Critical nucleotides in the interaction of CatR with the *pheBA* promoter: conservation of the CatR-mediated regulation mechanisms between the *pheBA* and *catBCA* operons

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The promoter of the plasmid-borne *pheBA* genes encoding enzymes for phenol degradation resembles the *catBCA* promoter and is activated by CatR, the regulator of the chromosomally encoded catechol-degradative *catBCA* genes in *Pseudomonas putida*. In this study, site-directed mutagenesis of the *pheBA* promoter region was performed. The interrupted inverted repeat sequence of the CatR recognition binding site (RBS) of the *pheBA* promoter is highly homologous to that of the *catBCA* promoter. However, the RBS was shown not to be the sole important feature for high-affinity binding of CatR to this site. Mutagenesis of the activation binding site (ABS) of CatR, which overlaps the –35 hexamer sequence TTGGAT of the promoter, revealed that the two G nucleotides in this sequence are important for promoter activity but not for CatR binding. All other substitutions made in the ABS negatively affected both the promoter activity and CatR binding. The spacer sequence of the *pheBA* and *catBCA* promoters between the –10 and –35 hexamers is 19 bp, which is longer than optimal. However, reducing the spacer region of the *pheBA* promoter was not sufficient for CatR-independent promoter activation. An internal binding site (IBS) for CatR is located downstream of the transcriptional start site of the *catBCA* genes and it negatively regulates the operon. A similar IBS was identified in the case of the *pheBA* operon and tested for its functionality. The results indicate a conservation of CatR-mediated regulation mechanisms between the *pheBA* promoter and the *catBCA* promoter. This universal mechanism of CatR-mediated transcriptional activation could be of great importance in enabling catechol-degrading bacteria to expand their substrate range via horizontal transfer of the phenol degradative genes.

Keywords: *Pseudomonas putida*, transcription activation, CatR, *pheBA* and *catBCA* operons, evolution of catabolic pathways

INTRODUCTION

A comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that these compounds are transformed by different enzymes to a limited number of central intermediates, such as proto-

catechuate and (substituted) catechols (Harayama & Timmis, 1989). In *Pseudomonas* species, unsubstituted phenols and haloaromatic compounds like chlorocatechols are usually metabolized by an *ortho*-cleavage pathway in which catechol is cleaved by catechol 1,2-dioxygenase. The cleavage product *cis,cis*-muconate (CCM) is transformed via β -ketoadipate-enol-lactone to succinate and acetyl coenzyme A (Ornston, 1966). Genes encoding catechol degradation enzymes (*cat* genes) are located on the chromosome of *Pseudomonas putida* (Aldrich & Chakrabarty, 1988). The genes of the modified *ortho*-cleavage pathways, including *clcABD*

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Abbreviations: ABS, activation binding site; CCM, *cis,cis*-muconate; β -Gal, β -galactosidase; IBS, internal binding site; RBS, recognition binding site.

Fig. 1. (a) Organization of the *pheBA* operon on plasmid pAT1140 (Kasak *et al.*, 1993). The genes *pheB* and *pheA* are flanked by two IS elements, IS1472 and IS1411 (Kallastu *et al.*, 1998). The right-end sequences of the transposon Tn4652 (Tsuda *et al.*, 1989) are shown by hatched boxes. IRR indicates the 46 bp terminal inverted repeat of Tn4652. Black boxes show the locations of the *pheBA* genes and the transposase genes (*tnpA*) of IS1472 and IS1411. The open boxes represent the intergenic region. The promoter of the *pheBA* operon (designated p_i) is located upstream of IS1472. The arrow indicates the direction of transcription of the genes. (b) Nucleotide sequences of the *pheBA* and *catBCA* promoter regions. The bracketed sequences represent nucleotides protected by CatR from cleavage by DNase I (Parsek *et al.*, 1995; Chugani *et al.*, 1997). The protected regions are designated as RBS and ABS. The nucleotides of the interrupted inverted repeat within the RBS are underlined. The boldface italic represents the T and A residues of the conserved T-N₁₁-A of the LysR family regulatory proteins binding motif present in the RBS (Goethals *et al.*, 1992). The *catBCA* promoter has a G instead of the conserved T. The nucleotides of the -35 and -10 elements of the promoters are in boldface and boxed. The transcriptional start sites are denoted by an asterisk. (c) The location of the CatR IBS in the *pheBA* operon. The CatR binding sites RBS, ABS and IBS are indicated by black boxes. The -35 and -10 hexamers of the *pheBA* promoter are marked by hatched boxes. The sequence of IBS is located in IS1472, between the left inverted repeat (indicated by a triangle) and the transposase gene *tnpA* (shown in black) of the element. The nucleotide sequence of the IBS region of the *pheBA* operon is shown below. The location of the nucleotides belonging to the IBS (indicated by a bracket) was originally proposed by Parsek *et al.* (1996). The sequence identical to one half of the interrupted inverted repeat of the RBS is underlined and the T and A residues of the conserved T-N₁₁-A of the LysR binding motif are in bold. The left inverted repeat of IS1472 is boxed and the putative translation initiator codon ATG of the *tnpA* gene is in boldface italics.

(Franz & Chakrabarty, 1987) and *tcbCDEF* (van der Meer *et al.*, 1991), have been characterized in bacteria capable of degrading chlorocatechols and are located on catabolic plasmids. Transcription of the *catBCA*, *clcABD* and *tcbCDEF* operons is controlled by the regulatory proteins CatR (Rothmel *et al.*, 1990), ClcR (Coco *et al.*, 1993) and TcbR (van der Meer *et al.*, 1991), respectively. These activators belong to the LysR family of bacterial regulators and utilize similar mechanisms for activating transcription (McFall *et al.*, 1997, 1998).

Many micro-organisms metabolize the toxic compound phenol via catechol as an intermediate. The catechol derived from phenol is usually metabolized by a different pathway known as the *meta* pathway (Herrmann *et al.*, 1988; Kukor & Olsen, 1990; Shingler *et al.*, 1992; Wigmore *et al.*, 1977). The *pheBA* genes specifying phenol degradation originate from multiplasmid *Pseudomonas* sp. strain EST1001 (Kivisaar *et al.*, 1990). *pheA* encodes phenol monooxygenase, which converts phenol to catechol, while *pheB* encodes catechol 1,2-dioxygenase. When the plasmid-borne *pheBA* operon (Fig. 1a) is introduced into a *P. putida* strain which is normally incapable of utilizing phenol, the bacteria acquire the ability to degrade phenol via the chromosomal *cat* pathway encoding catechol degradation in concert with the phenol to catechol or CCM conversion mediated by the *pheBA* operon (Kasak *et al.*, 1993). Transcription of the chromosomal *ortho* pathway genes *catBCA* and of the plasmid-encoded *pheBA* operon is activated by the same positive regulator, the chromosomally encoded LysR family protein CatR (Kasak *et al.*, 1993; Rothmel *et al.*, 1990). Despite the different origins of the *catBCA* and the *pheBA* promoters, the CatR-dependent transcriptional activation mechanism of these promoters seems to be similar. This is therefore an example of how evolution took a short-cut to simply evolve a couple of genes for phenol degradation, instead of a whole pathway with its cluster of structural and regulatory genes that normally evolves in micro-organisms.

A comparison of the nucleotide residues in the *pheBA* and *catBCA* promoters suggests that the two promoters are highly conserved and the DNase I protection patterns of CatR on these two promoter sequences are very similar (Chugani *et al.*, 1997; Parsek *et al.*, 1995) (Fig. 1b). CatR binds to the *pheBA* and the *catBCA* promoters in both the presence and absence of the inducer but activates transcription only in the presence of the inducer CCM (Parsek *et al.*, 1995; Rothmel *et al.*, 1991). In the absence of CCM, CatR binds to the recognition binding site (RBS). CCM allows CatR to bind to a second site, the activation binding site (ABS) and to activate transcription from the promoter. Binding of CatR to the ABS of the *catBCA* or the *pheBA* promoter is co-operative, requiring the presence of an intact RBS (Kasak *et al.*, 1993; Parsek *et al.*, 1992, 1995). Footprinting studies revealed that the ABS overlaps the -35 hexamer of the promoters (Chugani *et al.*, 1997; Parsek *et al.*, 1992, 1995). The C-terminal domain of the α -subunit of RNA polymerase is required for the expression of the

catBCA and the *pheBA* operons because RNA polymerases with truncated α -subunits were deficient in activation (Chugani *et al.*, 1997). Recently, an internal binding site (IBS) for CatR was identified within the *catB* structural gene. The IBS negatively regulates the expression of the *catBCA* promoter and the repression is CatR-mediated (Chugani *et al.*, 1998).

To study how similar or dissimilar the *pheBA* promoter region is to the *catBCA* promoter region, not just in terms of DNA homology but also in terms of functionality, we conducted site-directed mutagenesis in the RBS, the ABS and the putative IBS of the *pheBA* promoter. Comparing the effect of these mutations with those introduced into the *catBCA* promoter region (Parsek *et al.*, 1994) allowed us to determine critical nucleotides involved in direct interactions with CatR at both promoters. Data obtained in this study demonstrate that although some interactions of CatR with the RBS seem to be different between these two promoters, both the promoters utilize a very similar mechanism for transcriptional activation.

METHODS

Bacterial strains, plasmids and media. *Escherichia coli* TG1 (Carter *et al.*, 1985) was used for the DNA cloning procedures and *P. putida* PaW85 (Bayley *et al.*, 1977) was used as the host strain to study the expression of the *pheBA* promoter mutants. *E. coli* BL21(DE3) (Studier & Moffat, 1986) was used for inducible overexpression of CatR in the pET11 vector under the control of the T7 promoter. The DNA fragment containing the *catR* gene was subcloned as a ~1 kb *HindIII*-*EcoRI* fragment into pBluescript SK(+). To obtain the CatR-overproducing plasmid pET11CatR, the *catR* sequence was amplified with primers CatRNde 5'-AAG(NdeI)CATATG GAGCTGCGCCACTTGCGT-3' (complementary to the pET11 sequences containing the *NdeI* site and to the first seven codons for CatR) and the Reverse primer 5'-AACAGCTA-TGACCAG-3' [complementary to pBluescript SK(+) sequences flanking the multicloning sites in this plasmid]. The 950 bp PCR fragment was cleaved with *NdeI* and *BamHI* [the *BamHI* site in the amplified DNA region was derived from pBluescript SK(+) multicloning sequence] and inserted into the *NdeI*- and *BamHI*-cleaved protein expression plasmid pET11. The 158 bp DNA fragment containing the *pheBA* promoter region was amplified by PCR using the primers catRylem (5'-TCCGAATTC(*EcoRI*)TTGCCTTATCTCGG-CATAACCC-3') and catRall (5'-GGCATCGAT(*ClaI*)TGC-CTCCCAACTTTTAGTCTTG-3'), complementary to nucleotides -132 to -103 and +14 to +45, respectively, relative to the transcriptional start site of the *pheBA* promoter. The PCR-generated DNA fragment, cloned into pBluescript SK(+), was inserted with *BamHI* and *XhoI* ends into the promoter-probe vector pKTlacZ (Hörak & Kivisaar, 1998). The 795 bp DNA fragment carrying the *pheBA* promoter region with the CatR IBS was cloned as a *PstI*-*Eco47III* DNA fragment into pBluescript SK(+) and then, using the *BamHI* and *XhoI* ends, into pKTlacZ.

Bacteria were grown on Luria-Bertani (LB) medium (Miller, 1992) or on M9 minimal medium (Adams, 1959). Antibiotics were added at the following final concentrations: for *E. coli*, ampicillin 100 $\mu\text{g ml}^{-1}$; and for *P. putida*, carbenicillin at 1500 $\mu\text{g ml}^{-1}$. *P. putida* was incubated at 30 °C and *E. coli* at

37 °C. *E. coli* was transformed with plasmid DNA as described by Hanahan (1983) and DNA was electroporated into *P. putida* by the protocol of Sharma & Schimke (1996).

Site-directed mutagenesis of the *pheBA* promoter region and DNA sequencing. Site-directed mutagenesis of the *pheBA* promoter region and the IBS sequence was carried out using two-step PCR with mutant oligonucleotides containing the specific substitutions. In the first step of the PCR the point mutations were introduced by using the mutant oligonucleotide and oligonucleotide catRylem 5'-GGCATC-(*EcoRI*)GATTGCCTCCCAACTTTTAGTCTTG-3', which is complementary to nucleotides -84 to -113 relative to the transcriptional start site of the *pheBA* promoter. The first step of the PCR (25 cycles) was: 1 min at 95 °C, 1 min at 54 °C, 1 min at 72 °C. Then 2.5 U *ExoI* (Amersham) was added to the reaction mixture and the probes were incubated for 30 min at 37 °C to remove the oligonucleotides used in the first step of the PCR. PCR products of ~70 bp were purified and used as oligonucleotides in the second step of the PCR with the oligonucleotide catRall 5'-GGCATC(*Clal*)GATTGCCTCCCAACTTTTAGTCTTG-3', which is complementary to nucleotides +14 to +44 relative to the transcriptional start site of the *pheBA* promoter. The conditions of the second step of the PCR (25 cycles) were: 1 min at 95 °C, 3 min at 54 °C, 1 min at 72 °C. The 158 bp amplified DNA fragments were cloned using *EcoRI* and *BamHI* ends into pBluescript SK(+). All mutations were verified by dideoxy-sequencing with a Sequenase version 2.0 kit (Amersham). The *pheBA* promoter mutants were cloned into the promoter-probe vector pKTLacZ by using the *BamHI* and *XhoI* ends.

β -Galactosidase assay. Cells of *P. putida* PaW85 harbouring different *pheBA* promoter constructs were grown overnight to stationary phase. Sodium benzoate (final concentration 10 mM) was added to the growth medium to induce transcription from the *pheBA* promoter. The β -galactosidase (β -Gal) assay was performed as specified by Miller (1992). Protein concentration in crude lysates was measured by the method of Bradford (1976).

Overproduction of CatR and preparation of CatR lysate. The CatR protein was overexpressed in *E. coli* strain BL21(DE3) containing the expression plasmid pET11CatR (see above). Cells were grown in 1 litre M9 minimal medium at 20 °C. IPTG, final concentration 0.4 mM, was added at OD₅₈₀ 0.5, after which growth was allowed to continue for 4 h. Cells were centrifuged at 4600 g for 10 min and suspended in 10 ml CatR lysate buffer (50 mM Tris/HCl pH 7.5; 0.05 mM EDTA; 10 mM MgCl₂; 200 mM NaCl; 1 mM DTT; 0.05% Triton X-100; 10%, v/v, glycerol). The cell suspension was sonicated and centrifuged at 25000 g for 10 min.

Gel-mobility-shift assay. The primer CatRylem, labelled with [γ -³²P]dATP using T4 polynucleotide kinase, and the primer CatRall were used for PCR amplification of the 158 bp DNA fragments containing the wild-type or the mutant *pheBA* promoter region. The radiolabelled DNA fragments were purified by polyacrylamide gel electrophoresis. The CatR binding reaction was carried out in a volume of 20 μ l. DNA probes (200 c.p.m.) were incubated at 20 °C for 20 min with CatR lysate (0.001 mg or 0.01 mg), 1 $\times$ binding buffer (50 mM Tris/HCl pH 7.5; 5 mM EDTA; 250 mM KCl; 25 mM MgCl₂; 25 mM β -mercaptoethanol; 0.5% Triton X-100; 20%, v/v, glycerol), 1 μ g bovine serum albumin, 5 μ g salmon sperm DNA and 100 μ M of the inducer CCM. After incubation, the reaction mixture was loaded on a pre-run (20 min) 5% (w/v) nondenaturing polyacrylamide gel. Electrophoresis was carried out at 3 °C in 0.5 $\times$ Tris/borate/EDTA

buffer, pH 7.5, at 10 V cm⁻¹ for 2 h. The gel was exposed to a phosphorimager screen.

Determination of the CatR binding activity of the *pheBA* promoter mutants. The gel-shift assays were performed three or four times in the presence and absence of 100 μ M CCM with the two different concentrations of CatR lysate (as indicated above) which yielded less than 100% bound radioactive probe for the wild-type *pheBA* promoter region. The bound DNA relative to the unbound DNA was quantified by Phosphorimager (Image Quant 4.2a software, Molecular Dynamics). The relative CatR binding activity for the *pheBA* promoter mutants was expressed as a percentage of the wild-type *pheBA* promoter binding.

RESULTS

Site-directed mutagenesis of the *pheBA* promoter region bound by CatR

The DNA sequences bound by CatR within the *pheBA* promoter region have been localized by DNase I footprinting (Parsek *et al.*, 1995). In order to determine the critical nucleotides involved in DNA-protein interactions at the *pheBA* promoter, this DNA sequence was subjected to site-directed mutagenesis. Base substitutions were introduced into the RBS, the ABS and the 'hinge' region between the RBS and the ABS shown to be involved in DNA bending at the *pheBA* promoter (Parsek *et al.*, 1995) (Fig. 2). The DNA fragments carrying the *pheBA* promoter with mutated RBS, ABS or 'hinge' regions were cloned into the promoter-probe vector pKTLacZ (Hörak & Kivisaar, 1998). The effect of the mutations on promoter activity was tested by measuring the β -Gal activity in cells of *P. putida* PaW85 grown in LB in the presence or absence of benzoate. CCM, the inducer of the *pheBA* operon, is an intermediate of the benzoate degradation pathway and is generated from it. Gel-shift assays were used to determine the effect of the mutations on the binding efficiency of CatR. The results of the study of the effect of the mutations on the *pheBA* promoter activity and CatR binding are shown in Table 1.

Effect of mutations in the RBS region

The mutations which were introduced into the RBS and changed the nucleotides in the interrupted inverted repeat ATACC-N₅-GGTAT (see Fig. 2) negatively affected transcription from the *pheBA* promoter (Table 1). A drastic negative effect on β -Gal activity was observed in the case of the mutations 73AT, 72TG, 70CA, 69CA, 60AT and 59TG, and in the case of the double mutation 72TC/73AC in both the presence and absence of benzoate. The mutation 63GA, which also changed the inverted repeat sequence, had a less marked effect on the *pheBA* promoter activity (21% of the wild-type activity was retained in the presence of benzoate). The mutations 67CA and 66CA that were generated between the interrupted inverted repeat sequence exhibited almost wild-type levels of β -Gal activity in induced cells. The 74CT mutation lies upstream of the repeat sequence. However, it reduced the promoter

Fig. 2. Site-directed mutagenesis of the *pheBA* promoter region. The arrows mark the location of the mutations. The brackets indicate the location of the CatR-binding sites RBS and ABS. RBS and ABS are separated by the hinge region (not marked in the figure). The nucleotides of the interrupted inverted repeat are underlined and the boldface italic represents the T and A residues of the conserved T-N₁₁-A of the LysR binding motif. The nucleotides of the -35 and -10 elements of the *pheBA* promoter are in boldface and boxed. The deletion derivatives at positions -16 to -18 are referred to as del.

activity significantly in both the presence and absence of benzoate (only approximately 7% of the wild-type activity remained). The results of the gel-shift assay monitoring CatR binding to the RBS mutants were in good agreement with *in vivo* results: the mutations that negatively affected the expression of the *pheBA* also led to reduced CatR binding efficiency (Table 1).

Effect of mutations in the 'hinge' region

The CatR binding sites RBS and ABS are separated from each other by a 'hinge' region which has been postulated to be involved in DNA bending at the *pheBA* promoter (Parsek *et al.*, 1995). Site-directed mutagenesis in the 'hinge' region, where the G residues were substituted with A residues, was performed in order to generate a more flexible AT-rich region. The 52GA and 51GA mutations increased the efficiency of CatR binding in *in vitro* binding assays in the presence of CCM approximately 2.5- and 3.5-fold, respectively, and this positive effect was also observed without the inducer (Table 1). A 2.5-fold increase in CatR binding efficiency was also observed in the case of the double mutation 51/52GA, which generated a stretch of four A residues in the 'hinge' region. However, the effect of these mutations in β -Gal assays was not so marked. Only the 52GA

mutation demonstrated more than a twofold increase in expression of the *pheBA* promoter in the presence of benzoate; the level of expression of this mutant in the absence of inducer was enhanced 3.5-fold compared to the wild-type promoter (Table 1). The double mutation 51/52GA resulted in slightly elevated levels of expression and the 51GA mutation resulted in expression levels that were 80% of the wild-type levels in the presence of benzoate. Interestingly, the mutation 50AT, which was neither expected to influence the flexibility of the hinge region nor was previously shown to be involved in the CatR binding on the basis of DNase I footprint analysis (Parsek *et al.*, 1995), had a negative effect on *pheBA* promoter activity. This mutation also conferred a reduced CatR-binding efficiency, as observed in the gel-shift assay in the presence of CCM (Table 1).

Effect of mutations in the ABS region

The CatR ABS overlaps the -35 hexamers of the *pheBA* promoter and the *catBCA* promoter. In the absence of CCM, CatR binds to the RBS in the *catBCA* promoter. Presence of CCM results in the occupation of an additional adjacent binding site, the ABS, and an approximately twofold increase in CatR's DNA binding

Table 1. Effects of base substitutions in the *pheBA* promoter region on the CatR-mediated *in vivo* activation and *in vitro* binding activity of the mutants

Mutant	Activity and binding plus inducer		Activity and binding minus inducer	
	<i>In vivo</i> activity of <i>pheBA</i> promoter*	Percentage of wild-type CatR binding†	<i>In vivo</i> activity of <i>pheBA</i> promoter*	Percentage of wild-type CatR binding†
Wild-type	475.7 ± 55.2 (100)	100	2.6 ± 0.7 (100)	100
RBS				
74CT	31.7 ± 9.2 (6.7)	25	0.2 ± 0.05 (7.4)	15
73AT	0.12 ± 0.03 (0.6)	ND	0.13 ± 0.02 (5.2)	ND
72TC/73AC	2.7 ± 0.3 (0.02)	ND	0.12 ± 0.01 (4.7)	ND
72TG	11.9 ± 1.5 (2.5)	ND	0.2 ± 0.02 (8.5)	ND
70CA	5.5 ± 0.4 (1.1)	ND	0.2 ± 0.04 (8.5)	ND
69CA	26.7 ± 2.2 (5.6)	ND	0.3 ± 0.04 (11)	ND
67CA	451.6 ± 29.5 (95)	115	1.9 ± 0.2 (75)	130
66CA	410.7 ± 27.5 (86)	150	1.3 ± 0.3 (52)	140
63GA	99.2 ± 7.5 (21)	40	0.2 ± 0.02 (6.6)	80
60AT	38.2 ± 4.3 (8)	ND	0.2 ± 0.02 (9)	ND
Hinge				
59TG	15.8 ± 2.9 (3)	ND	0.2 ± 0.01 (10)	ND
52GA	1056.5 ± 154.0 (222)	280	9.8 ± 1.4 (375)	120
51/52GA	637.2 ± 82.6 (134)	260	2.8 ± 0.6 (107)	130
51GA	378.2 ± 47.4 (80)	370	2.2 ± 0.1 (84)	150
50AT	56.6 ± 2.2 (12)	40	0.5 ± 0.1 (20)	105
ABS				
46AC	0.15 ± 0.01 (0.03)	55	0.17 ± 0.03 (6.8)	90
45AC	0.13 ± 0.03 (0.03)	60	0.13 ± 0.03 (5.2)	135
42AC	0.1 ± 0.02 (0.02)	55	0.15 ± 0.01 (6)	102
41GA	0.1 ± 0.02 (0.02)	40	0.17 ± 0.01 (6.5)	135
40TC	16.4 ± 1.5 (3.5)	50	0.17 ± 0.03 (6.8)	120
39AG	7.0 ± 1.1 (1.5)	50	0.3 ± 0.05 (14)	80
38TG	7.2 ± 0.8 (1.5)	75	0.8 ± 0.1 (31)	130
36GC	9.9 ± 0.7 (2)	110	0.2 ± 0.01 (10)	130
35GA	4.9 ± 1.3 (1)	110	0.2 ± 0.03 (8.5)	120
34AC	277.7 ± 47.6 (58)	75	0.7 ± 0.06 (26)	110

* Specific activity of β -Gal [nmol *o*-nitrophenol formed min⁻¹ (mg protein)⁻¹] measured in *P. putida* PaW85 cells grown in the presence or absence of benzoate (inducer). Data (means ± standard deviations) of at least five independent experiments are presented. The *in vivo* expression of the mutant promoters relative to the wild-type promoter (%) is shown in parentheses.

† *In vitro* CatR binding data expressed as the percentage of the wild-type binding activity in the presence or absence of CCM (inducer). ND, Not detectable.

affinity (Parsek *et al.*, 1994, 1995). Binding of CatR to the ABS is cooperative and requires an intact RBS (Parsek *et al.*, 1992, 1994). Thus, substituting the nucleotides necessary for the CatR binding to the ABS would result in a decrease in CatR's binding efficiency to the *pheBA* promoter region to half of that detected with the original promoter sequence in the presence of CCM. As expected, the base substitutions 39AG, 40TC, 41GA, 42AC and 46AC that were introduced upstream of the -35 element of the *pheBA* promoter led to approximately 50% CatR-binding efficiency to the *pheBA* promoter region in the presence of CCM as compared

with the wild-type promoter (Table 1). In comparison to these substitutions, the effect of the mutations 45AC, 38TG and 34AC on CatR binding to the ABS was weaker (60–75% of the CatR-binding efficiency to the wild-type promoter region was retained) and no negative effect was observed in the case of the mutations 36GC and 35GA (Table 1).

The *in vivo* study of the expression of the *pheBA* promoter carrying different mutations in the ABS region revealed a negative effect of all positions substituted. All mutations except 34AC led to greatly reduced levels of β -

Gal activity in the presence of benzoate (Table 1). The base substitutions in the ABS region also negatively affected the basal level of expression of the *pheBA* promoter (see the β -Gal activity in cells grown without benzoate in Table 1).

Effect of the IBS on expression of the *pheBA* operon

An additional binding site for CatR downstream of the *catBCA* promoter, called the internal binding site, IBS, has been identified (Chugani *et al.*, 1998). The IBS negatively regulates the expression of the *catBCA* promoter. Occupation of the IBS by CatR was facilitated in the presence of the RBS and the ABS on the same DNA fragment, and the maintenance of phasing between the promoter and the IBS was important for the IBS-mediated repression (Chugani *et al.*, 1998). On the basis of these data it was proposed that CatR bound to the DNA at the *catBCA* promoter, through formation of a DNA loop, could interact with CatR bound to the IBS, and that this interaction could cause impaired transcriptional activation from the *catBCA* promoter (Chugani *et al.*, 1998). On the basis of DNase I footprint data a weak CatR-binding site downstream of the transcriptional start site of the *pheBA* operon (+204 to +221) was found as well (Parsek *et al.*, 1996). In order to examine the effect of the *pheBA* IBS (see Fig. 1c) on the expression of the *pheBA* operon, this potential CatR binding site was deleted. The resulting construct, pIBS18del, contains an 18 bp deletion of the IBS sequence and is replaced by an 8 bp foreign sequence. The 795 bp *Eco47III*–*PstI* fragment containing the *pheBA* promoter with the IBS sequence (designated as pIBS) and the deletion variant lacking this sequence were cloned into the plasmid pKTLacZ upstream of the promoterless *lacZ* gene. The expression of the *lacZ* transcriptional fusions in *P. putida* PaW85 grown in the presence of benzoate revealed an approximately twofold higher level of β -Gal activity in the case of the IBS deletion construct pIBS18del when compared with the original construct pIBS: 621 ± 96 versus 348 ± 38 nmol min⁻¹ (mg protein)⁻¹ (means $\pm$ SD, $n = 5$). This indicates that the IBS of the *pheBA* operon could function as the *cis*-acting repressing element analogous to the IBS of the *catBCA* operon. However, the effect observed in this study was somewhat less (a twofold effect in comparison with the three- to fourfold effect found in the case of the *catBCA* system).

The IBS of the *catBCA* promoter closely matches the consensus sequence of the CatR-binding site RBS (Chugani *et al.*, 1998). The IBS region of the *pheBA* operon contains the sequence ATACC at positions +207 to +211, which is identical to one half of the interrupted inverted repeat of the RBS sequence (Fig. 1). The location of an A at position +220 (11 nt from the T of the sequence ATACC) matches the LysR-binding consensus T-N₁₁-A motif. We generated two mutations in the IBS region of the *pheBA* operon: the 208TC mutation, which substituted the T residue in the IBS to a C residue, and the 220A-GG mutation, which replaced the A residue at position +220 with two G residues (Fig.

1c). The effect of these mutations was tested using the pKTLacZ reporter system (constructs pIBS208TC and pIBS220A-GG, respectively) with cells grown in the presence of benzoate. Only a slight increase of the β -Gal activity was observed when the 208TC mutation was compared with the wild-type sequence: 397 ± 35 versus 348 ± 38 nmol min⁻¹ (mg protein)⁻¹. However, the 220A-GG mutation resulted in a twofold increase in the expression of the β -Gal activity [to 607 ± 54 nmol min⁻¹ (mg protein)⁻¹] in comparison with the wild-type. The twofold positive effect of the IBS deletion and mutation 220A-GG was observed also in cells that were grown without the inducer (data not shown).

Effect of the length of the spacer sequence between the –35 and –10 hexamers of the *pheBA* promoter on CatR-dependent transcriptional activation of the *pheBA* promoter

The optimal distance between the –35 and –10 hexamers of the RNA polymerase σ^{70} -recognized promoters is usually 17 bp (Stefano & Gralla, 1982). The spacer sequence between the –35 and –10 hexamers of the *pheBA* promoter is abnormally long, 19 bp. The 19 bp spacer adds an additional twist angle of at least 34° and the two hexamers may be out of phase with respect to each other. This raised the question whether optimizing the distance between the –35 and –10 elements of the *pheBA* promoter could compensate for the requirement of CatR for transcriptional activation of this promoter. Using PCR, we made deletions in the spacer sequence of the *pheBA* promoter that reduced the distance between the hexamers from 19 bp to 18, 17 or 16 bp and cloned the mutant promoters into the plasmid pKTLacZ (constructs pDEL18, pDEL17 and pDEL16, respectively; Fig. 2). Like cells with the wild-type promoter, the deletion mutants exhibited only a low basal level β -Gal activity both in the wild-type and in the CatR⁻ background when bacteria were grown without the inducer (data not shown). When benzoate was added to the growth medium the 18 bp spacer mutant showed a higher level of β -Gal activity than the control: 735 ± 10 versus 365 ± 19 nmol min⁻¹ (mg protein)⁻¹. Reducing the distance between the –35 and –10 elements to 17 bp or 16 bp had a negative effect on transcriptional activation of the *pheBA* promoter: pDEL17 showed a twofold lower level of β -Gal activity [184 ± 20 nmol min⁻¹ (mg protein)⁻¹] than the construct carrying the wild-type promoter with the 19 bp spacer, and pDEL16 demonstrated only the basal level of activity. Thus, optimizing the distance between the –35 and –10 hexamers of the *pheBA* promoter is not sufficient for CatR-independent transcriptional activation of this promoter.

DISCUSSION

The promoter of the plasmid-encoded phenol-degradative operon *pheBA* shows sequence similarity to the promoter of the chromosomal catechol-degradative *catBCA* genes in *P. putida* (Kasak *et al.*, 1993). The chromosomal *catR* product positively regulates the

expression of both promoters (Kasak *et al.*, 1993; Parsek *et al.*, 1995). Site-directed mutagenesis of the *pheBA* promoter region performed in this study revealed critical nucleotides involved in protein–DNA interactions at the *pheBA* promoter. Mutations in the interrupted inverted repeat in the RBS had drastic effects on CatR binding and activation of the *pheBA* promoter (Fig. 2, Table 1). Interestingly, base substitutions 67GA and 66CA between the repeat sequences did not have any effect. This indicates that the nucleotides in the interrupted inverted repeat may play an important role in the sequence-specific recognition of the RBS by CatR. Similar results were seen in a previous site-directed mutagenesis study of the *catBCA* promoter (Parsek *et al.*, 1994). The mutation 74CT, which does not lie in the repeat, had a negative effect on the *in vivo* activity of the promoter and resulted in a lower CatR binding efficiency. Some mutations outside the inverted repeat of the *catBCA* promoter also had negative effects on the *in vivo* activity of the promoter (Parsek *et al.*, 1994). The RBS sequence of the *catBCA* promoter contains the imperfect inverted repeat AGACC-N₅-GGTAT. It harbours a G-N₁₁-A motif instead of the consensus T-N₁₁-A of the LysR-family binding motifs. The *pheBA* promoter has a perfect inverted repeat ATACC-N₅-GGTAT and CatR binds the *pheBA* promoter with greater affinity than the *catBCA* promoter region (Parsek *et al.*, 1995). Changing the G to the consensus T in the *catBCA* RBS resulted in slightly elevated levels of the CatR binding and the promoter activation. However, the mutation 72TG, which made the repeat sequence of the *pheBA* RBS identical to that of the *catBCA* promoter, abolished CatR binding and only 2% of the wild-type promoter activity was observed under activating conditions. The possibility of different CatR–DNA interactions at the RBS of the *pheBA* and the *catBCA* promoter regions is also supported by the CatR-induced DNA bending data demonstrating a different degree of bending between the *pheBA* and the *catBCA* promoter regions in the absence of the inducer (Parsek *et al.*, 1995). Moreover, the CatR-binding motif AGACC-N₅-GGTAT is conserved between the RBS and the low-affinity binding site IBS of the *catBCA* promoter (Chugani *et al.*, 1998). This finding also indicates that the conserved interrupted inverted repeat sequence is not the sole important feature for high-affinity binding of CatR.

Under activating conditions, in the presence of the inducer CCM, CatR binds to the *pheBA* promoter and the *catBCA* promoter as a tetramer. One dimer binds to the RBS and the second dimer binds cooperatively to the ABS (Parsek *et al.*, 1994, 1995). The CatR binding site ABS encompasses the promoter –35 element. Since the CatR recognition elements are oriented on the opposite face of the DNA helix to the –35 element, both CatR and RNA polymerase may simultaneously interact with the same sequences from opposite sides of the DNA helix. Mutations in the ABS of the *pheBA* promoter fall into two groups on the basis of their effects: (1) mutations that affect both the promoter activity and CatR binding, and (2) mutations that affect negatively

only the expression of the promoter (Fig. 2, Table 1). For example, the mutations 36GC and 35GA, which encompass the –35 hexamer sequence TTGGAT of the *pheBA* promoter, drastically reduced the level of promoter expression in both the presence and the absence of the inducer but did not affect CatR binding. This indicates that these two nucleotides may be involved in interaction of RNA polymerase with the promoter sequence. The –35 hexamer sequence TTGGAT of the *pheBA* promoter deviates from the –35 consensus sequence TTGACA at three positions. Therefore, it was unexpected that changing the nucleotide from G to A at position –35 relative to the *pheBA* transcriptional start (mutation 35GA) would inactivate the promoter, since such a change made the promoter sequence similar to the σ^{70} -recognized promoter consensus. Comparison of the sequences of the –35 elements of the *pheBA* promoter and the *catBCA* promoter reveals that they are highly conserved (sequences TTGGAT and TTGGAC, respectively). The –35 hexamer of the promoter of the chlorocatechol-degradative genes *clcABD* is identical to that of the *catBCA* promoter and it was shown that CatR and ClcR activate transcription via a conserved mechanism (McFall *et al.*, 1997). The elimination of expression from the *pheBA* promoter as a result of the 35GA mutation and the conservation of a G nucleotide instead of the consensus nucleotide A in these three promoters indicate that this G nucleotide is important for RNA polymerase interactions with the CatR- and ClcR-regulated promoters.

A 19 bp spacer separates the –35 and –10 hexamers of the *pheBA* promoter and the *catBCA* promoter. The promoter of the mercury-resistance operon *mer* also deviates from the σ^{70} -recognized promoter consensus in that there is a 19 bp spacer region separating the two hexamers of the promoter (Parkhill & Brown, 1990). Mercury-dependent activation of this promoter brings the –10 and –35 elements into a better helical alignment through a MerR-mediated untwisting effect at the spacer DNA (Ansari *et al.*, 1992; O'Halloran *et al.*, 1989). Single and double base-pair deletions in the interhexamer spacer of the *mer* operon promoter resulted in MerR-independent transcriptional activation of this promoter (Parkhill & Brown, 1990). In the case of the promoter of the *mom* gene (encoding a DNA-modification function of the Mu phage), the 19 bp suboptimal spacer region is also known to function (Bolker *et al.*, 1989). C-protein-mediated torsional changes reorient the –10 and –35 elements to a favourable conformation for RNA polymerase binding at this promoter (Basak & Nagaraja, 1998). The deletions made by us to optimize the length of the spacer region of the *pheBA* promoter did not compensate for CCM-dependent CatR-mediated activation. However, these data show that the exact orientation of the CatR- and RNA-polymerase-binding elements on the DNA helical face is important. Deletion of 2 bp or more from the spacer sequence had a negative influence on the promoter activity: the deletion variant with the 16 bp spacer was not activated at all.

CatR induces a DNA bend in the 'hinge' region between the CatR-binding sites RBS and ABS of the *pheBA* and *catBCA* promoters (Parsek *et al.*, 1995). In the presence of CCM, the DNA bending angle of the *catBCA* promoter is partially relaxed. In the case of the *pheBA* promoter, CatR bends the DNA in the presence or absence of inducer to the relaxed bending angle of the *catBCA* promoter when CCM is present. Although the fixed DNA bending angle may be important for the CatR-mediated transcriptional activation of both the promoters, some other inducer-dependent CatR-mediated conformational changes at the promoter-RNA polymerase complex are required as well. The requirement of the carboxy-terminal domain of the α -subunit (α -CTD) of RNA polymerase was demonstrated for the activation of the *pheBA* and the *catBCA* promoters (Chugani *et al.*, 1997). The pattern of activation of these promoters resembles the pattern of activation for upstream enhancer element (UP element)-dependent activation more closely than cyclic AMP receptor protein (CRP)-dependent activation (Murakami *et al.*, 1996). Therefore, it was suggested that the α -CTD might interact directly not only with CatR but also with the DNA at the putative UP element (Chugani *et al.*, 1997).

The substitution of an A nucleotide with a G nucleotide (52AG mutation) in the *catBCA* promoter 'hinge' region lowered the activation level of the promoter approximately fivefold but did not affect CatR binding (Parsek *et al.*, 1994). It was thought that this change would alter the flexibility of the 'hinge' region of the promoter (Parsek *et al.*, 1994, 1995). The *in vivo* and *in vitro* effects of the base substitutions that were made in the 'hinge' region of the *pheBA* promoter were somewhat unexpected (Table 1). The binding efficiency of CatR as determined by the gel-shift assay was increased approximately threefold by the substitution of G nucleotides with A nucleotides. Concurrently, the increase in the *in vivo* expression of the promoter (twofold) was seen only in one particular case (mutation 52GA). The reason for such a discrepancy is unclear. According to the model presented by Chugani *et al.* (1997, 1998), both CatR and RNA polymerase may simultaneously interact with the same sequences from opposite sides of the helix and the α -CTD most likely interacts with the nucleotides located between the RBS and ABS. Therefore, it is tempting to speculate that the mutation 52GA improved the binding of α -CTD to the putative UP element sequence of the *pheBA* promoter for its activation, thereby enhancing the level of transcriptional activation from the promoter.

The third CatR binding site, IBS, was localized within the *catB* structural gene (Chugani *et al.*, 1998). The cooperativity observed in DNase I protection studies and the phasing dependence of IBS function indicated that the CatR dimers, bound to the RBS and ABS, interact with the CatR molecules bound to the IBS, with the looping out of the intervening DNA (Chugani *et al.*, 1998). This interaction resulted in reduced transcriptional activation from the *catBCA* promoter. The presence of the CatR low-affinity binding site has also

been suggested in the case of the *pheBA* operon at positions +204 to +221 with respect to the transcriptional start site of the operon (Parsek *et al.*, 1996). In this study we have examined the possible biological effect of this site on the regulation of the *pheBA* operon. A study of the effect of base substitutions in the IBS region and the deletion of this site indicated that the IBS of the *pheBA* operon affects transcription from the *pheBA* promoter in a similar manner as in the case of IBS-mediated repression of the *catBCA* promoter.

The genetic organization of the *pheBA* operon is unusual: besides IS1411, which flanks the *pheBA* genes downstream, a second IS element, IS1472, is located between these genes and their promoter (Fig. 1a). A very similar IS element, IS1384, which shares approximately 99% homology at the DNA level to IS1472, has been found in phenol-degrading *Pseudomonas* sp. strain H isolated from soil in Germany (GenBank accession number AF052751). The IBS of the *pheBA* operon is located between the left IR and the transposase gene *tnpA* of the IS element IS1472 (Fig. 1c). A sequence identical to the IBS of the *pheBA* operon is also present in IS1384. Thus, the location of the IBS of the *pheBA* operon in IS1472 demonstrates that, in principle, any sequence could play a role of IBS if this sequence can function as a binding site for CatR and has an appropriate location relative to the CatR-regulated promoter. It is also a good example of how mobile DNA elements can modulate the expression of neighbouring genes.

Comparative studies of the interaction of CatR at the promoters of the *pheBA* and *catBCA* operons have revealed that the CatR-mediated activation mechanism is well conserved despite the different origins of these operons. The *pheBA* gene cluster appears to be flanked by transposable elements (Kasak *et al.*, 1993; Kallastu *et al.*, 1998). This in turn could allow a rapid movement of these genes from one DNA molecule to another. After the release of the laboratory *P. putida* strain carrying the *pheBA* genes on a plasmid into a phenol-contaminated mining area in Estonia, horizontal transfer of the *pheBA* operon and its expression in different soil bacteria was observed (Peters *et al.*, 1997). In all isolates degrading phenol via the *ortho* pathway and harbouring the *pheBA* genes integrated into other plasmids, the original *pheBA* promoter sequence was present as before. Thus, the universal mechanism of CatR-mediated transcriptional activation could be of great importance in enabling catechol-degrading soil bacteria to expand their substrate range via horizontal transfer of the phenol-degradation genes without the need for subsequent extensive genetic rearrangements.

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