

## Differential DNA Binding of Transcriptional Regulator PcaU from *Acinetobacter* sp. Strain ADP1

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**Transcriptional regulator PcaU from *Acinetobacter* sp. strain ADP1 governs expression of genes for protocatechuate degradation (*pca* genes) as a repressor or an activator depending on the levels of the inducer protocatechuate and of its own gene. PcaU is a member of the IclR protein family. Here the DNA binding properties of the purified protein are described in terms of the location of the binding sites and the affinity to these sites. Native PcaU was purified after overexpression of the *pcaU* gene in *Escherichia coli*. It is a dimer in solution. The binding site in the *pcaU-pcaI* intergenic region is located between the two divergent promoters covering 45 bp, which includes three perfect 10-bp repetitions. A PcaU binding site downstream of *pcaU* is covered by PcaU across two palindromic sequence repetitions. The affinity of PcaU for the intergenic binding sites is 50-fold higher (dissociation constant [ $K_d$ ], 0.16 nM) than the affinity for the site downstream of *pcaU* ( $K_d$ , 8 nM). The binding of PcaU was tested after modifications of the intergenic binding site. Removal of any external sequence repetition still allowed for specific binding of PcaU, but the affinity was significantly reduced, suggesting an important role for all three sequence repetitions in gene expression. The involvement of DNA bending in the regulatory process is suggested by the observed strong intrinsic curvature displayed by the *pcaU-pcaI* intergenic DNA.**

The  $\beta$ -ketoacid pathway is of central importance for growth of soil bacterium *Acinetobacter* sp. strain ADP1 on aromatic compounds, which comprise a considerable part of the biomass on earth in the form of lignin (21). In two separate sets of enzymatic reactions it catalyzes the successive breakdown of the starting metabolites protocatechuate and catechol into the common metabolites of the central carbon metabolism acetyl-coenzyme A (CoA) and succinyl-CoA, and the two branches of the pathway appear to underlie sophisticated specific regulation enabling efficient gene expression (5, 17, 35). Enzymes for the breakdown of protocatechuate are encoded by the *pca* genes, which form a cluster on the chromosome of *Acinetobacter* sp. strain ADP1. This cluster could be shown to form a supraoperonic chromosomal region by the discovery of other gene clusters adjacent to it encoding enzymes for pathways funneling more-complex aromatic compounds to protocatechuate (8, 9, 12, 13). The *pca* genes are likely to be expressed as a polycistronic transcript of at least 8 kbp from promoter *pcaIp* upstream of *pcaI*, the first structural gene as judged from the effect of a *pcaIp* mutation on *pcaHG* expression (16). This promoter is controlled in a complex manner by transcriptional regulator PcaU (17). Addition of protocatechuate or precursors thereof leads to a PcaU-dependent activation of gene expression, the level of which depends on the nature of the carbon source added (42). At the same promoter PcaU exerts a repressing function when no effector is added (42). In addition it is involved in regulation of its own gene expression. The *pcaU* gene is located upstream of the *pca* gene

cluster and is transcribed in the opposite direction, forming a 282-bp intergenic region (Fig. 1). Promoter *pcaUp* is repressed 6- to 11-fold when cells contain functional PcaU (42).

The PcaU protein has similarities in amino acid sequence to a number of proteins of wide distribution including proteins of members of the gram-positive bacteria, the *Thermotogales*, and the *Archaea*, indicating a well-conserved protein family. In all cases the function of these proteins as transcriptional regulators has either been shown or is predicted. The closest homologues are PcbR from *Acinetobacter* sp. strain ADP1 (50% identical residues [8]) and PcaR from *Rhodococcus opacus* (33% identical residues [14]), from *Pseudomonas aeruginosa* (33% identical residues, GenBank accession no. AE004453), and from *Pseudomonas putida* (31% identical residues [36]). Specific binding of PcaU contained in an *Escherichia coli* cell extract to a DNA fragment consisting of part of the *pcaU-pcaI* intergenic region and to a DNA fragment containing DNA directly downstream of *pcaU* has been shown. Both fragments contain three sequence repetitions; it has been suggested that these are recognized and bound by PcaU (17). A first indication that full transcriptional activation requires the presence of all three sequence repetitions comes from the recent identification of spontaneous mutations in the *pcaU-pcaI* intergenic region (7). Nothing is known about the way in which PcaU interacts with this unusual potential binding site and how communication with RNA polymerase is accomplished. The only homologue that has been analyzed in more detail in this respect is PcaR from *P. putida* (18). Despite the high similarity of the two proteins and their potential (for PcaU) or proven (for PcaR) binding sites, they probably differ in activity, as judged from the location of the binding sites with respect to the regulated promoters. PcaR binding covers parts or all of the promoter signature (for the *pcaR* or *pcaII* promoter), whereas the suggested PcaU binding site is separated from the struc-

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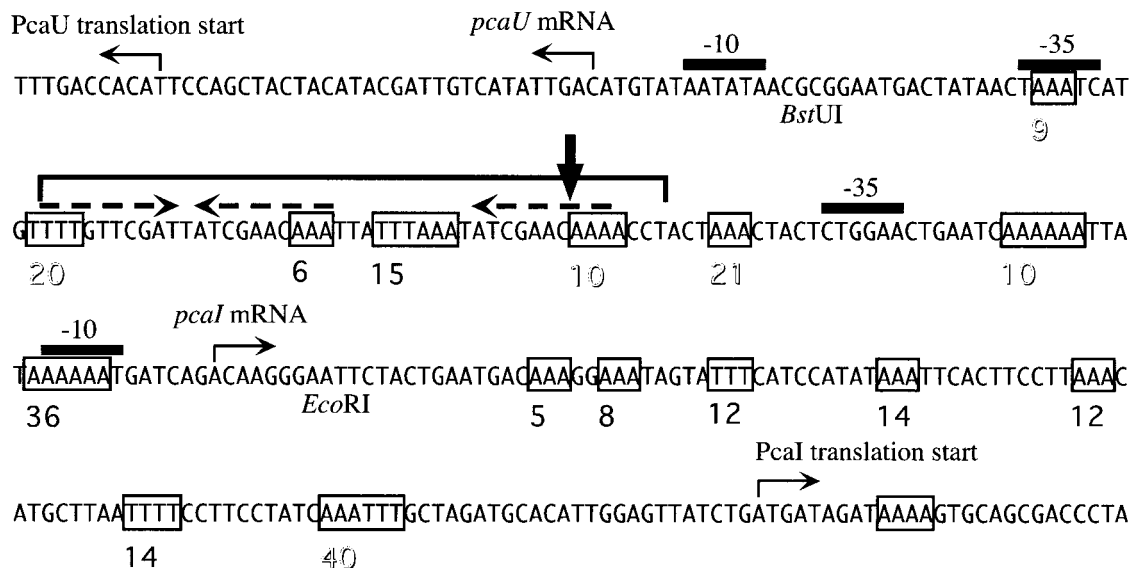


FIG. 1. Intergenic region between *pcaU* and *pcaI* from *Acinetobacter* sp. strain ADP1. Dashed arrows, three 10-bp sequence repetitions located in the binding site of regulator protein PcaU; bracket, region protected from DNase I footprinting by PcaU; boxes, stretches of at least three successive adenine residues. The distance from each stretch of As to the next one is given by a number underneath the sequence (printed open if the distance corresponds to one or multiple DNA helix turns). Vertical arrow, bending center for the 128-bp *BstUI-EcoRI* fragment.

tural gene promoter (−35 signature) by 14 bp and from the *pcaU* gene promoter (−35 signature) by 4 bp (17, 18). Here, we describe the purification of the native PcaU protein and, qualitatively and quantitatively, the interaction with its DNA binding sites. Reducing the binding site by one of the external sequence repetitions reduced the affinity of PcaU for these modified sites but did not eliminate specific binding. The question of the biological function of the PcaU binding site downstream of the *pcaU* gene is addressed by determining the effect of its deletion on structural gene expression. Furthermore, the possible contribution to the process of gene expression by the

topology of DNA within the *pcaI-pcaU* intergenic region is suggested by presenting data showing that this area is severely intrinsically bent.

MATERIALS AND METHODS

**Bacteria, plasmids, and growth conditions.** Bacteria and plasmids used in this study are listed in Table 1. Strains of *E. coli* were grown in Luria-Bertani medium (37) with efficient aeration at 37°C. Antibiotics were added as required (ampicillin at 100 µg/ml, rifampin at 200 µg/ml, kanamycin at 50 µg/ml). *Acinetobacter* strains were grown at 30°C on mineral medium as described earlier and in the presence of antibiotics if required (ampicillin at 200 µg/ml, kanamycin at 12 µg/ml) (42).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>Acinetobacter</i> strains		
ADP1	Wild type (strain BD413, ATCC 33305)	26
ADPU6	PcaU binding site downstream of <i>pcaU</i> replaced by Km <sup>r</sup> cassette	This study
<i>E. coli</i> strains		
DH5α	General-purpose cloning strain	20
BL21(DE3)	F <sup>−</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>−</sup>m<sub>B</sub><sup>−</sup>) gal dcm</i> (DE3); gene for T7 RNA polymerase under control of <i>lacUV5</i> integrated into the chromosome	39
Plasmids		
pBend5	Vector designed for circular permutation assay based on pBluescript SK(−)	46
pET-21(+)	Transcription vector with T7 promoter	39
pKOK6	Contains promoterless <i>lacZ-Km<sup>r</sup></i> cassette	28
pZR15	7-kbp <i>HpaII</i> fragment containing <i>pcaU</i> inserted into pUC19	17
pZR17	2,792-bp <i>EcoRI</i> fragment from pZR15 cloned into pUC19	17
pAC7	2,792-bp <i>EcoRI</i> fragment from pZR15 cloned into pET-21(+), T7 promoter upstream of <i>pcaU</i>	This study
pZR18	2,618-bp <i>HindIII-HpaII</i> fragment containing <i>pcaU</i> inserted into pUC19	17
pAC25	Corresponds to pZR18 after removal of 218-bp <i>SwaI-Eco47III</i> fragment	This study
p5/128	128-bp <i>EcoRI-BstUI</i> fragment of the <i>pcaU-pcaI</i> intergenic region in <i>HpaI</i> site of pBend5	This study
pAC29	pZR17 with 130-bp <i>BstBI</i> fragment replaced by Km <sup>r</sup> cassette from pKOK6	This study
pAC48	Contains <i>pcaI'-pcaU</i> , <i>lacZ-Km<sup>r</sup></i> cassette from pKOK6 fused to <i>pcaI'</i> on vector pRK415	P. Patz and U. Gerischer, unpublished results
pAC52	130-bp <i>BstBI</i> fragment deleted from pAC48	This study
p5DR	Intergenic PcaU binding site without the external inverted 10-bp sequence repetition on vector pBend5	This study

#### Gel retardation assay, determination of $K_d$ for the protein-DNA interaction.

The DNA probes (a 131-bp *Bst*BI fragment from plasmid pZR17 for the binding site downstream of the *pcaU* gene and a 214-bp *Xmn*I-*Eco*RI fragment from plasmid pZR17 for the intergenic binding site) were generated by restriction digestion and radiolabeled at 3' ends with the Klenow fragment of DNA polymerase or at 5' ends with T4 DNA polymerase. The labeled fragments were purified with the NucleoSpin extract kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The probe, at 10,000 to 15,000 cpm (0.3 to 0.6 nM in the assay) was incubated with various amounts of protein in a mixture containing 50 mM Tris-HCl, pH 8.0, 50 mM potassium chloride, 5 mM magnesium chloride, 1 mM EDTA, 0.01% (wt/vol) bovine serum albumin, 10% (vol/vol) glycerol, 1 mM dithiothreitol, and 2  $\mu$ g of poly(dI-dC) with a total volume of 20  $\mu$ l for 15 min at 30°C. For all interactions tested, controls for specificity were included. Addition of 1  $\mu$ g of nonspecific DNA did not alter the retardation, whereas addition of 0.1  $\mu$ g of unlabeled probe prevented the shift. The samples were separated on a 1.2 or 1.5% (wt/vol) agarose gel in 0.5 $\times$  TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8). After electrophoresis, the gel was dried and radioactivity was detected with a Bio Imager Fujix BAS (Fuji Photo Film Co., Ltd., Tokyo, Japan). The software MacBAS (Fuji Photo Film Co., Ltd.) served to quantify the signals. For the determination of the dissociation constant ( $K_d$ ) the unretarded part of the probe as a percentage of the total probe employed in one assay was plotted against the concentration of PcaU protein in the assay. The  $K_d$  was the PcaU concentration where 50% of the probe was retarded. This value was visually determined from the curves. The concentration of the probe was significantly lower than the PcaU concentration (4). Each  $K_d$  determination was repeated at least three times, and the standard deviation was 50% at maximum.

**DNase I footprinting.** Radiolabeled DNA probes for the intergenic PcaU binding site were prepared as described above. For the binding site downstream of *pcaU* a 127-bp *Ssp*I-*Xba*I fragment was isolated after restriction enzyme cleavage of plasmid pZR17. Footprinting reactions were carried out in a total volume of 200  $\mu$ l with 10,000 to 15,000 cpm of probe and 5 to 500 ng of protein in a mixture containing 25 mM Tris-HCl, pH 8.0, 25 mM potassium chloride, 15 mM magnesium chloride, 5 mM calcium chloride, 1 mM EDTA, 0.01% (wt/vol) bovine serum albumin, 1 mM dithiothreitol, and 2  $\mu$ g of poly(dI-dC). This mixture was incubated for 30 min at 30°C before the addition of 5  $\mu$ l of a solution of 5 to 10 ng of DNase I in 50 mM Tris-HCl, pH 7.2–10 mM magnesium sulfate–1 mM dithiothreitol–25% (vol/vol) glycerol. After 2 min at room temperature digestion was stopped by adding 700  $\mu$ l of 90% (vol/vol) ethanol, 350 mM ammonium acetate, and 5  $\mu$ g of tRNA. DNA was precipitated for 20 min at –20°C, centrifuged, washed twice with 70% (vol/vol) ethanol, and dried. The pellet was dissolved in loading buffer (95% [vol/vol] formamide, 10 mM EDTA, 0.1% [wt/vol] bromophenol blue, 0.1% [wt/vol] xylene cyanol) and denatured for 10 min at 90°C prior to electrophoresis. Samples were run alongside a dideoxy sequencing ladder of the same fragment on a 6 or 8% polyacrylamide gel containing 8 M urea with 1 $\times$  TBE buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA). The gel was soaked for 10 min in 10% (vol/vol) acetic acid and dried, and radioactivity was detected with a Bio Imager Fujix BAS (Fuji Photo Film Co., Ltd.).

**Overexpression of native PcaU.** A 2,792-bp *Eco*RI fragment containing the *pcaU* gene and 175 bp of upstream DNA was isolated from plasmid pZR15 and cloned into vector pET-21(+). In the resulting plasmid, pAC7, the T7 promoter of the vector was located upstream of the *pcaU* gene. Transformation of plasmid pAC7 into *E. coli* BL21(DE3) enabled expression of the chromosomally encoded T7 RNA polymerase, which is under the control of *lacUV5*, and thereby specific expression of PcaU. In initial purification attempts copurification of DNase activity was observed. To suppress the simultaneous expression of *E. coli* proteins, the bacterial RNA polymerase was inhibited by addition of rifampin to the medium. The cells were grown in Erlenmeyer flasks in Luria-Bertani medium. IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) was added at an optical density at 600 nm of 0.8; addition of rifampin was 30 min after the induction. The cultures were incubated for another 3 h, cooled on ice for 10 min, and then harvested by centrifugation. The cells were washed with 50 mM Tris-Cl, pH 8.0–2 mM EDTA–5% (wt/vol) glycerol and stored at –20°C if not processed immediately.

**Purification of PcaU.** Cells were suspended in 50 mM Tris-HCl, pH 8.0–2 mM EDTA–5% (wt/vol) glycerol–0.5 mM phenylmethylsulfonyl fluoride and disrupted by ultrasonication. The first two chromatography steps were performed on a BioCAD perfusion chromatography workstation (Perseptive Biosystems, Framingham, Mass.). The extract was cleared by centrifugation (50,000  $\times$  g, 15 min, 4°C) and injected onto an anion exchange column (POROS 20 QE; Perseptive Biosystems) that had been equilibrated with 50 mM Tris-HCl, pH 8.0. The column was washed with the same buffer, and the proteins were eluted by using a gradient of 0 to 1 M NaCl within 50 column volumes. PcaU-containing

fractions were identified by performing the gel retardation assay using a 216-bp *Xmn*I-*Eco*RI DNA fragment with the presumptive *pcaU-pcaI* intergenic PcaU binding site. They were desalted by dialysis and then subjected to chromatography on immobilized heparin (POROS 20 HE), which had been equilibrated with 50 mM Tris-HCl, pH 8.0. After a washing with 10 column volumes, elution was done in two steps at 0.2 and at 0.5 M NaCl. After being identified, PcaU-containing fractions were desalted and concentrated by dialysis against a mixture containing 10% (wt/vol) polyethylene glycol 20000, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 10% (vol/vol) glycerol and stored at –20°C. Finally the PcaU-enriched preparation was subjected to gel filtration by fast-protein liquid chromatography on Superdex 75 (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) using 50 mM Tris-HCl, pH 8.0–150 mM KCl–5% (vol/vol) glycerol for elution. That the PcaU protein was the major peak was confirmed by a gel retardation assay. The protein solution was concentrated using Microcon centrifugal filter devices (YM-10; Millipore, Eschborn, Germany) and was stored at –20°C after being adjusted to 50% (vol/vol) glycerol–2 mM dithiothreitol–1 mg of glycine/ml.

**Protein determination, SDS-PAGE, and size determination of the native protein in solution.** Protein concentrations were determined by using the methods of Markwell et al. (32) or Bradford (3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (30). Gel filtration on Superdex 75 (Amersham Pharmacia Biotech Europe GmbH) was used for size determination of the native PcaU protein. The system was calibrated by determining the elution volume for globular standard proteins RNase (molecular weight [MW], 13,700), chymotrypsinogen A (MW, 25,000), ovalbumin (MW, 43,000), and albumin (MW, 67,000) (Amersham Pharmacia Biotech Europe GmbH) in 50 mM Tris-HCl, pH 8.0.  $K_{av}$  values were calculated according to equation  $K_{av} = V_e - V_0/V_t - V_0$ , where  $V_e$  is the elution volume for the protein,  $V_0$  is the column void volume (equal to the elution volume for blue dextran 2000), and  $V_t$  is the total bed volume.

**Deletion of the PcaU binding site downstream of the *pcaU* gene.** *Acinetobacter* sp. strain ADPU6 with a deletion of the PcaU binding site downstream of the *pcaU* gene was constructed as follows. Plasmid pZR17 was incubated with restriction endonuclease *Bst*BI, and the biggest fragment was isolated. A DNA fragment carrying a  $Km^r$  gene and a terminator from phage *fd* was prepared by treating plasmid pKOK6 with restriction endonucleases *Bam*HI and *Eco*RI and was gel purified. Both fragments were treated with the Klenow fragment of DNA polymerase I to produce blunt ends and incubated together with T4 DNA ligase. After transformation in *E. coli* DH5 $\alpha$ , selection for ampicillin and kanamycin resistance led to identification of plasmid pAC29, which was verified by restriction analysis. pAC29 was cleaved with *Sna*BI and *Msc*I, releasing a fragment containing the modified site with flanking ADP1 wild-type sequences (169 and 724 bp, respectively). *Acinetobacter* sp. strain ADP1 was transformed with the fragment mixture, and transformants were selected on plates with succinate mineral agar containing kanamycin. Sensitivity to ampicillin was tested to exclude cointegration of the vector. PCR with oligonucleotides 5'-TGAATCAGA TCGTATGGC-3' and 5'-AAACCACCAATCAGGATG-3' was used to verify new strain ADPU6. In a second approach, the PcaU binding site downstream of the *pcaU* gene was deleted from a plasmid (pAC48) that contained the complete *pcaU* gene plus 1,410 bp downstream of *pcaU*, the *pcaU-pcaI* intergenic region, the first 92 bp of *pcaI*, and a *lacZ-Km^r* cassette for determination of the activity of *pcaI*. pAC48 was treated with *Bst*BI, and the resulting fragment mixture was exposed to T4 DNA ligase. After transformation of the ligation products into *E. coli*, strains with the desired plasmid, pAC52, were identified by the absence of the *Xba*I restriction site, which was contained on the 130-bp *Bst*BI fragment to be deleted.

**Modification of the *pcaI-pcaU* intergenic PcaU binding site.** Plasmid pAC25 containing a PcaU binding site without the direct repetition of the 10-bp sequence found three times within the PcaU binding site was created by removal of a 218-bp *Swa*I-*Eco*47III fragment from plasmid pZR18. Plasmid pBend5DR was created by hybridizing oligonucleotide 5'-TATCGAACAAATTATTTAAA TATCGAACAAAACCTACTAACTAC-3' to the respective complementary oligonucleotide and cloning the product into the *Hpa*I cloning site of vector pBend5 after phosphorylation using T4 polynucleotide kinase. The resulting plasmids were confirmed by restriction analysis or sequence analysis.

**Determination of DNA curvature.** DNA curvature was determined in the circular permutation assay (43). A 128-bp *Eco*RI-*Bst*UI fragment including most of the DNA between the two transcriptional start sites of *pcaI* and *pcaU* was cloned into the *Hpa*I site of bending vector pBend5 after converting the single-stranded DNA resulting from the *Eco*RI cut into double-stranded DNA with the Klenow fragment of DNA polymerase I. The construct was verified and its orientation was determined by sequence analysis. Permuted fragments were produced by digestion with the restriction enzymes contained in the permutation

element and analyzed on 8% acrylamide gels in  $1 \times$  TBE buffer, pH 7.5, at 4°C and a voltage of 7 V/cm. The relative mobilities of the fragments (mobility of a fragment divided by mobility of an unbent control fragment of identical length) were plotted against the number of base pairs of permutation element DNA fused to the test DNA in the direction of the *Hind*III restriction site of pBend5. Polynomials of fourth order best described the plotted data, and tangents were laid into their turning points. The crossing of these tangents was at the distance of the fragment that had the bending center in the middle of the fragment. The bending angle ( $\alpha$ ) was determined according to the empirical relationship  $\mu_M/\mu_E = \cos(\alpha/2)$ , where  $\mu_M$  is the mobility of a fragment with a central curvature and  $\mu_E$  is the mobility with the bend at the end (41). Since the mobility of a fragment with the bend at the end is comparable to the mobility of an unbent fragment, relative mobility was used for  $\mu_M/\mu_E$ . Computational analysis of the DNA topology was done using the program DIAMOD (11).

**DNA sequence determination.** DNA sequence determination was performed with a SequiTherm Excel Long-Read DNA sequencing kit LC (Biozym Diagnostik GmbH, Oldendorf, Germany) by using primers with the fluorescent label IRD800 (MWG-Biotech GmbH, Ebersberg, Germany). Electrophoresis was done on a LI-COR DNA sequencer 4000L, and the accompanying software was used for annotation (MWG-Biotech GmbH). Sequence reactions used as a size standard for DNase I footprints were performed with a T7 sequencing kit (Amersham Pharmacia Biotech Europe) and  $^{35}$ S-dATP (15).

## RESULTS

**Overexpression and purification of native PcaU.** To obtain stable overexpression of PcaU, T7 polymerase-based expression was used (39, 40). The *pcaU* gene was cloned into transcription vector pET-21(+) under the control of the T7 promoter, creating plasmid pAC7. After transformation into host strain *E. coli* BL21(DE3) expression of PcaU could be directed by activation of the *lacUV5* promoter controlling the gene for T7 RNA polymerase by addition of IPTG. Cell extracts prepared from such induced cultures contained significant amounts of an additional 28,000-Da protein (Fig. 2) and showed specific binding of a 216-bp *Xmn*I-*Eco*RI DNA fragment containing the PcaU binding site (data not shown). The recombinant PcaU protein was purified to near homogeneity in a three-step procedure employing anion exchange chromatography, affinity chromatography on heparin, and gel filtration as detailed in Materials and Methods (Fig. 2). The native protein had a molecular mass of 54,000 Da as determined by using globular reference proteins for calibration (Fig. 2). From these data it is concluded that the oligomeric structure of the PcaU protein in solution is a homodimer.

**Determination of the binding sites of PcaU.** A previous investigation had revealed two PcaU binding sites, one in the 282-bp intergenic region between *pcaI* and *pcaU* (detected by retardation of a 216-bp *Xmn*I-*Eco*RI DNA fragment) and a second one downstream of the *pcaU* gene (detected by retardation of a 300-bp PCR fragment) (17). Identification of a sequence motif within the intergenic DNA, which consisted of three perfect 10-bp sequence repetitions two of which formed a palindrome, led to the hypothetical formulation of a PcaU binding site. The presumption was strengthened by the finding that DNA directly downstream of the *pcaU* gene contained a motif which showed high similarity to the intergenic motif (33 out of 43 nucleotides identical after introduction of one gap corresponding to 1 nucleotide). In addition, DNA bound by the PcbR protein, necessary for expression of the *pobA* gene, also contained a similar motif (22 out of 37 nucleotides identical after introduction of one gap corresponding to 1 nucleotide) (Fig. 3). The purified PcaU protein was used to reveal its binding site in the *pcaU-pcaI* intergenic region as well as down-

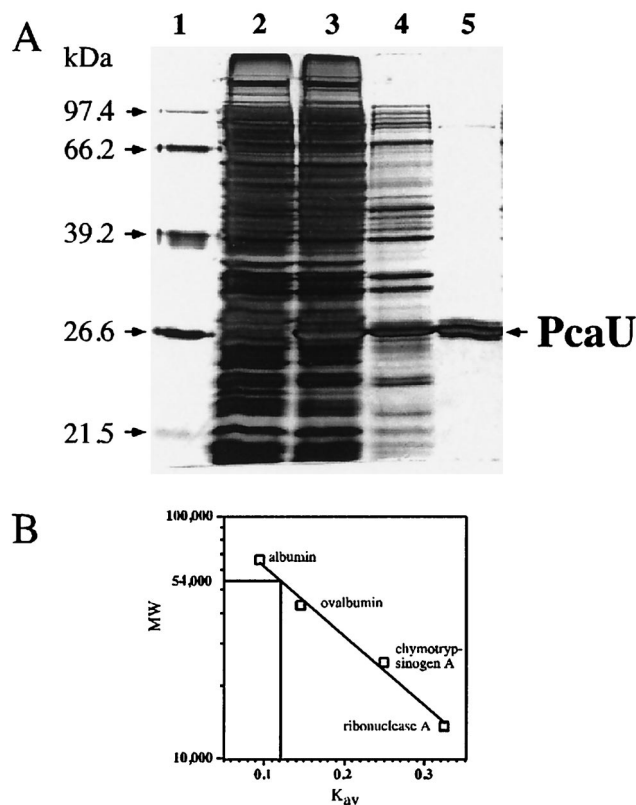


FIG. 2. (A) Denaturing SDS-PAGE from fractions of the purification of native PcaU after overexpression in *E. coli*. Lane 2, cell extract of the host strain with pET-21(+) without insert; lane 3, cell extract of the strain overproducing PcaU; lane 4, fraction after anion exchange chromatography; lane 5, fraction after affinity chromatography on heparin resin; lane 1, size standard. A low-molecular-weight protein still present in the preparation after chromatography on heparin was separated from PcaU by gel filtration (not shown). (B) Size determination of the native PcaU protein by gel filtration on Superdex 75. Squares,  $K_{av}$  values for reference proteins of known size; vertical line, respective value for PcaU.

stream of *pcaU*. In both cases the presumed sites could be verified (Fig. 4). Bound PcaU covered 45 bp within the *pcaU-pcaI* intergenic DNA including the three 10-bp sequence repetitions (Fig. 1 and 3). For the binding site downstream of *pcaU* the footprint of PcaU was found to include the palindrome but did not extend to include the direct sequence repetition (Fig. 3 and 4).

**Different affinities of PcaU for the two binding sites upstream and downstream of its gene.** Despite the high similarity between the two recognized binding sites, they probably differ in their biological functions. Here, the affinities of PcaU for the sites were compared by employing a DNA retardation assay. The  $K_d$  of PcaU for the *pcaU-pcaI* intergenic binding site was 0.16 nM, and the  $K_d$  for the PcaU binding site downstream of its gene was 8 nM (Fig. 5). Thus the affinity of PcaU for the downstream binding site was 50 times lower than that for the *pcaU-pcaI* intergenic binding site.

**The PcaU binding site downstream of the *pcaU* gene does not significantly influence the expression of the structural gene cluster.** Identification of a second functional binding site downstream of the *pcaU* gene poses the question of its biolog-

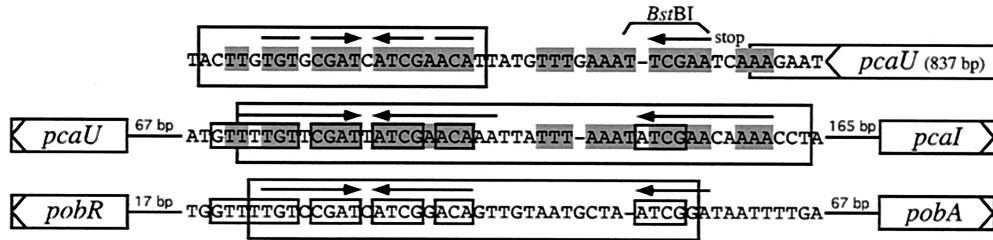


FIG. 3. Comparison of the two PcaU binding sites characterized here and the binding site of the closest homologue of PcaU, PobR from *Acinetobacter* sp. strain ADP1. Genes are represented as boxes with arrowheads inside indicating the direction of transcription. Arrows above sequence, sequence repetitions. Sequence identity between the two PcaU binding sites is highlighted by shaded boxes; identity between the *pca* and *pob* intergenic binding sites is shown by small open boxes. Large open boxes indicate the areas protected by the respective regulators in DNase I footprinting experiments.

ical function. Most likely would be an involvement of PcaU binding to this site in the regulation of the structural gene cluster or of the *pcaU* gene itself. To explore the first possibility, a strain that differed from the wild type in that the downstream binding site was removed on a 130-bp fragment and replaced with a kanamycin resistance cassette was constructed (see Materials and Methods). *pca* gene expression by that strain was compared with that by the wild type on a medium that required the respective gene products (mineral medium with quinate as the carbon source). There was no significant

difference in growth between the two strains (data not shown). To get a more detailed picture of the expression level itself, we used a heterologous system of monitoring PcaU-dependent *pca* gene expression. PcaU expressed in *E. coli* in small amounts functions in concert with *E. coli* RNA polymerase at *pcaI* in the same way it does in *Acinetobacter* sp. strain ADP1 as shown by using a transcriptionally fused *lacZ* cassette (P. Patz and U. Gerischer, unpublished results). For these studies a fragment of *Acinetobacter* DNA which contained the complete *pcaU* gene and 1,785 bp downstream of it, the complete *pcaU-pcaI*

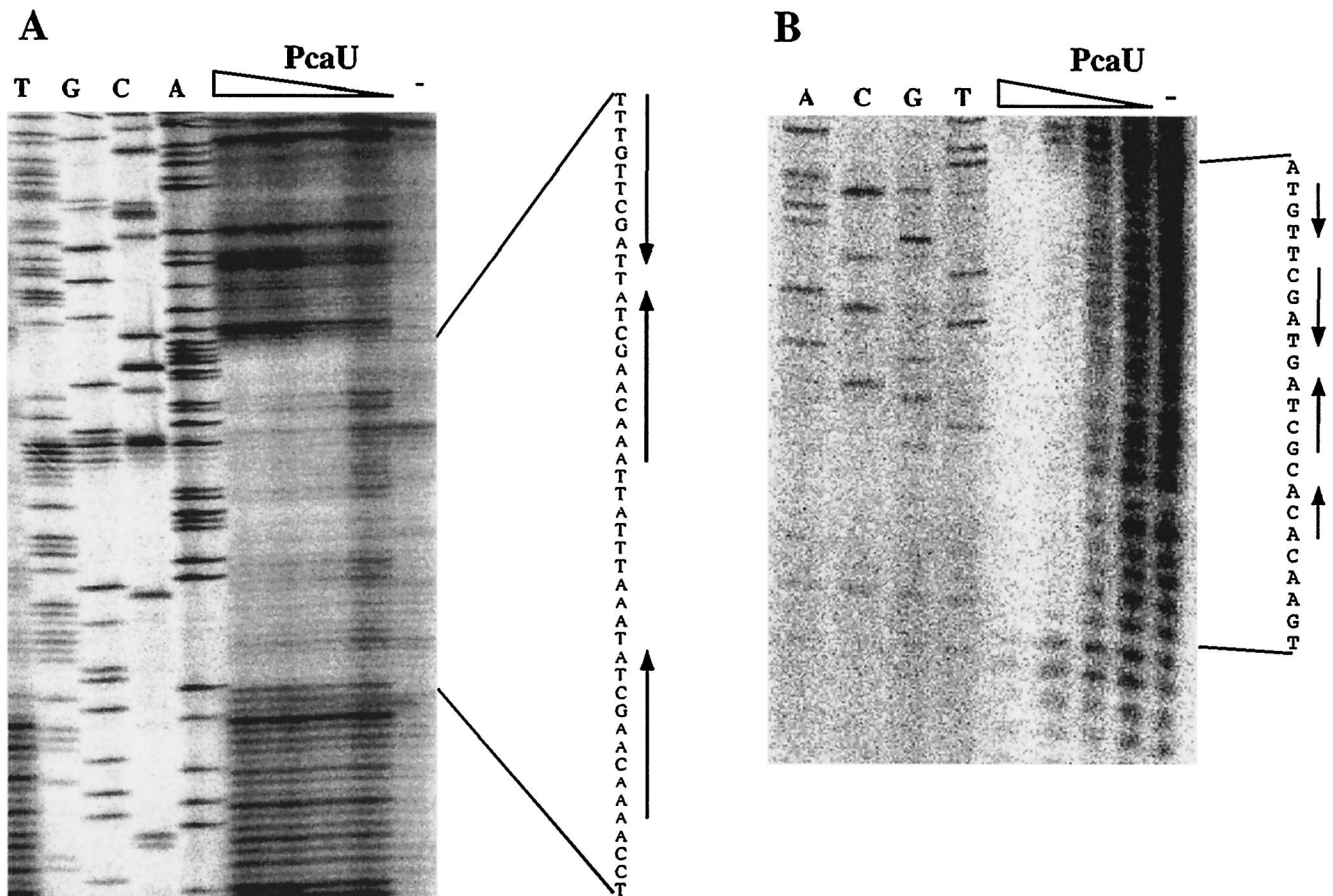


FIG. 4. Determination of the PcaU binding sites by DNase I footprinting in the *pcaU-pcaI* intergenic region (A) and downstream of *pcaU* (B). PcaU was added in various amounts (20, 10, 5, and 2.5 ng)

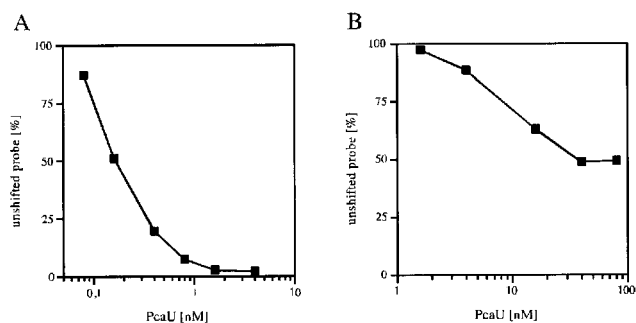


FIG. 5. Determination of the  $K_d$  for PcaU binding to the *pcaU-pcaI* intergenic region (A) and to the binding site downstream of *pcaU* (B). The  $K_d$  was the PcaU concentration at which 50% of the DNA was bound. For the downstream binding site, a maximum of 50% of the total probe was bound under any condition; thus the  $K_d$  was the PcaU concentration at which 25% of the DNA was bound.

intergenic region, and the first 92 bp of the first structural gene, *pcaI*, was used. The *pcaI* gene was transcriptionally fused with reporter gene *lacZ*. A 130-bp fragment of DNA containing the downstream PcaU binding site was removed from the reporter construct. The expression pattern of this construct (pAC52)

was indistinguishable from that of the respective wild-type construct (data not shown).

**Removal of the external sequence repetitions does not eliminate the specific interaction of PcaU with the binding site but decreases its affinity.** The PcaU binding sites contain three 10-bp sequence repetitions, two in a palindromic order and, separated by 10 bp, a direct repetition. To investigate the influence of the removal of one of these repeated sequences on PcaU binding, modified DNA fragments were constructed and tested for PcaU binding. A 212-bp *Bam*HI-*Xmn*I fragment containing only the palindrome was isolated from plasmid pAC25. A 287-bp *Hind*III-*Eco*RI fragment from plasmid pBend5DR contained the PcaU binding site without the external palindromic sequence repetition. Gel retardation assays with the purified PcaU protein were performed. In both cases PcaU still specifically bound the modified binding sites. For both modifications the affinity of PcaU for the DNA site decreased considerably (Fig. 6).  $K_d$  for the direct sequence repetition was 16 nM, and that for the palindrome was 8 nM. Thus  $K_d$ s of both shortened PcaU binding sites are in a range comparable to the  $K_d$  for the binding site downstream of the *pcaU* gene.

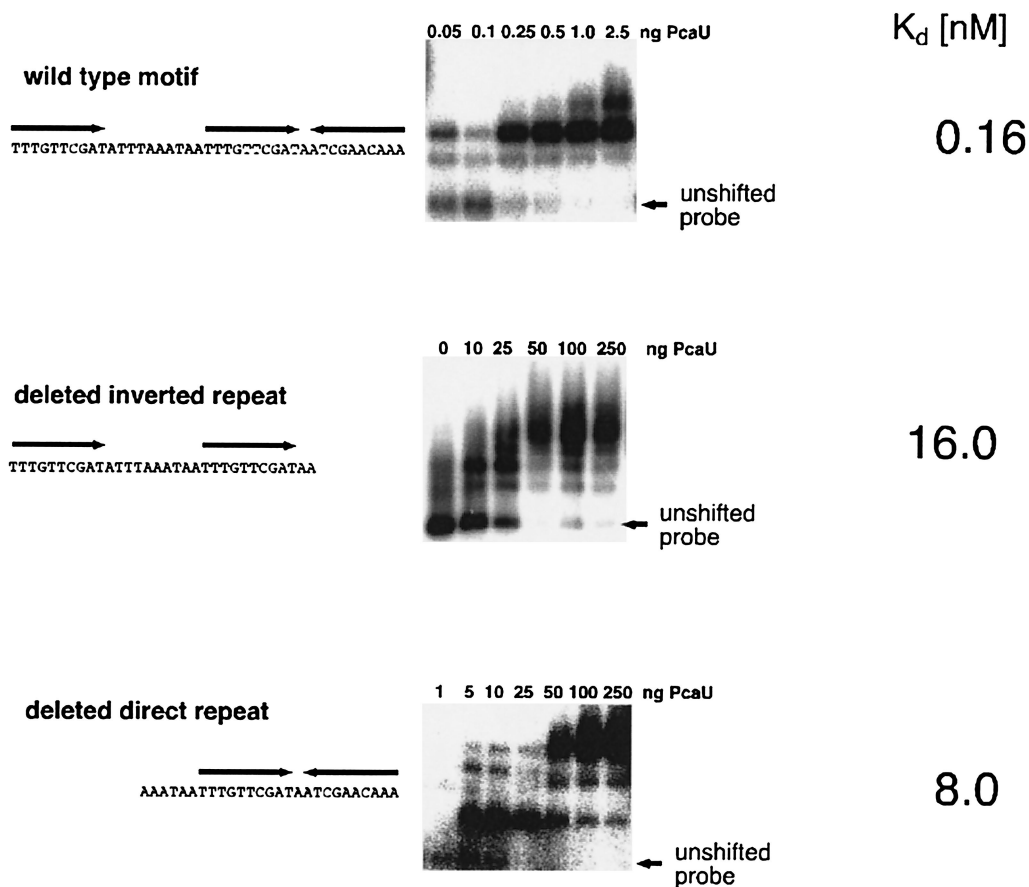


FIG. 6. Qualities of purified PcaU binding to modified intergenic binding sites. The relevant part of the respective DNA fragment used for the gel retardation assay is shown on the left. The wild-type motif was contained on a 214-bp *Eco*RI-*Xmn*I fragment, the motif containing only the direct sequence repetitions was contained on a 45-bp fragment cloned into plasmid pBend5, and the motif containing only the palindrome was contained on a derivative of pZR18 after removal of the 218-bp *Swa*I-*Eco*47III fragment containing the direct sequence repetition. Results of the respective gel retardation assays are shown on the right. The amount of unshifted probe was plotted against the respective PcaU concentration and served for determination of  $K_d$ .

**The *pcaI-pcaU* intergenic region displays strong intrinsic bending.** The intergenic DNA between the *pcaU* gene and the *pcaI* gene contains multiple polyadenosine nucleotides (Fig. 1). Such sequences are known to cause intrinsic bending of DNA, particularly when multiple poly(A) stretches (with each individual one at least 4 nucleotides long) are in phase with the turns of the DNA helix (10.5 bp) (19). Previous data indicated that there may be physical deviation from straight topology in this region (A. Segura and L. N. Ornston, unpublished results). Therefore the *pcaI-pcaU* intergenic region was investigated in more detail in this respect by employing the circular permutation assay (43). This assay is based on the observation of retardation of a bent DNA fragment through a native polyacrylamide gel in comparison with that of an unbent fragment of identical length (31). The extent of retardation depends on the position of the bend, and this can be exploited to determine the bending angle and the bending center (41, 43). Positioning the DNA sequence under investigation at different positions on a series of fragments of identical lengths is simplified by the use of special vectors (46). Here a 128-bp fragment encompassing roughly the region between promoters *pcaIp* and *pcaUp* was cloned into vector pBend5 and used for the determination of DNA curvature as detailed in Materials and Methods. This DNA displayed strong intrinsic bending centered within the direct sequence repetition of the PcaU binding site with a bending angle of 72° (Fig. 7). Computational calculation of the topology of the respective 128-bp fragment also resulted in the prediction of strongly bent DNA with multiple bending centers (data not shown).

## DISCUSSION

As a prerequisite for a study of the interaction of transcriptional regulator PcaU from *Acinetobacter* sp. strain ADP1 with its DNA targets the protein was purified in its native state after overexpression in *E. coli*. On the basis of the appearance of the protein in denaturing polyacrylamide gels and upon gel filtration, it is suggested that the protein is a dimer in solution. This is an observation that is made frequently for regulatory proteins, such as PcaR from *P. putida* and the Gal repressor from *E. coli* (18, 25). Both binding sites for PcaU on DNA upstream and downstream of the *pcaU* gene, suggested earlier based on the threefold repetition of a sequence motif which is also found in the binding sites of other regulatory proteins, could be verified by DNase I footprinting experiments. In the *pcaU-pcaI* intergenic region, all three sequence repetitions are included in the DNA covered by PcaU whereas the motif directly downstream of the *pcaU* gene was protected from DNase I digestion only in the palindromic area (Fig. 3). The fact that the sequence repetitions are not as well preserved as in the intergenic DNA may be the reason for this observation and may also explain the lower affinity of PcaU for the downstream motif than for the intergenic PcaU binding motif. No specific binding of PcaU to the binding site for the closely related PobR protein located in the *pobR-pobA* intergenic region from the same organism could be detected (data not shown). Taking into consideration that the downstream PcaU binding site binds PcaU specifically without involvement of a third direct sequence repetition, specificity for PcaU binding must be determined by the palindromic structure alone. The sequences of

the palindrome in the downstream PcaU binding site differ in 3 out of 20 positions from that of the *pcaU-pcaI* intergenic binding site; the palindrome of the PobR binding site displays the same number of mismatches, two of which are in the same positions as mismatches between the two PcaU binding sites (Fig. 3). Thus it is suggested that DNA sequence requirements are pronounced. In this context the discovery of a protein that binds to both the intergenic PcaU binding site and to the PobR binding site appears remarkable in that the protein is a derivative of PobR with the single-amino-acid substitution T57A producing a stretch of six residues that are identical within the presumed helix-turn-helix motifs of PcaU and PobR (27).

The  $K_d$  value determined for the intergenic PcaU binding site is comparable to the  $K_d$  found for PcaR binding to the *pcaII* promoter (0.16 and 0.13 nM, respectively), whereas the affinity of PcaU for the binding site downstream of the *pcaU* gene is 50-fold lower. The existence of an additional regulator binding site is reminiscent of what is found for other regulatory systems where multiple binding sites for the same regulator exist and contribute to gene expression, as in the well-characterized systems governed by AraC and LacI (33, 38) and the regulation brought about by DeoR from *E. coli* (6). In all these cases, simultaneous binding of two or more DNA binding sites by the respective regulator protein consisting of a dimer or oligomer and loop formation of the intervening DNA as a consequence have been described. Here we explored the possibility of a contribution of the downstream binding site to the regulated expression of the *pca* genes by deleting it. In two different approaches we could not detect any difference in *pca* gene expression or in growth between situations in which the downstream binding site was present and those in which it was absent. The nature of the downstream binding site is very unlikely to be random due to the site's complexity. The downstream binding site may have a function which affects *pca* gene expression but which is so subtle that it could not be detected in the two approaches we chose. Alternatively the downstream binding site may contribute to the strong autoregulatory repression observed for the *pcaU* gene, which has not been tested (42).

The intergenic PcaU binding site contains three perfect 10-bp sequence repetitions, which are all protected from DNase I digestion by PcaU as demonstrated here (Fig. 3 and 4). The PcaU binding site downstream of its gene displays a similar structure with less-well-preserved sequence repetitions, and there only binding of PcaU to the palindrome could be demonstrated. To determine the function of the individual sequence repetitions, we tested the binding of PcaU to modified intergenic PcaU binding sites missing the direct sequence repetition or the external inverted repetition. Sites with either modification still bound PcaU specifically but with a lower affinity. Indication for a physiological function of these external sequence repetitions comes from the observed growth phenotypes of two spontaneous mutant strains, one containing a C→T base substitution within the external sequence repetition and the second one with a deletion of the direct sequence repetition. The respective strains show a cold-enhanced block in protocatechuate catabolism (7, 42). Thus both PcaU binding and gene expression are impaired when one of the external sequence repetitions is modified or deleted, and possibly *pca* gene expression is not inducible at all. The arrangement of three sequence repetitions in one palindrome and one direct repetition spaced by 10 bp from the palindrome is unusual for

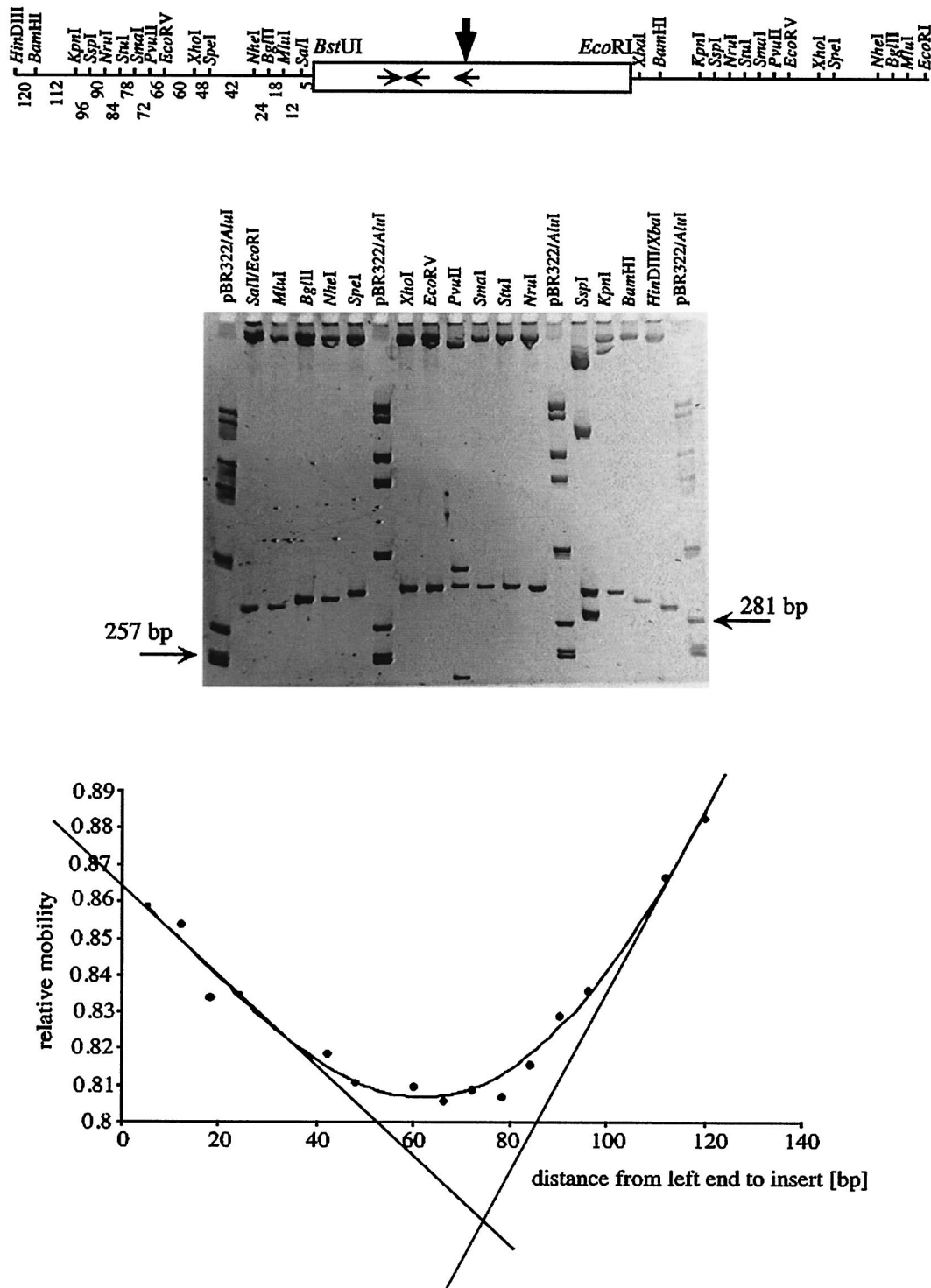


FIG. 7. Determination of DNA bending within the 128-bp *BstUI-EcoRI* fragment from the *pcaU-pcaI* intergenic region (Fig. 1) by a circular permutation assay using bending vector pBend5. (Top) The relevant part of plasmid p5/128 is shown with the restriction sites of the permutation element and the test DNA as a box in the middle. Horizontal arrows, locations of the PcaU binding sites; vertical arrow, bending center revealed in this experiment. Numbers under the left half of the permutation element, distances in base pairs between the *BstUI* site of the test DNA and the left end of the fragment resulting from restriction digestion with the respective enzymes. These numbers were used as the distances from left end to insert in the graph (bottom). (Middle) Example of a gel containing samples of plasmid p5/128 after restriction cleavage with the enzymes indicated. The permuted fragments had a length of 255 bp; for comparison two fragments of the standard are indicated. (Bottom) Plot of the relative mobilities of the fragments against the distance between the left end of the permuted fragments and the test DNA enables the determination of the bending center. Relative mobilities are the averages of six gels; the error was between 0.7 and 1.5%.



regulator binding sites in that the most abundant motif is a twofold-repeated sequence arranged as a palindrome. A direct repetition of two sequences as observed for *araOI* and *araI* is rare (22, 34). Binding sites structured similarly to the PcaU binding sites are found only for regulators with close homology to PcaU such as the PcbR and PcaR binding sites (Fig. 3) (10, 18). We are aware of one other example of a similarly structured regulator binding site. The DeoR repressor from *Bacillus subtilis*, regulating genes required for deoxyribonucleoside and deoxyribose utilization, was shown to protect an area of 43 bp that contains three repetitions of a 6-bp sequence arranged in a palindrome and a direct sequence repetition from DNase I digestion (45). For the interaction of this regulator, which shows no homology to PcaU, with its binding site all three sequence repetitions are required (44, 45). This differs from the DNA binding quality of PcaU described here. Such data are not available for proteins closely related to PcaU (PcbR and PcaR) (10, 18), but comparing the architectures of the respective regulatory regions reveals significant differences for each individual regulator (18). These differences may be an indication of differences in the way in which the regulators interact with RNA polymerase.

We show here that the stretch of DNA between the two promoters of the *pcaU-pcaI* intergenic DNA is intrinsically bent by 72° and that the center of this strong curvature is within the external sequence repetition of the PcaU binding motif. The assay used can only reveal the sum of the effects of multiple bends if there is more than one bend. The DNA fragment assayed was 128 bp long and contained multiple polyadenosine stretches spaced by roughly one (10 or 11 bp) or two helix turns (20 to 22 bp) (Fig. 1). Thus it is possible that this stretch of DNA contains multiple curved regions. This assumption is supported by the computational prediction, which revealed three bends, one close to the -10 site of *pcaI* and two near the PcaU binding site. This observation is in agreement with the prediction of intrinsic bending for a substantial fraction of all promoters or the respective upstream regions of many mesophilic bacteria (2). The nonlinear DNA conformation may, for example, be necessary to correct for a nonoptimal spacing of the -10 and the -35 regions of *pcaI*, a mechanism found to apply for SoxR and MerR and proposed to apply to PcaU homologue PcaR (1, 18, 23). Alternatively, the intrinsic DNA curvature may support a potential DNA looping brought about by the two PcaU binding sites as discussed above.

In addition to the potential function of curved DNA in the promoter area, PcaU-dependent regulation has other features in common with regulation by metalloregulator MerR and by redox-regulated SoxR of *E. coli*. These proteins act as repressors or as activators of the respective structural gene promoters, depending on the absence or presence of the respective inducing signal, an unusual quality of a regulatory protein shared by PcaU (24, 29, 42). In contrast to the binding of MerR and SoxR, which overlap the structural gene promoters creating an unusual situation for activator proteins, PcaU binding between positions -48 and -92 with respect to the transcriptional start of the structural genes is observed. The specific binding of PcaU to the intergenic binding site was observed in the absence or presence of the inducer protocatechuate (100 μM), thus allowing the speculation that both repression and activation at *pcaI* are based on a specific interaction between

PcaU and RNA polymerase. The binding of the inducer must change the nature of this interaction in a way that leads from a repression to a strong activation of RNA polymerase at this promoter. The mechanism leading to repression by PcaU at *pcaUp* may be similar to that leading to repression at *pcaIp* since the PcaU binding site (positions -41 to -85 with respect to the transcriptional start site of *pcaUp*) does not overlap with this promoter either.

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