the transcription initiation site as well as the −10 sequence for recognition of the σ70 RNA polymerase (Fig. 1B) is in agreement with the observed repressor role of BzdR at the PN promoter. Nevertheless, to precisely understand the molecular basis of the BzdR-mediated repression at the PN promoter, more work needs to be done.

### FIG. 7. Activity and purification of the His6-BzdR protein. A, E. coli AFMCPN (P_N: lacZ) cells harboring plasmids pQE32-His6BzdR (His6-bzdR), pCK01BzdA (bsdA), or the control plasmid pQE32 were grown anaerobically in glycerol-containing minimal medium in the absence (empty blocks) or presence (filled blocks) of 1 mM benzoate. Values for β-galactosidase activity (in Miller units) were determined as indicated under “Experimental Procedures.” Results of one experiment are shown, and values were reproducible in three separate experiments with S.D. values of <10%. B, analysis on a 12.5% SDS-PAGE of the purification of His6-BzdR from E. coli M15 (pREP4, pQE32-His6BzdR) cells. Lane 1, molecular mass markers (in kDa); lane 2, soluble fraction of the crude extract from E. coli M15 (pREP4, pQE32-His6BzdR) cells obtained as described under “Experimental Procedures”; lane 3, extract that flows through the nickel-nitrilotriacetic acid-agarose column; lane 4, washing step; lane 5, purified His6-BzdR protein.

### FIG. 8. Gel retardation analysis of His6-BzdR binding to the PN promoter. Gel retardation analyses were performed as indicated under “Experimental Procedures.” A, lane 1 shows the free PN probe; lanes 2–7 show retardation assays containing 2.5, 5, 10, 25, 50, and 100 nM concentrations, respectively, of purified His6-BzdR protein. B, lanes 1–5 show retardation assays containing 50 nM of purified His6-BzdR in the presence of 0, 0.25, 0.5, 1, or 2 mM benzoyl-CoA (Sigma), respectively; lane 6, retardation assay containing 50 nM purified His6-BzdR protein in the presence of 2 mM benzoate. The PN probe and the PN-BzdR complexes (complexes 1 and 2) are indicated by the arrows.

### Modeling of BzdR and Docking Simulations of Benzoyl-CoA Binding—With the aim of building three-dimensional models of BzdR, we carried out a 10-fold recognition approach for the N-terminal region, from residue 25 to 87 (N-BzdR), and the C-terminal region, residues 131–298 (C-BzdR), of the protein. The five threading methods used in this work found potential templates with high scores, always above the 95% certainty level (see “Experimental Procedures”). The threading results for N-BzdR revealed that all of the templates found belong to the same SCOP superfamily, the repressor-like DNA-binding domains. Pairwise comparisons of the Cα atoms of the crystal structures of all of the templates found, as calculated with the program O (35), yield a maximum root mean square deviation of 1.3 Å and correspond to a five-helix closed bundle folding (Fig. 10A). The template selected for building a three-dimensional model of N-BzdR was SinR of B. subtilis (1b0n) (Table II), since
it shares the highest identity. The presence within this fold of an HTH (residues 38–58 of BzdR) (Figs. 2 and 10A), a motif found in numerous DNA-binding proteins (51), provides a reasonable structural basis for suggesting N-BzdR as the region directly interacting with the target DNA.

The threading results obtained for C-BzdR also provided consistent results, since all of the hits found share the same SCOP fold, the P-loop-containing nucleoside triphosphate hydrolase fold, almost all of them belonging to the shikimate kinase family (see “Experimental Procedures”). The template used to build a full-atom three-dimensional model of C-BzdR was the shikimate kinase I of E. coli (52, 53) (Fig. 2). Interestingly, the amino acid sequence identity shared between C-BzdR and the selected template (23%) is similar to that observed between the two different isoenzymes of E. coli, shikimate kinases I and II (30% identity) (39). This observation indicates that the protein fold of shikimate kinases is highly versatile, since it can accommodate a wide array of sequences without significant structural departures. As shown in Fig. 10B, C-BzdR presents a canonical mononucleotide-binding fold (54) found in a number of structurally diverse proteins (55), which is constituted by a five-stranded parallel β-sheet, strand order 23145, flanked by eight α-helices. Within this highly conserved fold, a phosphate-binding loop (P-loop) or Walker-A motif (56, 57) can be observed between β1 and α5 (Fig. 10B). The Walker-A motif (consensus sequence: GLRGAGK(T/S)) is
highly conserved in all BzdR orthologues (Fig. 2), and, in fact,
the suggested three-dimensional structure of C-BzdR reveals
that the environment around the P-loop is also essentially
conserved in all of these proteins. Moreover, the strictly con-
served Gly present in the Walker B-motif of purine nucleotide-
binding proteins (56), usually located around the
strand (55), is also present in BzdR (Gly-209) and its orthologues
(Fig. 2).

The three-dimensional model of C-BzdR permits us to envis-
age a putative three-dimensional model for the complex be-
tween C-BzdR and the effector molecule benzoyl-CoA. The fea-
sibility of this model is accentuated by the striking structural
similarity between both ends of the benzoyl-CoA molecule
(namely the ADP and the benzoyl moieties) and the two sub-
strates of shikimate kinases (ATP and shikimate, respectively).
Manual docking of the ADP moiety of benzoyl-CoA into the
C-BzdR structure is immediate, since the C-BzdR fold has a
conserved nucleotide-binding site (Fig. 10). On the other
hand, the pantothenate and β-mercaptoethylamine units fit a
deep groove (Fig. 10) whose walls are formed by the loops
between residues 163–158 and 231–271 (52, 55). The groove
ends in a cavity, equivalent to the shikimate-binding site de-
described for shikimate kinases (52, 55), that perfectly hosts the
benzoyl moiety of the effector molecule (Fig. 10). It has been
proposed that the enzymatic activity of shikimate kinases pro-
ceeds through an induced fit mechanism, since the loops that
constitute the walls of the groove suffer important conforma-
tional changes upon substrate binding (52). In this sense, bind-
ing of benzoyl-CoA to BzdR might involve a similar confor-
mational change at C-BzdR that would then trigger structural
modifications at the DNA-binding region. The crystallization of
BzdR and the BzdR-benzoyl-CoA complex is currently under
way, since the resolution of their three-dimensional structures
will undoubtedly permit the elucidation of the structural basis
that determines the biological action of this prototype of a new
subfamily of regulatory proteins.

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**TABLE II**

| Method       | N-BzdR        | C-BzdR        |
|--------------|---------------|---------------|
| BIOINBGU     | 1r69 (48.7)   | 1zug (34.2)   |
|              | 1b0n-A (30.7) | 1l4u-A (9e-25) |
|              | 114u-A (7e-23) | 2shk-B (2e-18) |
|              | 3a0h (2e-23)  | 3adk (2e-18)  |
| FFAS         | 1adr (25.45)  | 2r63 (24.40)  |
|              | 2shk-B (23.73) | 3cro-R (27.17) |
|              | 1shk (10.20)  | 2cmk-A (10.11) |
|              | 1kag (18.40)  | 1nks-F        |
| FUGUE        | 1r69 (12.08)  | 2cro (12.08)  |
|              | 1adr (24.68)  | 1shk (24.68)  |
|              | 1dag (18.40)  | 1shk (18.40)  |
| HMM          | 1b0n (7.35e-12)| 2or1 (1.06e-10) |
|              | 1adr (3.00e-10) | 1dag (4.41e-24) |
|              | 16e-A (4.29e-21) | 1via-A (5.14e-21) |
|              | 1kag-A (5.14e-21) | 1via-A (5.14e-21) |
| 3D-PSSM      | 1b0n (3.1e-4) | 1adr (0.330) |
|              | 1dag (0.581)  | 114u-A (0.007) |
|              | 1dag (0.011)  | 1kag (0.014)  |
|              | 1via (0.014)  | 1via (0.014)  |

* In these cases, the E-score is indicated, since the -fold recognition step was not activated because BIOINBGU found good hits in the preliminary search with PSI-BLAST.
* In this case, E values are indicated (E values < 3e-5 are typical of close homologues (28).
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