Bacterial Degradation of Benzoate

CROSS-REGULATION BETWEEN AEROBIC AND ANAEROBIC PATHWAYS

We have studied for the first time the transcriptional regulatory circuit that controls the expression of the box genes encoding the aerobic hybrid pathway used to assimilate benzoate via coenzyme A (CoA) derivatives in bacteria. The promoters responsible for the expression of the box cluster in the β-proteobacterium *Azooarcus* sp., their cognate transcriptional repressor, the BoxR protein, and the inducer molecule (benzoyl-CoA) have been characterized. The BoxR protein shows a significant sequence identity to the BzdR transcriptional repressor that controls the bzd genes involved in the anaerobic degradation of benzoate. Because the box*R* gene is present in all box clusters so far identified in bacteria, the BoxR/benzoyl-CoA regulatory system appears to be a widespread strategy to control this aerobic hybrid pathway. Interestingly, the paralogous BoxR and BzdR regulators act synergistically to control the expression of the box and bzd genes. This cross-regulation between anaerobic and aerobic pathways for the catabolism of aromatic compounds has never been shown before, and it may reflect a biological strategy to increase the cell fitness in organisms that survive in environments subject to changing oxygen concentrations.

Aromatic compounds comprise one-quarter of the earth’s biomass and are the second most widely distributed class of organic compounds in nature next to carbohydrates. Some microorganisms have evolved a complex machinery for the mineralization of aromatic compounds, and therefore, they become crucial in the biogeochemical cycles and in the sustainable development of the biosphere (1–4). There are two major microbial strategies to degrade aromatic compounds depending on the presence or absence of oxygen. In the aerobic catabolism, molecular oxygen is not only the final electron acceptor but also an essential cosubstrate of oxygenases involved in the hydroxylation (activation) and cleavage (dearomatization) of the aromatic ring (5, 6). The anaerobic catabolism, however, relies on a completely different strategy based on coenzyme A (CoA)-dependent activation of the aromatic ring followed by reductive dearomatization and then hydrolytic ring cleavage (3, 4). A third degradation strategy has been described for the aerobic mineralization of some aromatic compounds (e.g. benzoate, phenylacetate, and 2-aminobenzoate) that incorporates features of both the classical aerobic and anaerobic pathways. These aerobic hybrid pathways start with the CoA-dependent activation of the aromatic acids, but then the dearomatization step requires molecular oxygen and the mechanism of ring cleavage is hydrolytic rather than oxygenolytic (3, 7–9).

Benzoate has been widely used as a model compound for the study of the bacterial catabolism of aromatic compounds (4, 10). The anaerobic degradation of benzoate by either facultative or strict anaerobes is initiated by its activation to benzoyl-CoA by the action of an ATP-dependent (AMP-forming) benzoate-CoA ligase. Benzoyl-CoA is then subject of aromatic ring reduction and a modified β-oxidation pathway that ends with an aliphatic C₇-dicarboxyl-CoA derivative (Fig. 1A) (3, 4, 11). On the contrary, the classical aerobic benzoate degradation in bacteria relies on the hydroxylation of the aromatic ring to produce catechol, which is then dearomatized (cleaved) by a dioxygenase (Fig. 1A) (12). A third mechanism to degrade benzoate is via an aerobic hybrid pathway (box pathway) that initiates the activation of benzoate to benzoyl-CoA by a benzoyl-CoA ligase (BclA). Then, a benzoyl-CoA 2,3-epoxidase (BoxAB) and a BoxC dihydrodralase are responsible for the dearomatization and ring-cleavage steps, respectively. The 3,4-dehydrodralyl-CoA semialdehyde formed becomes converted to succinyl-CoA and acetyl-CoA by a β-oxidation-like metabolism (box lower pathway) (Fig. 1A) (8, 13–22).

A search in the bacterial genomes revealed that the box genes are present in many α- and β-proteobacteria and in some δ-proteobacteria (14, 20). Moreover, several bacterial strains...
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FIGURE 1. Major biochemical strategies for benzoate degradation and genetic arrangements of the box clusters in proteobacteria. A, schemes of the first biochemical steps of the classical aerobic (white box) and anaerobic (gray box) benzoate degradation pathways and that of the aerobic hybrid box pathway (black box) are shown. The activation and the deamination/ring-cleavage steps are indicated by white and striped arrows, respectively. The lower pathway that funnels the dehydrodiphenyl-CoA semialdehyde into the central metabolism is represented by a dotted arrow. OX, benzoate dioxygenase; DH, benzoate dihydrodiphenyl dehydrogenase; DOX, catechol dioxygenase (ortho (intradiol) or meta (extradiol) cleavage). The Box enzymes responsible for the activation and deamination/ring-cleavage reactions are indicated: BclA, benzoate-CoA ligase; BoxA, NADPH-dependent reductase; BoxB, benzoate-CoA 2,3-epoxidase; BoxC, 2,3-epoxybenzoate-CoA dihydrodolase. B, schemes of the major arrangements of the functional modules within the box clusters from proteobacteria are shown. The activation (C1, bclA gene), deamination/ring-cleavage (C2, boxABC genes), and lower pathway (C3) catabolic modules are shown by white, striped, and dotted arrows, respectively. The putative regulatory module (boxR gene) is shown by black arrows. It should be noted that the gene composition and arrangement within the C3 module can differ among strains of the same genus. Type 1 box clusters are present in many β-proteobacteria, e.g. strains of the Azoarcus/Aromatoleum, Burkholderia, Delftia, Leptothrix, Magnetospirillum, Comamonas, Achromobacter, Rhodopseudomonas, Rhodopseudomonas, Rhodobacter, and Rhodobacter genera, or in some β-proteobacteria where the C3 module can be missing. Type 3 box clusters are present in other α-proteobacteria where the C3 module contains only one gene and the C2 module lacks the boxA gene, e.g. strains of the Rhodopseudomonas and Bradyrhizobium genera, or in some β-proteobacteria, e.g. Thauera aromatica, where the C3 module is not found between the C1 and C2 modules. In the genome of some δ-proteobacteria, e.g. Sorangium cellulosum, there is a boxR gene divergently oriented to the C2 module (boxBC and boxAB genes).

Contain both the classical and the hybrid pathway for aerobic benzoate degradation. The box pathway was suggested to be specially active at reduced oxygen tension (20, 23, 24). An in silico search among the available bacterial genomes revealed that most box clusters are organized into at least two major functional catabolic units: 1) the activation module (encoded by the bclA gene) and 2) the deamination and ring-cleavage module encoded by the boxAB and boxC genes, respectively. A lower pathway module (encoded by other box genes) can be physically associated or not to the activation and deamination/ring-cleavage module (Fig. 1B). Although the box pathway becomes a major route for aerobic benzoate degradation and its biochemistry and genetics have been investigated in some bacteria, thus far there is no information about the regulatory mechanism that controls the inducible expression of the box genes (14, 22, 24). Interestingly, a common feature of most box clusters is the presence of a boxR gene, first described in Azoarcus strains (14, 25), that encodes a protein that might be a member of the BzdR subfamily of prokaryotic transcriptional regulators (26, 27) and, therefore, might constitute the regulatory unit of the box cluster (Fig. 1B).

Some Azoarcus/Aromatoleum strains (β-proteobacteria) are able to degrade benzoate both aerobically and anaerobically (4, 13, 25). The regulation of the bzd genes involved in the anaerobic benzoate degradation has been studied in Azoarcus sp. CIB (26–28). On the other hand, Azoarcus sp. CIB is also able to degrade benzoate aerobically (26), although the genes involved have not been yet described. In this paper we have identified the box cluster of Azoarcus sp. CIB and studied for the first time the specific regulatory circuit that controls the expression of the box genes in bacteria. Moreover, we have shown the existence of an unexpected transcriptional cross-regulation between the aerobic and anaerobic benzoate degradation pathways.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The Escherichia coli and Azoarcus strains as well as the plasmids used for this study are indicated in Table 1, and the oligonucleotides employed for PCR amplification of the cloned fragments and other molecular biology techniques are summarized in Table 2. To construct the plasmids pSj3P_X and pSj3P_D,
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### Table 1: Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant genotype or phenotype | Ref. or source |
|-------------------|-------------------------------|---------------|
| **E. coli strains** |                               |               |
| CC118             | Ap<sup>r</sup>, orGrE1, T5 promoter lac operator, A<sub>Lac</sub> E. coli rmbB T1 terminators, N-terminal His<sub>6</sub> boxR | Qiagen        |
| DH10B             | Ap<sup>r</sup>, pUC19 harboring a 7.1 kb EcoRI DNA fragment containing the bzdRNA genes | Qiagen        |
| M15               | K<sup>r</sup>, Azoarcus sp. strain CIB with a disruption of the bzdR gene | This work     |
| **Azoarcus sp. strains** |                               |               |
| CIB               | Wild type strain | 26 |
| CIBdboxR          | K<sup>r</sup>, Azoarcus sp. strain CIB with a disruption of the boxR gene | This work     |
| CIBdboxRhboxR     | K<sup>r</sup>, Azoarcus sp. strain CIB with a disruption of bzdR and boxR | This work     |
| CIBdboxA          | K<sup>r</sup>, Azoarcus sp. strain CIB with a disruption of the boxA gene | This work     |
| **Plasmids**      |                               |               |
| pQE32             | Ap<sup>r</sup>, oriColE1, T5 promoter lac operator, A<sub>Lac</sub> E. coli rmbB T1 terminators, N-terminal His<sub>6</sub> boxR | Qiagen        |
| pQE32-3HisboxRdR  | Ap<sup>r</sup>, pQE32 derivative harboring the His<sub>6</sub>-boxR gene | This work     |
| pQE32-HisBoxR     | Ap<sup>r</sup>, pQE32 derivative harboring the His<sub>6</sub>-boxR gene | This work     |
| pREP4             | K<sup>r</sup>, plasmid that expresses the lac repressor | Qiagen        |
| pCER03           | Ap<sup>r</sup>, pUC19 harboring a 7.1 kb EcoRI DNA fragment containing the bzdRNA genes | Qiagen        |
| pKl8mob          | K<sup>r</sup>, oriColE1 Mob<sup>+</sup> lacZ<sup>+</sup>, used for directed insertional disruption. | 26 |
| pKl8mobddboxR    | K<sup>r</sup>, 499 bp blunt-ended boxR internal fragment cloned into Smal-digested pk18mob | 27 |
| pKl8mobddboxA    | K<sup>r</sup>, 578 bp bclA internal fragment cloned into EcoRI/Smal-digested pk18mob | 27 |
| psJSJ1          | Ap<sup>r</sup>, psf3 derivative carrying the translational fusion P<sub>bzdR</sub>boxR | This work     |
| psJSJ2          | Ap<sup>r</sup>, psf3 derivative carrying the translational fusion P<sub>bzdR</sub>boxR | This work     |
| pKNG101          | Sm<sup>r</sup>, oriR6K, Mob<sup>+</sup>, suicide vector used for directed insertional disruption | 35 |
| pKNG101boxR      | Sm<sup>r</sup>, 325 bp BamHI/SpeI boxR internal fragment cloned into BamHI/SpeI double digested pKNG101 vector | This work     |
| pCK01           | Cm<sup>r</sup>, oriPlc101, low copy number cloning vector with polylinker flanked by NorI sites | 36 |
| pCK01Bzldr    | Cm<sup>r</sup>, pCK01 derivative harboring a 1.6 kb DNA fragment from pECOR7 containing the boxR gene under control of Plac | This work     |
| pCK01BoxR      | Cm<sup>r</sup>, pCK01 derivative harboring a 1.0 kb DNA fragment from pQE32-HisboxR containing the His<sub>6</sub>-boxR gene under control of Plac | This work     |

<sup>a</sup> Ap<sup>r</sup>, ampicillin-resistant; Cm<sup>r</sup>, chloramphenicol-resistant; K<sup>r</sup>, kanamycin-resistant; Rf<sup>r</sup>, rifampicin-resistant; Sm<sup>r</sup>, spectinomycin-resistant. 36 |

### 775-bp PCR-amplified fragments that include the boxD-boxR intergenic region were obtained by using Azoarcus sp. CIB genomic DNA as template and the oligonucleotides 5′-boxR/3′-boxR (P<sub>x</sub> fragment) and 5′-boxD/3′-boxD (P<sub>p</sub>) fragments (Table 2). The P<sub>x</sub> and P<sub>p</sub> fragments were digested with KpnI and XbaI restriction enzymes and ligated to the KpnI/XbaI double-digested psJS1 promoter-probe vector, rendering plasmids psJS3<sub>Px</sub> and psJS3<sub>Pp</sub>, respectively (Table 1). The correct lac<sub>T</sub> translational fusions were confirmed by nucleotide sequence analysis. The pQE32-His<sub>6</sub>boxR plasmid was constructed by cloning into BamHI/PstI double-digested pQE32 plasmid (Table 1) a 903-bp BamHI/PstI fragment harboring the boxR gene obtained by PCR amplification of Azoarcus sp. CIB genomic DNA with oligonucleotides 5′-Hisbox and 3′-Hisbox (Table 2). The recombinant plasmid pQE32-His<sub>6</sub>boxR expresses under control of the T5 promoter and two lac operator boxes the protein His<sub>6</sub>-boxR that carries 13 amino acids (MRGSHHHHHSHGIL) fused to its N terminus (second amino acid). To construct plasmid pCK01BoxR (Table 1), the His<sub>6</sub>-boxR gene cloned into pQE32-His<sub>6</sub>boxR plasmid was EcoRI/BamHI double-digested and subcloned into an EcoRI/BamHI double-digested pCK01 plasmid. E. coli cells were grown at 37 °C in Luria-Bertani (LB) medium (37). When required, E. coli cells were grown aerobically or anaerobically (using 10 mM nitrate as the terminal electron acceptor) in M63 minimal medium (38) at 30 °C using the corresponding necessary nutritional supplements and 20 mM glycerol as carbon source. Azoarcus strains were grown aerobically or anaerobically (using 10 mM nitrate as the terminal electron acceptor) at 30 °C in MC medium as described previously (26). Where appropriate, antibiotics were added at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), and streptomycin (50 μg/ml).

**Molecular Biology Techniques**—Recombinant DNA techniques were carried out by published methods (37). Plasmid DNA was prepared with High pure plasmid isolation Kit (Roche Applied Science). DNA fragments were purified with Gene Clean Turbo (Q-BIOgene). Oligonucleotides were supplied by Sigma. All cloned inserts and DNA fragments were confirmed by DNA sequencing with an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Transformation of E. coli was carried out by using the RbCl method or by electroporation (Gene Pulser, Bio-Rad) (37). Plasmids were transferred from E. coli S17–1λpir or E. coli SM10λpir (donor strains) into Azoarcus sp. CIB (recipient strains) by biparental filter mating as described previously (26). Proteins were analyzed by SDS-PAGE (39).

**Sequence Data Analyses**—The nucleotide sequence of the box cluster from Azoarcus sp. CIB has been submitted to the GenBank with accession number HE589495. Nucleotide sequence analyses were done at the National Center for Biotechnology Information (NCBI) server (available at World Wide Web at www.ncbi.nlm.nih.gov). Open reading frames searches were performed with the ORF Finder program at the NCBI server (www.ncbi.nlm.nih.gov). Gene cluster search was performed at the KEGG server. The codon adaptation index
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TABLE 2

| Primers | Sequence (5′–3′)* | Use |
|---------|------------------|-----|
| 5′ Hisbox | CGGATCTGCTTACGTTGACAGACGAA (BamHI) | 903-bp boxR fragment cloned into BamHI/PstI double digested pQE32 to generate plasmid pQE32-His6BoxR |
| 3′ Hisbox | AAATCTTGTCACGATGTCACGTTGGC (PstI) | |
| 5′ pboxD | GGCGTTACACAGCTCGAGATGTCAG (EcoRI) | 775-bp boxDR intergenic region cloned into the KpnI/XbaI digested pSJ3 vector to give rise to plasmid pSJ3boxR |
| 3′ pboxD | GCTTTAAGTTGCGTTTTCGCGTTGC (XbaI) | 775-bp boxDR intergenic region cloned into the KpnI/XbaI digested pSJ3 vector to give rise to plasmid pSJ3boxR |
| 5′ pboxR | GCTTTAAGTTGCGTTTTCGCGTTGC (XbaI) | |
| 3′ pboxR | CGGATCTGCTTACGTTGACAGACGAA (BamHI) | |
| FPAdh 5′ | CGGATCTGCTTACGTTGACAGACGAA (EcoRI) | 234-bp boxDR intergenic fragment for footprinting assays |
| FPAdh 3′ bis | AAAATCTTGTCACGATGTCACGTTGGC (PstI) | 266-bp boxDR intergenic fragment for gel retardation and footprinting assays |
| 5′ FPAdhRev | AAAATCTTGTCACGATGTCACGTTGGC (PstI) | 525-bp boxR internal fragment cloned into BamHI/SpeI digested pKG101 vector to generate plasmid pKG101boxR |
| 3′ FPAdhRev | AAAATCTTGTCACGATGTCACGTTGGC (PstI) | 152-bp boxR fragment for RT-PCR assays |
| 5′ boxRmut | GCTTTAAGTTGCGTTTTCGCGTTGC (BamHI) | 124-bp boxR fragment for RT-PCR assays |
| 3′ boxRmut | GCTTTAAGTTGCGTTTTCGCGTTGC (BamHI) | |
| 5′ boxRq | ATCTGCGGACGAGAAGATGCG | 153-bp P1 promoter fragment for RT-PCR assays |
| 3′ boxRq | ATCTGCGGACGAGAAGATGCG | |
| 5′ RtpN1 | GCGACGCTCGAGAGGATAATGCAGTAG | 141-bp P1 promoter fragment for RT-PCR assays |
| 3′ RtpN1 | GCGACGCTCGAGAGGATAATGCAGTAG | |
| 5′ boxDext | GGACTAGTGCCATGTGTTCTTCCGGCTGC | Used to analyze by PCR disruption insertions with pK18mob |
| 3′ boxDext | GGACTAGTGCCATGTGTTCTTCCGGCTGC | Used to analyze by PCR disruption insertions with pK18mob |
| Lac 57 | GGTTACGGTTGATACGGGTCAGACG | Used to analyze by PCR disruption insertions with pKNG101 |
| F24 | GGTTACGGTTGATACGGGTCAGACG | Used to analyze by PCR disruption insertions with pKNG101 |
| R24 | GCGATTTGACATATTCACTACAGA | 166-bp internal fragment of dnaE (encoding the subunit of DNA polymerase III) used as internal control in RT-PCRs |
| FpKNG101 | GTCCGCCGCTCGATACCTTCGCG | 578-bp bzdA internal fragment cloned into EcoRI/SmaI digested pK18mob vector to generate pK18mobbzdA |
| RpkKNG101 | CTGCCGCGCCGCGATACTTGC | |
| 5′POLIIIHK | CGAAACTCTGGATCGAAGCACCG | | |
| 3′POLIIIHK | CGAAACTCTGGATCGAAGCACCG | | |
| 5′LigO21055 | GCCGATTTGAAATGCGACACATCCTTCATGCG (EcoRI) | |
| 3′LigO21633 | GCCGATTTGAAATGCGACACATCCTTCATGCG (SmaI) | |

* Engineered restriction sites are underlined, and the corresponding restriction enzyme is shown in parentheses.

(CAI) was determined at the CAIcal server (40) using the Azoarcus sp. CIB whole genome nucleotide sequence. The amino acid sequences of the open reading frames were compared with those present in databases using the TBLASTN algorithm (41) at the NCBI server (blast.ncbi.nlm.nih.gov). Pairwise and multiple protein sequence alignments were made with the ClustalW program (42) at the EMBL-EBI server. Phylogenetic analysis of BoxR-like proteins was carried out according to the neighbor-joining method (43) of the PHYLIP program (45). Protein secondary structure prediction was performed by using the Jpred3 program (46).

Construction of Azoarcus sp. CIBboxR, Azoarcus sp. CIBbclA Strains—For insertional disruption of the boxR gene through single homologous recombination, a 525-bp internal fragment of the boxR gene was PCR-amplified with primers 5′ boxRmut and 3′ boxRmut (Table 2), and it was cloned into the BamHI/SpeI digested pKNG101 plasmid. The resulting construct, pKNG101boxR (Table 1), was transferred from E. coli SM10pir (donor strain) into Azoarcus sp. CIB (recipient strain) by biparental filter mating. An exconjugant, Azoarcus sp. CIBboxRbzdR, was isolated aerobically on streptomycin- and kanamycin-containing MC medium harboring 10 mM glutarate as the sole carbon source for counterselection of donor cells. The mutant cells were analyzed by PCR to confirm the disruption of the target gene. For insertional disruption of the boxD gene through single homologous recombination, a 578-bp intergenic region cloned into the KpnI/XbaI digested pSJ3 vector to give rise to plasmid pSJ3boxD, a 578-bp boxDR intergenic region cloned into the KpnI/XbaI digested pSJ3 vector to give rise to plasmid pSJ3boxDR, and an 5′-end, under control of the P1promoter and two lac operator.
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boxes. The His-tagged BoxR and BzdR proteins were overproduced in E. coli M15 strain-harboring plasmids pQE32-His5BoxR and pQE32-His5BzdR, respectively, and the pREP4 plasmid (Table 1) that produces the LacI repressor to strictly control gene expression from pQE32 derivatives in the presence of isopropyl-1-thio-β-D-galactopyranoside. E. coli M15 (pREP4, pQE32-His5BoxR) and E. coli M15 (pREP4, pQE32-His5BzdR) cells were grown at 37 °C in 100 ml of ampicillin- and kanamycin-containing LB medium until the cultures reached midexponential growth phase. Overexpression of the His-tagged proteins was then induced during 5 h by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested at 4 °C, resuspended in 10 ml FP buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM β-mercaptoethanol, and 50 mM KCl), and disrupted by passage 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 25 min at 4 °C. The protein concentrations in cell extracts were determined following the method of Bradford (47) using bovine serum albumin as the standard. The amount of His5-BoxR and His5-BzdR proteins in the cell extracts was estimated by densitometry of the corresponding bands in a Coomassie Brilliant Blue-stained 12.5% SDS-PAGE, and it was about 12 and 10% of the total protein, respectively (supplemental Figs. S1).

Gel Retardation Assays—The PNa probe was obtained as described previously from plasmid pECOR7 (27). The boxDR probe was PCR-amplified from pSJ3Pr (Table 1) by using oligonucleotides 5′FPAdhRev and 3′FPAdhRev (Table 2). The amplified DNA was then digested with Scal and EcoRI restriction enzymes and labeled by filling in the overhanging EcoRI-digested end with [α-32P]dATP (6000 Ci/mmol; PerkinElmer Life Sciences) and the Klenow fragment of E. coli DNA polymerase I as described previously (37). The retardation reaction mixtures in FP buffer contained 0.5 μM DNA probe, 500 μg/ml bovine serum albumin, 25 μg/ml herring sperm (competitor) DNA, and His5-BoxR cell extracts in a 9-μl final volume. After incubation of the retardation mixtures for 20 min at 30 °C, the reactions were analyzed by electrophoresis in 5% polyacrylamide-urea gels. The gels were dried on Whatman No. 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences).

DNase I Footprinting Assays—The boxD-boxR intergenic fragments were obtained by PCR amplification from pSJ3PrD and pSJ3PrR plasmids (Table 1) with the oligonucleotide pairs FPAdh5′/FPAdh3′bis and 5′FPAdhRev/3′FPAdhRev (Table 2), respectively. To label the fragment at the boxD end, the amplified DNA was digested with Scal and EcoRI restriction enzymes, and the resulting 234-bp fragment was single-end labeled by filling in the overhanging EcoRI-digested end with [α-32P]dATP (6000 Ci/mmol; PerkinElmer Life Sciences) and the Klenow fragment of E. coli DNA polymerase I, as described previously (27). To label the fragment at the boxR end, the PCR amplification reaction was performed with the 3′FPAdhRev primer previously labeled at its 5′-end with phage T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences). For DNase I footprinting assays, the reaction mixture contained 2 nM DNA probe, 1 mg/ml bovine serum albumin, and cell extracts in 15 μl of FP buffer (see above). This mixture was incubated for 20 min at 30 °C, after which 3 μl (0.05 unit) of DNase I (Amersham Biosciences) (prepared in 10 mM 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 0.3 μg/ml glycogen. After phenol extraction, DNA fragments were analyzed as previously described (27). A + G Maxam and Gilbert reactions (48) were carried out with the same fragments and loaded on the gels along with the footprinting samples. The gels were dried on Whatman No. 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences).

RT-PCR and Real-time RT-PCR Assays—Total RNA was extracted from Azoarcus sp. CIB cells grown aerobically or anaerobically in MC medium harboring the appropriate carbon source. Cells were then harvested at the mid-exponential phase of growth and stored at −80 °C. Pellets were thawed, and cells were lysed in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 5 mg/ml lysozyme. RNA was extracted using the RNaseasy mini kit (Qiagen), including a DNase I treatment according to the manufacturer instructions, precipitated with ethanol, washed, and resuspended in RNase-free water. The concentration and purity of the RNA samples were measured 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 1 μg 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-PCR reactions, the cDNA was amplified with 1 unit of AmpliTaq DNA polymerase (Biotools) and 0.5 μM concentrations of the corresponding primer pairs. Control reactions in which reverse transcriptase was omitted from the reaction mixture ensured that DNA
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products resulted from the amplification of cDNA rather than from DNA contamination. For real-time RT-PCR assays, the cDNA was purified using GeneClean Turbo kit (MP Biomedicals), and the concentration was measured using a ND100 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 pairs of oligonucleotides used to amplify the mRNA driven by the PboxD (boxD) and PboxN (boxN) promoters and that of the boxR and bzdR genes were 5'pbboxDQ/3'pbboxDQ, 5'RTpN1/3'RTpN2, 5'boxRq/3'boxRq, and 5'bdzRq/3'bdzRq, respectively, and their sequences are detailed in Table 2. 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'POLIIIH2/3'POLIIIH2 (Table 2) and used to normalize the sample data. 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; annealing temperature, 60 °C, 1 min; and elongation and signal acquisition, 72 °C, 30 s). Each reaction was performed in triplicate. After the PCR melting curve was generated to confirm the amplification of a single product. For relative quantification of the fluorescence values, a calibration curve was constructed for each amplicon by 5-fold serial dilutions of an Azoarcus sp. CIB genomic DNA sample ranging from 0.5 ng to 0.5 × 10^{-4} ng. This curve was then used as a reference standard for extrapolating the relative abundance of the cDNA targets within the linear range of the curve. Results were normalized relative to those obtained for the dnaE internal control.

β-Galactosidase Assays—The β-galactosidase activities were measured with permeabilized cells when cultures reached mid-exponential phase as described by Miller (38).

Cell Viability Assays—Azoarcus sp. CIB, Azoarcus sp. CIBboxR, Azoarcus sp. CIBbdzRd, and Azoarcus sp. CIBboxRhbdzR strains were grown anaerobically in MC medium (in the absence of antibiotics) with 10 mM glutarate as the sole carbon source until the cells reached an A_{600} of 0.1. The number of viable cells in four replicates was determined on MC medium agar plates containing 10 mM glutarate as the unique carbon source, and no antibiotic was added to the medium to avoid its negative effect on the growth yield. The plates were incubated for 72 h at 30 °C, and then the number of colony forming units (CFUs) was calculated.

RESULTS AND DISCUSSION

boxR Gene Encodes a Transcriptional Regulator of box Genes in Azoarcus sp. CIB—During the course of a genome sequenc- ing project of Azoarcus sp. CIB, we identified a set of 16 genes that show a high identity (>90%) and a similar organization to the box cluster previously characterized in Azoarcus evansii (14) and also a significant identity (>80%) with that predicted in Aromatoleum aromaticum EbN1 (25) and Azoarcus sp. BH72 (49), strongly suggesting the existence of identical benzoate-degradation pathways in these bacteria (supplemental Figs. S2). The construction of an Azoarcus sp. CIBbdzA strain harboring a disrupted bclA gene (Table 1) and the observation that this mutant strain lacked the ability to grow aerobically on benzoate as the sole carbon source (data not shown) confirmed the participation of the identified box cluster in the aerobic degradation of benzoate in Azoarcus sp. CIB.

As occurs in A. evansii (14), the box cluster from Azoarcus sp. CIB is arranged in at least two divergent operons driven by the P_{box} and P_{box} promoters (supplemental Figs. S2). The boxR gene from Azoarcus sp. CIB, which corresponds to off10 in the box cluster from A. evansii (14), encodes a protein of 300 amino acids that shows an overall 47% sequence identity and a similar domain organization than the BzdR transcriptional repressor that controls the bzd genes responsible for the anaerobic degradation of benzoate in this strain (27). Thus, whereas the N-terminal region of BoxR (residues 1–93) exhibits significant similarity with transcriptional regulators of the HTH-XRE family, the C-terminal domain of BoxR (residues 133–300) presents high identity with shikimate kinases (Fig. 2). The central region of BoxR (residues 94–132) corresponds to the linker region of BzdR involved in the transmission of the conformational change from the C-terminal effector binding domain to the N-terminal DNA binding domain (50). Interestingly, the central regions of both regulators show the lowest amino acid sequence identity (24%) (Fig. 2).

To demonstrate whether boxR regulates the box pathway, the expression of the box genes was monitored by RT-PCR analysis in the wild-type strain and in the Azoarcus sp. CIBboxR mutant strain that harbors a disruption insertion of the boxR gene (Table 1). Whereas the wild-type strain showed a benzoate-inducible expression of the boxD gene, this catabolic gene was efficiently expressed both in the presence or in the absence of benzoate in the boxR mutant strain (Fig. 3A), thus suggesting that boxR encodes a transcriptional repressor of the box genes.

To further investigate the regulatory role of the BoxR protein on the expression of the box genes, the activity of the P_{box} and P_{box} promoters was monitored in a heterologous host, i.e. E. coli, in the absence or presence of the boxR gene. To accomplish this, the boxD-boxR intergenic region was PCR-amplified and cloned in both orientations in the pSJ3 promoter probe vector, giving rise to plasmids pSJ3P_{box} and pSJ3P_{box} that express the P_{box}::lacZ and P_{box}::lacZ translational fusions, respectively (Table 1). On the other hand, the boxR gene was cloned under the control of the heterologous Plac promoter-producing plasmid pCK01BoxR (Table 1). The β-galactosidase assays of permeabilized E. coli CC118 (pSJ3P_{box}) and E. coli CC118 (pSJ3P_{box}) cells grown aerobically in glycerol-containing minimal medium revealed that both promoters were active, although P_{box} was about 3.5-fold more active than P_{box} (Fig. 3B). Remarkably, the activity of both promoters was drastically reduced in the presence of the boxR gene as shown by the low β-galactosidase levels measured in permeabilized E. coli CC118 (pSJ3P_{box}, pCK01BoxR) and E. coli CC118 (pSJ3P_{box}, pCK01BoxR) cells (Fig. 3B), suggesting that the regulatory circuit also works in the heterologous host. Therefore, these results confirm that P_{box} and

5 The abbreviations used are: CFU, colony forming units; HTH, helix-turn-helix.
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are functional promoters whose activity becomes negatively regulated by the product of the \textit{boxR} gene. All these results taken together reveal that the \textit{boxR} gene product behaves as a transcriptional repressor of the \textit{box} genes in \textit{Azoarcus} sp. CIB, and most probably a similar regulatory system may account for the transcriptional control of the \textit{box} genes in other bacteria. A phylogenetic tree of all members of the BzdR subfamily present in the databases reveals a good correlation between the taxonomical position of the organism, i.e. \(\alpha\), \(\beta\), and \(\gamma\)-proteobacteria, and the level of identity among BoxR homologues (supplemental Figs. S3). The phylogenetic analysis also suggests that in the evolution of

\[\text{FIGURE 2.} \] Amino acid sequence comparison between BzdR and BoxR proteins from \textit{Azoarcus} sp. CIB. The amino acid sequences of BzdR (AAQ08805) and BoxR (CCD33120) were aligned using the multiple sequence alignment program ClustalW. The amino acid residues of each sequence are numbered at the right. Amino acids are indicated by their standard one-letter code. Dark gray shows identical residues in the two sequences, whereas light gray indicates functional similarity between residues. The \(\alpha\)-helices and \(\beta\)-strands predicted for the BzdR (top) and BoxR (bottom) proteins are also drawn. The N terminus, linker, and C-terminal regions of both proteins are indicated at the top. The predicted helix-turn-helix (HTH) motif for DNA binding is marked within the N-terminal region.

\[\text{FIGURE 3.} \] The \textit{boxR} gene encodes a transcriptional repressor of the \textit{box} genes. A, agarose gel electrophoresis of RT-PCR products is shown. Total RNA was isolated from \textit{Azoarcus} sp. CIB (CIBwt) or \textit{Azoarcus} sp. CIB\textit{boxR} (CIB\textit{boxR}) cells grown in alanine (0.4%)-containing MC medium in the presence (lanes 1 and 5) or in the absence (lanes 3 and 7) of 1 mM benzoate (Bz). RT-PCRs were performed as indicated under “Experimental Procedures” with the primer pair 5’\textit{boxD}Q/3’\textit{boxD}Q (Table 2) that amplifies a 153-bp fragment of the \textit{boxD} gene (arrow). Lanes 2, 4, 6, and 8, control reactions in which reverse transcriptase was omitted from the reaction mixture. Lane M, molecular size markers (HaeIII-digested \(\Phi\)X174 DNA). Numbers on the right represent the sizes of the markers (in base pairs). B, shown is \(\beta\)-galactosidase activity of \textit{E. coli} CC118 cells grown aerobically in glycerol-containing minimal medium and harboring plasmids pSJ3PD (PD::\textit{lacZ}) (white bars) or pSJ3PX (PX::\textit{lacZ}) (black bars) and the pCK01\textit{BoxR} (BoxR) or the control plasmid pCK01 (\textit{−}). Values for \(\beta\)-galactosidase activity (in Miller units) were determined as indicated under “Experimental Procedures.” Each value is the average from three separate experiments; error bars indicate S.D.

\(P_x\) are functional promoters whose activity becomes negatively regulated by the product of the \textit{boxR} gene.

All these results taken together reveal that the \textit{boxR} gene product behaves as a transcriptional repressor of the \textit{box} genes in \textit{Azoarcus} sp. CIB, and most probably a similar regulatory system may account for the transcriptional control of the \textit{box} genes in other bacteria. A phylogenetic tree of all members of the BzdR subfamily present in the databases reveals a good correlation between the taxonomical position of the organism, i.e. \(\alpha\), \(\beta\), and \(\gamma\)-proteobacteria, and the level of identity among BoxR homologues (supplemental Figs. S3). The phylogenetic analysis also suggests that in the evolution of
the BzdR-like regulators, the widespread BoxR proteins might have evolved and give rise to the anaerobic BzdR regulators that are found so far only in some Azoarcus/Aroma-
toleum strains.

Identification of BoxR Binding Sites and Benzoyl-CoA as Inducer Molecule—To further study the interaction of the BoxR protein with the PD and PX promoters, we first mapped the transcription start sites of the promoters and overproduced the regulatory protein in recombinant E. coli cells. Primer extension analyses were performed with total RNA isolated from exponentially grown E. coli CC118 (pSJ3PD) and E. coli CC118 (pSJ3PX) cells (Figs. 4, A and B). The transcription start site at the PD promoter was mapped in a cytosine located 85 nucleotides upstream of the GTG translation initiation codon of the boxD gene. The transcription start site at the PX promoter was mapped in a cytosine located 34 nucleotides upstream of the ATG translation initiation codon of the boxR gene (Fig. 4C). An identical −35 box (TTGACG) and two very similar −10 boxes (TATT (C or G) T) that resemble the consensus −35 and −10 boxes typical of σ^{32}-dependent promoters (51) were identified in the PD and PX promoters (Fig. 4C).

To overproduce the BoxR protein, the boxR gene was cloned into the pQE32 vector under the control of the strong P_{T5} promoter to render plasmid pQE32-His_{6}BoxR (Table 1), and gene expression was induced in E. coli as detailed under “Experimental Procedures.” Unfortunately, the His_{6}-BoxR protein purified through affinity chromatography in nickel-nitrilotriacetic acid columns forms inactive aggregates, and therefore, we have been obliged to use crude cell extracts of E. coli M15 (pQE32-His_{6}BoxR) as the source of an active BoxR protein.

To demonstrate in vitro that the BoxR regulatory protein directly interacts with the PD and PX promoters, gel retardation assays were performed using as probe the boxD-boxR intergenic region (boxDR probe). As expected, BoxR was able to retard the migration of the boxDR probe in a protein concentration-dependent manner (Fig. 5A). Because the addition of the unlabeled DNA probe prevented the formation of the pro-
tein-DNA complex (Fig. 5C), the BoxR binding was shown to be specific.

To determine the BoxR binding regions in the P_\_D and P_\_X promoters, we performed DNase I footprinting assays. The BoxR protein protected nucleotide sequences throughout the entire intergenic boxD-boxR region (Fig. 6). Moreover, binding of BoxR induces changes in the DNA structure as revealed by several phosphodiester bonds that become hypersensitive to DNase I cleavage (Fig. 6). The DNase I-hypersensitive sites were spaced at ∼10-nucleotide intervals (Fig. 6), corresponding to about 1 helix turn, which suggests binding of BoxR to one side of the double helix. The BoxR binding regions in P_\_D and P_\_X promoters span the transcription initiation sites as well as the −10 and −35 sequences for recognition of the σ^70-dependent RNA polymerase (Figs. 4C and 6), which is in agreement with the observed repressor role of BoxR at both promoters (Fig. 3). The protected regions usually contain direct repetitions of the TGCA sequence that, in some cases, is located within longer palindromic structures (Fig. 4C). This promoter architecture based on short direct repeats resembles that of promoters regulated by other members of the HTH-XRE family of transcriptional regulators (52). In this sense the TGCA direct repeats have been shown to be present also at the P_\_X promoter controlling the anaerobic boxD operon in Azoarcus sp. CIB, and they were proposed to be the BzdR-recognition sites (27, 50). This observation is in agreement with the fact that both BoxR and BzdR proteins contain highly similar helix-turn-helix (HTH) DNA binding domains (Fig. 2), thus suggesting an analogous DNA recognition mechanism for these two regulators.

As indicated above, the C-terminal region of BoxR shows a significant similarity to the C-terminal domain of BzdR (Fig. 2) that has been proposed to interact with the inducer molecule benzoyl-CoA (50). Because benzoyl-CoA is also the first intermediate in the aerobic degradation of benzoate via the box pathway (Fig. 1A), it was tempting to speculate that benzoyl-CoA could be also the inducer molecule that switched on the box genes. To check this assumption, different concentrations of benzoyl-CoA were added to the gel retardation assays, revealing that 2 mM inhibited binding of BoxR to the boxDR probe (Fig. 5B). On the contrary, the addition of 2 mM benzoate or phenylacetyl-CoA did not prevent the formation of the protein-DNA complex (Fig. 5B). These results were also confirmed by DNase I footprinting assays. As expected, the specific protection of the target promoter by the BoxR protein could not be observed in the presence of benzoyl-CoA (Fig. 6, A, lanes 9 and 10, and B, lane 11), suggesting that this molecule alleviates the BoxR-DNA interaction. In contrast, the addition of other benzoyl-CoA analogues, such as phenylacetyl-CoA, or the CoA-free benzoate did not avoid BoxR binding to the intergenic boxD-boxR region (Fig. 6, A, lanes 11 and 12, and B, lanes 9 and 10). These results suggest that benzoyl-CoA rather than benzoate may be the specific inducer molecule that interacts with the BoxR repressor, avoiding its binding to the promoters that drive the expression of the box genes when Azoarcus sp. CIB grows in benzoate.

All these results taken together show for the first time that BoxR and benzoyl-CoA act as the specific regulator and inducer, respectively, that control the expression of the box genes in Azoarcus. Because the boxR gene is present in all box clusters so far identified (Fig. 1B), the BoxR/benzoyl-CoA regulatory system may also be a widespread strategy in bacteria for the transcriptional control of the aerobic degradation of benzoate via the box hybrid pathway.

**Oxygen-independent Expression of box Cluster in Azoarcus sp. CIB**—It is well known that superimposed upon the specific regulation there is an additional control that links the induction of aromatic catabolic clusters to the environmental changes (4, 53–56). To determine whether oxygen controls the expression of the box genes in Azoarcus sp. CIB, we checked by real time RT-PCR analyses the expression of the box genes in Azoarcus sp. CIB. We observed that the expression of the box genes was even higher in cells grown aerobically than in cells grown aerobically (Fig. 7), oxygen does not appear to play a major role in the activity of the P_\_D and P_\_X promoters. To confirm that the P_\_D and P_\_X promoters are also active under anaerobic conditions, the expression of the P_\_D::lacZ and P_\_X::lacZ translational fusions was analyzed in recombinant E. coli CC118 cells grown anaerobically in glycerol-containing minimal medium. As observed above in the presence of oxygen (Fig. 3B), both promoters were active in the absence of oxygen, and they were also efficiently repressed by the boxR gene product (supplemental Figs. S4), revealing that P_\_D and P_\_X are oxygen-independent promoters.
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As shown above, the boxR gene is expressed both under aerobic and anaerobic conditions in Azoarcus sp. CIB (Fig. 7). On the other hand, the bzdR gene encoding the specific transcriptional regulator of the anaerobic bzd genes is also expressed both under aerobic and anaerobic conditions (57) and at levels that do not differ significantly from those of the boxR gene (Fig. 7). Moreover, because BoxR and BzdR share similar DNA binding features and use benzoyl-CoA as the inducer molecule, a similar gene repression strategy for the cognate catabolic promoters could be suggested, and transcriptional cross-regulation between the aerobic and anaerobic benzoate degradation pathways could be anticipated.

To experimentally demonstrate that BoxR and BzdR were able to directly interact with the anaerobic PN promoter and with the $P_N/P_D$ promoters of the aerobic box genes, respectively, we first accomplished an in vitro approach. To study the interaction of BoxR with the $P_N$ promoter, gel retardation assays were performed using the $P_N$ probe and increasing concentrations of the BoxR protein. As shown in Fig. 8A, the BoxR...
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![Diagram](image_url)  
**FIGURE 8.** Cross-interaction between BoxR and $P_N$ and between BzdR and $P_D$ promoters. Gel retardation analyses of BoxR binding to the $P_N$ promoter (A) and BzdR binding to the boxD-boxR intergenic region (B) and $P_D$ promoter (C) were performed as indicated under "Experimental Procedures." Lanes 1, free probes. Lanes 2–6 show retardation assays containing 25, 50, 100, 200, and 400 ng, respectively, of E. coli M15 (pREP4, pQE32-His$_{6}$BoxR) cell extract harboring His$_{6}$-BoxR protein (A) or E. coli M15 (pREP4, pQE32-His$_{6}$BzdR) cell extract harboring His$_{6}$-BzdR protein (B and C). Lane c (A), retardation assay containing 400 ng of E. coli M15 (pREP4, pQE32) control cell extract. The $P_N$ and boxDR probes as well as the $P_D$/BoxR, boxDR/BzdR and $P_D$/BzdR complexes are indicated by the arrows. D, E, and F, expression shown is of the $P_N$/lacZ, $P_D$/lacZ, or $P_D$/lacZ translational fusions, respectively. D. E. coli AFMCP$_N$ cells, which harbor a chromosomal $P_N$/lacZ insertion, carrying plasmid pCK01BoxR (BoxR), pCK01BzdR (BzdR) or the control plasmid pCK01 (–) were grown anaerobically in glycerol-containing minimal medium. E and F, E. coli CC118 (pSJ3PX) ($P_D$/lacZ) and E. coli CC118 (pSJ3PX) ($P_D$/lacZ) cells, respectively, carrying plasmid pCK01BzdR (BzdR) or the control plasmid pCK01 (–) were grown aerobically in glycerol-containing minimal medium. Values for $\beta$-galactosidase activity (in Miller units) were determined when cultures reached mid-exponential phase as indicated under "Experimental Procedures." Each value is the average from three separate experiments; error bars indicate S.D.

The boxR regulator was able to shift the $P_N$ probe in a concentration-dependent manner and with a similar efficiency than that observed with the cognate boxDR probe (Fig. 5). On the other hand, the BzdR protein was able to retard the migration of the boxDR probe (Fig. 8B) with similar efficiency to that shown with its cognate $P_N$ probe (Fig. 8C). Therefore, these results indicate that BoxR and BzdR are able to efficiently bind to their heterologous $P_N$ and $P_D/P_D$ promoters, respectively, supporting the hypothesis that they may act as repressors of their counterpart promoters. To demonstrate the last assumption, we monitored the activity of the $P_N$ $P_N$ and $P_D$ promoters in recombinant E. coli strains that contain the $P_N$/lacZ, $P_D$/lacZ, and $P_D$/lacZ translational fusions, respectively, in the presence or absence of the boxR or bzdR genes. To this end, the E. coli AFMCP$_N$ strain, which harbors a translational $P_N$/lacZ fusion integrated into the chromosome (27), was transformed with plasmids pCK01BoxR, pCK01BzdR (expresses the bzdR gene), or the control plasmid pCK01 (Table 1). On the other hand, the E. coli CC118 strain containing plasmids pSJ3PX ($P_X$/lacZ) or pSJ3PD ($P_D$/lacZ) was also transformed with plasmids pCK01BzdR or the control plasmid pCK01. The E. coli strains were grown in MC minimal medium supplemented with 20 mM glycerol as sole carbon source under anaerobic conditions (AFMCP$_N$-derived strains) or aerobic conditions (CC118 derived strains). The activity of the promoters was monitored by measuring the $\beta$-galactosidase levels of permeabilized cells. As shown in Fig. 8D, under anaerobic conditions the BoxR regulator was able to inhibit the activity of the $P_N$ promoter in an analogous manner to that shown by the cognate BzdR repressor. Similarly, under aerobic conditions the BzdR regulator was able to inhibit the expression of the $P_X$ (Fig. 8E) and $P_D$ (Fig. 8F) promoters as previously observed with the cognate BoxR repressor (Fig. 3B). All these results indicate that BoxR and BzdR are able to repress both the anaerobic $P_N$ promoter and the $P_D$ promoters, and they may act synergistically controlling the expression of the box and bzd clusters in Azoarcus sp. CIB.

To confirm the cross-regulation of the BoxR/BzdR regulators and the catabolic $P_N$ and $P_D$ promoters, we measured the expression of the bzdN and boxD genes in the wild-type Azoarcus sp. CIB strain and in the mutant strains Azoarcus sp. CIBboxR (boxR gene disrupted), Azoarcus sp. CIBbzdR (bzdR gene disrupted), and Azoarcus sp. CIBboxRbzdR (boxR and bzdR genes disrupted) (Table 1). Interestingly, in the Azoarcus sp. CIBboxRbzdR double mutant strain, the activity of the $P_N$ and $P_D$ promoters under non-inducing conditions (alanine) was similar to that found in the wild-type strain growing under inducing conditions (benzoate), and this activity was about 100-fold higher than that in the wild-type strain growing in
aline (Fig. 9). Thus, these results confirm that BoxR and BzdR are the key regulators that control the efficient repression of the two catabolic promoters in Azoarcus sp. CIB. Nevertheless, the \( P_N \) promoter is more strictly repressed by the BzdR regulator than by the BoxR regulator (Fig. 9), suggesting that BzdR is the major repressor of \( P_N \) in Azoarcus sp. CIB. On the other hand, although the repression of the \( P_D \) promoter is significantly alleviated in the Azoarcus sp. CIBdoubleR strain, the activity levels of \( P_D \) in the Azoarcus sp. CIBdoubleR strain indicates a BzdR-mediated 10-fold repression of \( P_D \) (Fig. 9), which suggests that the BzdR- and BoxR-mediated control of \( P_D \) are both physiologically relevant and necessary for the tight regulation of the box pathway.

All these results taken together reveal the existence of a transcriptional cross-regulation between the anaerobic and the aerobic benzoate degradation pathways in Azoarcus sp. CIB. Although there are some previous reports on cross-regulation between aromatic degradation pathways within the same organism (58–62), a cross-regulation between anaerobic and aerobic degradation pathways has never been shown before.

Adaptive and Evolutionary Considerations about Presence of boxR and bzdR Paralogs in Azoarcus sp. CIB—The BoxR and BzdR proteins from Azoarcus sp. CIB are homologous BzdR-type transcriptional regulators with distinct functions. However, both proteins respond to the same inducer, benzoyl-CoA, and can recognize the same target promoters. This redundancy raises questions about the need for both regulators. As we have shown above, the BzdR and BoxR regulators act synergistically to tightly control the expression of the \( bzd \) and \( box \) clusters (Fig. 9). Moreover, it is known that functional and/or genetic redundancy of regulators is a straightforward solution toward the robustness of the regulatory network mitigating the effect of noise during gene regulation (63, 64).

The retention of the \( bzdR \) and \( boxR \) paralogs in the genome of Azoarcus sp. CIB may favor the bacterial fitness under some growth conditions when the cells do not metabolize benzoate. To check this assumption, we measured the relative fitness of the wild-type strain versus that of the single and double mutant strains when the cells were grown in a non-aromatic compound as the carbon source and under non-optimal conditions, e.g. in solid medium. To accomplish this, cells of Azoarcus sp. CIB wild-type strain, Azoarcus sp. CIBdoubleR, and Azoarcus sp. CIBdoubleD single mutants and the Azoarcus sp. CIBdoubleRh double mutant were grown in glutarate-containing MC liquid medium. When these cultures reached an optical density of 0.1, they were plated on glutarate-containing MC solid medium, and the number of viable cells was recorded. Whereas \( 2 \times 10^6 \) CFU/ml were obtained from the culture of the wild-type strain, \( 10^6 \) and \( 9 \times 10^7 \) CFU/ml were obtained from the cultures of the \( boxR \) and \( bzdR \) mutant strains, respectively, and only \( 3 \times 10^6 \) CFU/ml were obtained from the culture of the Azoarcus sp. CIBdoubleRh double mutant. These results clearly show that the cell fitness becomes remarkably reduced in the \( boxR/bzdR \) double mutant strain. However, although the single \( boxR \) or \( bzdR \) mutants presented a decreased viability with respect to that of the wild-type strain, the remaining regulator partially compensates for the loss of the other. Therefore, all these results taken together suggest that the presence of the \( boxR \) and \( bzdR \) genes in the genome of Azoarcus sp. CIB contributes to the fitness of the cell avoiding the constitutive expression of the \( box/bzd \) genes in a medium lacking benzoate and constitutes an adaptive advantage when the occasional failure of one regulator is backed by the functionality of the other regulator. Nevertheless, because the \( boxR \) gene is also present in some bacteria that do not harbor a box cluster, such as Rhodopseudomonas palus-
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tris CGA009 (65) or some Methylobacterium spp. (www.ncbi.nlm.nih.gov), we cannot discard the fact that this regulator may control additional functions in the cell that can also compromise the bacterial fitness.

Some bacteria, e.g. Thauera and Magnetospirillum strains, that degrade benzoate both aerobically via the box pathway and anaerobically via the bzd pathway share the same benzoate-CoA ligase (bclA gene) for both pathways, and they have a single boxR gene associated to the box cluster in their genomes (22, 66). On the contrary, Azoarcus sp. CIB and A. aromaticum EbN1 strains contain two different regulator/ligase couples associated to the aerobic (BoxR regulator/BclA ligase) and anaerobic (BzdR regulator/BzDA ligase) pathways (25, 27). This observation suggests that Azoarcus strains may have recruited an additional regulatory circuit homologous to that of the box pathway to tightly control the bzd genes. In this sense we have shown above that the BoxR repressor is not able to efficiently repress the anaerobic P_N promoter, and BzdR repressor behaves as the main regulator of the bzd genes in strain CIB (Fig. 9). Despite the fact that the aerobic BoxR and the anaerobic BzdR proteins belong to the same BzdR subfamily of regulators, the anaerobic BzdR regulators from the Azoarcus/Aromaticum strains cluster together in a branch of the phylogenetic tree separated from that of the BoxR regulators from these bacteria (supplemental Figs. S3). This finding suggests that although both types of regulators may have a common ancestor, they have subsequently diverged and adapted to the corresponding catabolic pathways. Interestingly, the nucleotide sequences of boxR and boxR genes from Azoarcus sp. CIB differ by 379 nucleotides among the total 894 nucleotides, suggesting that either duplication of these regulatory genes in the Azoarcus cell did not occur very recently or that both genes have different bacterial origins. In this sense, it is worth mentioning that whereas the GC content of the boxR gene (66.4%) matches the average GC content of the whole genome (65.8%) and it shows a good codon adaptation index (0.81), the GC content (62.8%) of bzdR differs from that of the Azoarcus sp. CIB chromosome, and this gene shows a low codon adaptation index (0.66). Interestingly, the bzdA gene also shows a codon adaptation index (0.69) that is significantly lower than that of other bzd genes and the homologous bclA gene (>0.8). Because such variations in GC content and codon adaptation index are taken as indicators of horizontal gene transfer events (40), it seems likely that the bzdR gene and perhaps also the bzdA gene might have been evolutionarily recruited by the bzd catabolic cluster of some Azoarcus strains from the box cluster of a different organism. The recruited bzdR gene could then evolve to tightly regulate the anaerobic benzoate degradation but still partially retain the control of the aerobic box pathway, which would explain the observed cross-regulation reported in this work. The cross-regulation between the aerobic and anaerobic benzoate degradation pathways may reflect a biological strategy to increase the cell fitness in organisms that drive in environments subject to changing oxygen concentrations.

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