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Involvement of the Global Crp Regulator in Cyclic AMP-Dependent Utilization of Aromatic Amino Acids by *Pseudomonas putida*

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The *phhAB* operon encodes a phenylalanine hydroxylase involved in the conversion of L-phenylalanine into L-tyrosine in *Pseudomonas putida*. The *phhAB* promoter is transcribed by RNA polymerase sigma-70 and is unusual in that the specific regulator PhhR acts as an enhancer protein that binds to two distant upstream sites (−75 to −92 and −132 to −149). There is an integration host factor (IHF) binding site that overlaps the proximal PhhR box, and, consequently, IHF acts as an inhibitor of transcription. Use of L-phenylalanine is compromised in a *crp*-deficient background due to reduced expression from the *phhAB* promoter. Electrophoretic mobility shift assays and DNase I footprinting assays reveal that Crp binds at a site centered at −109 only in the presence of cyclic AMP (cAMP). We show, using circular permutation analysis, that the simultaneous binding of Crp/cAMP and PhhR bends DNA to bring positive regulators and RNA polymerase into close proximity. This nucleoprotein complex promotes transcription from *phhA* only in response to L-phenylalanine.

The zone of soil surrounding the roots of plants, known as the rhizosphere, is a niche rich in nutrients due to the continuous secretion of root exudates by plants. Recent global transcriptomic studies have revealed that microbial survival in this niche requires a dedicated oxidative stress response and a highly regulated system for the control of food utilization, since compounds in the rhizosphere vary depending on the type of plant, plant growth state, and soil physicochemical parameters (23, 25). In root exudates, proteogenic amino acids are abundant, and *Pseudomonas putida* KT2440 (Table 1), a model rhizosphere microorganism, uses a number of amino acids, including tyrosine, glutamine, aspartate, proline, and glutamate, as both C and N sources, while phenylalanine is metabolized exclusively as a nitrogen source (8, 29). In *P. putida*, a hierarchy exists for the use of amino acids that is under the control of the global regulator Crc, which organizes the assimilation of preferred amino acids over nonpreferred ones (2, 30, 33, 34, 41, 42). Crc exerts this control posttranscriptionally by binding to the 5′ end of the target mRNAs, thereby inhibiting translation (31, 32, 33, 34).

Utilization of certain D- and L-amino acids by *P. putida* appears to involve the global regulator Crp, since growth with D- and L-lysine, D-alanine, and L-phenylalanine as N sources was compromised in *crp* mutants (8, 27). In contrast with other microorganisms, it should be noted that the characterization of a *P. putida* *crp*-deficient mutant revealed that the profile of C sources used by this mutant is identical to that of the wild-type strain (8, 27). In *P. putida*, the *crp* gene product (PP0424) is annotated as a cyclic AMP (cAMP) receptor protein, with high similarity to Crp of *Escherichia coli* (62% identity) and Vfr of *Pseudomonas aeruginosa* (82% identity). In *E. coli*, Crp is a key global regulator of C metabolism that orchestrates glucose-induced catabolite repression control and directly or indirectly controls a number of transporters, catabolic enzymes, and stress response proteins (15), while Vfr in *P. aeruginosa* seems to be involved in the control of a virulence program, and it is not required for catabolite repression (46). Therefore, it appears that Crp of *P. putida* has been recruited to control a set of functions involved in the utilization of amino acids as N sources, rather than in catabolism of sugars or virulence.

We have previously analyzed the metabolism of L-phenylalanine by *P. putida* KT2440 (19), and a key pathway for its utilization as an N source was found to involve its initial hydroxylation to yield L-tyrosine in a reaction mediated by the *phhAB* gene products (Fig. 1). Subsequently, the amino group of tyrosine is transferred to α-ketoglutarate to yield *p*-hydroxyphenylpyruvate and glutamate, which channels organic nitrogen in the cells. The *phhAB* genes are transcribed divergently with respect to the *phhR* gene, which encodes the positive regulator of the *phhAB* operon. Expression of *phhR* is constitutive, while that of the *phhAB* operon occurs only in response to phenylalanine or tyrosine (17). Expression of the *phhAB* operon is mediated by the PhhR regulator and occurs only in the presence of L-phenylalanine (Fig. 1). Therefore, L-phenylalanine catabolism in *P. putida* is likely subject to control through the global Crc and Crp regulators and through activation by the specific local regulator PhhR. The *phhAB* promoter is transcribed by RNA polymerase sigma-70 and is unusual in that for this promoter the PhhR protein functions as an enhancer that binds to two upstream enhancer binding sequences that are centered at positions −83 and −141 from the +1 transcriptional start point with a stoichiometry of one dimer per binding site (19) (Fig. 1). Both target sequences are indispensable for transcription from the *phhAB* promoter. In addition, overlapping the proximal enhancer site is an integration host factor (IHF) binding site (19). Transcription assays with a P_{phhA}::*lacZ* fusion, electrophoretic mobility shift assays (EMSAs), and footprint assays showed that IHF acts as a negative regulator and that in a *himD* mutant background, expression from this promoter in response to

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TABLE 1 Plasmids and strains used in this study

Strain or plasmid	Genotype[a]	Reference or source
Strains		
P. putida		
KT2440	Cm^r	11
phhR strain	Km^r	19
crp strain	Cm^r Km^r *crp*::mini-Tn*5*	8, 9
cya strain	Cm^r Km^r *cya*::mini-Tn*5*	8, 9
E. coli		
BL21(DE3)	F$^-$ *ompI hsdS*$_B$ (r_B^- m_B^-) *gal dem met*	Novagen
DH5αF′	F′ *hsdR17 recA1 gyrA*	49
Plasmids		
pMP220	′*lacZ* IncP Tc^r	45
pMCA	P$_{phhA}$::*lacZ* Tc^r	19
pET28a (+)	Km^r protein expression vector	Novagen
pBend2	Ap^r circular permutation vector	24
pCR2.1	Ap^r Km^r	Invitrogen
pMCPhhR	Km^r *phhR*	19
pCRP1	Ap^r Km^r *crp*	This study
pCRP2	Ap^r Km^r Crp expression	This study
pCRP3	Ap^r Km^r P$_{PhhAB}$	This study
pMCCrp	Km^r *crp*	This study
pCRP3	Ap^r	This study
pGEMT-*phhA*-v	Ap^r, mutant, *phhA* variant, deleted of the Crp binding site	This study
pPhhRBend	Ap^r	This study

[a] Ap^r, Cm^r, Km^r, and Tc^r, resistance to ampicillin, chloramphenicol, kanamycin, and tetracycline, respectively.

L-phenylalanine was maximal (19). Therefore, the *phhAB* promoter appears to be the target of multiple regulators, which is relatively unusual in prokaryotes (20).

In this study, we provide insight into the role of Crp/cAMP as a coactivator in the control of L-phenylalanine catabolism, and we show that this global regulator binds to the *phhAB* promoter at a site located between the two PhhR enhancer sites. Crp/cAMP and PhhR act synergistically and induce DNA bending, which favors the interaction between PhhR and the RNA polymerase. We discuss a model in which a nucleoprotein complex is formed in which Crp, PhhR, and RNA polymerase are involved, but the transcriptional machinery is active only in response to L-phenylalanine. This is the first report that implicates cAMP as an important signaling molecule for amino acid catabolism in *P. putida*.

MATERIALS AND METHODS

Strain, plasmids, and culture media. The bacterial strains, cloning vectors, and plasmids used in this study are shown in Table 1. *Pseudomonas putida* KT2440 and its mutant derivatives were grown in M9 minimal medium supplemented with glucose (0.5% [wt/vol]) as the carbon source (1). When indicated, M8 minimal medium (M9 medium without NH_4Cl) was used with either L-phenylalanine or L-tyrosine at a concentration of 5 mM. Cultures were incubated at 30°C and shaken on an orbital platform operating at 200 strokes per minute. When required, antibiotics were used at the following final concentrations (in micrograms per milliliter): ampicillin, 100; chloramphenicol, 30; kanamycin, 50; and tetracycline, 20. *Escherichia coli* strains were grown at 37°C in LB medium with shaking. *E. coli* DH5α was used for gene cloning, and *E. coli* BL21(DE3) was used for protein expression.

β-Galactosidase assays. The pMCA plasmid bears a fusion of the promoter of the *phhAB* operon to a promoterless ′*lacZ* gene in the pMP220 low-copy-number vector (19). The wild-type *P. putida* KT2440 strain and isogenic mutants were grown overnight in M9 medium with glucose plus tetracycline. The cultures were then diluted 100-fold in the same medium and grown to a turbidity of about 0.8 at 660 nm. Aliquots were prepared and incubated in the absence or presence of 5 mM effector at 30°C for 2 h with shaking. β-Galactosidase activity was assayed in permeabilized whole cells according to Miller's method (28) described elsewhere (13). Assays were run in triplicate and were repeated for at least three independent experiments.

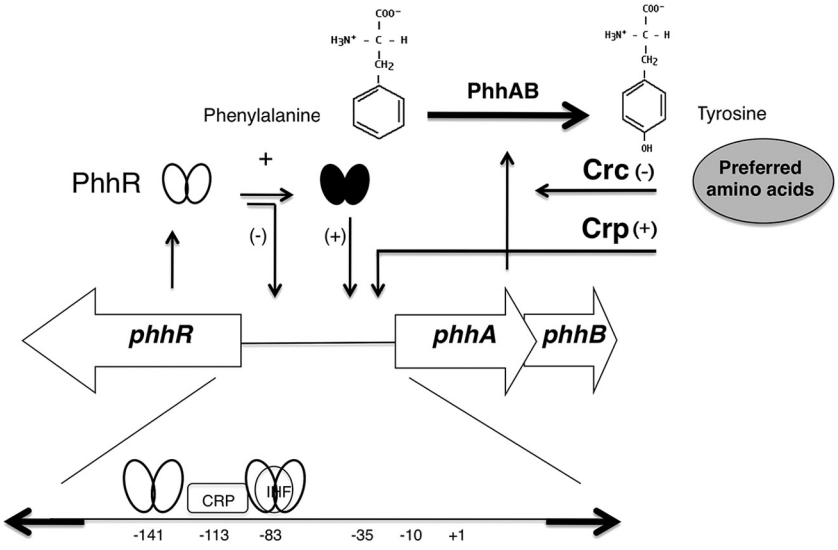

FIG 1 First step in the catabolic pathway of phenylalanine in *Pseudomonas putida* and organization of the genes encoding the phenylalanine hydroxylase. L-Phenylalanine is converted into L-tyrosine by the action of phenylalanine hydroxylase, made of the *phhA* and *phhB* gene products. The physical organization of the *phh* genes was established by Herrera and Ramos (19), and gene regulation by PhhR was as established by Herrera et al. (18). Expression of *phhR* is constitutive, and the ellipses represent PhhR without effector (open); once phenylalanine or tyrosine enters the cell, PhhR with the amino acid (black) is active. Crc and Crp are global regulators that influence negatively (−) or positively (+) the level of expression from the *phhAB* promoter. The bottom shows the physical sites to which PhhR, IHF, and Crp bind in the intergenic *phhA-phhR* region.

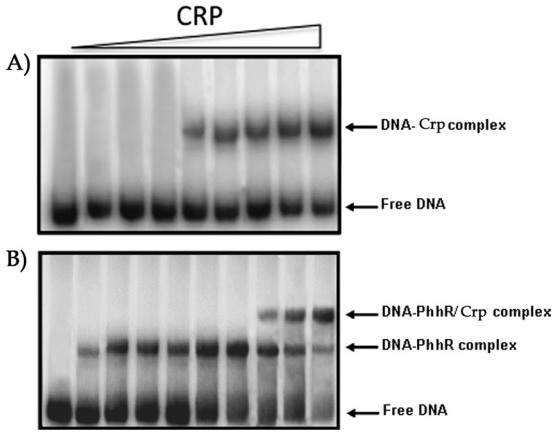

FIG 2 EMSA of Crp with the *phhA* operator intergenic region. The whole region was amplified by PCR and end labeled with ^{32}P and 2 nM the *phhAB* operator was incubated with 1 mM cAMP in the absence or in the presence of increasing concentrations of Crp (from left to right, 0, 2, 4, 6, 8, 10, 15, 20, and 25 μM) and electrophoresed as indicated in Materials and Methods (A). (B) DNA binding assays were carried out using 20 μM purified PhhR protein, 2 nM the *phhAB* operator region amplified by PCR as described above, and 1 mM cAMP. Then, increasing concentrations of Crp (from left to right starting with the third lane, 0, 2, 4, 6, 8, 10, 15, and 20 μM) were added. The first lane on the left is control DNA with no added protein, and the second lane is DNA with 20 μM Crp.

Overexpression and purification of His-tagged Crp. The *crp* gene was amplified by PCR to produce pCRP2 (Table 1), which was used to express Crp protein with a 6× histidine tag at its amino-terminal end as described previously (17–19). His_6-tagged Crp was purified by nickel affinity chromatography using a Ni^+-Sepharose matrix (Amersham-Biosciences) as described by the supplier, and the bound protein was eluted with an imidazole gradient (10 to 500 mM) in 50 mM phosphate buffer (pH 7.6) with 300 mM NaCl. Homogeneous peak fractions were pooled, dialyzed against 50 mM sodium phosphate buffer (pH 7.6), 300 mM NaCl, and stored at −80°C. Protein concentrations were determined spectrophotometrically using molecular extinction coefficients (12).

Electrophoresis mobility shift assays. A 374-bp DNA fragment containing the *phhAB* promoter region was amplified by PCR from the *P. putida* chromosome as described previously (18). A variant of the *phhA* promoter in which the Crp binding site (5′-CGAATTTTCCGACACT T-3) sequence was replaced by a random sequence (5′-AAGTCAGGTTT TTAATA-3′) was obtained by overlapping PCR mutagenesis as described previously (10). The amplified fragment was cloned in pGEMT, and a clone was used to confirm the sequence of the *phhA*-variant promoter. This *phhA* variant was also amplified by PCR for EMSA. To this end, amplified fragments were separated in agarose gels and subsequently extracted from this matrix. The fragments were end labeled with [γ-^{32}P]dATP using the T4 polynucleotide kinase, and about 1 nM (10^5 cpm) was incubated with increasing amounts of Crp-His_6 for 20 min at 25°C in 10 μl of binding buffer containing 20 μg of poly(dI-dC)/ml, 200 μg/ml of bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM dithiothreitol (DTT), 20 μM cAMP, and 1 mM EDTA (Fig. 2; see Fig. S1 in the supplemental material for the *phhA*-variant promoter). Reaction mixtures were then electrophoresed in a nondenaturing 4.5% (wt/vol) polyacrylamide gel in 50 mM Tris buffer (pH 7.5), 50 mM borate, and 20 μM cAMP. For the EMSA (Fig. 2), a 10-μl sample containing about 2 nM labeled DNA (1.5×10^4 cpm) was incubated with increasing concentrations of purified Crp and a constant level of PhhR protein for 1 h in 10 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1 mM DTT, and 20 mM cAMP) containing 20 μg/ml of poly(dI-dC) and 200 μg/ml of bovine serum albumin. The DNA-protein complexes were resolved by electrophoresis in 4% (wt/vol) nondenaturing polyacrylamide gels in TBE (Tris-borate-EDTA) buffer supplemented with cAMP. The results were analyzed with a model GS525 molecular imager (Bio-Rad).

DNase I footprinting. The *phhAB* promoter region generated by PCR (374-bp fragment) was used for footprinting analysis. To this end, we amplified DNA using primers 5′-TGAATTCACCAGCAGGTTGA-3′ (end labeled with [γ-^{32}P]ATP as described above) and 5′-ATCTGCAGA TAAAACCATGC-3′. About 5 nM concentrations (10^4 cpm) of each labeled probe were incubated in 10-μl reaction mixtures of 20 μM PhhR with increasing concentrations of His_6-tagged Crp protein (5, 15, and 20 μM). cAMP was added to reach a concentration of 1 mM. Reaction mixtures were incubated for 20 min at 25°C, before being treated with 1.4×10^{-4} U/μl of DNase I diluted in 10 mM Tris-HCl (pH 7) supplemented with 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, and 50 mM KCl. After 4 min at 30°C, reactions were stopped with 2 μl of 0.5 mM EDTA and the sample was extracted with phenol. The DNA was precipitated with 2 volumes of ethanol and resuspended in 5 μl of water and 2.5 μl of loading dye. Equal amounts of DNA (5,000 to 6,000 cpm) were heated at 90°C for 3 min and electrophoresed through a 6.5% (wt/vol) denaturing polyacrylamide gel. Sequencing ladders were generated in each case with the corresponding labeled primer, using a T7 DNA polymerase sequencing kit (USB-Amersham) and the pCRP3 plasmid (Table 1).

Plasmid construction and circular permutation gel retardation assays. A 257-bp DNA fragment containing the divergent *phhR* and *phhAB* promoters was amplified from the *P. putida* KT2440 chromosome by PCR using primers with XbaI and SalI restriction sites at their 5′ ends. The fragment was isolated and subcloned between the XbaI and SalI restriction sites of DNA bending vector pBend2 to yield pPhhRBend. Purified pPhhRBend plasmid was digested with BglII, NheI, ClaI, XhoI, DraI, SmaI, NcoI, and BamHI restriction enzymes to generate DNA fragments having a circularly permuted *phhA-phhR* intergenic region. The digested fragments were purified and end labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase. The labeled DNA fragments were mixed with saturated amounts of Crp-His_6 and/or PhhR-His_6 for 20 min at 25°C in 10 μl of binding buffer containing 20 μg of poly(dI-dC)/ml, 200 μg/ml of bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM DTT, 20 μM cAMP, and 1 mM EDTA. The reaction mixtures were electrophoresed using a nondenaturing 4.5% (wt/vol) polyacrylamide gel in 50 mM Tris buffer (pH 7.5), 50 mM borate, and 20 μM cAMP. The magnitude of the protein-induced bending was calculated using the empirical equation (47) $\mu_M/\mu_E = \cos(\alpha/2)$, where μ_E and μ_M are the mobility of the protein-DNA complex with the binding site at the ends and at their centers, respectively, and α is the bend angle.

Molecular representation of bending of *phhA* promoter. The generation of the three-dimensional (3D) structural bending model of the double-stranded DNA (*phhA* promoter) was generated from the 3D-Dart server (3DNA-Driven DNA Analysis and Rebuilding Tool) (48), introducing a 120° bending angle on the DNA, as calculated by circular permutation gel retardation assays. The Crp tridimensional structure shown in Fig. 5 corresponds to the crystal structure of DNA binding protein Vfr of *Pseudomonas aeruginosa* (Protein Data Bank [PDB] accession number 2OZ6), which presents a high identity to the *P. putida* Crp sequence, and was determined by the Geno3D program Automatic Modeling of Proteins Three-Dimensional Structure (http://geno3d-pbil.ibcp.fr). The representation of RNA polymerase subunits has been constructed by joining of the crystal domain structures of *E. coli* RNA polymerase (PDB accession number 3LU0, molecular model of the core; PDB accession number 1COO, COOH terminus of the alpha subunit) by using PyMOL software.

The prediction of PhhR tertiary structure was done by homology modeling using the EasyPred 3D server (automated homology modeling program using neural networks) and Swiss-Model server (fully automated protein structure homology-modeling server), accessible via the Expasy Proteomics server.

TABLE 2 Growth of KT2440 and its mutants with ammonium, L-phenylalanine, and L-tyrosine as the sole N source[a]

Genotype	Doubling time (h)		
	NH_4^+	L-Phenylalanine	L-Tyrosine
Wild type	2 ± 0.1	8 ± 0.5	1.8 ± 0.1
crp	2.1 ± 0.1	16.3 ± 1.5	6.5 ± 0.7
cya	2.2 ± 0.4	18 ± 3	7.3 ± 3
phhR	2.1 ± 0.1	26.1 ± 2.5	3.2 ± 0.2

[a] Bacterial cells were grown overnight on M9 minimal medium with glucose. On the following morning, cultures were diluted 100-fold in M8 minimal medium with glucose (0.5% [wt/vol]) and 5 mM the indicated N source. Doubling times were measured in cells growing exponentially. Data shown are the average of 3 independent assays carried out in duplicate.

TABLE 3 Expression of P_{phhA} in the parental and in *phhR*- and *cya*-deficient backgrounds[a]

Strain	P_{phhA}::'*lacZ* β-galactosidase activity (Miller units) with:		
	Medium alone	Phe	Tyr
Wild type	50 ± 3	2,780 ± 215	630 ± 20
crp strain	70 ± 2	832 ± 92	234 ± 38
cya strain	40 ± 3	1,109 ± 68	272 ± 32
phhR strain	50 ± 3	55 ± 3	70 ± 4

[a] *Pseudomonas putida* (wild type) and its isogenic strains (*crp* deficient, *cya* deficient, and *phhR* deficient) were transformed with P_{phhA}::'*lacZ* in the pMCA plasmid. Bacterial strains bearing pMCA were grown on M9 minimal medium with glucose in the absence (medium alone) or in the presence of 5 mM L-phenylalanine or 5 mM L-tyrosine. β-Galactosidase activity was determined when turbidity of cultures was about 0.8 at 600 nm. Values are the average of three independent experiments carried out in duplicate.

RESULTS

Maximal expression of the *phhAB* operon requires Crp and cAMP. To provide insight into the potential role of the *P. putida* KT2440 Crp protein in the metabolism of L-phenylalanine, we first determined the growth rate of KT2440 and its isogenic *crp* mutant in minimal medium with ammonium, L-phenylalanine, or L-tyrosine as the N source. Both the wild type and the mutants grew rapidly with ammonium, exhibiting a 2- to 2.2-h doubling time (Table 2). With phenylalanine the doubling time of the wild type was 8 h, while the *crp* mutant had a doubling time of over 16 h. We also found that the rate of growth of the *crp* mutant with L-tyrosine as the sole N source was much slower than that of the parental strain (Table 2). It is known that L-tyrosine (but not L-phenylalanine) is used by *P. putida* KT2440 as a sole carbon source (19); therefore, we tested the growth of the *crp* mutant on L-tyrosine as the sole carbon source and ammonium as an N source. We found that the mutant used L-tyrosine as a carbon source as efficiently as the parental strain (i.e., doubling times were in the range of 3.2 to 3.4 h). We therefore concluded that the *crp* gene product is involved in the utilization of these aromatic amino acids as nitrogen sources. These results are in agreement with those reported by Milanesio et al. (27), which showed that *crp* mutations do not affect the panel of C compounds that can be used by *P. putida* as growth substrates and that a *crp* mutant was impaired in the use of certain dipeptides as an N source.

The only source of cAMP in *P. putida* is the conversion of ATP into cAMP through the activity of the enzyme adenylate cyclase (Cya) (27). To verify that the Crp protein in *P. putida* requires cAMP for its regulatory function, we tested growth of a *cya*-deficient mutant with L-phenylalanine as an N source. We found that this mutant was also impaired in the use of L-phenylalanine and L-tyrosine as an N source (Table 2). Therefore, synthesis of cAMP is also required for optimal transcriptional regulation of the *phhAB* operon. It should be noted that we have previously shown that a *phhR* mutant did not grow on L-phenylalanine as an N source. These results suggest a positive effect of PhhR and Crp on the utilization of L-phenylalanine by *P. putida* KT2440.

To expand our understanding of the role of the Crp/cAMP in the transcriptional control of the *phhAB* operon, we measured β-galactosidase activity, in exponential phase, using a P_{phhAB}::'*lacZ* fusion in the wild-type and Crp and Cya mutant backgrounds. Experiments were performed in the presence and in the absence of L-phenylalanine or L-tyrosine in M9 minimal medium with glucose as a C source. We found that the expression levels in cells growing with L-phenylalanine or L-tyrosine were lower in both mutant backgrounds, being about one-third of those measured in the wild type (Table 3). In the PhhR mutant (Table 3) or a double PhhR/Crp mutant, expression from P_{phhA} was practically undetectable. The set of growth results and the results of transcriptional assays using '*lacZ* fusion support the idea that PhhR and Crp/cAMP have a positive effect on the activation of expression of the *phhAB* operon. Expression of *phhR* was constitutive, and its level was only slightly lower in the *crp* mutant background than in the parental strain (see Table S1 in the supplemental material).

Crp recognizes the target DNA sequence in the *phhAB* promoter in the presence of cAMP. Previous work has shown that in *E. coli* cAMP acts as an allosteric effector, which increases the affinity of the Crp protein for DNA (50). To test if the *P. putida* Crp protein binds to the *phhAB* promoter, we carried out electrophoretic mobility shift assays (EMSAs), using a 374-bp *phhAB* promoter region and purified *P. putida* Crp protein in the presence and in the absence of cAMP. In the absence of cAMP, no retardation was observed (data not shown), while EMSA revealed that the Crp protein bound to the *phhAB* promoter when cAMP was present (Fig. 2A). We also carried out EMSAs of the *phhAB* promoter region in the presence of PhhR at a fixed concentration with increasing levels of purified Crp in the presence of cAMP (Fig. 2B). The results showed that PhhR is able to bind to and retard the migration of the *phhA* operator region specifically in the absence of Crp (Fig. 2B); however, upon the addition of increasing concentrations of purified Crp in the presence of cAMP, the formation of a second complex, combined with the gradual disappearance of the PhhR-DNA complex, was observed. This suggests a potential cooperativity in binding of these two proteins to target DNA.

To confirm that cAMP/Crp plays a role in the regulation of the *phhAB* promoter, DNase I footprinting was carried out in the presence or absence of cAMP/Crp. There was no DNA protection by Crp (20 μM) alone. Addition of cAMP/Crp afforded some protection of a region from −103 to −119, which is located between both PhhR boxes (Fig. 3A). In this region, a potential inverted repeat (uppercase), 5′-AAtTtTCcGAcAcTT-3′, was found. Interestingly, the protected region within the *phhAB* promoter did not match well with the reported consensus for the *E. coli* cAMP/Crp binding sequence (5′-TGTGA-N_6-TCACA-3′). To test if this site is recognized by Crp of *P. putida*, we carried out EMSA with a 250-bp *phhA* variant in which the Crp binding site was replaced by

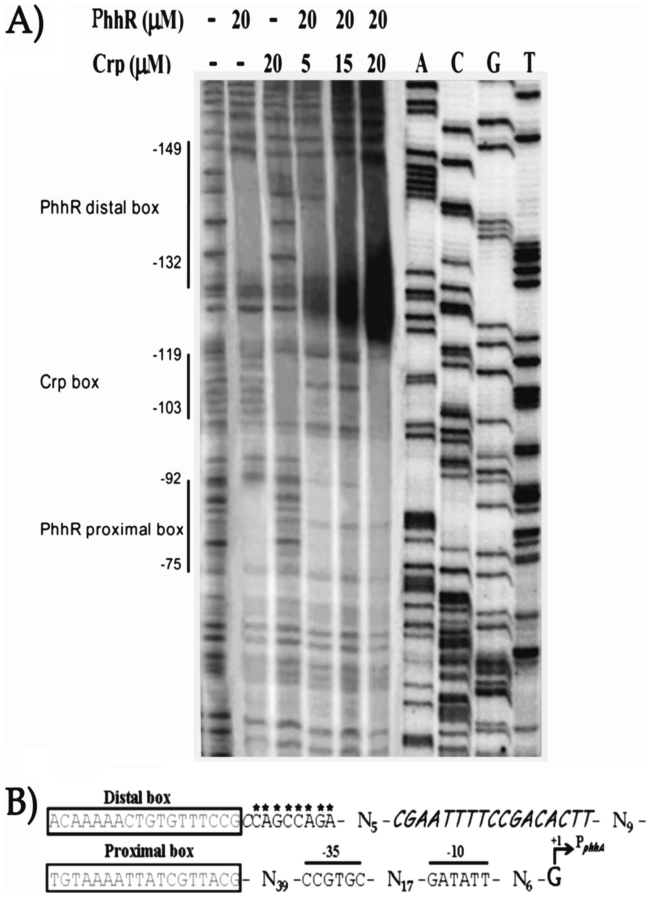

FIG 3 (A) DNase I footprint of PhhR and Crp in the *phhA* operator intergenic region. Assays were carried out as described in Materials and Methods. First lane on the left, control in the absence of PhhR and without Crp and 1 mM cAMP; second to sixth lanes, footprint in the presence of PhhR (20 μM); third lane, footprint in the presence of Crp; fourth lane, footprint in the presence of a fixed concentration of PhhR (20 μM), 1 mM cAMP, and increasing concentrations of Crp, as indicated (5, 15, and 20 μM). The regions protected by PhhR and Crp are indicated by vertical lines. (B) Relevant motifs in the intergenic *phhA-phhR* region. The distal and proximal PhhR boxes are indicated. The Crp binding site is marked in italics, and the bases that exhibit hypersensitivity to DNase I are indicated by asterisks.

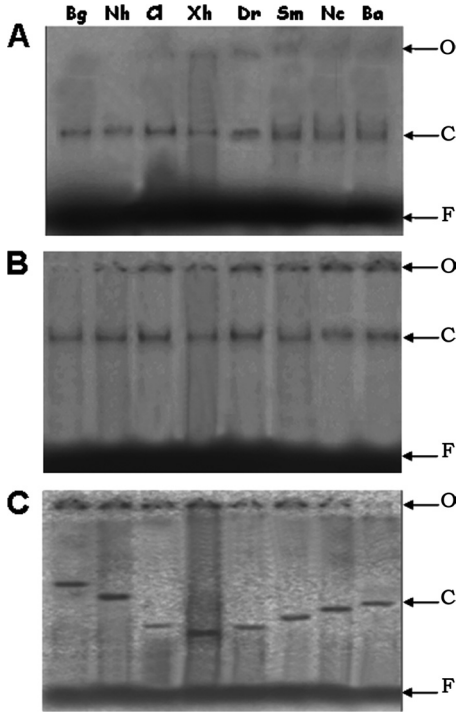

FIG 4 Electrophoresis mobility of circularly permuted DNA fragments complexed to the PhhR and Crp proteins. The fragments were generated from the digestion of the PhhRBend construct with restriction enzymes BglII (Bg), NheI (Nh), ClaI (Cl), XhoI (Xh), DraI (Dr), SmaI (Sm), NcoI (Nc), and BamHI (Ba). O, origin of the wells; F, unbound DNA; C, the PhhR-DNA complex (A), the cAMP/Crp-DNA complex (B), and the PhhR-cAMP/Crp-DNA complex (C). Bending angle (α) was calculated by the equation described by Thompson and Landy (47): $\mu_M/\mu_E = \cos(\alpha/2)$, where μ_E and μ_M are the mobility of the protein-DNA complex with the binding site at the ends and at their center, respectively.

a random sequence (see Materials and Methods), but the PhhR binding site was retained. For EMSA we used homogeneous Crp and PhhR protein preparations. We found that while the PhhR protein shifted the *phhA* variant, Crp was not able to retard the band in either the absence or presence of cAMP (see Fig. S1 in the supplemental material). This result suggests that the mutated site is a specific target of the *P. putida* Crp protein.

We also performed footprint assays with PhhR and Crp/cAMP simultaneously, leading to an expansion of the protected area, which covered both PhhR boxes and a more pronounced protection of the Crp site. We also observed, for Crp/cAMP and PhhR, that the DNase I hypersensitivity of the sequence between the Crp and the distal PhhR site increased with increasing concentration of Crp/cAMP (indicated by asterisks in Fig. 3B). This suggests that Crp/cAMP along with PhhR forms a nucleoprotein complex and that the bending induced by the combination of Crp and PhhR creates a distortion in the DNA helix that widens the DNA minor grove, making it more sensitive to DNase I.

To further elucidate the structural effect of PhhR/Crp-cAMP binding to the *phhAB* promoter, we used circular permutation analysis (35, 50) to measure the curvature of DNA when PhhR or Crp/cAMP, or both, was added to target DNA (Fig. 4). We found that neither PhhR nor Crp/cAMP alone provoked DNA curvature; however, the simultaneous presence of both regulators induced a marked curvature in the DNA with an angle of 120° (Fig. 5).

DISCUSSION

Transcriptional synergy involving multiple activators is a common occurrence in gene regulation in eukaryotes, and a number of examples of synergy have been reported in prokaryotic organisms, although much less frequently (6, 7, 16, 26, 38). The *phhAB* promoter of *P. putida* appears to be the target of at least two global regulators, IHF and Crp, and a specific regulator, PhhR. The positive action of PhhR occurs after binding to effector molecules so that the protein, like other positive transcriptional activators, acquires a modified confirmation and binds with higher affinity to its target sites (14, 16, 20). The global regulators IHF and Crp can enhance or inhibit transcription of a number of promoters (3, 5, 43). Our previous studies (19) and the present study suggest that at the *phhAB* promoter, IHF and Crp have opposite roles in the maximal PhhR-mediated activation of transcription. From previous studies it is known that PhhR binds to the *phhAB* promoter at

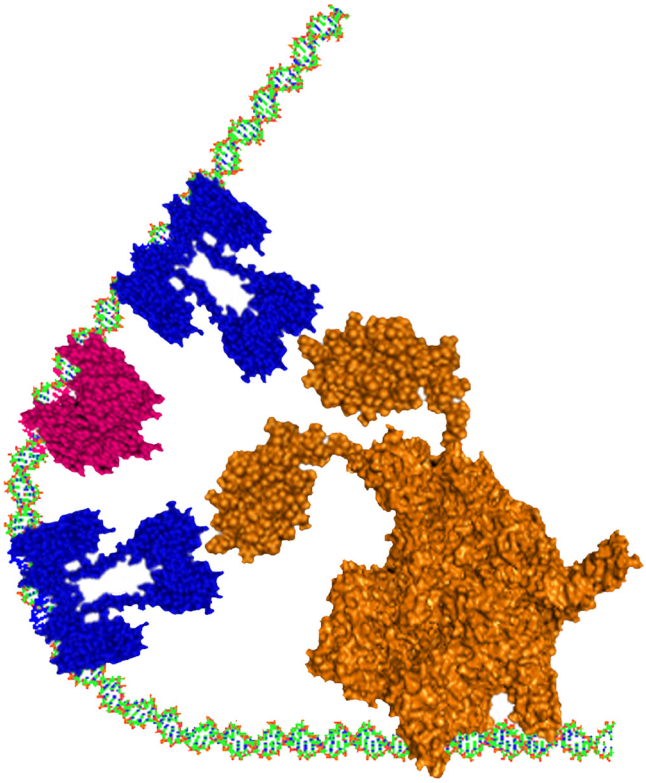

FIG 5 Schematic representation of the bending of *phhA* promoter induced by the specific binding of two PhhR dimers and Crp protein. The PhhR dimers are colored in blue and Vfr is in magenta; RNA polymerase is in orange. The PhhR proteins are bound to the distal and the proximal *phhR* binding boxes, and Crp is bound to its binding site, allowing the PhhR dimers to be in close proximity to RNA polymerase.

two similar, yet nonidentical distal and proximal enhancer sites that are centered at positions −83 and −141 upstream from the +1 position, respectively (17–19). The regulatory function of these boxes appears to be obligatory to the function of the *phhA* promoter, since elimination of either of them results in a silent promoter. Overlapping the proximal PhhR box is an IHF binding site, and in an IHF-deficient background, expression from the *phhA* promoter was maximal (18). This then suggests that IHF competes with PhhR and that IHF binding introduces a bend that prevents contact with RNA polymerase and limits local PhhR concentration. Here, we have shown that maximal expression from *phhA* requires Crp/cAMP. Synergistic coactivation by Crp and another regulator has been described with the LuxR homolog SmcR for activation of the *vvpE* promoter in *Vibrio* (21, 22, 24) and, in the case of MalT, in the activation transcription from *malEp* and *malKp* promoters in *E. coli* (39).

It is surprising that the cAMP-Crp complex is a transcriptional activator of the *phhAB* promoter for L-phenylalanine utilization as a nitrogen source, given the fact that Crp is a generally recognized global regulator of gene expression of carbon sources in *E. coli* and other microbes (4–6); however, our data clearly show that Crp/cAMP recognizes the *phhA* operator and that it recognizes a specific binding site in the *phhAB* promoter located between the proximal and distal PhhR boxes centered at position −109. From several studies, it has been firmly established that both Crp and IHF induce DNA bending near their respective target sites (36, 40, 44, 49, 50). Presumably, protein-induced bending allows protein-protein and/or protein-DNA interactions to occur from a distance, thereby optimizing the appropriate transcriptional response (37). Our results show that the simultaneous binding of Crp/cAMP and PhhR provokes a strong DNA distortion that results in the hypersensitivity of the sequence between the distal site and the *crp* site to DNase I. The induced curvature likely promotes expression by bringing the activators into close proximity with RNA polymerase.

Taken together, our data suggest a model in which the formation of a higher-order complex containing the two dimers of PhhR is the key feature in the transcriptional activation of *phhA*. We hypothesized that cAMP/Crp, together with PhhR, induces the bending of DNA to increase the local concentration of PhhR dimers within the vicinity of RNA polymerase. The possibility of direct interaction between cAMP/Crp and the PhhR has not yet been addressed. It is not clear whether PhhR and Crp physically interact to achieve optimal promoter expression, but our results show that the simultaneous occupancy of DNA binding sites is a critical feature. DNase I footprinting experiments in the presence of the two proteins PhhR and Crp indicate cooperative effects in DNA distortion. We have previously shown that the α-CTD (carboxy-terminal domain) arms of RNA polymerase were involved in interactions with PhhR, and in the model proposed in Fig. 5, we suggest that each of the α-CTD arms interacts with a PhhR dimer. The binding of the two regulators and RNA polymerase results in a scaffold that remains in a transcriptionally closed state until L-phenylalanine or L-tyrosine is recognized by the PhhR regulator.

The PhhR protein of *P. putida* is known to regulate the expression of a number of genes involved in the biosynthesis and transport of aromatic amino acids (17). In the *hmg* and PP2827 promoters, PhhR binds to a single binding sequence located at −9 and −66, respectively, and, in contrast with *phhA*, expression is maximal under an IHF-proficient background. Our inspection of the *hmg* and PP2827 promoters did not reveal the existence of any Crp sites, and, accordingly, we found that expression of *hmg* and PP2827 promoters was unchanged under a Δ*crp* background. Therefore, the mechanism of PhhR regulation seems to vary with specific promoters, and the degree of activation of the promoters is critically related to the location of the PhhR enhancer site(s) and the formation of specific DNA-protein interactions that lead to the creation of active transcriptional scaffolds.

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