AccR Is a Master Regulator Involved in Carbon Catabolite Repression of the Anaerobic Catabolism of Aromatic Compounds in Azoarcus sp. CIB*

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Background: Mechanisms underlying carbon catabolite repression (CCR) control of the anaerobic degradation of aromatic compounds have previously remained elusive. Results: Phosphorylated AccR was identified as a transcriptional repressor of aromatic degradation operons expressed under anaerobic conditions. Conclusion: The response regulator AccR controls the succinate-dependent CCR in Azoarcus sp. CIB. Significance: AccR is a master regulator that controls anaerobic CCR in bacteria.

Here we characterized the first known transcriptional regulator that accounts for carbon catabolite repression (CCR) control of the anaerobic catabolism of aromatic compounds in bacteria. The AccR response regulator of Azoarcus sp. CIB controls succinate-responsive CCR of the central pathways for the anaerobic catabolism of aromatics by this strain. Phosphorylation of AccR to AccR-P triggers a monomer-to-dimer transition as well as the ability to bind to the target promoter and causes repression both in vivo and in vitro. Substitution of the Asp60 phosphorylation target residue of the N-terminal receiver motif of AccR to a phosphomimic Glu residue generates a constitutively active derivative that behaves as a superrepressor of the target genes. Acc-R-P binds in vitro to a conserved inverted repeat (ATGCA-N6-TGCAT) present at two different locations within the P

Promoter of the benzaldehyde degradation genes for anaerobic benzoate degradation. Because the DNA binding-proficient C-terminal domain of AccR is monomeric, we propose an activation mechanism in which phosphorylation of Asp60 of AccR alleviates interdomain repression mediated by the N-terminal domain. The presence of AccR-like proteins encoded in the genomes of other β-proteobacteria of the Azoarcus/Thauera group further suggests that AccR constitutes a master regulator that controls anaerobic CCR in these bacteria.

Carbon catabolite repression (CCR) is widespread in bacteria and imparts a competitive advantage and improved fitness by establishing priorities in carbon utilization. This enables bacteria to optimize their growth rates in natural environments that provide complex mixtures of nutrients. The regulatory mechanisms of CCR operate through subverting expression of genes required for the uptake and/or metabolism of secondary (less preferred) carbon sources in the presence of a preferred substrate (1). Such secondary carbon sources include aromatic compounds, which, after carbohydrates, are the most widely distributed class of organic compounds in nature.

Where studied, CCR mechanisms impact expression of pathways for the degradation of aromatic compounds. In contrast to enterics, such as Escherichia coli, which utilize glucose as the preferred carbon source, soil bacteria (e.g. Pseudomonas) metabolize many organic acids or amino acids in preference to sugars (2). However, not all organic acids mediate CCR; nor do all organic acids provoke the same effect in different microorganisms (3, 4).

The regulatory elements involved in CCR of aerobic catabolism of aromatic compounds in different bacteria are diverse (2, 4–8). In E. coli, the cAMP-responsive CRP transcriptional regulator imparts CCR control over catabolism of aromatic compounds (8). In Pseudomonas strains, the Crc protein exerts CCR on the assimilation of certain aromatic compounds (9–11) as well as some sugars and amino acids (12–14). In contrast to CRP, Crc acts post-transcriptionally (3, 4, 15). Other proteins described to be involved in CCR control of aerobic catabolism of aromatics in Pseudomonas include PtsN and PtsO (nitrogen phosphotransferase system) (6, 16) and the redox-responsive o-type terminal oxidase CyoB (17, 18), whereas in Acidovorax sp. KKS102 CCR of biphenyl degradation in response to some organic acids is mediated by the response regulator BphQ (7).

Far less is known about CCR control of anaerobic catabolism of aromatic compounds, although cases of this phenomenon have been reported. For example, some organic acids cause CCR of genes involved in the anaerobic degradation of aromatic compounds in Thauera aromatica (19) and Azoarcus sp. CIB (20), whereas in closely related strains, such as Aromatoleum aromaticum EbN1, aromatic compounds are preferred carbon sources in nature.
sources over aliphatic organic acids (21). However, the mechanism(s) underlying CCR in these cases are still unknown.

Elucidating the molecular mechanism(s) underlying CCR is important for both basic understanding of how metabolism is regulated in the environment and for potential biotechnological applications, such as optimizing bioremediation strategies and the design of tailor-made biocatalysts/biosensors (4). This is particularly relevant for aromatic compounds that are difficult to degrade and tend to accumulate in the environment under both aerobic and anaerobic conditions. In this work, we sought to use the denitrifying β-proteobacterium Azoarcus sp. CIB to analyze the mechanism(s) underlying CCR of anaerobic pathways for the dissimilation of aromatic compounds. Azoarcus sp. CIB is able to degrade aromatic compounds both aerobically and anaerobically (20, 22), and it has been used as a model organism to study the specific regulation of the benzoyl-CoA central pathways for the aerobic (box genes) and anaerobic (bdz genes) degradation of benzoate (20, 23–27).

The Azoarcus sp. CIB bdz genes responsible for the anaerobic degradation of benzoate are clustered and consist of the P$_N$ promoter–driven bdzNOPQ MSTUV WYZA catabolic operon and the bdzR regulatory gene (20). BdzR-mediated repression of P$_N$ is alleviated by the inducer molecule benzoyl-CoA, the first intermediate of the catabolic pathway (23, 24). However, activity of the P$_N$ promoter also strictly requires the AcpR transcriptional activator, an ortholog of the E. coli Fnr global regulator, which specifically drives expression of the bdz catabolic operon in an oxygen-dependent manner (25). In addition to this dual repressor/activator control, the P$_N$ promoter is also subject to control by the benzoyl-CoA dependent BoxR repressor, a BdzR paralog that regulates the expression of the box genes, indicating the existence of cross-regulation between the aerobic and anaerobic benzoate degradation pathways of Azoarcus sp. CIB (27).

In our previous work (20), we found that anaerobic expression of the bdz genes from the P$_N$ promoter is additionally subject to CCR control in response to organic acids, such as succinate, malate, and acetate, although the mechanism(s) underlying this level of control was not determined. In this work, we report the characterization of AccR as a response regulator that mediates anaerobic CCR control on the P$_N$ promoter. Based on our finding that AccR exerts anaerobic CCR control on central pathways for catabolism of other aromatics in Azoarcus sp. CIB, AccR constitutes the first master regulator described that controls CCR of anaerobic catabolism of aromatic compounds in bacteria. Because of the existence of AccR orthologs within the genomes of closely related Azoarcus/Thauera strains, we suggest that AccR is probably the global mediator of CCR control for anaerobic assimilation of aromatic compounds in this group of β-proteobacteria.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—Bacterial strains and plasmids used are listed in Table 1. Plasmids were constructed by standard molecular techniques (35) with the fidelity of all PCR-derived DNA (Table 2) verified by sequencing. E. coli cells were routinely grown at 37 °C in lysogeny broth (LB) medium (35). When required, E. coli AFMCP$_N$ was grown aerobically or anaerobically (using 10 mm nitrate as terminal electron acceptor) at 30 °C in M63 minimal medium (36), supplemented with 0.1 mg/ml thiamine and 20 mM glycerol as the carbon source. Azoarcus strains were similarly grown aerobically or anaerobically at 30 °C in MC medium containing the indicated carbon source(s) as described previously (20). Where appropriate, antibiotics were added at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (7.5 μg/ml), and streptomycin (50 μg/ml). An AccR null derivative of Azoarcus sp. CIB was generated by homologous recombination using the R6K-based suicide plasmid pKNG101ΔaccR (Table 1), which allows positive selections of double site recombinants using the sacB gene of Bacillus subtilis (31). Conjugation of pKNG101ΔaccR from replication-permissive E. coli SM10 Apri was used to introduce the plasmid into Azoarcus sp. CIB. Derivatives containing first site recombinants were selected on streptomycin-containing MC medium with 10 mM glutarate as the sole carbon source. Second site recombination was selected by selecting on the same medium supplemented with 5% sucrose. Correct allelic exchange in sucrose-resistant and streptomycin-sensitive derivatives was verified using primers 5′AccRmut (BamHII) and 3′AccRmut (SphI) (Table 2).

**Sequence Analyses**—The nucleotide sequence of the accR gene of Azoarcus sp. CIB has been submitted to GenBank™ with accession number KF233996. Pairwise and multiple sequence alignments of AccR-like proteins were made with the ClustalW (37) program at the EMBL-EBI server. Phylogenetic analysis, carried out according to Kimura’s two-parameter method (38), was reconstructed using the neighbor-joining method (39) PHYLIP program (40).

**β-Galactosidase Assays**—The β-galactosidase activities from promoter-lacZ reporter fusions were measured with permeabilized cells, when cultures reached mid-exponential phase, as described by Miller (36).

**Purification of AccR Derivatives**—His-tagged AccR derivatives, all with a 13-amino acid (MRGSHHHHHHGIL) N-terminal fusion, were expressed from the isopropyl-1-thio-β-D-galactopyranoside-inducible T5 promoter of the pQE32 expression vector (His$_6$-AccR from pQE32-His$_6$-AccR, His$_6$-AccRD60E from pQE32-His$_6$-AccRD60E, and His$_6$-CAccR from pQE32-His$_6$-CAccR; Table 1). Proteins were purified from E. coli M15 that additionally harbored pREP4, a LacI expression plasmid that aids isopropyl-1-thio-β-D-galactopyranoside-dependent expression. Cultures were grown at 37 °C until the mid-exponential growth phase in LB containing appropriate antibiotics, and then expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (0.1 mM). After a further 5-h incubation, cells were harvested from 1 liter of culture at 4 °C, resuspended in 25 ml of lysis buffer (50 mM NaH$_2$PO$_4$, pH 8.0, 300 mM KCl, 20 mM imidazole), and disrupted by passage through a French press (Aminco Corp.) operated at a pressure of 20,000 p.s.i. Cell lysates were clarified by centrifugation (26,000 × g for 25 min at 4 °C) prior to loading on nickel-nitrilotriacetic acidagarose columns (Qiagen) equilibrated with lysis buffer. Columns were washed at 4 °C with 50 volumes of lysis buffer, and the His-tagged proteins were subsequently eluted with elution buffer (50 mM NaH$_2$PO$_4$, pH 8.0, 300 mM KCl, 75 mM imidazole). Peak fractions were pooled,
dialyzed at 4 °C into modified FP buffer (20 mM Tris-HCl, pH 7.5, 5% glycerol, and 50 mM KCl), and stored as independent aliquots at −20 °C.

In Vitro Phosphorylation of AccR Derivatives—AccR and its derivatives were phosphorylated by treatment with 20 mM cambamoyl phosphate in phosphorylation buffer (20 mM Pipes, 10 mM magnesium acetate, 10 mM KCl, 170 mM NaCl, and 5% (v/v) glycerol, pH 7.5) for 90 min at 28 °C.

Mass Spectrometry Analyses—Mass spectrometry experiments with the AccR and AccR-P proteins (20 μM) were performed on an Autoflex III MALDI-TOF-TOF instrument (Bruker Daltonics, Bremen, Germany) with a smartbeam laser. Spectra were acquired using a laser power just above the ionization threshold. Typically, 1000 laser shots were summed into a single mass spectrum. External calibration was performed using bovine albumin (Sigma), covering the range from 30,000 to 70,000 Da. A saturated solution of sinapinic acid in 3:1 water/acetonitrile and 0.1% trifluoroacetic acid was used as the matrix. For sample preparation, the sample solution and the matrix were premixed (1:1, v/v), and then 1 μL of the mixture was spotted on the stainless steel target and allowed to dry at room temperature.

Analytical Ultracentrifugation Methods—Analyses were performed using several protein concentrations (from 5 to 40 μM) equilibrated with 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 5% glycerol buffer. Sedimentation velocity runs (48,000 rpm at 20 °C) were carried out in an XL-I analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with UV-visible optic detection system, using an An50Ti rotor and 12-mm double sector centerpiece. Sedimentation profiles were registered every 1–5 min at 280 nm in the experiments with His6-AccR and His6-AccR-P, and 235 nm in the experiments with His6-AccR.

TABLE 1
Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli</strong> strains**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH10B</td>
<td>F', mcrA Δ(mrr bsdRMS-mcrBC) 480Δlac</td>
<td>Invitrogen</td>
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<tr>
<td></td>
<td>ΔlacX74 deoR recA araD139 Δ ara-leu7697</td>
<td></td>
</tr>
<tr>
<td></td>
<td>galU galK rpsL (Sm') endA1 nupG</td>
<td></td>
</tr>
<tr>
<td>M15</td>
<td>Strain for regulated high level expression with pQE vectors</td>
<td>Qiagen</td>
</tr>
<tr>
<td>MC4100</td>
<td>araD139 (argF-lac)ΔU169 rpsL150 (Sm') relA1 blb5301 deoC1 psdF25 rbsR</td>
<td>28</td>
</tr>
<tr>
<td>A8IF1ΔN</td>
<td>Km' R' MC4100 spontaneous rifampicin-resistant mutant harboring a chromosomal insertion of the P2-lacZ translational fusion</td>
<td>23</td>
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<tr>
<td>SM10 Δpir</td>
<td>Km' thi-1 for leu tonA lacY supC rcsA;carries a 636-bp BamHI to PstI fragment generated using primers 5'HisCaccR and 3'HisaccR</td>
<td>29</td>
</tr>
<tr>
<td>S17-Δpir</td>
<td>Tp' Sm' recA thi hsdR34M2 Pst4-2-Tc::Mu Km Tn7 Δpir lysiogen</td>
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<tr>
<td>XLI-Bae MRA (P2)</td>
<td>Δ(mcrA)183, Δ(mcrCB-hsdSMR-rrn)173, endA1, gyrA96, supF44, relA1, thi-1, lac, P2 lysiogen</td>
<td>Stragatene</td>
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<td><strong>Azoroc sp. CIB</strong> strains</td>
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<td></td>
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<tr>
<td>CIB</td>
<td>Wild-type strain CIB</td>
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<tr>
<td>CIBΔaccR</td>
<td>Azoroc sp. strain CIB with a deletion of the accR gene</td>
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**Plasmids**

<table>
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<th>Plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>pQE32</td>
<td>Ap', oriColE1, T5 promoter lac operator, λ DE3. coli rmbB T1 terminators, N-terminal His6</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQE32-His6-AccR</td>
<td>Ap', pQE32 derivative for expression of His6-AccR carries a 636-bp BamHI to PstI fragment generated using primers 5'HisCaccR and 3'HisaccR (Table 2)</td>
<td>This work</td>
</tr>
<tr>
<td>pQE32-His6-AccR</td>
<td>Ap', pQE32 derivative for expression of His6-AccR carries a 232-bp BamHI to PstI fragment generated using 5'HisCaccR and 3'HisaccR (Table 2)</td>
<td>This work</td>
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<tr>
<td>pQE32-His6-AccRD60E</td>
<td>Ap', pQE32 derivative for expression of His6-AccRD60E; carries a 636-bp BamHI to PstI fragment generated using flanking primers 5'HisCaccR and 3'HisaccR and overlapping PCR mutagenesis primers 5'AccRD60E and 3'AccRD60E</td>
<td>This work</td>
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<tr>
<td>pREP4</td>
<td>Km', plasmid that expresses the lacI repressor</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pECOR7</td>
<td>Ap', pUC19 harboring a 7.1-kb EcoRI fragment containing the bsdRNA genes</td>
<td>20</td>
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<tr>
<td>pNKG101</td>
<td>Sm', oriR6K, Mob'. Suicide vector with a lacI selection marker for gene replacement by double site homologous recombination</td>
<td>31</td>
</tr>
<tr>
<td>pNKG101ΔaccR</td>
<td>Sm', pNKG101 derivative carrying the ΔaccR allele as a 1.4-kb BamHI to SpeI fragment assembled using the four AccRmut primers listed in Table 2</td>
<td>This work</td>
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<tr>
<td>pIZ1016</td>
<td>Gm', pIZ1016 derivative expressing accR from the Ptac promoter; carries a 3.3-kb SalI to SpeI fragment amplified using primers 5'AccR and 3'AccR (Table 2)</td>
<td>This work</td>
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<tr>
<td>pIZ4AccR</td>
<td>Gm', pIZ4AccR derivative expressing the accR gene from the Plac promoter; carries a 679-bp BamHI to Xbal fragment generated using primers 5'AccExt and 3'AccExt (Table 2)</td>
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</tr>
<tr>
<td>pBBR1MCS-5</td>
<td>Gm', pBBR1MCS-5 derivative expressing a 5-kb accR gene from the Plac promoter; carries a 697-bp BamHI to Xbal fragment generated using primers 5'AccExt and 3'AccExt (Table 2)</td>
<td>This work</td>
</tr>
<tr>
<td>pBBR5AccR</td>
<td>Gm', pBBR5AccR derivative expressing the accR gene from the Ptac promoter; carries a 697-bp BamHI to Xbal fragment generated using primers 5'AccExt and 3'AccExt (Table 2)</td>
<td>This work</td>
</tr>
<tr>
<td>pPBBRSPN</td>
<td>Gm', pBBR1MCS-5 derivative expressing a 5-kb accR gene from the Plac promoter; carries a 697-bp BamHI to Xbal fragment generated using primers 5'AccExt and 3'AccExt (Table 2)</td>
<td>This work</td>
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<tr>
<td>pPBBRP5N1</td>
<td>Gm', pBBR5AccR derivative expressing a 5-kb accR gene from the Plac promoter; carries a 697-bp BamHI to Xbal fragment generated using primers 5'AccExt and 3'AccExt (Table 2)</td>
<td>This work</td>
</tr>
<tr>
<td>pIZP1</td>
<td>Gm', pIZ1016 derivative harboring a 585-bp EcoRI fragment that includes the P2 promoter</td>
<td>34</td>
</tr>
<tr>
<td>pICD-PN</td>
<td>Ap', pICD01 derivative harboring a 585-bp EcoRI fragment that includes the P2 promoter</td>
<td>25</td>
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</tbody>
</table>

* Ap', ampicillin-resistant; Gm', gentamicin-resistant; Km', kanamycin-resistant; R', rifampicin-resistant; Sm', streptomycin-resistant.
corresponding base-line offset. The measured low speed equilibrium concentration (signal) gradients of His\textsubscript{5} AccR, His\textsubscript{5} AccR-P, and His\textsubscript{5} CAccR were fitted using an equation that characterizes the equilibrium gradient of an ideally sedimenting solute (using a MATLAB program, kindly provided by Dr. Allen Minton, National Institutes of Health) to obtain the corresponding buoyant signal average molecular weight.

**Gel Retardation Assays**—Gel retardation reaction mixtures (9 μl) contained FP buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM β-mercaptoethanol, 50 mM KCl), 0.05 nM [\textsuperscript{32}P]dATP (6,000 Ci/mmol; U.S. Biochemical Corp.), 200 nM His\textsubscript{5} Fnr\* activator (a constitutively active Fnr derivative that is able to promote transcription in the presence of oxygen) (25, 44), and different concentrations of purified AccR or AccR-P proteins. Reactions were equilibrated for 10 min at 37 °C prior to initiation of transcription by the addition of nucleotides (final concentrations: 500 μM of ATP, CTP, and

**TABLE 2**

Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence*</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′HisaccR</td>
<td>5′-CGGATCTTTATCGCACCCTCGCCGCTTA (BamHI)</td>
<td>Amplification of the 5′-end of accR; used to construct His-tagged accR and accRD60E genes</td>
</tr>
<tr>
<td>3′HisaccR</td>
<td>3′-AAATCAGTGCAGAGCAGTCATG</td>
<td>Amplification of the 3′-end of accR; used to construct the accR, CaccR, and accRD60E genes</td>
</tr>
<tr>
<td>5′HisAccR</td>
<td>5′-CGGATCTTTATCGCACCCTCGCCGCTTA (BamHI)</td>
<td>Amplification of the 5′-end of CaccR; used to construct His-tagged CaccR</td>
</tr>
<tr>
<td>3′AccRD60E</td>
<td>3′-CATGGCAGCTGAGACGACGAG</td>
<td>Internal accR primers used to introduce the accRD60E substitution</td>
</tr>
<tr>
<td>5′AccSR</td>
<td>5′-ACGGTCTAGCTGACCTGAGATGGAACCTTGCG (SalI)</td>
<td>Used to amplify and clone the accR genes into plasmid pZAcCsr</td>
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<tr>
<td>3′AccRext</td>
<td>3′-CGGATCTTAGATATTACTGAGTGAGGCTTACATCTTCTC (BamHI)</td>
<td>Amplification of the accR gene into plasmid pBRSAccR</td>
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<tr>
<td>5′AccRmut(BamHI)</td>
<td>5′-CGGATCTCCCGAGGCTCCGAGCCAGC (BamHI)</td>
<td>Amplification of a 762-bp BamHI/Xbalm fragment, spanning the upstream region of accR, for constructing the ΔaccR allele in pKNG101ΔaccR</td>
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<tr>
<td>3′AccRmut(Xbal)</td>
<td>3′-CGGATCTCATGAGAGGAACTCCCTTGG (Xbal)</td>
<td>Amplification of a 642-bp Xbal/SpeI fragment, spanning the downstream region of accR, for constructing the ΔaccR allele in pKNG101ΔaccR</td>
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<tr>
<td>3′AccRmut(SpeI)</td>
<td>3′-CGGATCTCATGAGAGGAACTCCCTTGG (SpeI)</td>
<td>RT-PCR amplification of cDNA, a 1,536-bp product that reports RT-PCR amplification of cDNA, a 1,664-bp product of dnaE used as internal control in RT-PCR assays</td>
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<tr>
<td>5′pboxQ</td>
<td>5′-CAATCCAGGCTGGCTGCGCC</td>
<td>Amplification of a 234-bp boxD intergenic fragment, P$_{\text{boxD}}$ probe</td>
</tr>
<tr>
<td>3′pboxQ</td>
<td>3′-GAGGAGGACAACTGAGGTAAGC</td>
<td>5′-end of P$<em>{\text{boxD}}$ promoter, used with 3′IVTPN to amplify the 376-bp P$</em>{\text{boxD}}$ probe</td>
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<td>5′RtpN1</td>
<td>5′-GCTTCAAGAGAGGAACTCCCTTGG</td>
<td>3′-end of P$<em>{\text{boxD}}$ promoter, used to amplify the P$</em>{\text{boxD}}$, P$<em>{\text{N2}}$, and P$</em>{\text{N2mut}}$ probes</td>
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<td>3′RtpN2</td>
<td>3′-GCTTCAAGAGAGGAACTCCCTTGG</td>
<td>Amplification of the 93-bp P$_{\text{boxD}}$ probe</td>
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<td>5′POLI HK</td>
<td>5′-CGGATCTCATGAGAGGAACTCCCTTGG</td>
<td>Used with 3′IVTPN to amplify the 162-bp P$_{\text{boxD}}$ probe</td>
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<tr>
<td>5′POLI HK</td>
<td>5′-CGGATCTCATGAGAGGAACTCCCTTGG</td>
<td>Used with 3′IVTPN to amplify the 165-bp P$_{\text{boxD}}$ Probe</td>
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<tr>
<td>3′IVTPN</td>
<td>3′-GGAACTCTGCAATATGATCGGCCGAG</td>
<td>Amplification of the 251-bp P$_{\text{boxD}}$ probe</td>
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</table>

*Engineered restriction sites are underlined, and the corresponding base-line offset is shown in parentheses.

**DNase I Footprinting Assays**—DNase I footprinting reactions (15 μl) in FP buffer contained 2 nm radiolabeled P$_{\text{boxD}}$ probe (see above), 1 mg/ml bovine serum albumin, and the indicated concentration of in vitro phosphorylated His\textsubscript{5} AccR (AccR-P). Reactions were incubated for 20 min at 25 °C prior to the addition of 3 μl (0.05 units) of DNase I solution (prepared in 10 mM CaCl$_2$, 10 mM MgCl$_2$, 125 mM KCl, and 10 mM Tris-Cl, pH 7.5). After a 20-s incubation at 37 °C, reactions were quenched by the addition of 180 μl of a solution containing 0.4 mM sodium acetate, 2.5 mM EDTA, 50 μg/ml calf thymus DNA, and 0.3 μg/ml glycogen. DNA fragments were phenol-extracted, precipitated, and analyzed as previously described (23). An A+G reaction (43) was carried out with the same fragment. Images were obtained as described for gel retardation assays.

**In Vitro Transcription Experiments**—Multiple-round in vitro transcription reactions (50 μl) were performed essentially as described previously (25) in a buffer consisting of 40 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl$_2$, 0.1 mM bovine serum albumin, and 10 mM dithiothreitol. Reactions contained 5 nm supercoiled plasmid pCD-P$_{\text{boxD}}$ as the DNA template, 50 nm E. coli σ$_{70}$ RNA polymerase (1 unit/μl; U.S. Biochemical Corp.), 200 nm His$_{5}$ Fnr$\*$ activator (a constitutively active Fnr derivative that is able to promote transcription in the presence of oxygen) (25, 44), and different concentrations of purified AccR or AccR-P proteins. Reactions were equilibrated for 10 min at 37 °C prior to initiation of transcription by the addition of nucleotides (final concentrations: 500 μM of ATP, CTP, and
The AccR Response Regulator from Azoarcus sp. CIB

GTP; 50 μM UTP; and 1 μCi of [α-32P]UTP (3,000 Ci/mmol; PerkinElmer Life Sciences)). After incubation for 20 min at 37 °C, reactions were terminated by the addition of an equal volume of a solution containing 50 mM EDTA, 350 mM NaCl, and 0.5 mg/ml of carrier tRNA. Ethanol-precipitated transcripts were dissolved in loading buffer (7 M urea, 1 mM EDTA, 0.6 M glycerol, 0.9 mM bromphenol blue, and 1.1 mM xylene cyanol); resolved by electrophoresis on a denaturing 7 M urea, 4% polyacrylamide gel; and visualized by autoradiography.

**Real-time RT-PCR Assays**—Total RNA was extracted and DNase I-treated using reagents provided with the RNeasy mini-kit (Qiagen) according to the manufacturer’s instructions. The concentration and purity of the RNA samples were assessed using an ND1000 spectrophotometer (Nanodrop Technologies) according to the manufacturer’s protocols. Synthesis of total cDNA was performed in 20-μl reactions containing 1 μg of RNA, a 0.5 mM concentration of each dNTP, 200 units of SuperScript II reverse transcriptase (Invitrogen), and 5 μM random hexamers in the buffer provided by the manufacturer. Samples were initially heated at 65 °C for 5 min and then incubated at 42 °C for 2 h. Reactions were terminated by incubation at 70 °C for 15 min, and the cDNA was further purified using GeneClean Turbo kit (MP Biomedicals). An IQ5 multicolor real-time PCR detection system (Bio-Rad) was used for real-time PCR of triplicate samples. Reactions (25 μl) contained 5 ng of cDNA, 0.2 μM two target-specific primers, and 12.5 μl of SYBR Green Mix (Applied Biosystems). Primer pairs used to amplify transcripts from the P_P promoters and the transcript of the dnaE gene (encoding the α-subunit of DNA polymerase III) as an internal control to normalize the sample data are given in Table 2. Amplifications were carried with one denaturation cycle (95 °C for 4 min), followed by 30 cycles of amplification (95 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 s). After amplification, melting curves were generated to confirm amplification of a single product. For relative quantification, a calibration curve was constructed for each ampiclon by using 5-fold serial dilutions of Azoarcus sp. CIB genomic DNA ranging from 2.5 ng to 1.6 × 10^{-6} ng. These standard curves were used to extrapolate 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.

**RESULTS**

The accR Gene Is Required for CCR Control of the bzd Genes in Azoarcus sp. CIB—In silico analysis of the unpublished draft Azoarcus sp. CIB genome sequence identified a gene, hereafter referred to as accR (aromatic catabolic control regulator; accession number KF233996), whose product shows 47% amino acid sequence identity with the response regulator BphQ involved in CCR control of the biphenyl degradation pathway in Acidovorax sp. KKS102 (7). Orthologs of accR are also found in the genomes of other β-proteobacteria Azoarcus and Thauera strains (supplemental Fig. S1). These observations prompted us to test AccR as a potential candidate for CCR control of the assimilation of aromatic compounds in Azoarcus sp. CIB. To this end, we generated an AccR null derivative (Azoarcus sp. CIBΔaccR; Table 1), and measured the activity of the P_P promoter, which drives the anaerobic expression of the bzd genes, in comparison with P_P activity in the wild-type strain. Transcripts from P_P were monitored by real-time RT-PCR using RNA extracted from cultures grown until mid-exponential phase, and the activity of the P_P promoter (in arbitrary units) was measured by real-time RT-PCR. Graphed values are the average of three independent experiments ± S.D. (error bars).

**FIGURE 1.** The accR gene is required for CCR control of the P_P promoter. Azoarcus sp. CIB (CIB), its AccR null counterpart (CIBΔaccR), and CIBΔaccR harboring plasmid pBBR5AccR, which expresses AccR from the Plac promoter (CIBΔaccR/accR), were grown anaerobically in minimal medium supplemented with 3 mM benzoate (black bars) or a mixture of 3 mM benzoate and 0.4% succinate (white bars). Total RNA was isolated from cells grown until mid-exponential phase, and the activity of the P_P promoter under control of the Plac promoter. Hence, these results support the idea that AccR acts to repress the P_P promoter under CCR conditions.

**AccR Possess in Vitro Properties of Response Regulators**—Sequence comparisons indicated that AccR belongs to the FixJ/NarL family of response regulators (45–47). AccR exhibits the typical two-domain architecture of this class of proteins, consisting of an N-terminal domain (NAccR; residues 1–122) and a C-terminal region (CAccR; residues 145–212) connected by a 23-residue-long linker (supplemental Fig. S1A). The NAccR domain contains the highly conserved receiver motif (REC motif) (residues 20–122) (supplemental Fig. S1A) that is involved in phosphorylation and dephosphorylation reactions of response regulators (48). The CAccR domain contains a NarL/FixJ helix-turn-helix DNA-binding motif (residues 162–189) responsible for interaction with the DNA (supplemental Fig. S1A).

To determine whether AccR behaves as a typical response regulator in vitro, we expressed and purified a N-terminally His-tagged version of the AccR protein (His6-AccR). Response regulators usually receive a phosphate group from a cognate histidine kinase in intact cells; however, REC motifs can also catalyze autophosphorylation using small phosphodonor molecules, such as carbamoyl phosphate or acetyl phosphate (48). Hence, we analyzed purified AccR before and after treatment with carbamoyl phosphate. Mass spectrometry analysis of
untreated AccR revealed a single peak that corresponds to the theoretical mass of monomeric AccR (24,629 Da; Fig. 2A). When carbamoyl phosphate-treated AccR was similarly analyzed, two peaks were resolved: one corresponding to the unadorned monomer and the other compatible with the addition of a phosphate group (24,708.6 Da; Fig. 2B). We conclude from these results that AccR becomes phosphorylated (AccR-P) in the presence of carbamoyl phosphate.

Analysis of the REC motif suggested that autophosphorylation of the AccR protein would occur at the highly conserved Asp60 residue that is located at the C-terminal end of β3-strand (supplemental Fig. S1A). To experimentally verify this supposition, we purified a His-tagged mutant variant with Asp60 substituted to Glu (AccRD60E), a substitution that serves as a phospho-mimic in some response regulators (49, 50). In contrast to the wild-type protein, AccRD60E exhibited no change in mass upon treatment with carbamoyl phosphate, supporting the idea that Asp60 is the target residue for AccR autophosphorylation.

AccR Undergoes a Monomer-to-dimer Shift upon Phosphorylation—Phosphorylation of some response regulators results in a change in their oligomeric state (51). To determine if such a change occurs with AccR, we performed analytical ultracentrifugation analyses with different concentrations (5–40 µM) of untreated and carbamoyl phosphate-treated AccR. Sedimentation velocity analysis of 10 µM untreated AccR revealed a single species with an s value of 1.5 S (Fig. 2C). Carbamoyl phosphate treatment resulted in two species of similar abundance, one with an s value of 1.5 S and another with an s value of 2.1 S (Fig. 2D), suggesting that ~50% of AccR becomes phosphorylated under the experimental conditions used.
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The molecular mass of the 1.5 S species, as measured by sedimentation equilibrium (24,000 ± 500 Da), is compatible with the mass of the AccR monomer (Fig. 2E, squares). However, because the frictional ratio $f/f_0$ of untreated AccR was 1.7, the shape deviates from that expected for a globular protein and suggests an elongated monomer. Sedimentation equilibrium experiments of the carbamoyl phosphate-treated AccR sample indicated a molecular mass (38,500 ± 400 Da) between that of a monomer and a dimer of the protein (Fig. 2E, circles), which is in agreement with the co-existence of approximately equal amounts of the 1.5 S (monomer) and 2.1 S (dimer) species within this preparation (Fig. 2D). Together, these results demonstrate that unmodified AccR exists primarily as an elongated monomer, whereas phosphorylation induces a monomer-to-dimer transition but does not significantly change the global shape of the protein that retains a frictional ratio $f/f_0$ of 1.7.

**DNA Binding by AccR Is Phosphorylation-dependent**—Having established that AccR undergoes a phosphorylation-dependent conformational change (Fig. 2) and influences expression of the bzd genes through the $P_N$ promoter (Fig. 1), we next determined the ability of AccR to bind to DNA. For this, we used a 376-bp DNA fragment spanning from -293 to +83 with respect to the transcription start site of the $P_N$ promoter ($P_N$ probe) in gel retardation assays. The AccR protein was unable to bind to the $P_N$ probe unless phosphorylated by prior carbamoyl phosphate treatment (Fig. 3A). In contrast, the Asp60 to Glu substitution in AccR60E rendered the protein DNA binding-competent in its non-phosphorylated state (Fig. 3C). These results show that the DNA binding capacity of AccR is dependent on its phosphorylated state, which can be mimicked by an Asp to Glu substitution. Because phosphorylation induces a monomer-to-dimer transition, it appears likely that AccR binds DNA primarily as a dimer.

**AccR Binds to Palindrome Operator Sites within $P_N$ to Confer CCR Control**—To further characterize the DNA binding sites of AccR-P within the $P_N$ promoter, we performed DNase I footprinting assays. As shown in Fig. 4A, AccR-P protected a DNA region spanning from position -34 to -153 with respect to the transcription start site of the $P_N$ promoter. This protected region contains two repetitions of the palindromic sequence (ATGCA-N$_6$-TGCAT), denoted OR1 (from -67 to -82) and OR2 (from -130 to -145) (see Fig. 4B). To determine if AccR-P was able to recognize the two palindromic regions independently, we used two probes ($P_N$, which spans OR1, and $P_N$ which spans OR2) (Fig. 4B) in gel retardation assays. AccR-P bound to both probes with an affinity similar to that observed with the complete $P_N$ probe (compare Fig. 4, C and D, with Fig. 3B), indicating that AccR-P is able to recognize and bind to two different operator regions within the $P_N$ promoter. To verify the role of the ATGCA-N$_6$-TGCAT palindromic binding in AccR binding to $P_N$, we constructed a mutant derivative of the PN2 probe (PN2mut) in which the left-half site of OR1 (ATGCA) was replaced by the sequence CCCTG. As shown in Fig. 4E, AccR-P was unable to bind to the PN2mut probe. Similarly, AccR-P was unable to bind to a truncated $P_N$ probe lacking the left-half site of OR1 (data not shown).

The results above demonstrate that AccR-P independently binds to two palindromic operator regions within the $P_N$ promoter. To confirm that the AccR operator regions are the ones responsible for carbon catabolite control of $P_N$, we constructed a truncated $P_N$ promoter that lacks the AccR binding regions ($P_N$: -61 to +79) in a $P_N$::lacZ fusion reporter plasmid and compared its activity with that of an otherwise identical wild-type $P_N$::lacZ reporter plasmid in Azoarcus sp. CIB. As anticipated, the $P_N$::lacZ fusion was essentially immune to CCR control in response to succinate (Fig. 4F).

**AccR Acts as a Repressor of the $P_N$ Promoter in Vitro**—Our in vivo experiments (Figs. 1 and 4F) suggested a repressor role for AccR on transcription from the $P_N$ promoter, which is consistent with the location of the AccR-P binding region spanning -35 to -153 (Fig. 4). To confirm this hypothesis, we performed in vitro transcription assays using a supercoiled DNA template bearing the $P_N$ promoter. Within these assays, activity from $P_N$ was elicited by constant concentrations of RNA polymerase and the Fnr$^+$ activator, an E. coli homolog of the Acpr activator of $P_N$ that retains activity in the presence of oxygen (25). The effect of different concentrations of AccR or AccR-P on $P_N$ output was then assessed. Whereas the formation of the expected 184-nucleotide transcript due to the activity of the $P_N$ promoter was unperturbed by the presence of AccR (Fig. 5, lanes 5–7), the levels of this transcript decreased in the presence of increasing amounts of AccR-P (Fig. 5, lanes 2–4). These data confirm the repressor role of AccR and demonstrate that...
its inhibitory action on \( P_N \) activity is strictly dependent on the phosphorylated status of AccR.

CAccR Functions as an Independent Monomeric DNA Binding Domain—As outlined above, Asp\(^{60} \) within the REC motif of the NAccR domain is involved in autophosphorylation, and CAccR contains the NarL/FixJ helix-turn-helix DNA-binding motif predicted to mediate DNA binding. To determine if CAccR could function independently in this capacity, we purified a His-tagged version of CAccR (His\(_{6}\)-CAccR) and monitored its DNA binding capacity in gel retardation assays using the PN probe (as in Fig. 3). The data in Fig. 6 demonstrate that CAccR binds to the PN probe in a concentration-dependent manner, experimentally verifying that this domain functions as an independent DNA binding domain.

Because phosphorylation of AccR provokes a monomer-to-dimer transition, we also determined the oligomeric status of CAccR. As shown in Fig. 6A, CAccR-P binds to two palindromic operator sequences within the \( P_N \) promoter. A, DNase I footprinting analyses of the \( P_N \) probe with 0–12 \( \mu \)M carbamoyl phosphate-treated His\(_{6}\)-AccR (AccR-P) as described under “Experimental Procedures.” Lane AG shows an A + G Maxam and Gilbert sequencing reaction with the location of the −10 and −35 boxes and the transcription initiation site (+1) indicated to the left. The AccR-P-protected region between positions −153 and −34 is highlighted by a bracket, with phosphodiester bonds mildly hypersensitive to DNase I cleavage indicated by asterisks. B, expanded view of the \( P_N \) promoter sequence. Inferred −10 and −35 promoter elements and the transcription start site (+1) are indicated in italic type. The ribosome-binding site (RBS) and the \( \text{bzdN} \) ATG initiation codon are underlined. The region protected by AccR-P (as in A) is boxed with the location of the palindrome operator sequences (OR1 and OR2) indicated by convergent arrows. Nucleotides corresponding to the P\(_{\text{max}}\) and P\(_{\text{mini}}\) probes used in C and D are underlined with discontinuous and continuous lines, respectively. C–E, gel retardation analyses of the indicated probes as in Fig. 3B. Gel retardation assays were performed using increasing concentrations (0–12 \( \mu \)M) of carbamoyl phosphate-treated His\(_{6}\)-AccR-P. The free probes and the major DNA-AccR-P complex are indicated by arrows. F, effect of succinate on the activity of the native \( P_N \) promoter and the truncated \( P_{NI} \) promoter that lacks the OR1 and OR2 operators. Azotobacter sp. CIB cells harboring the \( P_N::\text{lacZ} \) translational reporter plasmid pBBR5PN or the \( P_{NI}::\text{lacZ} \) translational reporter plasmid pBBR5PNI were grown anaerobically in minimal medium containing 3 mM benzoate (black bars) or a mixture of 3 mM benzoate and 0.2% succinate (white bars) until the mid-exponential phase. Values are the average of three independent experiments ± S.D. (error bars) and are graphed setting the β-galactosidase activities obtained for each promoter in the absence of succinate (22,000 and 2,500 Miller units for \( P_N \) and \( P_{NI} \), respectively) as 100%.

FIGURE 5. AccR-P represses transcription from the \( P_N \) promoter in vitro. Multiple-round in vitro transcription reactions, as detailed under “Experimental Procedures,” contained 5 nm supercoiled pJCD-PN plasmid DNA (which produces a 184-nucleotide mRNA from \( P_N \) and a 105-nucleotide control mRNA), 40 nm \( E. \) coli RNA polymerase, and 200 nm His\(_{6}\)-Fnr* activator. Reactions were carried out in the absence of AccR or in the presence of increasing concentrations of phosphorylated His\(_{6}\)-AccR (AccR-P) or unphosphorylated His\(_{6}\)-AccR (AccR).
CACC as described previously for full-length AccR. Sedimentation velocity experiments revealed a single species of CACC with an s value of 1.1 S (Fig. 6B). The molecular weight of the 1.1 S species, as measured by sedimentation equilibrium of CACC at different concentrations, is compatible with the mass of a monomer (Fig. 6C). We conclude from this data that, under our experimental conditions, DNA binding-proficient CACC is a monomer in solution. As expanded upon under “Discussion,” this suggests that dimerization of AccR after phosphorylation is carried out through the N-terminal domain and that dimerization is not a prerequisite for DNA binding per se.

AccRD60E Acts as a Superrepressor—The Asp⁶⁰ → Glu substitution renders AccR constitutively active (i.e. phosphorylation-independent) in terms of its DNA binding capacity (Fig. 3C). Therefore, we were interested to test if it also exhibited constitutive repressor activity in vivo. To this end, we used the heterologous E. coli AFMCPR strain, which carries a PA::lacZ translational fusion integrated into its chromosome (Table 1) and would be expected to lack a histidine kinase capable of phosphorylating AccR. Similar activities from the PA promoter were found in E. coli AFMCPR harboring either a vector control or a plasmid expressing AccR, whereas PA activity was reduced ~4-fold in the strain harboring a plasmid expressing AccRD60E (Fig. 7A).

The results outlined above demonstrate that the unphosphorylated AccRD60E protein is able to effectively bind and repress PA promoter activity in E. coli. To test if this was also the case in Azoarcus sp. CIB, we monitored the effect of expressing AccRD60E on the ability of the AccR null strain to grow anaerobically at the expense of benzoate. Whereas Azoarcus sp. CIBΔaccR harboring a plasmid expressing AccRD60E could not (Fig. 7B, triangles), Azoarcus sp. CIBΔaccR harboring a plasmid expressing AccRD60E protein could not (Fig. 7B, squares). These results strongly support the idea that AccRD60E is constitutively active in Azoarcus and behaves as a superrepressor of the bzd cluster through inhibiting the activity of the PA promoter.

AccR Is a Master Regulator Controlling All Central Pathways for the Anaerobic Catabolism of Aromatics in Azoarcus sp. CIB—In addition to the bzd-pathway, the Azoarcus sp. CIB mbd pathway for anaerobic assimilation of 3-methylbenzoate/m-xylene is also subject to CCR control in response to some organic acids (34). To explore whether AccR also controls expression of the mbd genes, we monitored the activity of the PB promoter that drives expression of the mbd genes using a
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CIB. Our evidence that AccR can be considered as a master regulator of the mbd genes in Azoarcus sp. CIB.

A third distinct central pathway for anaerobic degradation of aromatics in Azoarcus sp. CIB is the hbd pathway devoted to 3-hydroxybenzoate catabolism (22). To determine whether AccR also controls the expression of the genes for 3-hydroxybenzoate catabolism, Azoarcus sp. CIBΔAccR harboring a plasmid expressing wild-type AccR or AccRD60E was grown anaerobically in minimal medium containing 3-hydroxybenzoate as the sole carbon source. As was the case for growth at the expense of benzoate (Fig. 7B), cells expressing AccR were able to grow on 3-hydroxybenzoate, whereas those expressing the constitutively active AccRD60E superrepressor could not. Hence, these results strongly suggest that the genes responsible for the anaerobic catabolism of 3-hydroxybenzoate are also negatively controlled by AccR. Together with the impact of AccR on the bdz and mbd pathways, the results provide additional support for the idea that AccR is a master regulator of the anaerobic catabolism of aromatic compounds in Azoarcus sp. CIB.

The AccR-mediated CCR Control Is Only Operational under Anaerobic Growth Conditions—To explore whether AccR also mediates CCR control of aerobic degradation of aromatic compounds, we used real-time RT-PCR to monitor the activity of the P_{D\alpha} promoter, which drives the expression of the box genes for the aerobic degradation of benzoate in Azoarcus sp. CIB (27). When the cells were grown aerobically in benzoate, succinate significantly inhibited the activity of P_{D\alpha} promoter; however, this inhibition was not attributable to AccR because it was also observed in the AccR null strain (Fig. 9A). Because we have previously shown that the box genes can be also expressed under anaerobic growth conditions (27), we tested whether such anaerobic expression was controlled by AccR. In contrast to the results obtained under aerobic growth conditions, when the cells were grown anaerobically in the presence of benzoate, succinate-mediated inhibition was found in the wild-type but not in the AccR null strain (Fig. 9B). As is the case for the P_{N} and P_{D\alpha} promoters, the P_{D\beta} promoter was bound by AccR-P in gel retardation assays (Fig. 9C) and also contains a sequence that bears homology to the ATGCA-N_{6}-TGCAT motif identified on the basis of AccR-P binding to P_{N}. (Fig. 9D). Taken together, these results indicate that the AccR regulatory circuit operates through binding to common DNA motifs and is only operational under anaerobic growth conditions.

**DISCUSSION**

When faced with a mixture of alternative carbon sources, bacteria usually strategically utilize the substrate that yields the highest energy return. The utilization of preferred carbon sources is controlled by global regulatory mechanisms, termed CCR, which depend on the energy status of the cell. CCR control systems are also responsive to other environmental conditions, such as growth temperature and carbon source concentrations, and thus confer a competitive advantage in natural environments (4, 52, 53). Aromatic compounds usually constitute a secondary (non-preferred) carbon source for many bacteria. Diverse mechanisms underlie CCR control in aerobic metabolism of aromatic compounds (6–11, 16–18). Here we report the first known mechanism that accounts for CCR control of anaerobic catabolism of aromatic compounds in bacteria, namely the transcriptional repressor AccR of Azoarcus sp. CIB. Our evidence that AccR can be considered as a master regulator of anaerobic catabolism of aromatics is based on the finding that this protein controls expression of all known central anaerobic aromatic catabolic pathways of this strain. These include the bdz pathway for dissimilation of benzoate, the 3-methylbenzoate mbd pathway, the 3-hydroxybenzoate hbd pathway, and the benzoate box pathway as expressed under anaerobic conditions.
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**FIGURE 9.** *AccR* controls the activity of the *P* ~N~ promoter under anaerobic conditions. A and B, *Azoarcus* sp. CIB (*CIB*) and *Azoarcus* sp. CIBΔaccR (*CIBΔaccR*) cells were grown aerobically (A) or anaerobically (B) in minimal medium containing 3 mM benzoate (black bars) or a mixture of 3 mM benzoate and 0.2% succinate (white bars) until mid-exponential phase. Transcripts from the *P* ~N~ promoter were measured by real-time RT-PCR. Graphed values (in arbitrary units) are the average from three independent experiments ± S.D. (error bars). C, gel retardation analyses of *P* ~N~ probe as in Fig. 3B. Assays were performed with increasing concentrations (0–12 μM) of carbamoyl phosphate-treated His6-AccR (*AccR-P*). The free *P* ~N~ probe and the major *P* ~N~-AccR-P complex are indicated by arrows. *D*, sequence alignment of AccR binding regions in different catabolic promoters of *Azoarcus* sp. *CIB*: *P* ~N~ (OR2), operator region 2 of the *P* ~N~ promoter; *P* ~D~ (OR1), operator region 1 of the *P* ~D~ promoter; *P* ~B1~ *P* ~B2~, promoter of the *box* cluster; *P* ~B3~ *P* ~B4~ promoter of the *mbd* cluster. A consensus sequence of the AccR operator is shown at the bottom. Nucleotides identical to the consensus are shown in boldface type.

expression of the box pathway is only affected by the lack of AccR under anaerobic but not aerobic conditions (Fig. 9), we suggest that the AccR circuitry is only operational under anaerobic growth conditions.

*In silico* analysis assigns AccR to the FixJ/NarL family of response regulators (supplemental Fig. S1), whose members participate in the control of a wide variety of biological functions (45–47). We found that, like some previously characterized members of this family (e.g. FixJ, OmpR, PhoB, and StyR (54–56)), phosphorylation of AccR to AccR-P with high energy small molecule phosphodonors (e.g. carbamoyl phosphate) (Fig. 2B) triggers a monomer-to-dimer transition (Fig. 2, D and E); however, this reconfiguration does not change the apparent overall elongated shape of the AccR protomers. Under our *in vitro* conditions, only ~50% phosphorylation of AccR was achieved (Fig. 2, B and D). This could be attributable to partial phosphorylation or spontaneous dephosphorylation; alternatively, it may be due to an innate phosphatase activity of AccR. In this respect, it is well established that despite extensive structural and chemical similarities, the half-life of the phosphorylated state of response regulators varies extensively (from seconds to hours), depending on both their innate autophosphorylation and autodephosphorylation kinetics (42).

In addition to subunit configuration, phosphorylation controls the ability of AccR to bind DNA (Fig. 3) and its capacity to cause repression of promoter output in *in vitro* transcription assays (Fig. 5). Conversely, substitution of the highly conserved Asp60 residue of the N-terminal REC motif of AccR to a phosphomimic Glu residue (AccRD60E) renders the protein constitutively active (phosphorylation-independent) both in terms of its DNA binding ability (Fig. 3C) and its capacity to repress promoter output in intact cells (Fig. 7), suggesting that Asp60 is the target residue of phosphorylation. Thus, AccR behaves like a typical response regulator that upon activation represses promoter output both *in vivo* and *in vitro*. The lack of anaerobic growth in benzoate of an AccR null derivative of *Azoarcus* sp. CIB expressing the AccRD60E protein (Fig. 7B) is consistent with the role of this mutant regulator as a superrepressor of the *bdz* central pathway.

The phosphorylation-triggered monomer-to-dimer transition of AccR suggests that the AccR-P would bind DNA as a dimer, a configuration consistent with our identification of the inverted repeat ATGCA-N6-TGCAT as the AccR DNA binding site within the *P* ~N~ promoter. The *P* ~N~ promoter harbors two of these sites (denoted OR1 and OR2), which can each be bound independently by AccR with approximately equal affinity (Fig. 4), whereas the *P* ~B1~ and *P* ~D~ promoters, which are also targeted by AccR (Figs. 8 and 9), possess one copy of this motif (Fig. 9D). A truncated *P* ~N~ promoter that lacks the AccR binding regions (P~N~) did not show succinate-dependent CCR control (Fig. 4F), which illustrates how a rational design of a target promoter can be an interesting strategy to circumvent CCR in bacteria. The location of the AccR binding region within *P* ~N~ (spawning positions −35 to −153 relative to the transcriptional start site) is consistent with the observed repressor role of AccR. We can envisage two plausible and non-mutually exclusive means by which DNA binding by AccR-P could inhibit promoter output: (i) inhibition of closed complex formation by occluding RNA polymerase and/or (ii) inhibition of open complex formation by preventing binding of the AcpR activator, whose operator region spans from −42.5 to −39.5 (25).

In contrast to the apparent dimerization-dependent DNA binding of intact AccR-P, the DNA binding-proficient C-termi-
nal domain of AccR (CAccR) is monomeric in solution (Fig. 6). These findings suggest that (i) dimerization per se is not a prerequisit for DNA binding, and (ii) dimerization is carried out through the N-terminal domain, as exemplified by NarL (46). Further, the results suggest a regulatory scenario in which phosphorylation of Asp60 of AccR alleviates interdomain repression mediated by the N-terminal domain to allow reconfiguration to a DNA binding-proficient configuration. Because linker regions can be crucial for correct interdomain communication (57), it is plausible that the long linker region of AccR may play a role in transmitting the phosphorylation-dependent conformational changes (from the NAccR domain to the CAccR domain) to result in derepression and thus activation of its capacity to bind to target promoters. However, like a potential autophosphatase activity of AccR, these mechanistic possibilities require experimental verification.

As described in the Introduction, among the diverse proteins that have been found to be involved in CCR control of aerobic catabolism of aromatic compounds, only one, BphQ, is a response regulator. A search for accR homologs in bacterial genomes revealed the presence of AccR-like proteins (amino acid sequence identity higher than 70%) encoded in the genomes of other Azoarcus and Thauera strains (supplemental Fig. S1A). These AccR-like regulators cluster in a branch of the phylogenetic tree different from that of the BphQ-like regulators (supplemental Fig. S1B), which suggests that both types of proteins, although evolutionarily related, constitute different subgroups of response regulators. In this respect, it is worth noting that AccR from Azoarcus sp. CIB and BphQ from Acidovorax sp. KKS102 have completely different regulatory features; whereas BphQ is a transcriptional activator that binds to target promoters in the absence of phosphorylation (7), AccR is a repressor that requires phosphorylation in order to bind to a target operator that is completely different from that described for BphQ.

Many β-proteobacteria Azoarcus and Thauera strains are able to anaerobically metabolize aromatic compounds. The presence of accR orthologs within their genomes raises the possibility that AccR constitutes a new global regulator that controls anaerobic carbon catabolite repression in this group of organisms. Our observation that the accR genes are always located adjacent to a gene that encodes a putative sensor histidine kinase strongly suggests that the latter would be responsible for maintaining AccR in its active phosphorylated state in the presence of preferred carbon sources, such as succinate. The work presented here should greatly facilitate our future efforts aimed at characterizing this predicted two-component regulatory system and deciphering the environmental signals that trigger the activation/inactivation status of AccR.

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