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PcaR-mediated activation and repression of *pca* genes from *Pseudomonas putida* are propagated by its binding to both the −35 and the −10 promoter elements

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Summary

Degradation of protocatechuate in *Pseudomonas putida* is accomplished by the products of the *pca* genes (*pcaH*,*G*, *pcaBDC*, *pcaI*, *J* and *pcaF*). In *P. putida*, all these genes (with the exception of *pcaH*,*G*) are activated by the regulatory protein PcaR, in association with the pathway intermediate β-ketoadipate. Having previously cloned and characterized the *pcaR* locus, we have overexpressed and purified the PcaR protein to homogeneity. The purified PcaR protein was shown to form a homodimer in solution and to bind specifically to its own promoter, as well as to the promoter regions of *pcaI*, *J* and *pcaF*. Subsequent footprint analyses demonstrated that the binding of PcaR to its own promoter occurs within a footprint that extends from the −20 to the +4 position. In contrast, PcaR appeared to interact with the inducible *pcaI*, *J* promoter as a dimer of dimers; binding in tandem within a dual footprint encompassing both the '−35′ and the '−10' regions of the promoter sequence. The similarities and differences between the two binding patterns correlate well with the very different effects that PcaR has upon transcription at each of the promoter sequences. The interactions at the *pcaI*, *J* promoter are reminiscent of those exhibited by the MerR family of regulatory proteins. However, as PcaR bears very little primary sequence homology to any of the regulatory proteins within this family, the results presented here reveal the possible existence of a new series of functionally related transcriptional inducers.

Introduction

A number of recent articles, featuring a variety of prokaryotic regulatory proteins, has helped to dispel the commonly held misconception that all negative transcriptional regulators act by merely providing a physical barrier to the binding of RNA polymerase (RNAP) to the promoter (Williams, 1993; Ansari *et al*., 1995; Monsalve *et al*., 1996; Nègre *et al*., 1998). While such a passive promoter occlusion model (readily exemplified by lambda phage CI repressor; Ptashne, 1992) still applies to a number of repressor systems (Williams, 1993; Schlax *et al*., 1995), the idea that negative regulators may also influence transcription through more intricate protein–RNAP interactions is quickly becoming established. These more complex regulatory proteins are not limited to the inhibition of RNAP binding *per se*, but are able to moderate any one or more of the different stages involved in transcriptional initiation (Williams, 1993; Ansari *et al*., 1995). Such findings, when coupled to the discovery that some transcriptional activators can even be converted into transcriptional repressors through relatively small changes in protein–DNA or protein–RNAP interactions (Lobell and Schleif, 1990; Choy *et al*., 1995), have seemingly revitalized interest in the mechanisms that underlie transcriptional regulation of bacterial genes (Hochschild and Dove, 1998; Mooney *et al*., 1998). To this end, we introduce the regulatory protein, PcaR, as an example of one of the more unusual transcriptional regulators. PcaR interacts with its designate promoter sequences through protein–DNA contacts within the critical −35 and −10 elements. In so doing, it is able to serve as a transcriptional repressor and activator.

Aerobic degradation of aromatic compounds in *Pseudomonas putida* is ultimately co-ordinated by the action of PcaR. The protein has been shown to be responsible for the inducible expression of all but one of the enzymes that constitute the *pca* branch of the convergent β-ketoadipate pathway (Houghton and Shanley, 1994; Harwood and Parales, 1996). PcaR is expressed in tandem with the downstream genes, *pcaK*, *pcaF* and the *pcaBDC* operonic gene cluster, which (collectively) provide the major portion of the *pca* regulon (Hughes *et al*., 1988; Romero-Steiner *et al*., 1994; Nichols and Harwood, 1997). A recent characterization of *pcaK* (which encodes for a pathway-related transport system) and *pcaF* promoter sequences has indicated that expression from each promoter can be induced approximately 10-fold by the action of PcaR in the presence of β-ketoadipate (Nichols and Harwood, 1997). This level of induction is very similar to that demonstrated previously for the *pcaI*, *J* operon (Parales and Harwood, 1993) and for the rest of the *pca* genes

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(Hughes *et al*., 1988; Parales and Harwood, 1992), which was found to range between nine- and 18-fold.

Comparison of the variously characterized promoter sequences (including the *pcaR* promoter itself) has revealed the conservation of a 15 bp, partially palindromic sequence (5′-GTTCGATAAtCGCAC-3′). This sequence has been implicated in the binding of the PcaR regulatory protein through both deletional analyses as well as DNA sequence comparisons (Parales and Harwood, 1993; Romero-Steiner *et al*., 1994). Curiously, the relative position of this highly conserved cluster of bases within the inducible *pcaI*, *J* promoter region is identical to its location within the negatively autoregulated *pcaR* promoter: encompassing the −10 hexameric region of each promoter (Romero-Steiner *et al*., 1994). In order to confirm the role of this conserved sequence and to determine how PcaR interacts specifically with the promoter sequences of the *pca* genes, this report details an account of the purification of the PcaR protein, together with an analysis of its specific interactions with fragments of DNA encompassing a subset of the PcaR-dependent promoter sequences. Moreover, in an effort to refine our understanding as to how PcaR regulates expression from these promoters, a more thorough biochemical characterization of PcaR binding to the *pcaI*, *J* and the *pcaR* promoter sequences has also been undertaken.

Results

Previous genetic characterizations of the *pca* gene loci have shown that PcaR is responsible for regulating the expression of the *pcaBDC*, *pcaF* and *pcaI*, *J* genes (Hughes *et al*., 1988; Parales and Harwood, 1993; Romero-Steiner *et al*., 1994). To complete this characterization, a $pcaR_{po}$*::lacZ* fusion was constructed (pZHRP1) to demonstrate the effect that PcaR has upon its own expression. This plasmid was transformed into the wild-type *P. putida* background (PRS2000), as well as into a $PcaR^-$ strain (PRS3015; Hughes *et al*., 1988), in which the *pcaR* locus had been transpositionally inactivated. Comparisons of β-galactosidase levels in these two strains (grown under a variety of potentially inducible/repressible conditions) indicated that the *pcaR* promoter was negatively regulated (up to four-fold) by its own gene product (Table 1). As with a number of similar negatively autoregulated systems (Schell, 1993), the autoregulation of PcaR was shown to be independent of any response to the presence of the co-inducer molecule, β-ketoadipate, as well as to the presence of any gratuitous inducer, such as adipate (Table 1; Parke and Ornston, 1976; Hughes *et al*., 1988).

Construction, overexpression, purification and protease cleavage of a His-tagged PcaR fusion protein

A detailed, biochemical characterization of the role that PcaR plays in controlling the *pca* regulon has awaited purification of the PcaR regulatory protein. Unusual difficulty has been encountered in previous attempts (using more conventional means) to overexpress the native PcaR protein and to obtain a sufficiently purified sample. To overcome these problems, we cloned the *pcaR* structural gene into the pTrcHisB vector (Table 2), so that a histidine-tagged PcaR fusion protein would be produced to facilitate the purification of the PcaR protein (Fig. 1D). One of the potential disadvantages of producing such a modified protein is the likelihood that the additional, charged residues at the N-terminus of the fusion protein would affect the regulatory function of the modified PcaR protein. To address this concern, one of the oligonucleotides used to construct the gene fusion was designed to encode a unique Factor Xa protease cleavage sequence directly upstream of the PcaR peptide sequence (Fig. 1D).

Having constructed the His-tag PcaR fusion vector (pZHisR), the plasmid was transformed into *Escherichia coli* strain DH5α (Table 2). Expression of the *pcaR* fusion in one of the resulting transformants was induced by the addition of IPTG to the medium. SDS–PAGE analyses

Table 1. β-Galactosidase activities in *P. putida* PRS2000 and PRS3015 carrying a *pcaR–lacZ* fusion on pZHRP1.

Growth medium[b]	β-Galactosidase specific activity (units mg^{-1} protein)[a]		Repression ratio caused by:	
	PRS2000 (wild type)	PRS3015 (*pcaR*::Tn*5*)	Strain[c]	Media[d]
Glc	258 (62)	793 (49)	3.1	–
Glc + BEN	232 (23)	698 (81)	3.0	1.11
Glc + POB	181 (10)	820 (259)	4.5	1.43
Glc + ADI	191 (16)	885 (144)	4.6	1.35

a. Standard deviations are given in parentheses.
b. Cells are grown with 0.04% glucose or 0.04% glucose plus 5 mM benzoate (BEN), 5 mM p-hydroxybenzoate (POB) or 20 mM adipate (ADI).
c. Repression ratio = activity in PRS3015/activity in PRS2000.
d. Repression ratio = activity in PRS2000 grown in glucose plus another carbon source/activity in PRS2000 grown in glucose.
$n = 4$, two single colonies with duplication.

Table 2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relative genotype[a] or phenotype[b]	Source or reference
P. putida		
PRS2000	Wild-type derivative of PRS1 (ATCC 12633)	Stanier *et al*. (1966)
PRS3015	*pcaR*::Tn5 BEN$^-$ POB$^-$	Hughes *et al*. (1988)
E. coli		
DH5α	F$^-$ ϕ80Δ*lacZ* Δ(*lacZYA*$^-$*argF*$^-$) U169 *endA1 recA1 hsdR17* (r_k^- m_k^+) *thi-1 supE44 gyrA96 relA1*	Gibco BRL
Plasmids		
pT7-6	Ap^r; T7 polymerase (Φ10); ColE1 origin	Tabor and Richardson (1985)
pTrcHisB	Ap^r; *trc* promoter; N-terminal His-tag; ColE1 origin	Invitrogen
pGEM-T	Ap^r; vector for the cloning of PCR products	Promega
pQF50	Ap^r; broad-host-range plasmid carrying promoterless *lacZ*; pMB1 and pRO1600 origins	Farinha and Kropinski (1990)
pHS104	Ap^r; pT7-6 carrying 1.7 kbp *Eco*RI fragment containing *pcaR* from PRS2000	Romero-Steiner *et al*. (1994)
pZHisR	Ap^r; pTrcHisB carrying 932 bp PCR product containing *pcaR* from PRS2000	This study
pZHRP1	Ap^r; pQF50 carrying 254 bp PCR product containing *pcaR* promoter *Eco*RI fragment from pHS104	This study
pGEMT-I, J	Ap^r; pGEM-T carrying 287 bp PCR product containing *pcaI, J* promoter from PRS2000	This study
pGEMT-F	Ap^r; pGEM-T carrying 375 bp PCR product containing *pcaF* promoter from PRS2000	This study

a. Genetic designations in the text are in parentheses.
b. Ap, ampicillin; Ben, benzoate; Pob, *p*-hydroxybenzoate.

of the cellular proteins that were expressed from this clonal isolate revealed the inducible overexpression of a discrete protein band. This protein band exhibited an apparent molecular weight of 38 kDa, which corresponded quite well to the predicted size of the His-tag PcaR fusion protein (≈36 kDa). Subsequently, this overexpressed fusion protein was purified through successive MonoQ, Superdex chromatography columns, and finally over a phenyl Sepharose column. In this way, high yields of the His-tagged PcaR protein (PcaR*) fusion were purified to homogeneity (>90%; Fig. 1A).

The vector-encoded N-terminal histidine residues were removed by cleaving the purified protein with the protease Factor Xa, which yielded a 32 kDa protein: the deduced size of PcaR approximates 31.8 kDa. (Romero-Steiner *et al*., 1994). The cleaved protein product was purified further by gel filtration, which was also used to show that the native form of the protein in solution is a dimer (Fig. 1C). The first nine residues (SDETLVNDP–) of the N-terminal portion of this cleaved protein were sequenced and confirmed to be identical to those that had been determined previously for the wild-type PcaR protein (data not shown), in which the N-terminal methionine had also been removed (Fig. 1D).

Affinity of the PcaR protein for pca *promoter sequences*

DNA mobility shift analyses were used (Fig. 2) to demonstrate that the purified PcaR protein was able to bind specifically to the promoters of known *pca* genes: *pcaF*, *pcaI*, *J* and to its own promoter sequence, *pcaR*. In this way, the direct involvement of PcaR in the expression of each of these genes can be inferred. Furthermore, to refine the potential location of the PcaR binding site within each of these promoter sequences, PcaR was also shown to bind specifically within a synthetic 39 bp DNA fragment (derived from the *pcaI*, *J* promoter region), which closely bracketed the almost ubiquitous 15 bp sequence.

More quantitative measurements of the affinity of PcaR for its designate binding sites were conducted using fragments of the *pcaR* and *pcaI*, *J* promoter sequences as examples of promoters that are either negatively or positively regulated by PcaR respectively. The apparent equilibrium dissociation constants (K_D) of PcaR with each of these promoter fragments was determined by mobility shift assays (Fig. 3A–D) and found to be 0.087 nM and 0.13 nM for the *pcaR* and *pcaI*, *J* promoters respectively. Such measurements were determined as a result of additional experiments that were undertaken to estimate the proportion of 'active' PcaR protein (data not shown). This proportion was calculated to be approximately 87% of the total protein concentration, as defined by Fried and Crothers (1983).

As demonstrated previously (Fig. 2), whether in relative excess or in the presence of the pathway inducer molecule (β-ketoadipate), PcaR formed only a single, principal protein–DNA complex (R1) with its own promoter (Fig. 2, lane 8, Fig. 3A and B). Similarly, the addition of a limited amount of PcaR to the *pcaI*, *J* promoter DNA produced a comparable, single R1 protein–DNA complex. As more protein was added to the reaction mixture, however, a second binding complex (R1–R2) emerged (Fig. 3C and D). Interestingly, the addition of the co-inducer molecule (β-ketoadipate) had no discernible effect on the affinity of PcaR for either promoter (Fig. 3).

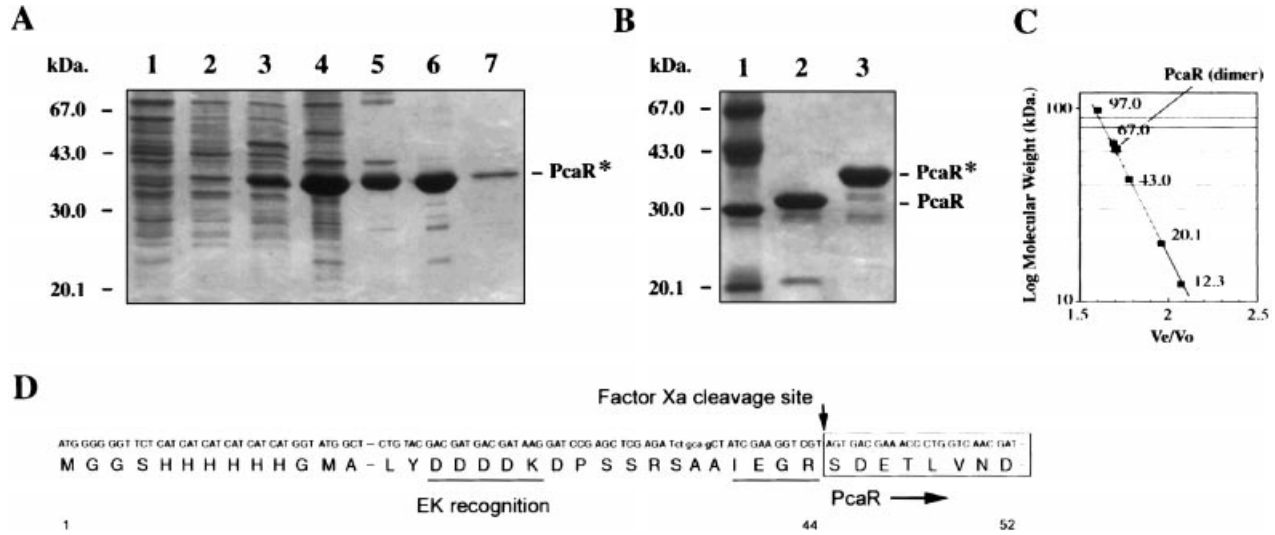

Fig. 1. Purification of PcaR protein.
A. Purification of PcaR fusion protein (PcaR*). Lane 1, *E. coli* DH5α crude lysate containing pTrcHisB vector; lane 2, PcaR* crude lysate without IPTG induction; lane 3, PcaR* crude lysate after induction with 0.1 mM IPTG for 4 h; lane 4, 30–40% $(NH_4)_2SO_4$ precipitation of induced PcaR* crude lysate; lane 5, MonoQ column peak; lane 6, Superdex column peak; lane 7, phenyl Sepharose column peak.
B. Cleavage of His-tag from PcaR*. Lane 1, molecular weight marker; lane 2, PcaR from Factor Xa treated-PcaR*; lane 3, PcaR* before Factor Xa treatment. PcaR was purified further by passage through a Superdex column.
C. Determination of the native size of PcaR by gel filtration. Standard proteins are: phosphorylase b (97.4 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), trypsin inhibitor (20.1 kDa) and cytochrome *c* (12.3 kDa).
D. Primary sequence and attendant DNA sequence of the N-terminus of the PcaR* fusion protein, showing the fusion juncture between the modified His-tag region of the plasmid and the N-terminal sequence of PcaR. Also shown are the amino acid sequence (underlined residues) that defines the EK recognition site and the Factor Xa cleavage site.

DNA footprint analysis of PcaR with the pcaI, J *and* pcaR *promoter sequences*

To determine whether the pattern of protein–DNA interactions that PcaR exhibited with both the promoter sequences could be distinguished further, DNase I footprint analyses were performed on both promoter fragments. Consistent with its negative autoregulation, the footprint of PcaR with its own promoter was found to bracket the −10 and +1 regions of this promoter sequence, extending from −20 to +4 (Fig. 4A and B, Fig. 6). Similar analyses with the *pcaI, J* promoter showed that the footprint of PcaR extended over a significantly larger portion of DNA, but still bracketed the −10 and +1 regions, ranging from the −38 to the +1 positions of the promoter sequence (Fig. 5A and B and Fig. 6). Again, the presence of β-ketoadipate had no discernible effect on the footprint of either promoter fragment.

Additional premethylation footprinting experiments confirmed the enhanced range of specific interactions that PcaR maintains with the *pcaI, J* promoter (Fig. 5C and D, Fig. 6). These studies also provided evidence that PcaR binds preferentially to both promoters within the major groove, as the premethylation of specific guanine residues gave rise to the major distinctions between the bound and unbound footprints (Hendrickson and Schleif, 1984; Lu *et al*., 1992). With the exception of a modified adenine residue at position −6, the distinctive pattern of the modified guanines that apparently affects the preferential interactions of PcaR with the $pcaR_{po}$ was matched precisely by that observed for PcaR in its R1 complex with the *pcaI*, J_{po}, strongly suggesting that PcaR makes similar protein–DNA contacts within and around the −10 hexamer of both its repressive and potentially inductive state. Indeed, the enhanced pattern of affected guanine residues on the

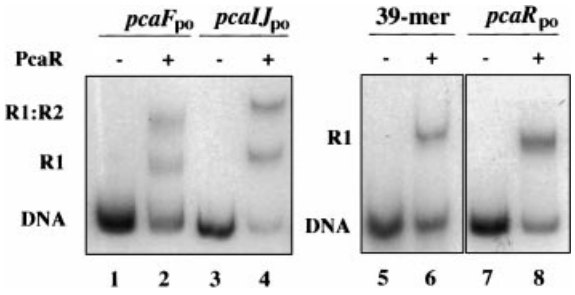

Fig. 2. Mobility shift analyses of PcaR interacting with various examples of *pca* promoter sequences. ^{32}P end-labelled promoters of *pcaF*, *pcaI, J*, *pcaR* and a 39-mer DNA (0.1 pM) were incubated with PcaR (0.1 nM) at 24°C for 20 min. DNA, R1 and R1–R2 designate unbound probe, higher mobility PcaR–DNA complexes and lower mobility PcaR–DNA complexes respectively.

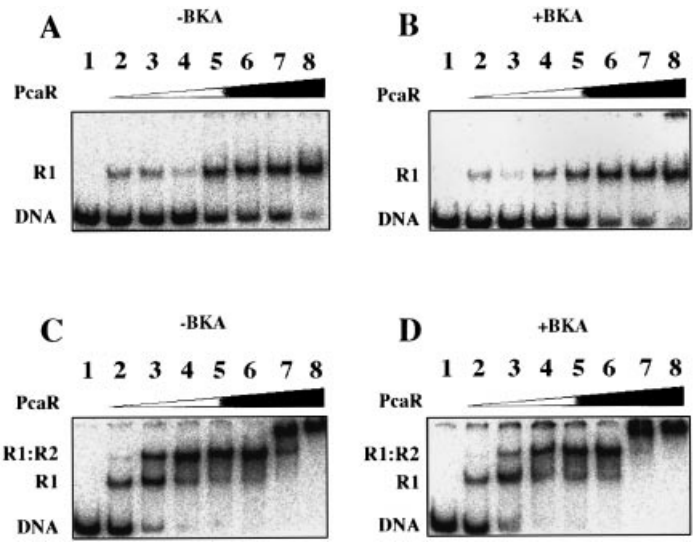

Fig. 3. Determination of the equilibrium dissociation constants of PcaR for *pcaR* and *pcaI*,J promoters. Various amounts of PcaR were incubated with ^{32}P end-labelled promoters of *pcaR* (A and B) and *pcaI, J* (C and D) in the absence (A and C) or presence (B and D) of 0.1 mM β-ketoadipate.
A and B. PcaR was added in the following amounts; 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1 and 2 nM (lanes 1–8).
C and D. PcaR was added in the following amounts; 0, 0.1, 0.5, 1, 2, 3, 5 and 10 nM (lanes 1–8).
Subsequent calculations to determine the K_D values for PcaR assumed an 87% activity rating for the PcaR protein, as defined by binding studies according to Fried and Crothers (1983) (data not shown).

template strand of DNA within the R1–R2 complex seems to build upon this spatial pattern of the R1 footprint in such a way as to indicate that the protein–DNA interactions within the R1–R2 complex potentially involve the binding of an additional PcaR moiety to the −35 region of the *pcaI*, *J* promoter. The differential binding patterns that are observed in the various footprint analyses tend to reinforce the distinctive banding patterns that were resolved in the mobility shift analyses (Fig. 3), i.e. preferential binding of PcaR to the $pcaR_{po}$ in a single R1 complex, compared with its binding to the $pcaI, J_{po}$ as both an R1 and a larger R1–R2 complex (Fig. 6).

Cohabitation of the PcaR regulatory protein with E. coli *RNA polymerase*

In the absence of any discernible changes to the binding of PcaR to the *pcaI*, *J* promoter region in the presence of β-ketoadipate, the potential requirement for some additional heterotropic protein–protein interactions of PcaR with RNAP was addressed. RNAPs from *Pseudomonas* spp. and *E. coli* share considerable primary sequence and functional similarities (Gao and Gussin, 1991). Indeed, the consensus promoter sequences that are recognized by the respective σ^{70}-directed holoenzymes of both polymerases show a high degree of conservation (McLean *et al*., 1997). Consequently, it was considered that the more readily available σ^{70} holoenzyme from *E. coli* could be used for preliminary *in vitro* characterizations of any potential interactions between PcaR and its attendant RNAP.

The use of the *E. coli* holoenzyme ($RNAP_{Ec}$) was found to be quite appropriate, as the holoenzyme was shown to bind specifically to both promoter sequences *in vitro* (although using similar amounts of DNA from both promoter

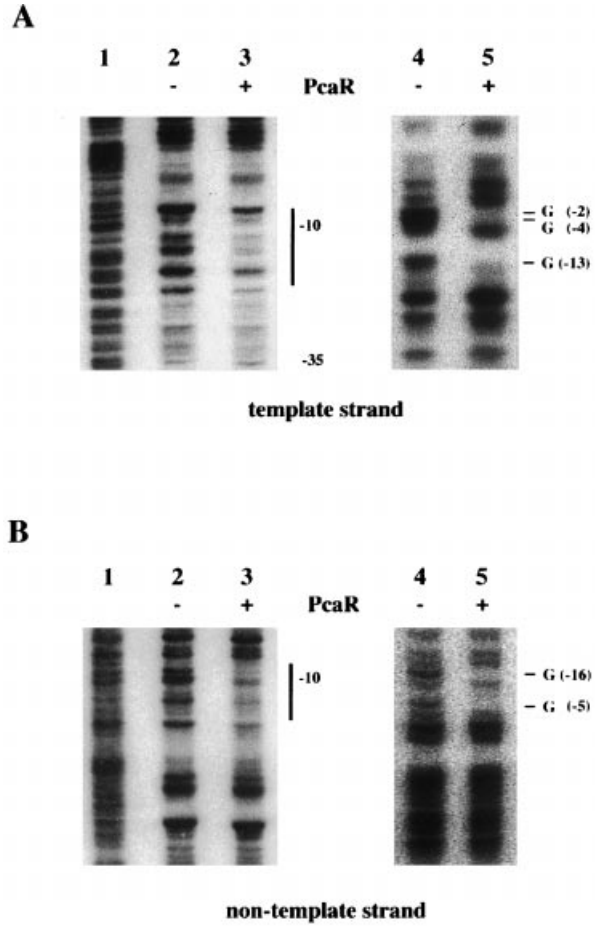

Fig. 4. DNase I footprint and premethylation interference footprint analyses of the *pcaR* promoter. A 254 bp fragment containing the *pcaR* promoter was singly ^{32}P end-labelled at template strand (A) or non-template strand (B). In both (A) and (B): lane 1, G + A sequencing ladder; lanes 2 and 3 represent the DNase I footprint sequence pattern in the absence and presence of PcaR (40 nM) respectively. Lanes 4 and 5 are the premethylation footprint sequences of 'unbound' and 'bound' DNA (R1) respectively.

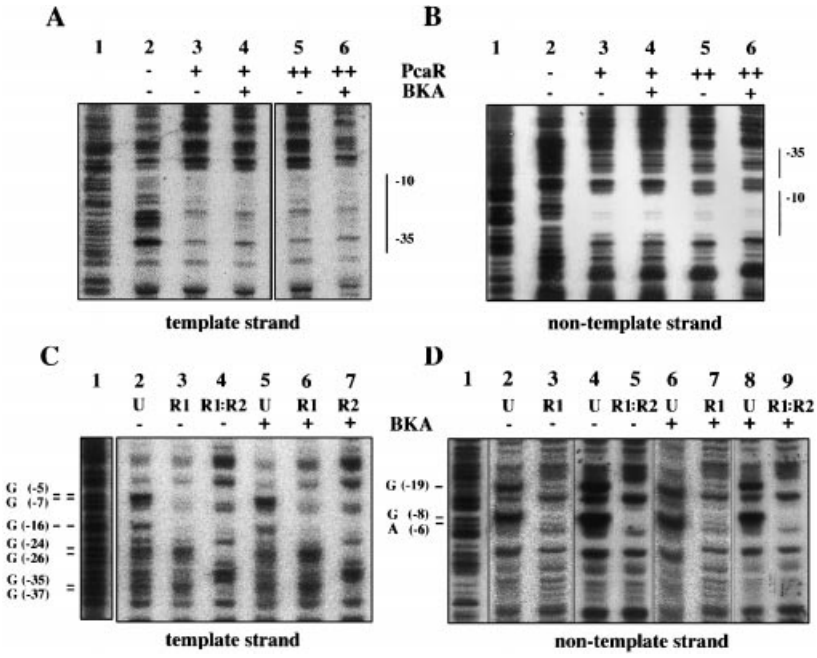

Fig. 5. DNase I footprint and premethylation interference footprint analyses of the *pcaI*, *J* promoter. The template and non-template strands of a 287 bp fragment, containing the *pcaI, J* promoter, were labelled independently using ^{32}P.
A and B. The results of DNase I footprint analyses of these strands. Lane 1, G + A sequencing ladder; lanes 2, 3 and 5 are the DNase I protection patterns in the absence and presence of 40 nM and 80 nM PcaR respectively; lanes 4 and 6 are similar to lanes 3 and 5, except that the reactions occurred in the presence of 0.1 mM β-ketoadipate (BKA).
C and D. The results of premethylation interference analyses of these same strands.
C. Lane 1, G + A sequencing ladder; lanes 2–4 are the premethylation footprint sequence analyses of 'unbound', R1 and R1–R2 complexed DNA respectively; lanes 5–7 are similar to lanes 2–4 except for the presence of 0.1 mM BKA.
D. Lane 1, G + A sequencing ladder; lanes 2 and 4 are the premethylation footprint sequences of 'unbound' fractions in the absence of BKA, while lanes 3 and 5 represent R1 and R1–R2 complex DNA footprints under similar conditions. Likewise, lanes 6 and 8 are the premethylation footprint sequences of 'unbound' fractions in the presence of 0.1 mM β-ketoadipate, while lanes 7 and 9 represent R1 and R1–R2 complex premethylation footprints under similar conditions.

fragments, the affinity of $RNAP_{Ec}$ for the *pcaI*, *J* promoter was seen to be considerably less than that for the $pcaR_{po}$; Fig. 7A–D, lanes 1–4). Moreover, the $RNAP_{Ec}$ formed a discernible and specific multimeric complex with the PcaR-bound *pcaI*, *J* promoter fragment (Fig. 7C and D, lanes 6–8), retarding the mobility of the slightly smaller $RNAP_{Ec}$–DNA complex. Such a PcaR–$RNAP_{Ec}$–DNA complex was confirmed by DNase I footprinting analysis (data not shown). Significantly, there was no corresponding change in the mobility of the $RNAP_{Ec}$–DNA complex for

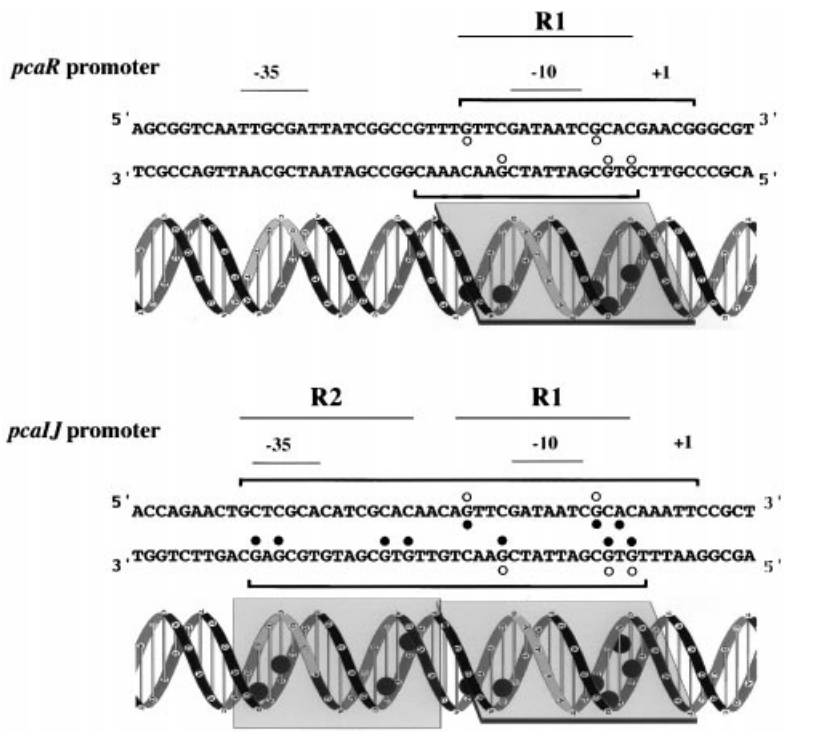

Fig. 6. Two-dimensional and three-dimensional representations of the promoter/operator regions of *pcaR* and *pcaI, J*. The schematics depict the protection afforded by the PcaR regulatory protein against DNase I cleavage (thick lines above and below the two-dimensional DNA sequences) and also the specific residues (open and filled circles) whose premethylation has been shown to alter the cleavage pattern in the bound and unbound fractions of DNA (see Figs 4 and 5). R1 and R2 designate the regions that encompass the consensus-like PcaR binding sequences. Open circles represent the premethylation of guanines that were defined for both *pcaR* and the *pcaI, J* promoter fragments bound in the R1 complex, while filled circles define similarly modified purines that affected the DNA bound in the R1–R2 complexes. The shaded trapezia approximate the relative planes of interaction that each PcaR dimer might be expected to adopt when bound to the R1 and R2 regions of DNA. The respective −35 and −10 hexamers are denoted as such above the two-dimensional DNA sequences and are defined in the backbone-ribbon of the double helices as areas of lighter shading.

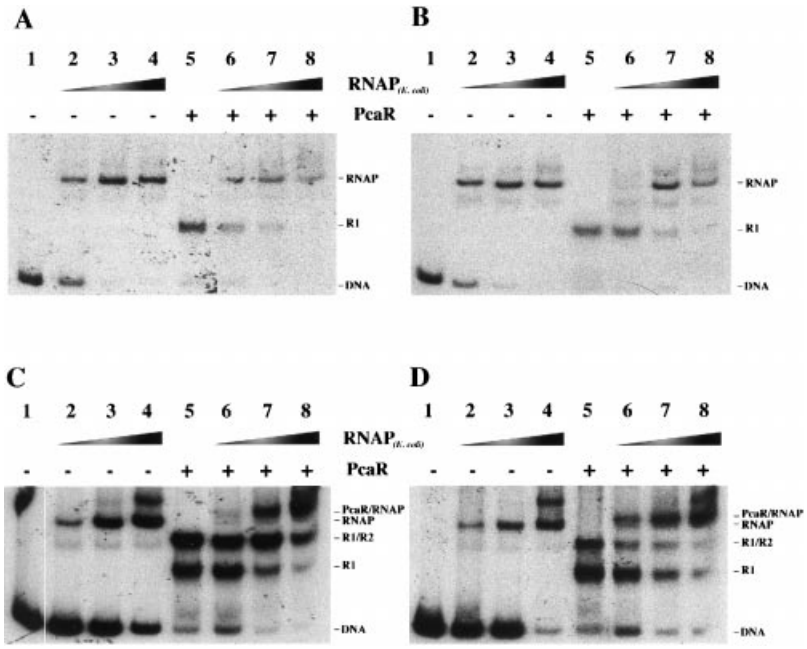

Fig. 7. Cohabitation of PcaR with *E. coli* RNA polymerase. Various amounts of *E. coli* RNAP holoenzyme $RNAP_{Ec}$ were incubated with ^{32}P end-labelled promoter fragments of *pcaR* (A and B) or *pcaI, J* (C and D). These reactions took place either in the absence (A and C) or in the presence of 0.1 mM β-ketoadipate (BKA; B and D). Lane 1, free DNA; lane 2, 22.6 nM RNAP; lane 3, 37.6 nM RNAP; lane 4, 56.5 nM RNAP; lanes 5–8 are similar to lanes 1–4, except that 2 nM PcaR was added in the reactions of the former. Similar concentrations of both labelled promoter fragments (approximating 1×10^{-13} M) were used in each reaction.

the $pcaR_{po}$ fragment in the presence of PcaR (Fig. 7A and B, lanes 6–8). This would indicate that PcaR is unable to form a multimeric PcaR–$RNAP_{Ec}$–DNA complex with the $pcaR_{po}$. Indeed, PcaR seemingly interferes with the binding of $RNAP_{Ec}$ to this promoter (Fig. 7A and B, lanes 2–4 versus lanes 6–8).

In addition, the formation of the various protein–DNA complexes with the *pcaI*, J_{po} (in response to the presence of β-ketoadipate) is also shown to be significantly different from that with the $pcaR_{po}$. The addition of β-ketoadipate to the *pcaI*, J_{po} reaction mixture enhanced the formation of the PcaR–$RNAP_{Ec}$–DNA complex (Fig. 7D, lanes 6–8 versus Fig. 7C, lanes 6–8). Moreover, in the presence of β-ketoadipate, the increasing concentration of the PcaR–$RNAP_{Ec}$–DNA complex (resulting from an increased presence of $RNAP_{Ec}$) was apparently matched by the concomitant disappearance of the R1–R2 band (Fig. 7D, lanes 4–8). Densitometric analysis confirmed that the apparent decrease in the intensity of the R1–R2 band in the presence of β-ketoadipate (Fig. 7C and D, lanes 5–8) was more rapid than the corresponding decrease in the R1–R2 complex in the absence of inducer (data not shown). In contrast, the disappearance of the R1 DNA complex remained relatively constant in both induced and uninduced conditions.

Discussion

In this study, we report that PcaR is able to regulate its own transcriptional expression negatively by up to fourfold (Table 1). This is in addition to its proven capacity as an inducer protein for the remaining genes within the PcaR-dependent, *pca* regulon (Hughes *et al*., 1988; Parales and Harwood, 1993; Nichols and Harwood, 1997). While such a duality of function is no longer considered to be too uncommon a regulatory feature (Schell, 1993; Nègre *et al*., 1998), the manner in which PcaR is seemingly able to undertake both types of regulation would appear to be so.

The PcaR regulatory protein has been considered to have a distinct preference for a series of residues 5′-GTTCGATAAtCGCAC-3′, the sequence of which is heavily conserved within those *pca* promoters that have been characterized to date (Parales and Harwood, 1993; Romero-Steiner *et al*., 1994; Guo and Houghton, 1998). The specific affinity of PcaR for this sequence of DNA was affirmed by mobility shift analyses, using various *pca* promoter fragments (Fig. 2), and by the ability of PcaR to bind to the much smaller 39 bp DNA fragment (Fig. 2, lanes 5 and 6). The observation that this sequence was both necessary and sufficient for PcaR binding was subsequently confirmed through DNase I and premethylation footprinting analyses of the *pcaR* promoter sequence. These studies defined the general region of the promoter that potentially interacts with PcaR and, more specifically, the purine residues within this conserved sequence that appear to be particularly important for PcaR binding (Figs 5 and 6).

Similar studies using the entire *pcaI*, J_{po} fragment showed that this minimal PcaR footprint (termed R1) could be expanded to include an additional PcaR binding site (R2), which has a similar DNA sequence (5′-GcTCGcacATCG-CAC-3′) and is located slightly upstream of the originally

defined R1 sequence (Figs 5 and 6). Binding of PcaR to this additional R2 site, however, is not identical to its binding to the R1 sequence, as only the assigned guanines on the 'template strand' appear to be directly involved in binding PcaR (Fig. 6). Moreover, binding of PcaR to this upstream site is seen to be entirely dependent upon the protein making simultaneous contacts with the R1 binding site, which has been slightly modified, presumably to accommodate for the binding of the additional PcaR dimer (Fig. 5D and Fig. 6). Such a requisite binding to R1 has also been confirmed by additional, concentration-dependent DNase I footprinting analyses of PcaR with the *pcaI*, J_{po} fragment (data not shown).

By far the most striking feature of the two PcaR binding sites (R1 and R2) within the inducible *pcaI*, *J* promoter is their relative positions within the promoter sequence itself, in that they occlude both the −35 and the −10 hexameric promoter sequences, extending the combined footprint from −38 to +1. Such an overlap of a regulatory protein binding site with the binding site of RNAP is not too common among bacterial transcriptional activators. However, precedence for such interactions has been established by MerR and SoxR, which provide conspicuous examples of this somewhat unusual subset of transcriptional activators/repressors (Summers, 1992; Ansari *et al*., 1995; Caslake *et al*., 1997; Hidalgo and Demple, 1997). The different promoters that are recognized and induced by MerR and SoxR (*merTPCAD* and *soxS* respectively) closely approximate consensus, except that their −35 and −10 promoter elements are separated by an excessively long and suboptimal 19 bp (Summers, 1992; Ansari *et al*., 1995; Hidalgo and Demple, 1997). A number of studies have indicated that σ^{70} from *E. coli* and *Pseudomonas* is able to 'sense' the spacing between these two promoter elements (Dombroski *et al*., 1996; McLean *et al*., 1997) and that altering the number of bases between the −35 and −10 promoter elements from the optimal 17 bp to either 16, 18 or 19 bp is similarly deleterious to promoter activity (for a review, see deHaseth *et al*., 1998). Both MerR and SoxR have been shown to optimize the spacing between these two promoter elements by inducing a bending and untwisting of the DNA within the *merTPCAD* and *soxS* promoters respectively (Ansari *et al*., 1995; Caslake *et al*., 1997; Hidalgo and Demple, 1997). This reconfiguration of each promoter is thought to realign the different −35 and −10 elements in such a way as to enhance the spatially specific interactions of the various RNAP subunits with the promoter DNA, and so promote the formation of the 'open transcriptional complex'.

A number of the interactions that PcaR exhibits with the *pcaI*, *J* promoter presented here are highly reminiscent of those displayed by both MerR and SoxR. In addition to their mutual and quite exceptional preference for binding within the −35 and −10 regions of their designate promoter sequences, all three regulatory proteins have been shown to bind the promoter DNA simultaneously with RNAP (Fig. 7C and D; Ansari *et al*., 1995; Hidalgo, and Demple, 1997), forming specific multimeric protein–DNA complexes. Binding of MerR, SoxR or PcaR to their respective co-inducer molecules has negligible, if any, discernible effects upon either their affinity for their binding sites or their specific DNA footprints (Figs 4 and 5; Hidalgo and Demple, 1994; Ansari *et al*., 1995). This is a somewhat unusual characteristic for transcriptional inducers (Schell, 1993) and suggests that the effect of the co-inducer is dissipated through a higher order of regulatory constraints within the RNAP complex itself (Caslake *et al*., 1997). Finally, all three sets of promoter sequences that are induced by MerR, SoxR and PcaR include a less than optimal spacing between the −35 and −10 hexamers. For the *merTPCAD* and *soxS* promoter sequences, this spacing is 19 bp (Park *et al*., 1992; Hidalgo, and Demple, 1997). In a slight but potentially significant contrast, the distance between the two promoter elements in the *pcaI*, *J* promoter is only 16 bp (Fig. 6).

Despite the similarities that are readily apparent among these three regulatory proteins, there are also some important distinctions. First, PcaR shares little primary sequence similarity to any members of the MerR family of regulatory proteins. Secondly, both MerR and SoxR bind to their promoter sequences as dimers. Even though PcaR has been shown to form a dimer in solution (and presumably binds to the $pcaR_{po}$ as a dimer), the potentially inducible form of PcaR bound to the *pcaI*, J_{po} should be considered to be a dimer of dimers, in which binding to the R2 site is contingent upon binding to the downstream R1 site. Moreover, in contrast to the binding at R1, specific base interactions within this R2 binding sequence were found to be exclusive to the template strand of the promoter DNA sequence (Fig. 6). Thirdly, as a consequence of these two similar but distinct binding sites, the overall footprint of PcaR within the *pcaI*, *J* promoter is far more extensive than that shown for either MerR or SoxR. Indeed, even though the combined PcaR-R1:R2 footprint is centred between the −35 and −10 promoter elements (like MerR and SoxR), the precise contacts that are apparently maintained by PcaR within these two regions of the *pcaI*, *J* promoter are considerably more intrusive with respect to potential RNAP binding and cohabitation (Caslake *et al*., 1997).

As a result of the similarities and differences that we have discerned among these three regulatory proteins, the less than optimal −35 sequence that is apparent in the *pcaI*, *J* promoter, together with the suboptimal separation from its associated −10 element, suggests two different models for PcaR induction of transcription at the *pcaI*, *J* promoter. The first of these would be that PcaR simply enhances the formation of an RNAP–promoter 'closed complex' by enhancing RNAP contacts with the

non-consensus −35 sequence, in a manner similar to that described for the phage ϕ29, P4 protein (Monsalve *et al*., 1997). The second is indicated by the location of the two bound PcaR dimers, which suggests that PcaR might act by optimizing the critical distance between the −35 and −10 promoter elements, thus potentially enhancing the formation of the 'open transcriptional complex' (as do MerR and SoxR). In this regard, it is worthy of note that the spacing between the two binding sites at R1 and R2 is such that, when bound, the two PcaR dimers would be slightly offset from one another by approximately 34° (Fig. 6). It is possible that, as a consequence of binding β-ketoadipate, the two regulatory dimers reorientate their interactions with the DNA in such a way as to lie along the same plane, giving rise to a potentially imperceptible twist in the DNA helix. This would result in a realignment of the −35 and −10 sequences, away from the less than desirable 16 bp, towards a more favourable consensus-like 17 bp separation.

Both models are consistent with the results of the footprinting and RNAP binding experiments (Figs 4, 5 and 7), which demonstrate that PcaR, when bound within the R1–R2 binding complex of the *pcaI*, J_{po}, does promote the formation of a larger PcaR–$RNAP_{Ec}$–DNA complex. Neither model necessarily excludes the other. However, the location of the R1 binding site and the finding that β-ketoadipate fails to have any discernible effect upon the binding of PcaR in the absence of RNAP would indicate that, to enhance RNAP interactions with the *pcaI*, *J* promoter, PcaR must first overcome the intrinsically negative effects of its own binding to the −10 sequence. This would suggest that the inductive potential of PcaR at the *pcaI*, J_{po} is slightly more complex than merely promoting the binding of RNAP to the −35 hexamer. Exactly how this induction is brought about is the focus of our current research in this area. The distinct possibility that PcaR from *P. putida* (in combination with RNAP and the co-inducer β-ketoadipate) provides a 'twisting' counterpart to the 'untwisting' actions of MerR and SoxR (Summers, 1992; Caslake *et al*., 1997) affords a particularly intriguing model for transcriptional induction that we are in the process of testing.

Experimental procedures

Bacterial strains, plasmids and growth media

Bacterial strains and plasmids used in this study are listed in Table 2. *P. putida* was grown at 30°C in either Luria broth or M9 minimal medium (Miller, 1972) supplemented with trace elements and appropriate carbon sources (Ornston, 1966). Unless otherwise specified, aromatic compounds were added at concentrations of 5 mM, with other sources of carbon being added at 10 mM. *E. coli* was grown at 37°C in Luria broth. When required for selection, the antibiotics ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}), and carbenicillin (1 mg ml^{-1}) were added.

β-Galactosidase activity analyses

The method of Miller (1972) was used for the determination of β-galactosidase activity, except that the cells were disrupted by sonication (using a Branson sonifier). Protein concentration was measured by the methods of Bradford (Bio-Rad kit) and Lowry *et al*. (1951).

Cloning and overexpression of a modified pcaR *construct: purification of PcaR**

In an effort to purify the PcaR protein to homogeneity by synthesizing a histidine-tagged fusion protein, the following primers were used to polymerase chain reaction (PCR) amplify and subsequently clone a slightly modified version of the *pcaR* gene from PRS2000: primer 1 (5′-GGGGctgcagCTATCGA-AGGTCGT**AGTGACGAAACCCTGGTCAACGATC-**3′) and primer 2 (5′-GTGGgaattcGGCTTGCCGACG-3′). Lower case letters represent either a *Pst*I site (primer 1) or an *Eco*RI site (primer 2). Underlined bases in primer sequence 1 denote the region encoding the attendant peptide sequence that is recognized and cleaved by Factor Xa. Emboldened letters in the same sequence denote the first 25 nucleotides of the *pcaR* structural gene, starting from the second amino acid.

The amplified PCR product was first cloned into pGEM-T vector (Promega) and then subcloned into the vector pTrcHisB, resulting in plasmid pZHisR. Overexpression of the cloned *pcaR* gene was achieved by transforming pZHisR into *E. coli* strain DH5α. The transformed culture was grown at 37°C to an optical density of 0.6 at 600 nm in Luria broth supplemented with 100 μg ml^{-1} ampicillin. IPTG was added to a final concentration of 0.1 mM in order to induce expression of the inserted gene. Cells were allowed to grow for another 4 h at 37°C. After harvesting the cells, the cell pellet was resuspended in 50 ml of 'breaking buffer' [20 mM NaH_2PO_4, Na_2HPO_4, pH 8.0, containing 1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (PMSF); Ausubel *et al*., 1992–98]. Cell extract was prepared by passing the cells through an Aminco French pressure cell twice at 14 000 psi. After centrifugation at 10 000 × *g* for 30 min, the supernatant was subjected to ammonium sulphate precipitation (Ausubel *et al*., 1992–98). $(NH_4)_2SO_4$ fractions of 0–30%, 30–40% and 40–70% were analysed for the presence of the PcaR by SDS–PAGE analysis. The fusion protein was found to be predominantly in the 30–40% $(NH_4)_2SO_4$ fraction. This protein was purified further to homogeneity by passage over a series of MonoQ (HR10/10; Pharmacia), Superdex 200 (HR10/30; Pharmacia) and phenyl Sepharose (HR5/5; Pharmacia) fast protein liquid chromatography (FPLC) columns. Each stage of the purification process was monitored by SDS–PAGE analysis, and the presence of a functional PcaR protein was verified further by mobility shift analysis of the *pcaI*, *J* promoter fragment (Romero-Steiner *et al*., 1994).

Treatment of the PcaR fusion protein with Factor Xa and molecular weight determination of native PcaR

In order to remove the N-terminal, His-tagged region from the purified PcaR fusion protein, the PcaR* was treated with Factor Xa (Pharmacia Biotech) according to the supplier's recommendations. The resulting 'native' PcaR protein was

again purified by passing through Superdex 200 (HR10/30; Pharmacia). The molecular weight of this PcaR protein species was then determined by passing it over a Superdex 200 column in 0.1 M phosphate buffer (pH 7.6). The N-terminal sequence of the purified PcaR protein was confirmed by automated Edman degradation, which was undertaken in the DNA/protein Core facility at Georgia State University.

DNA mobility shift analyses

The DNA-binding ability of PcaR was confirmed by gel mobility shift assay according to previously published procedures (Lu *et al*., 1992). Plasmids containing the *pcaI*, *J*, *pcaR* and *pcaF* promoters (pGEMT-I,J, pHS104 and pGEMT-F respectively) were digested using *Eco*RI and *Bam*HI. The 287 bp, 254 bp and 375 bp fragments containing the *pcaI*, *J*, *pcaR* and *pcaF* promoters, respectively, were labelled at their 3′ ends using [α-^{32}P]-dATP and Klenow fragment (Biolabs). In addition to the larger promoter fragments, a smaller 39 bp DNA fragment that encompassed the -31 to $+5$ of the *pcaI*, *J* promoter region (including the 15 bp conserved binding sequence) was constructed using two primers: primer 3 (5′-tttCATCGCACAACAGTTCGATAATCGCACAAATTCCGC-3′) and primer 4 (5′-tttGCGGAATT-TGTGCGATTATCGAACT-GTTGTGCGATG-3′). Annealing of the two fragments was initiated by mixing the two primers in equal amounts and incubating them for 10 min at 65°C. The primer mixture was then slowly cooled to 25°C, and the double-stranded DNA fragment was labelled with [α-^{32}P]-dATP, as described previously. The labelled DNA fragments ($\approx 1 \times 10^{-13}$ M) were incubated with PcaR in 10 μl of 10 mM Tris-HCl buffer (pH 7.1) containing 95 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 0.125 mg ml^{-1} BSA, 20 μg ml^{-1} of poly-(dI–dC) and (when required) 0.1 mM β-ketoadipate. For quantitative definition of PcaR–DNA interactions, the binding affinity of PcaR for each of the DNA fragments was determined by measuring the intensity of the unbound DNA bands using a FUJIX BAS1000 image analysis program. In so doing, the apparent dissociation constants were defined as the point at which half of the labelled DNA remained unbound (Hendrickson, and Schleif, 1984; Lu *et al*., 1992). In studying the effect of PcaR on RNAP–DNA interactions, the labelled DNA probe was preincubated with or without PcaR (2 nM) in 10 μl of the above reaction buffer at 25°C for 10 min (as defined by Hidalgo and Demple, 1994). *E. coli* RNAP holoenzyme (1 μl; Boehringer Mannheim) was then added at various concentrations, and the incubation was continued at 25°C for 20 min.

DNase I footprinting and premethylation interference footprinting analyses

DNase I footprint and premethylation interference analyses were performed as described previously (Lu *et al*., 1992), with some slight modifications to the DNase I footprinting procedure. For DNase I footprinting reactions, the reaction mixture (50 μl) contained 1×10^{-10} M singly end-labelled restriction fragments, various amounts of purified PcaR, 10 mM Tris-HCl (pH 7.1), 95 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.125 mg ml^{-1} BSA, 1 μg poly-(dI–dC) and 0.1 mM β-ketoadipate (when necessary).

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References

Ansari, A.Z., Bradner, J.E., and O'Halloran, T.V. (1995) DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature* **374:** 371–374.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., *et al*. (1992–98) *Current Protocols in Molecular Biology*. New York: J. Wiley & Sons.

Caslake, L.F., Ashraf, S.I., and Summers, A.O. (1997) Mutations in the alpha and sigma-70 subunits of RNA polymerase affect expression of the *mer* operon. *J Bacteriol* **179:** 1787–1795.

Choy, H.E., Park, S.W., Aki, T., Parrack, P., Fujita, N., Ishihama, A., *et al*. (1995) Repression and activation of transcription by Gal and Lac repressors: involvement of alpha subunit of RNA polymerase. *EMBO J* **14:** 4523–4529.

Dombroski, A.J., Johnson, B.D., Lonetto, M., and Gross, C.A. (1996) The sigma subunit of *Escherichia coli* RNA polymerase senses promoter spacing. *Proc Natl Acad Sci USA* **93:** 8858–8862.

Farinha, M.A., and Kropinski, A.M. (1990) Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J Bacteriol* **172:** 3496–3499.

Fried, M., and Crothers, D.M. (1983) Equilibria and kinetics of *lac* repressor–operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res* **23:** 6505–6525.

Gao, J., and Gussin, G.N. (1991) RNA polymerase from *Pseudomonas aeruginosa* and *Pseudomonas syringae* respond to *Escherichia coli* activator proteins. *J Bacteriol* **173:** 394–397.

Guo, Z., and Houghton, J.E. (1998) Purification and characterization of the transcriptional regulator PcaR from *Pseudomonas putida*. In *Proceedings of the American Society for Microbiology Meetings*, Atlanta, GA. **H25**.

Harwood, C.S., and Parales, R.E. (1996) The β-ketoadipate pathway and the biology of self-identity. *Annu Rev Microbiol* **50:** 553–590.

deHaseth, P.L., Zupanic, M., and Record, M. (1998) RNA polymerase promoter interactions: the comings and goings of RNA polymerase. *J Bacteriol* **180:** 3019–3025.

Hendrickson, W., and Schleif, R.B. (1984) Regulation of the *Escherichia coli* L-arabinose operon studied by gel electrophoresis DNA binding assay. *J Mol Biol* **178:** 611–628.

Hidalgo, E., and Demple, B. (1994) An iron–sulfur center is essential for transcriptional activation by the redox-sensing SoxR protein. *EMBO J* **13:** 138–146.

Hidalgo, E., and Demple, B. (1997) Spacing of promoter regulates the basal expression of the *soxS* gene and converts SoxR from a transcriptional activator into a repressor. *EMBO J* **16:** 1056–1065.

Hochschild, A., and Dove, S.L. (1998) Protein–protein contacts that activate and repress prokaryotic transcription. *Cell* **92:** 597–600.

Houghton, J.E., and Shanley, M.S. (1994) Catabolic potential of pseudomonads: a regulatory perspective. In *Biological Degradation and Bioremediation of Toxic Chemicals*. Chaudhry, R. (ed.). Portland, OR: Discorides Press, pp. 11–32.

Hughes, E.J., Shapiro, M.J., Houghton, J.E., and Ornston, L.N. (1988) Cloning and expression of *pca* genes from *Pseudomonas putida* in *Escherichia coli*. *J Gen Microbiol* **134:** 2877–2887.

Lobell, R.B., and Schleif, R.F. (1990) DNA looping and unlooping by AraC protein. *Science* **250:** 528–532.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 269–275.

Lu, C.-D., Houghton, J.E., and Abdelal, A.T. (1992) Characterization of the arginine repressor from *Salmonella typhimurium* and its interactions with the *carAB* operator. *J Mol Biol* **225:** 11–24.

McLean, B.W., Wiseman, S.L., and Kropinski, A.M. (1997) Functional analysis of sigma-70 consensus promoters in *Pseudomonas aeruginosa* and *Escherichia coli*. *Can J Microbiol* **43:** 981–985.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Monsalve, M., Mencia, M., Rojo, F., and Salas, M. (1996) Activation and repression of transcription at two different phage f29 promoters are mediated by interaction of the same residues of regulatory protein p4 with RNA polymerase. *EMBO J* **15:** 383–391.

Monsalve, M., Mencia, M., Salas, M., and Rojo, F. (1997) Transcription activation or repression by phage f29 protein p4 depends on the strength of the RNA polymerase–promoter interactions. *Mol Cell* **1:** 99–107.

Mooney, R.A., Artsimovitch, I., and Landick, R. (1998) Information processing by RNA polymerase: recognition of regulatory signals during RNA chain elongation. *J Bacteriol* **180:** 3265–3275.

Nègre, D., Oudot, C., Prost, J.-F., Murakam, K., Ishihami, A., Cozzone, A.J., *et al*. (1998) FruR-mediated activation at the *ppsA* promoter of *Escherichia coli*. *J Mol Biol* **276:** 355–365.

Nichols, N.N., and Harwood, C.S. (1997) PcaK, a high affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. *J Bacteriol* **179:** 5056–5061.

Ornston, L.N. (1966) The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. II. Enzymes of the protocatechuate pathway. *J Biol Chem* **241:** 3787–3794.

Parales, R.E., and Harwood, C.S. (1992) Characterization of the genes encoding β-ketoadipate: succinyl-coenzyme A transferase in *Pseudomonas putida*. *J Bacteriol* **174:** 4657–4666.

Parales, R.E., and Harwood, C.H. (1993) Regulation of the *pcaI, J* genes for aromatic degradation in *Pseudomonas putida*. *J Bacteriol* **175:** 5829–5838.

Park, S.-J., Wireman, J., and Summers, A.O. (1992) Genetic analysis of the Tn21 *mer* promoter-operator. *J Bacteriol* **174:** 2160–2171.

Parke, D., and Ornston, L.N. (1976) Constitutive synthesis of the enzymes of the protocatechuate and of the β-ketoadipate uptake system in mutant strains of *Pseudomonas putida*. *J Bacteriol* **126:** 271–281.

Ptashne, M. (1992) *Phage l and Higher Organisms*. Oxford: Cell Press and Blackwell Scientific Publications.

Romero-Steiner, S., Parales, R., Harwood, C.S., and Houghton, J.E. (1994) Characterization of the *pcaR* regulatory gene for *Pseudomonas putida*, which is required for the complete degradation of p-hydrobenzoate. *J Bacteriol* **176:** 5771–5779.

Schell (1993) Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol* **47:** 597–626.

Schlax, P.J., Capp, M.W., and Record, M.T.J. (1995) Inhibition of transcription initiation by *lac* repressor. *J Mol Biol* **245:** 331–350.

Stanier, R.Y., Palleroni, N.J., and Douderoff, M. (1966) The aerobic *Pseudomonads*: a taxonomic study. *J Gen Microbiol* **48:** 159–271.

Summers, A.O. (1992) Untwist and shout: a heavy metal-responsive transcriptional regulator. *J Bacteriol* **174:** 3097–3101.

Tabor, S., and Richardson, C.A. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82:** 1074–1078.

Williams, D.R. (1993) Multifunctional repressor KorB can block transcription by preventing isomerization of RNA polymerase–promoter complexes. *Nucleic Acids Res* **21:** 1141–1148.