New insights into the BzdR-mediated transcriptional regulation of the anaerobic catabolism of benzoate in *Azoarcus* sp. CIB

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The expression of the *bzd* genes involved in the anaerobic degradation of benzoate in *Azoarcus* sp. CIB is controlled by the specific BzdR transcriptional repressor at the P_N promoter. This catabolic promoter is also subject to catabolite repression by some organic acids. *In vivo* and *in vitro* experiments have shown that BzdR behaves as a repressor of the P_R promoter by overlapping the transcription initiation site as well as the -35 and -10 boxes, benzoyl-CoA being the inducer molecule. In addition, by using a $P_N::lacZ$ fusion both in *Azoarcus* sp. CIB and in an isogenic strain lacking the *bzdR* gene, we have shown that the succinate-dependent catabolite repression requires participation of the BzdR repressor.

INTRODUCTION

Since aromatic compounds are widely distributed in the environment, they are a common carbon source for micro-organisms (Harwood & Parales, 1996). As many ecosystems are often anoxic, the anaerobic catabolism of aromatic compounds by micro-organisms is important in the global biogeochemical cycles (Gibson & Harwood, 2002; Lovley, 2003). Benzoate has been used as a good model compound for studying the anaerobic catabolism of aromatic compounds. Benzoate is a common carbon source in nature that is funnelled directly to the widely distributed benzoyl-coenzyme A (benzoyl-CoA) central pathway (Carmona & Díaz, 2005; Gibson & Harwood, 2002; Harwood *et al.*, 1999). As a general rule, micro-organisms first activate benzoate to benzoyl-CoA, which is then converted to aliphatic intermediates through aromatic ring reduction, β -oxidation-like reactions and ring cleavage as the main steps. The biochemistry of the benzoyl-CoA central pathway has been well studied in some facultative anaerobic bacteria such as *Rhodopseudomonas palustris*, *Azoarcus evansii* and *Thauera aromatica* (Boll, 2005; Gibson & Harwood, 2002; Harwood *et al.*, 1999) and to a lesser extent in strict anaerobes such as *Desulfococcus multivorans* (Peters *et al.*, 2004), *Geobacter metallireducens* (Carmona & Díaz, 2005; Wischgoll *et al.*, 2005) and *Syntrophus aciditrophicus* (Elshahed *et al.*, 2001; Peters *et al.*, 2007). Moreover, molecular microbial ecology studies suggest that the ability to degrade aromatic compounds

under anaerobic conditions is more widespread among bacteria than previously thought (Jahn *et al.*, 2005; Safinowski *et al.*, 2006; Song & Ward, 2005). In contrast to the current knowledge on the biochemistry of the anaerobic benzoyl-CoA central pathway, few studies have been conducted to unravel the regulatory circuits that control the expression of the cognate genes, being mainly restricted to the regulation of the *bad* genes in *Rhodopseudomonas palustris* and the *bzd* genes in *Azoarcus* sp. CIB (Barragán *et al.*, 2005; Durante-Rodríguez *et al.*, 2006; Eglund & Harwood, 1999, 2000; Peres & Harwood, 2006). In the latter organism, the *bzd* catabolic genes are organized as a single operon (*bzdNOPQMSTUVWXYZA*) controlled by the P_N promoter (López Barragán *et al.*, 2004) and the *bzdR* gene, which is located immediately upstream of the catabolic operon and encodes the transcriptional repressor BzdR (Barragán *et al.*, 2005). The BzdR-mediated repression of P_N is alleviated by the inducer benzoyl-CoA, the first intermediate of the catabolic pathway (Barragán *et al.*, 2005) (Fig. 1). The BzdR protein is the prototype of a new subfamily of transcriptional regulators that exhibit a peculiar modular architecture. Thus, whereas the N-terminal region of BzdR (residues 1–130) shows significant similarity with members of the helix–turn–helix (HTH)-XRE family of transcriptional regulators (SMART release; available at <http://smart.embl-heidelberg.de/>), the C-terminal region (residues 131–298) shows similarity with shikimate kinases (PFAM accession no. PF01202) (Barragán *et al.*, 2005). Although the BzdR subfamily of transcriptional regulators is actively growing, and currently BzdR-like proteins can be identified by sequence comparison analyses in benzoate degradation clusters of over 30

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Abbreviation: RNAP, RNA polymerase.

Fig. 1. Scheme of the *bzd* cluster and P_R promoter from *Azoarcus* sp. CIB. (a) The *bzdR* regulatory gene and the *bzdNOPQMSTUVWXYZ* catabolic operon are shown as black and white boxes, respectively. The P_R and P_N promoters are indicated as thick white and grey arrows, respectively. The BzdR protein is represented as a black ellipse; benzoyl-CoA is represented as a white rectangle. The minus signs indicate repression of P_R and P_N by the BzdR protein. The ellipse within the rectangle represents a BzdR protein that is not able to repress the P_R or the P_N promoters. In the anaerobic catabolism of benzoate, this molecule is first activated to benzoyl-CoA by a benzoate-CoA ligase (BzdA) and is channelled to the central metabolism of the cell through a central pathway that involves the products of the genes *bzdNOPQMSTUVWXYZ*. (b) Expanded view of the P_R promoter. The nucleotide sequence from position -241 to +42 is shown. The transcription start site (+1) and the inferred -10 and -35 boxes of the P_R promoter are indicated. The ribosome-binding site (RBS) and the ATG start codon of the *bzdR* gene are shown in italic and bold letters, respectively. The BzdR-binding region (operator) is boxed. The TGCA and GCAC sequences within the operator region are underlined with a discontinuous line. A short palindromic structure is indicated by convergent arrows.

denitrifying proteobacteria from the α and β -subgroups, to our knowledge there have been no reports on the expression of any of the *bzdR* genes.

It is well known that expression of aromatic catabolic clusters is usually tightly controlled. Superimposed upon the specific regulation there is an additional control that links the induction of the pathway genes to the physiological status of the cell (Cases & de Lorenzo, 1998, 2005; Díaz & Prieto, 2000; Morales *et al.*, 2004; Tropel & van der Meer, 2004). In this context, it has been recently shown that the P_N promoter activity in *Azoarcus* sp. CIB is strictly dependent on the AcpR transcriptional activator, an

orthologue of the Fnr global regulator of *E. coli* that specifically controls the oxygen-dependent expression of the *bzd* catabolic operon (Durante-Rodríguez *et al.*, 2006). On the other hand, carbon catabolite repression is one of the most fundamental environmental-sensing mechanisms in bacteria and imparts competitive advantage by establishing priorities in carbon metabolism. The term catabolite repression describes a number of regulatory processes that ensure that when the cell is exposed to a preferred carbon source, the catabolic pathways for other, non-preferred, substrates are not induced, even when the appropriate inducers are present (Magasanik, 1970). Whereas catabolite repression of pathways for the aerobic catabolism of

aromatic compounds is well documented (Collier *et al.*, 1996; Ohtsubo *et al.*, 2006; Prieto *et al.*, 2004; Rojo & Dinamarca, 2004), only a couple of examples of this phenomenon have been reported for the anaerobic degradation of aromatic compounds in *T. aromatica* (Heider *et al.*, 1998) and *Azoarcus* sp. CIB (López Barragán *et al.*, 2004), and there are no studies on the molecular mechanisms that control such catabolite repression.

In this work we have studied the regulatory circuit that drives the expression of a gene encoding a transcriptional regulator (BzdR) of a catabolic operon involved in anaerobic degradation of aromatic compounds. Moreover, we provide genetic evidence that the BzdR repressor is also required for the catabolite repression exerted by some carbon sources on the anaerobic benzoate-degradation pathway in *Azoarcus* sp. CIB.

METHODS

Strains, plasmids, and growth conditions. The *Escherichia coli* and *Azoarcus* strains, as well as the plasmids used in this work, are listed in Table 1. *E. coli* cells were grown at 37 °C on Luria–Bertani (LB) medium (Miller, 1972). *Azoarcus* strains were grown at 30 °C on MC medium as described previously (López Barragán *et al.*, 2004). Where appropriate, antibiotics were added at the following concentrations: ampicillin, 100 µg ml⁻¹; gentamicin, 7.5 µg ml⁻¹; kanamycin, 50 µg ml⁻¹.

Molecular biology techniques. Recombinant DNA techniques were carried out by published methods (Sambrook & Russell, 2001).

Plasmid DNA was prepared with a High Pure plasmid isolation kit (Roche Applied Science). DNA fragments were purified with Gene-Clean Turbo (Q-BIOgene). Oligonucleotides were supplied by Sigma. 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) (Sambrook & Russell, 2001). Plasmids were transferred from *E. coli* S17-1λpir (donor strain) into *Azoarcus* sp. recipient strains by biparental filter mating as described previously (López Barragán *et al.*, 2004). Proteins were analysed by SDS-PAGE (Laemmli, 1970). The protein concentrations in cell extracts were determined by the method of Bradford (1976) using BSA as the standard.

β-Galactosidase assays. β-Galactosidase activities were measured with permeabilized cells as described by Miller (1972). Experiments were repeated at least three times for each sample.

Sequence data analyses. Sequence comparison analyses with the *Azoarcus* sp. strain EbN1 complete genome dataset were done using the TBLAST algorithm (Altschul *et al.*, 1990) at the National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi>).

Purification of His₆-BzdR. The recombinant plasmid pQE32-His₆BzdR (Table 1) was used for the overproduction of the His-tagged BzdR protein in *E. coli* strain M15 harbouring plasmid pREP4 (Table 1) as described previously (Barragán *et al.*, 2005).

Gel retardation assays. The DNA fragment used for gel retardation assays (*P_R* probe) was PCR amplified from plasmid pECOR7 (Table 1) by using oligonucleotides 5'PR-ScaI (5'-AAAAGTACTCGCGGTTCATGACGTTTCATCTG-3'; an engineered *ScaI* site is underlined)

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant phenotype and/or genotype	Reference or source
<i>E. coli</i>		
DH5α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA(Nal^r) relA1 Δ(argF-lac)U169 depR φ80dlac Δ(lacZ)M15</i>	Sambrook & Russell (2001)
S17-1λpir	Tp ^r Sm ^r , <i>recA thi hsdR</i> M ⁺ RP4::2-Tc::Mu::Km Tn7 λpir phage lysogen	de Lorenzo & Timmis (1994)
M15	Strain for regulated high-level expression with pQE vectors	Qiagen
<i>Azoarcus</i> sp. CIB		
CIB	Wild-type strain	López Barragán <i>et al.</i> (2004)
CIBdbzdR	Km ^r , <i>Azoarcus</i> sp. CIB with a disruption of the <i>bzdR</i> gene	López Barragán <i>et al.</i> (2004)
CIBdbzdN	Km ^r , <i>Azoarcus</i> sp. CIB with a disruption of the <i>bzdN</i> gene; does not express the <i>bzd</i> catabolic operon	Barragán <i>et al.</i> (2005)
Plasmids		
pQE32-His ₆ BzdR	Ap ^r , pQE32 derivative harbouring the His ₆ - <i>bzdR</i> gene under the control of the T5 promoter <i>lac</i> operator	Barragán <i>et al.</i> (2005)
pECOR7	Ap ^r , pUC19 containing a 7.1 kb <i>EcoRI</i> DNA fragment from λBzd1	López Barragán <i>et al.</i> (2004)
pSJ3P _R	Ap ^r , <i>oriColE1</i> , contains the <i>P_R</i> promoter driving the expression of the <i>lacZ</i> gene (<i>P_R::lacZ</i> fusion)	López Barragán <i>et al.</i> (2004)
pBBR1MCS-5	Gm ^r , <i>ori</i> pBBR1MCS Mob ⁺ <i>lacZα</i> , broad-host-range cloning and expression vector	Kovach <i>et al.</i> (1995)
pBBR5P _N	Gm ^r , pBBR1MCS-5 derivative harbouring a <i>P_N::lacZ</i> translational fusion	Barragán <i>et al.</i> (2005)
pBBR5P _R	Gm ^r , pBBR1MCS-5 derivative harbouring the <i>P_R::lacZ</i> translational fusion as a 3.8 kb <i>EcoRI</i> – <i>HindIII</i> fragment from pSJ3P _R	This work
pREP4	Km ^r , expresses the <i>lacI</i> repressor	Qiagen
pJCD01	Ap ^r , <i>oriColE1</i> , polylinker of pUC19 flanked by <i>rpoC</i> and <i>rrnBT1T2</i> terminators	Marschall <i>et al.</i> (1998)
pJCD-P _R	Ap ^r , pJCD01 derivative harbouring a 298 bp <i>SmaI</i> – <i>EcoRI</i> fragment that includes the <i>P_R</i> promoter	This work

and 3'PR-EcoRI (5'-CGGAATTCGCCCAACATCAGCAGGTAG-TTG-3'; an engineered *EcoRI* site is underlined). The amplified DNA was then digested with *ScaI* and *EcoRI* restriction enzymes, and the resulting 298 bp fragment was singly 3'-end labelled by filling in the overhanging *EcoRI*-digested end with [α - ^{32} P]dATP and the Klenow fragment of *E. coli* DNA polymerase as reported previously (Barragán *et al.*, 2005). The retardation reaction mixtures in FP buffer (20 mM Tris/HCl, pH 7.5, 10 % glycerol, 2 mM β -mercaptoethanol and 50 mM KCl) contained 0.05 nM DNA probe, 500 μ g BSA ml $^{-1}$ and purified His $_6$ -BzdR protein in a final volume of 9 μ l. After incubation of the retardation mixtures for 20 min at 30 °C, the mixtures were analysed by electrophoresis in 5 % polyacrylamide gels buffered with 0.5 $\times$ TBE (45 mM Tris/borate, 1 mM EDTA). The gels were dried on Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences).

DNase I footprinting assays. The DNA probe used for DNase I footprinting assays was the same as that reported for the gel retardation assays (see above). For the assays, the reaction mixture contained 2 nM DNA probe, 1 mg BSA ml $^{-1}$ and purified protein in 15 μ l FP buffer (see above). This mixture was incubated for 20 min at 37 °C, after which 3 μ l (0.05 units) of DNase I (Amersham Biosciences) (prepared in 10 mM CaCl $_2$, 10 mM MgCl $_2$, 125 mM KCl and 10 mM Tris/HCl, pH 7.5) was added, and the incubation was continued at 37 °C for 20 s. The reaction was stopped by the addition of 180 μ l of a solution containing 0.4 M sodium acetate, 2.5 mM EDTA, 50 μ g calf thymus DNA ml $^{-1}$ and 0.3 μ g glycogen ml $^{-1}$. After phenol extraction, DNA fragments were analysed as previously described (Barragán *et al.*, 2005). A+G Maxam and Gilbert reactions (Maxam & Gilbert, 1980) were carried out with the same fragments and loaded on the gels along with the footprinting samples. The gels were dried on Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences).

In vitro transcription assays. Transcription assays were performed by a published procedure (Carmona & Magasanik, 1996). A 298 bp DNA fragment containing the P_R promoter was PCR amplified from plasmid pECOR7 (Table 1) by using oligonucleotides 5'PR-*ScaI* and 3'PR-EcoRI (see above), and it was cloned into the *SmaI/EcoRI*-restricted pJCD01 cloning vector (Marschall *et al.*, 1998), yielding plasmid pJCD- P_R (Table 1). Reactions (50 μ l mixtures) were performed in a buffer consisting of 50 mM Tris/HCl (pH 7.5), 50 mM KCl, 10 mM MgCl $_2$, 0.1 mM BSA, 10 mM dithiothreitol and 1 mM EDTA. Each DNA template (0.5 nM of supercoiled plasmid pJCD- P_R) was premixed with 50 nM σ^{70} -containing *E. coli* RNA polymerase (Amersham) and different amounts of purified His $_6$ -BzdR and benzoyl-CoA. For multiple-round assays, transcription was then initiated by adding a mixture of 500 nM (each) ATP, CTP and GTP; 50 mM UTP; and 2.5 μ Ci [α - ^{32} P]UTP (3000 mCi mmol $^{-1}$; 111 GBq mmol $^{-1}$). After incubation for 15 min at 37 °C, the reactions were stopped with an equal volume of a solution containing 50 mM EDTA, 350 mM NaCl and 0.5 mg carrier tRNA ml $^{-1}$. The mRNA produced was then precipitated with ethanol, redissolved in loading buffer (7M urea, 1 mM EDTA, 0.6 M glycerol, 0.9 mM bromophenol blue and 1.1 mM xylene cyanol), electrophoresed on a denaturing 7M urea-4 % polyacrylamide gel, and visualized by autoradiography. Transcript levels were quantified with a Bio-Rad Molecular Imager FX system. The reactions were done using as a template pJCD01 plasmid derivatives, which enabled an internal control mRNA of 105 nt to be obtained.

Primer extension analysis. *Azoarcus* sp. CIB cells harbouring plasmid pBBR5P $_R$ (P_R : *lacZ*) were grown anaerobically on benzoate-containing MC medium until the culture reached an OD $_{600}$ of 0.3. Total RNA was isolated by using an RNeasy Mini kit (Qiagen) according to the instructions of the supplier. Primer extension reactions were carried out with the avian myeloblastosis virus reverse

transcriptase (Promega) and 10 μ g total RNA as described previously (Martín *et al.*, 1996), using oligonucleotide Lac57 (5'-CGATTAAG-TTGGGTAACGCCAGGG-3') labelled at its 5'-end with phage T4 polynucleotide kinase and [γ - ^{32} P]ATP (3000 Ci, 111 TBq mmol $^{-1}$; Amersham Biosciences). To determine the length of the primer extension products, sequencing reactions of pBBR5P $_R$ were carried out with oligonucleotide Lac57 using the T7 sequencing kit and [α - ^{32} P]dCTP (Amersham Biosciences) as indicated by the supplier. Products were analysed on 6 % polyacrylamide-urea gels. The gels were dried onto Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences).

Real-time RT-PCR assay. For this assay, RNA was extracted from *Azoarcus* sp. CIB cells grown on benzoate (3 mM), succinate (7.5 mM), or benzoate (3 mM) plus succinate (7.5 mM). Cells were harvested at the exponential phase of growth (OD $_{600}$ 0.2) and stored at -80 °C. Pellets were thawed and cells lysed in TE buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA) containing 5 mg lysozyme ml $^{-1}$ by a series of freeze/thaw cycles. RNA was extracted using the RNeasy Mini kit (Qiagen), including a DNase treatment according to the manufacturer's instructions, precipitated with ethanol, washed and resuspended in 40 μ l RNase-free water. The concentration and purity of the RNA samples were measured by using an ND1000 spectrophotometer (Nanodrop Technologies) according to the manufacturer's protocols. Analyses of the transcript levels from the P_N or P_R promoters were carried out with 20 μ l reverse transcription reactions containing 2 μ g RNA, 0.5 mM of each dNTP, 200 U SuperScript II reverse transcriptase (Invitrogen) and 0.5 μ M primer 3RTpN2 (5'-GTGTAGGTACACATCGTTGC-3') or 3RTpR2 (5'-GAGGATGTCCAGGAAGGATTG-3'), respectively, in the buffer recommended by the manufacturer. The reactions were incubated at 42 °C for 2 h, terminated by incubation at 70 °C for 15 min, and the template RNA was removed by incubation at 37 °C in the presence of 20 U RNase H (Amersham Biosciences) for 20 min. The cDNA obtained was stored at -20 °C. The IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) was used for real-time PCR in a 25 μ l reaction containing 10 μ l diluted cDNA, 0.2 μ M 3RTpR2 primer, 0.2 μ M 5RTpR1 primer (5'-GCTGTCATCGTGCTTCACG-3') for the P_R promoter, or 0.2 μ M 3RTpN2 primer, 0.2 μ M 5RTpN1 primer (5'-GCAACACATCAGAGGAGATAG-3') for the P_N promoter, and 12.5 μ l SYBR Green mix (Applied Biosystems). PCR amplifications were carried out as follows: 1 initial cycle of denaturation (95 °C for 3 min), followed by 44 cycles of amplification (95 °C, 30 s; test annealing temperature, 57 °C, 30 s; elongation and signal acquisition, 72 °C, 30s). For relative quantification of the fluorescence values, a calibration curve of serial dilutions of the genomic DNA sample was made.

RESULTS AND DISCUSSION

BzdR auto-regulates its expression

Transcriptional regulators governing the expression of catabolic promoters from aromatic catabolic pathways usually also control their own expression (Díaz & Prieto, 2000; Tropel & van der Meer, 2004). However, there appear to be no studies on the auto-regulation of transcriptional regulators controlling anaerobic aromatic pathways. To gain some knowledge on this topic, we checked whether the BzdR regulator from the anaerobic *bzd* cluster of *Azoarcus* sp. CIB also controls the activity of its own P_R promoter. To accomplish this task, we fused the P_R promoter to the *lacZ* reporter gene to generate plasmid

Fig. 2. Expression of the *bzdR* gene under different growth conditions. (a) Activity of the P_R promoter. β -Galactosidase activity of *Azoarcus* sp. CIB (white bars), *Azoarcus* sp. CIBdbzdR (black bars) and *Azoarcus* sp. CIBdbzdN (grey bars) cells harbouring plasmid pBBR5P_R ($P_R::lacZ$) was assayed. Cells were grown anaerobically until mid-exponential phase on MC medium containing 3 mM benzoate (B), 7.5 mM succinate (S), or 3 mM benzoate plus 7.5 mM succinate (B+S). β -Galactosidase activity was measured as described in Methods. (b) β -Galactosidase activities of *Azoarcus* sp. CIB (white bars) or *Azoarcus* sp. CIBdacpR (hatched bars) cells grown aerobically (+O₂) or anaerobically (-O₂) until mid-exponential phase on MC medium containing 3 mM benzoate (B) or 7.5 mM succinate (S). (c) Activity of the P_R promoter measured by real-time RT-PCR was determined as detailed in Methods. Relative changes in the P_R transcript level are indicated as percentages of the values obtained from cells growing on benzoate plus succinate. Error bars in (a) and (b) indicate standard deviations ($n=3$).

pBBR5P_R (Table 1). We then analysed the β -galactosidase activity when *Azoarcus* sp. CIBdbzdR, a mutant strain carrying a disrupted *bzdR* gene (Table 1), harbouring plasmid pBBR5P_R ($P_R::lacZ$), was grown on benzoate, succinate, or benzoate plus succinate as carbon sources. In all conditions tested, the *bzdR* mutant cells showed similar levels of expression of the $P_R::lacZ$ fusion to those of the wild-type *Azoarcus* sp. CIB(pBBR5P_R) growing on benzoate (induction conditions) (Fig. 2a), which suggests that the *bzdR* gene product indeed behaves as a repressor of its own expression.

It has been shown before that benzoyl-CoA is the inducer molecule that relieves the repression of BzdR on the P_N promoter (Barragán *et al.*, 2005). To check whether benzoyl-CoA was also alleviating the BzdR-mediated repression of P_R , we monitored the expression of the $P_R::lacZ$ fusion in *Azoarcus* sp. CIBdbzdN, a mutant strain that is unable to grow anaerobically on benzoate and that does not form benzoyl-CoA because the expression of the entire transcriptional *bzd* unit has been interrupted (López Barragán *et al.*, 2004). *Azoarcus* sp. CIBdbzdN cells harbouring plasmid pBBR5P_R and grown on succinate or succinate plus benzoate showed the same levels of P_R activity as the wild-type strain grown in the presence of succinate, and about twofold lower levels of P_R activity than the wild-type strain grown in the presence of benzoate or benzoate plus succinate (Fig. 2a). These results, therefore, suggest that the repressor role of BzdR on the P_R promoter becomes relieved when the cells grow in the presence of benzoate and benzoyl-CoA is formed.

To confirm *in vitro* the regulation of the P_R promoter by the BzdR regulator, we first mapped the transcription start site of P_R by primer extension analysis performed with total

RNA isolated from cells of *Azoarcus* sp. CIB(pBBR5P_R) grown on benzoate. Transcription initiation was located 39 nt upstream of the ATG translation initiation codon of the *bzdR* gene (Fig. 3), showing putative -10 (CACTAT) and -35 (TTGCAA) boxes separated by 17 bp (Fig. 1) that match significantly the -10 (TATAAT) and -35

Fig. 3. Identification of the transcription start site in the P_R promoter. Total mRNA was isolated from *Azoarcus* sp. CIB cells harbouring plasmid pBBR5P_R ($P_R::lacZ$), as described in Methods. The size of the extended product (lane P_R) was determined by comparison with the DNA sequencing ladder (lanes A, C, G and T) of the P_R promoter region. Primer extension and sequencing reactions were performed with primer Lac57 as described in Methods. An expanded view of the nucleotides surrounding the transcription initiation site (asterisk) in the noncoding strand is shown.

Fig. 4. *In vitro* activity of the P_R promoter. Multiple-round *in vitro* transcription reactions were carried out by using pJCD- P_R , a plasmid template that produces mRNA from P_R of 242 nt. *In vitro* transcription reactions were performed with 50 nM *E. coli* RNAP in the absence of His₆-BzdR (lane 2), or in the presence of 50 nM (lane 3), 100 nM (lane 4) or 200 nM (lane 5) purified His₆-BzdR. In lane 6, 1 mM benzoyl-CoA was added to the transcription reaction containing 200 nM purified His₆-BzdR. Heat-inactivated His₆-BzdR (200 nM) was added as a control to the transcription reaction (lane 1).

(TTGACA) consensus sequences recognized by the σ^{70} subunit of RNA polymerase (σ^{70} -RNAP). We then performed *in vitro* transcription assays using purified

His₆-BzdR protein, RNAP and plasmid pJCD- P_R , which contains a DNA fragment including the P_R promoter (Table 1), as supercoiled DNA template. As shown in Fig. 4, the formation of the expected 242 nt transcript due to the activity of the P_R promoter was inhibited by increasing concentrations of His₆-BzdR (Fig. 4, lanes 3–5). In the presence of 200 nM His₆-BzdR, the addition of benzoyl-CoA was able to increase the formation of the P_R transcript (Fig. 4, lane 6), suggesting that this CoA derivative acts as an inducer avoiding the repression effect of BzdR. These results are in agreement with the previous *lacZ* gene fusion experiments (see above), and they reveal a similar pattern of benzoyl-CoA-dependent induction of the BzdR-mediated repression at both the P_R and P_N (Barragán *et al.*, 2005) promoters from *Azoarcus* sp. CIB.

Characterization of the BzdR- P_R interaction

To study the interaction of BzdR with the P_R promoter, we first performed gel retardation assays with purified His₆-BzdR protein and a P_R probe spanning from position –169 to +120 of the P_R promoter. The His₆-BzdR protein bound to the P_R promoter in a protein concentration-dependent manner (Fig. 5a), and this interaction was

Fig. 5. *In vitro* analysis of the interaction of BzdR with the P_R promoter. (a) Gel retardation analysis of His₆-BzdR binding to the P_R promoter, performed as indicated in Methods. Lane 1, free P_R probe; lanes 2–5, retardation assays containing 0.05, 0.1, 0.5 and 1 μ M, respectively, of purified His₆-BzdR. The P_R probe and the P_R -BzdR complex are indicated by arrows. (b) DNase I footprinting analysis of the interaction of purified His₆-BzdR with the P_R promoter region. The experiments were carried out using the P_R probe as indicated in Methods. Lane 1, footprinting assay in the absence of His₆-BzdR; lanes 2–5, footprinting assays containing 0.1, 0.2, 0.5 and 1 μ M purified His₆-BzdR, respectively; lane AG, A + G Maxam and Gilbert sequencing reaction. The protected region is marked by a bracket, and the phosphodiester bonds hypersensitive to DNase I cleavage are indicated by asterisks. The –35 and –10 boxes and the transcription initiation site (+1) of the P_R promoter are also shown.

specific because it was inhibited by adding unlabelled P_R probe and it was not affected by adding an unlabelled heterologous probe to the retardation assays (data not shown).

The BzdR operator at the P_R promoter was identified by using DNase I footprinting assays. As shown in Fig. 5(b), the His₆-BzdR protein protected a region spanning from position -33 to +25 of the P_R promoter. Three direct GCAC repetitions and a short palindromic structure located 2 nt downstream of the +1 site were observed (Fig. 1). The fact that the operator of BzdR spans the transcription initiation site as well as the -10 and -35 boxes of P_R (Figs 1 and 5b) is in agreement with the repressor role of BzdR on the activity of this promoter (see above). Since BzdR also overlaps the transcription initiation site as well as the -10 box at the catabolic P_N promoter (Barragán *et al.*, 2005), BzdR appears to act at both the P_R and P_N promoters using the same repression mechanism. Binding of BzdR to P_R induces changes in the DNA structure as revealed by several phosphodiester bonds that become hypersensitive to DNase I cleavage (Fig. 5b). The same effect was also observed in the BzdR- P_N interaction (Barragán *et al.*, 2005). However, the binding affinity of BzdR to P_N and P_R promoters is quite different. Thus, whereas 50 nM of purified His₆-BzdR was needed to protect the P_N promoter from DNase I digestion (Barragán *et al.*, 2005), protection of the P_R promoter was achieved with 0.5–1 µM of purified regulator (Fig. 5b). This result is also in agreement with the moderate (Fig. 2) and high repression (Barragán *et al.*, 2005) caused by BzdR at the P_R and P_N promoters, respectively. It should be noted that three BzdR operators were identified at the P_N promoter, and all of them showed a direct repetition of the TGCA sequence forming part of a long palindromic operator structure (Barragán *et al.*, 2005). This promoter architecture resembles that of promoters regulated by other members of the HTH-XRE family of transcriptional regulators such as the Cro and 434 repressors from the λ and 434 phages, respectively, which also bind to short repeated sequences that, in most cases, are located within palindromic regions that span the promoters (Cervin *et al.*, 1998; Koudelka & Lam, 1993; Mandic-Mulec *et al.*, 1995; Weickert & Adhya, 1992). In contrast, only one BzdR operator, which contains a sole TGCA sequence located at the 5'-end (positions -32 to -29) of the protected region, was identified at the P_R promoter. Therefore, the lower binding efficiency of BzdR to P_R might reflect the different architecture of the P_N and P_R promoters.

Role of oxygen and additional carbon sources in the expression of the *bzdR* gene

Since the *bzd* genes are responsible for the anaerobic metabolism of benzoate in *Azoarcus* sp. CIB, the presence or absence of oxygen could play an essential role in the expression of such genes. In agreement with this, we have shown previously that the activity of the catabolic P_N

promoter is inhibited by oxygen (Durante-Rodríguez *et al.*, 2006). To determine whether oxygen controls the expression of P_R , we checked the activity of the P_R promoter driving the expression of the *bzdR* gene when *Azoarcus* sp. CIB was cultivated in the presence or absence of oxygen. To this end, we determined the β -galactosidase activity in *Azoarcus* sp. CIB(pBBR5P_R) cells that had reached, under aerobic or anaerobic conditions, mid-exponential growth phase on benzoate-containing minimal medium. As shown in Fig. 2(a, b), although the activity of the P_R promoter was almost twofold higher in cells growing aerobically than in cells growing anaerobically, oxygen does not appear to play a major role in the expression of the P_R promoter. To confirm this result, we checked the expression of the P_R promoter in *Azoarcus* sp. CIB*dacpR*, a strain that harbours a disruption of the *acpR* gene required for the oxygen-dependent activity of the catabolic P_N promoter and, therefore, unable to grow anaerobically on benzoate (Durante-Rodríguez *et al.*, 2006). As shown in Fig. 2(b), the activity of the P_R promoter in *Azoarcus* sp. CIB*dacpR*(pBBR5P_R) cells growing anaerobically on succinate-containing minimal medium was similar to that observed in *Azoarcus* sp. CIB(pBBR5P_R) cells. Moreover, Western-blotting experiments using anti-BzdR antibodies revealed that the levels of BzdR protein in crude extracts of *Azoarcus* sp. CIB cells grown either aerobically or anaerobically were similar (data not shown). All these results taken together indicate that the P_R promoter is not subject to the AcpR/oxygen-dependent superimposed regulation that controls the activity of the catabolic P_N promoter in *Azoarcus* sp. CIB. The aerobic expression of the *bzdR* gene might contribute to the lack of expression of the *bzd* catabolic operon when the cells grow in the presence of oxygen, thus avoiding the production of some oxygen-sensitive enzymes such as benzoyl-CoA reductase (BzdNOPQ) (López Barragán *et al.*, 2004).

The simultaneous presence of different carbon sources plays a major role in the superimposed regulation of aromatic catabolic clusters (Cases & de Lorenzo, 2005; Díaz & Prieto, 2000; Carmona *et al.*, 2008). Whereas carbon catabolite repression of regulatory genes has been reported in aerobic degradation pathways (Canosa *et al.*, 2000; Müller *et al.*, 1996), such superimposed regulation of regulatory genes controlling aromatic anaerobic clusters has not been studied so far. As shown in Fig. 2(a), the activity of the P_R promoter in *Azoarcus* sp. CIB(pBBR5P_R) was almost twofold higher when the cells were grown on benzoate or benzoate plus succinate than when they were grown on succinate as sole carbon source. Therefore, in contrast to what had been observed for the *bzd* catabolic genes, whose expression becomes significantly reduced when the cells grow in the presence of benzoate and an additional carbon source such as succinate (López Barragán *et al.*, 2004), the expression of the *bzdR* gene does not decrease when succinate is provided as an additional carbon source. To confirm that the activity levels of the $P_R::lacZ$ fusion are indeed the result of the

transcriptional activity of the P_R promoter and are not due to a possible post-transcriptional regulation of the *lacZ* gene, we performed real-time RT-PCR experiments to monitor the P_R transcript levels directly. The results obtained using RNAs isolated from *Azoarcus* sp. CIB cells grown on succinate, benzoate or succinate plus benzoate were in good agreement with those reported by β -galactosidase assays with the $P_R::lacZ$ fusion (Fig. 2c). Therefore, all these results indicate that the expression of the *bzdR* gene is not subject to catabolite repression by organic acids, such as succinate, in *Azoarcus* sp. CIB.

BzdR is required for the catabolite repression of the *bzd* catabolic operon

As previously reported for *Azoarcus* sp. CIB*lacZ*, a strain that contains the $P_N::lacZ$ translational fusion integrated into its chromosome, the P_N promoter is subject to carbon catabolite repression by some organic acids such as succinate, malate and acetate (López Barragán *et al.*, 2004). Whereas the molecular basis of the carbon catabolite repression has been studied in some aromatic aerobic clusters, there are no data on the factor(s) involved in catabolite repression of aromatic anaerobic clusters. To try to identify some of the factors involved in such catabolite repression, we analysed the expression of the $P_N::lacZ$ translational fusion in *Azoarcus* sp. CIB and *Azoarcus* sp. CIB*dbzdR* strains harbouring plasmid pBBR5*P_N* ($P_N::lacZ$) (Table 1). As expected, the β -galactosidase activity levels in *Azoarcus* sp. CIB(pBBR5*P_N*) cells grown anaerobically in the presence of benzoate plus succinate were threefold lower than those obtained in cells grown on benzoate as the sole carbon source (Fig. 6a), which confirms the observed catabolite repression exerted by succinate at the P_N promoter when expressing the $P_N::lacZ$ fusion from the chromosome (López Barragán *et al.*, 2004). However, it is interesting to note a stronger catabolite repression (about 500-fold repression) using the chromosome-inserted $P_N::lacZ$ fusion (López Barragán *et al.*, 2004), suggesting that the element(s) responsible for the catabolite repression is present in *Azoarcus* sp. CIB at a concentration that cannot overcome the multicopy dosage effect of the $P_N::lacZ$ fusion in plasmid pBBR5*P_N*. To confirm that the activity levels of the $P_N::lacZ$ fusion are indeed the result of the transcriptional activity of the P_N promoter and they are not due to a possible post-transcriptional regulation of the *lacZ* gene, we performed real-time RT-PCR experiments to monitor the P_N transcript levels directly. The results obtained using RNAs isolated from *Azoarcus* sp. CIB cells grown on succinate, benzoate or succinate plus benzoate were in good agreement with those reported by β -galactosidase assays with the $P_N::lacZ$ fusion, and they confirmed that the catabolite repression caused by succinate on the P_N promoter is exerted at the transcriptional level (Fig. 6b).

Interestingly, the β -galactosidase activity levels in *Azoarcus* sp. CIB*dbzdR*(pBBR5*P_N*) cells grown in the presence of

Fig. 6. *In vivo* activity of the P_N promoter. *Azoarcus* sp. CIB cells were grown anaerobically on MC medium containing 3 mM benzoate (B), 7.5 mM succinate (S), or a mixture of 3 mM benzoate and 7.5 mM succinate (B+S), until the cultures reached mid-exponential phase. (a) Expression of the $P_N::lacZ$ reporter fusion. β -Galactosidase activity of *Azoarcus* sp. CIB (white bars) or *Azoarcus* sp. CIB*dbzdR* (black bars) cells harbouring plasmid pBBR5*P_N* ($P_N::lacZ$) was assayed as described in Methods. Error bars indicate standard deviations ($n=3$). (b) Activity of the P_N promoter measured by real-time RT-PCR. Total RNA was obtained from *Azoarcus* sp. CIB cultures, and real-time RT-PCR was performed as detailed in Methods. Relative changes in the P_N transcript levels are indicated as percentages of the values obtained from cells growing on benzoate. The results of one experiment are shown, and the values were reproducible in three separate experiments with standard deviations of $<10\%$ of the mean.

benzoate plus succinate were similar to those obtained with cells grown on benzoate (Fig. 6a). Moreover, when the cells were grown under non-inducing conditions, i.e. in the presence of succinate as sole carbon source, the expression of the $P_N::lacZ$ fusion in *Azoarcus* sp. CIB*dbzdR*(pBBR5*P_N*) was similar to that observed in wild-type *Azoarcus* sp. CIB(pBBR5*P_N*) cells growing in the presence of benzoate (Fig. 6a). All these data taken together indicate that the inactivated BzdR relieves the catabolite repression exerted by succinate at the P_N promoter.

Concluding remarks

There are several reports showing that the downregulation of promoters from catabolic pathways when the cells grow in the presence of the particular substrate and a preferred carbon source is mediated by shifting the levels of the cognate transcriptional regulators. Thus, catabolite repression of the alkane degradation pathway (*alk* genes) encoded in the OCT plasmid from *Pseudomonas putida* strain GPO1 parallels a decrease of the expression of the AlkS activator (Canosa *et al.*, 2000). Similarly, catabolite repression of phenol degradation (*phl* genes) in *P. putida* strain H occurs by interfering with the activating function of the PhlR transcriptional regulator (Müller *et al.*, 1996). In this work, however, we show that a specific transcriptional regulator whose expression is not subject to catabolite repression (see above) mediates the catabolite repression of the cognate catabolic operon. Moreover, our results suggest the existence of additional factors that, together with BzdR, might account for the molecular basis of the catabolite repression of the anaerobic benzoate degradation pathway in *Azoarcus* sp. CIB. A possible scenario for catabolite repression that would explain our results is that BzdR might recruit an additional regulatory factor that binds to the P_N promoter in response to succinate, thus preventing the basal levels of *bzd* readthrough that allow the effector benzoyl-CoA to be generated. In this context, proteins such as Crc (catabolite repression control) (Morales *et al.*, 2004), PtsN and PtsO (nitrogen phosphotransferase system) (Aranda-Olmedo *et al.*, 2005, 2006; Cases & de Lorenzo, 2005; Marqués *et al.*, 2006), and some *o*-type terminal oxidases (Cyo) that sense the redox state of the cell (Rojo & Dinamarca, 2004; Morales *et al.*, 2006; Petruschka *et al.*, 2001) have been reported to be involved in catabolite repression of several aromatic degradation pathways in *Pseudomonas*. Recently, a response regulator (BphQ) of a two-component regulatory system was shown to control catabolite repression of the *bph* operon for the degradation of polychlorobiphenyl/biphenyl in the β -proteobacterium *Acidovorax* sp. KKS102 (Ohtsubo *et al.*, 2006). Orthologues of these carbon-repression mediators have been found in the genome of *Azoarcus* sp. EbN1, another *Azoarcus* strain that degrades aromatic compounds and whose complete genome is known (Rabus *et al.*, 2005). Thus, *Azoarcus* sp. EbN1 has an orthologue of the Crc (EbA3323), CRP (EbA7043), PtsN/PtsO (EbA27937/EbA2794) and BphQ (EbA125) proteins, as well as three orthologues of the CyoB protein (EbA4228, EbA156 and EbA4554). The involvement of all or some of these proteins in carbon catabolite repression in *Azoarcus* sp. CIB, and the molecular mechanisms underlying the role of BzdR in the carbon catabolite repression, will be explored in future work and will allow us to increase our current view on the superimposed regulation of the *bzd* gene cluster, so far the best-studied regulatory system in anaerobic catabolism of aromatic compounds.

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