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# Molecular determinants of the *hpa* regulatory system of *Escherichia coli*: the HpaR repressor

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## ABSTRACT

The HpaR-mediated regulation of the *hpa-meta* operon (*Pg* promoter) of the 4-hydroxyphenylacetic acid catabolic pathway of *Escherichia coli* has been studied. The HpaR regulator was purified to homogeneity showing that it is able to bind selectively to 4-hydroxyphenylacetic, 3-hydroxyphenylacetic and 3,4-dihydroxyphenylacetic acids, which act as inducers of the system. The role of HpaR as a repressor and the requirement for cAMP receptor protein for maximal activity have been confirmed by *in vitro* transcription analyses. Two DNA operators, OPR1 and OPR2, have been identified in the intergenic region located between the *hpa-meta* operon and the *hpaR* gene. The OPR1 operator contains a perfect palindromic sequence overlapping the transcriptional +1 start site of the *Pg* promoter. The OPR2 operator shows a similar but imperfect palindromic sequence and is located far downstream of the +1 start site of the *Pr* promoter. The binding of HpaR to OPR2 displays a clear cooperativity with OPR1 binding. Based on the above observations and the results of permanganate footprinting experiments, a repression mechanism for HpaR is postulated. A 3-dimensional model of HpaR, generated by comparison with the crystal structures of the homologous regulators, MarR and MexR, suggests that HpaR is a dimer that contains a typical winged-helix DNA binding motif in each subunit.

## INTRODUCTION

The *hpa* cluster of *Escherichia coli* W codes for a group of proteins involved in the catabolism of 4-hydroxyphenylacetic acid (4HPA) (1) (Fig. 1). The *hpa* catabolic genes are organized in two operons: the *upper* operon (*hpaBC*) encoding the two-component 4HPA monooxygenase, which transforms 4HPA to 3,4-dihydroxyphenylacetic acid (3,4HPA) (2,3), and the *meta* operon (*hpaGEDFHI*) encoding the enzymes that cleave the aromatic ring of 3,4HPA and allows its complete mineralization (1,4). The *hpa* pathway is regulated by two proteins named HpaA and HpaR (1). Although the role of the

HpaA activator of the *hpaBC* operon has been analyzed in some detail (5), very few data are available on the function of HpaR, the putative regulator of the *hpa-meta* operon (6). It has been suggested that transcription of the homologous *hpc-meta* operon of *E.coli* C is repressed by the product of *hpcR* (4) and we have postulated that the homologous *hpaR* gene of *E.coli* W might play a similar role (1,6). Recently, we have demonstrated that the transcription from the *Pg* promoter of the *hpa-meta* operon is strictly controlled by a global regulatory system, which allows the expression of the *hpa* catabolic genes only in the absence of a preferred carbon source (6). This unusually strong catabolite repression control of the *Pg* promoter of the *hpa-meta* operon is mediated by the cAMP receptor protein (CRP). When *E.coli* W cells are grown on glucose plus 4HPA, *Pg* is only active in stationary phase and the activation mechanism requires the global regulator integration host factor (IHF) (6) (Fig. 1).

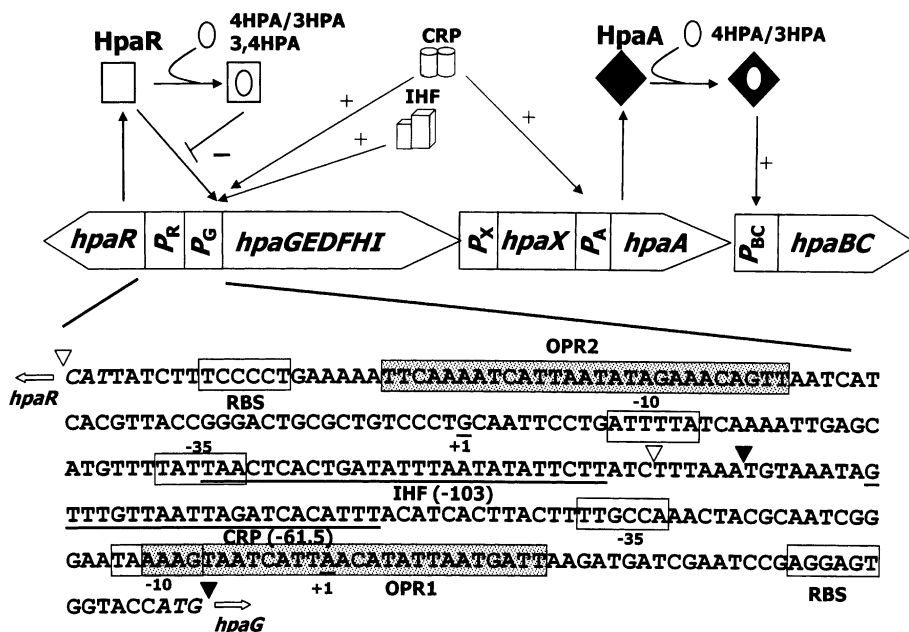
In this work we have used different genetic and biochemical approaches to characterize the interaction of HpaR with its target DNA and its cognate inducer molecules. We have also demonstrated that *hpaR* controls both the expression of the *hpa-meta* operon (*Pg* promoter) and its own expression (*Pr* promoter) through an unusual repression mechanism, suggesting that although HpaR is related in amino acid sequence and structure to the MarR family of regulators (7,8), it is not related in its mode of action.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and growth conditions

Bacteria were grown in LB medium (9) or M63 minimal medium (10) at 30°C supplemented with thiamine (1 µg/ml) and vitamin B<sub>12</sub> (1 µg/ml) and 20 mM glycerol as carbon source as described previously (6). When required, 1 mM appropriate aromatic compound was added to the medium. The aromatic compounds used as putative inducers were purchased from Sigma. The abbreviations used are: 4HPA, 4-hydroxyphenylacetic acid; 3,4HPA, 3,4-dihydroxyphenylacetic acid; 2HPA, 2-hydroxyphenylacetic acid; 3HPA, 3-hydroxyphenylacetic acid; PP, phenylpropionic acid; PA, phenylacetic acid; 2,5HPA, homogentisic acid. When needed, antibiotics were added at the following concentrations: ampicillin, 100 µg/ml; tetracycline, 10 µg/ml; kanamycin, 50 µg/ml; rifampicin, 50 µg/ml. The *E.coli* strains and

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**Figure 1.** Regulation of the *hpa* cluster and sequence of the *hpaR-hpaG* intergenic region. The organization of the catabolic (*hpaBCDEFGHI*) transport (*hpaX*) and the regulatory genes (*hpaA* and *hpaR*) of the *hpa* cluster, and their regulation by HpaA and HpaR and the global regulators CRP and IHF are represented. The open arrows indicate the directions of gene transcription.  $P_R$ ,  $P_G$ ,  $P_X$ ,  $P_A$  and  $P_{BC}$  are promoter regions. The white square indicates the active form of HpaR repressor; the black diamond indicates the inactive form of HpaA activator; the white circle represents the inducer. The lines ending in arrows or bars indicate positive or negative effects, respectively. The complete nucleotide sequence of the *hpaR-hpaG* intergenic region (PR-PG probe) is indicated. DNA regions located between triangles correspond to PG probe from -80 to +46 (filled triangles) and PR probe from -216 to -87 (empty triangles) numbered relative to the  $P_G$  transcription start, taken as +1. The -35 and -10 boxes of the  $P_G$  and  $P_R$  promoters, the ribosome binding sites (RBS), the transcription start site of  $P_G$  and  $P_R$  (+1) and the CRP and IHF sites are indicated. ATG start codons of *hpaR* and *hpaG* are shown in italic. - and + indicate transcriptional repression and activation, respectively. The OPR1 and OPR2 operators are indicated as dotted boxes.

plasmids used in this work are listed in Table 1. The pUTminiTn5 derivatives are mini-Tn5 delivery plasmids used for the insertion of genes into the chromosome of different *E. coli* strains by the filter-mating technique using *E. coli* S17- $\lambda$ pir as donor according to the method described previously (15).

### DNA manipulations and sequencing

Isolation of plasmid DNA, digestion with restriction enzymes, ligation with T4 DNA ligase and transformation were carried out as described elsewhere (9). DNA fragments were purified using the GeneClean Turbo Kit (BIO101 Inc.). Oligonucleotides were synthesized in an Oligo-1000M nucleotide synthesizer (Beckman Instruments). Nucleotide sequences were determined directly from plasmids by using the dideoxy chain termination method (9). The manufacturer's standard protocols for *Taq* DNA polymerase-initiated cycle sequencing reactions with fluorescently labeled dideoxynucleotide terminators (Applied Biosystems Inc.) were used.

### Construction of strains harboring a translational *Pr::lacZ* fusion in the chromosome

To construct a translational fusion of the  $P_R$  promoter region of *hpaR* and the *lacZ* reporter gene, a 314 bp DNA fragment covering this promoter region was amplified by PCR using 10 ng of plasmid pAJ40 (Table 1) as template and the primers PAR3' (5'-GCACGGATCCGTAATAGAAAAGGGGAC-3'; an engineered BamHI site is underlined) and PAR5' (5'-CCGAATTCCTTTCATGGTACCACTCC-3'; the start codon is indicated in bold and an engineered EcoRI site is

underlined). To create plasmid pBM2 (Table 1), the PCR amplified fragment was cut with EcoRI and BamHI endonucleases and ligated to the EcoRI and BamHI double-digested promoterless *lacZ* vector pUJ9 (Table 1). The correct fusion was verified by sequence analysis. Plasmids pPR13 and pPR14 were constructed by subcloning the NotI cassette of pBM2 into the mini-Tn5 delivery plasmids pUTminiTn5-Km and pUTminiTn5-Tc, respectively (Table 1), and they were used for insertion of the  $P_R::lacZ$  fusion into the chromosome of *E. coli* AF15 and AFMC (Table 1), respectively, by the filter-mating technique (15). The generated exconjugants containing the *lacZ* translational fusions inserted into their chromosomes were selected for the transposon marker, kanamycin, on rifampicin-containing LB medium to give the strain WPR13 and selected on tetracycline-containing LB medium for MCR14. In each case the final strain was selected from among three different exconjugants with similar expression levels and expression profiles of the reporter gene. The relevant genotypes of the resulting strains are indicated in Table 1.

### Overexpression and purification of HpaR

The *hpaR* coding sequence was amplified by PCR using oligonucleotides HpaR5' (5'-GGGAATTCCTAAATGAAGG-AGAAAGATAATGCACGACTC-3'; the start codon is indicated in bold and an engineered EcoRI site is underlined) and HpaR3' (5'-GGGTACCAAGCTTAGATACTAAAAAGTT-ATTC-3'; an engineered KpnI site is underlined) and plasmid pAJ40 (Table 1) as DNA template. The amplified DNA fragment was digested with EcoRI and KpnI and then inserted

**Table 1.** Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference
<i>Escherichia coli</i> K-12		
S17-1 $\lambda$ pir	Host for pUT-derived plasmids	(11)
MC4100	F <sup>-</sup> <i>araD319</i> $\Delta$ ( <i>argF-lac</i> ) <i>U169 relA1fbbB5301 deoC1 ptsF25 rbsR</i>	(5)
AFMC	MC4100 Rif <sup>r</sup>	(12)
MCR14	AFMC derivative, <i>Pr::lacZ</i> , Tc <sup>r</sup>	This work
MCR141	MCR14 derivative, <i>Pr::lacZ</i> , <i>hpa</i> complete pathway, Km <sup>r</sup> , Tc <sup>r</sup>	This work
MCR142	MCR14 derivative, <i>Pr::lacZ</i> , <i>Pr::hpaR</i> , Km <sup>r</sup> , Tc <sup>r</sup>	This work
<i>Escherichia coli</i> W		
W14	W derivative ( $\Delta$ <i>paa</i> )	(13)
AF15	W14 derivative ( $\Delta$ <i>lacZ</i> ), Rif <sup>r</sup>	(12)
WPG11	W14 derivative ( $\Delta$ <i>lacZ</i> , <i>Pg::lacZ</i> ), Km <sup>r</sup>	(6)
WPR13	W14 derivative ( $\Delta$ <i>lacZ</i> , <i>Pr::lacZ</i> ), Km <sup>r</sup>	This work
Plasmids		
pUJ9	Promoterless <i>lacZ</i> vector, Ap <sup>r</sup>	(14)
pAJ40	pUC18 derivative containing <i>hpa</i> pathway	(1)
pHCR2	pACYC184 derivative with an EcoRI DNA fragment containing <i>hpaR</i>	(1)
pUTminiTn5-Km	MiniTn5 delivery plasmid, Km <sup>r</sup> , Ap <sup>r</sup>	(14)
pUTminiTn5-Tc	MiniTn5 delivery plasmid, Tc <sup>r</sup> , Ap <sup>r</sup>	(14)
pUC18Not	Identical to pUC18 but with NotI sites flanking MCS	(15)
pUCR1	pUC18Not derivative overexpressing the <i>hpaR</i> gene under <i>Plac</i>	This work
pJCD01	pUC19 derivative, vector for <i>in vitro</i> transcription	(16)
pBF1	pJCD01 derivative containing <i>Pr-Pg</i> promoters	This work
pBM2	pUJ9 derivative, <i>Pr::lacZ</i> , Ap <sup>r</sup>	This work
pPR13	pUTminiTn5-Km derivative, <i>Pr::lacZ</i> , Km <sup>r</sup> , Ap <sup>r</sup>	This work
pPR14	pUTminiTn5-Tc derivative, <i>Pr::lacZ</i> , Tc <sup>r</sup> , Ap <sup>r</sup>	This work
pAJ402	pUTminiTn5-Km derivative containing <i>hpa</i> pathway	(1)
pBA1	pUCNot derivative, <i>Pr::hpaR</i> , Km <sup>r</sup> , Ap <sup>r</sup>	This work
pBA2	pUTminiTn5-Km derivative, <i>Pr::hpaR</i> , Km <sup>r</sup> , Ap <sup>r</sup>	This work

into vector pUC18Not (Table 1) under control of the *Plac* promoter. The resulting recombinant plasmid pUCR1 was transformed into *E.coli* MC4100 (Table 1). *Escherichia coli* MC4100 (pUCR1) cells overproduced a 17 kDa protein (17% of total protein) that corresponded to the expected size of HpaR (Fig. 2).

To purify HpaR, *E.coli* MC4100 (pUCR1) cells cultured overnight at 37°C in 500 ml of ampicillin-containing LB medium were harvested by centrifugation, washed with saline solution (0.9% NaCl) and, finally, resuspended in 25 ml of HpaR buffer (20 mM Tris-HCl pH 7.5, containing 10% glycerol, 2 mM  $\beta$ -mercaptoethanol, 1 mM EDTA and 100 mM KCl). All the steps were performed at 4°C. Cells were lysed by passage through a French press (Aminco Corp.) operated at a pressure of 20 000 p.s.i. The crude extract was spun in an ultracentrifuge at 150 000 *g* for 45 min and the supernatant, which contains the soluble HpaR, was collected. Nucleic acids present in the supernatant were removed by precipitation with 0.5% polyethyleneimine at pH 8.0. The solution was stirred gently over 15 min followed by centrifugation at 18 000 *g* for 15 min. Then, the DNA free supernatant was dialyzed for several hours against several changes of HpaR buffer (total volume 5 l). After dialysis the protein solution becomes cloudy due to the precipitation of a complex between HpaR and traces of remaining polyethyleneimine. The precipitate was recovered by centrifugation (18 000 *g* for 15 min) and solubilized in 30 ml of HpaR buffer supplemented with KCl to a concentration of 1 M. To eliminate the traces of polyethyleneimine, the solubilized material was loaded onto a phosphocellulose P11 (Whatman) column (10 ml), equilibrated with the same buffer. In these conditions

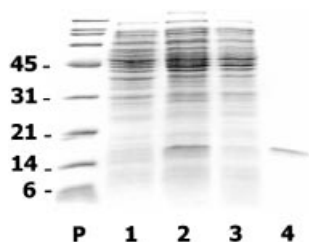
polyethyleneimine binds to the column while HpaR does not. Thus, the eluted unbound fraction, containing a polyethyleneimine-free protein of 17 kDa that corresponded to HpaR, was dialyzed against HpaR buffer. The purity of HpaR was estimated by SDS-PAGE and Coomassie staining (17). Using this procedure we obtained 2 mg of purified HpaR at a concentration of 0.2 mg/ml (Fig. 2). Further attempts to concentrate HpaR were unfruitful due to its low solubility.

### Phenyl-Sepharose chromatography

The DNA-free solution containing the HpaR protein was added to 8 ml of phenyl-Sepharose CL-4B (Amersham-Pharmacia Biotech AB) previously equilibrated with HpaR buffer plus 1 M KCl. Then, the matrix was washed with 24 ml of the same buffer without KCl to elute most of the retained proteins. After this washing step, HpaR remained adsorbed to the matrix by a specific interaction with the phenyl group. To determine the compounds that could compete with the phenyl group of the matrix releasing the HpaR protein, the washed matrix containing the HpaR protein was eluted with different solutions of 100 mM Tris-HCl pH 7.5 buffer containing one of the putative competing compounds (4HPA, 2HPA, 3HPA, 3,4HPA, PP, PA, 3-hydroxycinnamic acid or 2,5HPA) at a final concentration of 0.2 M. The corresponding eluted fractions were analyzed by SDS-PAGE and Coomassie staining.

### Gel retardation assays (EMSA)

The DNA fragments PR-PG, PR and PG of 314, 179 and 147 bp, respectively, used as probes were amplified by PCR using 10 ng of plasmid pAJ40 (Table 1) as template and the



**Figure 2.** Overexpression and purification of HpaR protein. SDS-PAGE analysis of HpaR purification from *E. coli* MC4100 (pUCR1) cells. Lane P, the molecular mass markers in kDa; lane 1, soluble control extract from *E. coli* MC4100 (pUC18Not); lane 2, soluble crude extract from *E. coli* MC4100 (pUCR1); lane 3, supernatant after polyethylimine precipitation and dialysis of crude extract from *E. coli* MC4100 (pUCR1); lane 4, purified HpaR protein by the method based on precipitation of HpaR under low ionic strength conditions followed by phosphocellulose chromatography.

following primers: PG3' (5'-GATAGTGGGATCCATGGTACCCTCCTCGGATTCGATC-3') and PGDE (5'-CCGG-AATTCTGTAAATAGTTTGTAAATTAG-3') for the PG fragment; PG5' (5'-AACGCAAGAATTCGTGAGTCGTGC-ATTATCTTTCCCC-3') and PRDE (5'-CCGGAATTC-GATAAGAATATATTAATATC-3') for the PR fragment. The DNA fragments were labeled at the 5'-end with phage T<sub>4</sub> DNA polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) (Amersham Pharmacia Biotech). These fragments were purified on a glassfiber column (High Pure PCR purification kit; Roche). Complexes with the labeled promoter region were formed for 20 min at room temperature in 10  $\mu$ l of buffer A (40 mM HEPES pH 8.0, 10 mM magnesium chloride, 100 mM potassium glutamate and 500  $\mu$ g/ml bovine serum albumin) using purified HpaR protein. The mixture was loaded into a 7.5% native polyacrylamide gel. The gel was fixed and dried before being quantified using a phosphorimager (Molecular Dynamics).

### KMnO<sub>4</sub> and DNase I footprinting

Activators (100 nM CRP or IHF), prepared as described (6), repressor HpaR (100 nM) and RNA polymerase (RNAP) (100 nM), prepared as described (16), were allowed to form complexes with the radioactively labeled PR-PG fragment for 20 min at 37°C in 15  $\mu$ l of buffer A. In one set of experiments 2.5  $\mu$ l of DNase I solution (1  $\mu$ g/ml in 10 mM Tris-HCl, 10 mM magnesium chloride, 10 mM calcium chloride, 125 mM potassium chloride) were added and incubated at 37°C for 20 s, or for 30 s when RNAP was present in the mixture. The reaction was stopped by addition of 200  $\mu$ l of a solution containing 0.4 M sodium acetate, 2.5 mM EDTA and 50  $\mu$ g/ml calf thymus DNA, and put on ice. In the other set, 2.5  $\mu$ l of KMnO<sub>4</sub> solution (40 mM) was added to the complexes for 30 s at 37°C. The reaction was stopped by adding 2.5 mM 2-mercaptoethanol (2 M). Then all the samples were phenol extracted and precipitated with ethanol. With the KMnO<sub>4</sub> samples, the ethanol precipitates were resuspended in 100  $\mu$ l of piperidine (1 M), heated at 90°C for 30 min and evaporated until dryness. Then 20  $\mu$ l of water was added and evaporated (twice). KMnO<sub>4</sub> and DNase I samples were resuspended in 5  $\mu$ l of loading buffer (20 mM EDTA in 80% v/v formamide containing xylene cyanol blue and

bromophenol blue) and loaded on a 7% denaturing polyacrylamide gel.

### Run-off transcription assays

Single round transcription by *E. coli* RNAP was carried out under standard conditions (16), using buffer B (40 mM Tris-HCl pH 8.0, 10 mM MgCl<sub>2</sub>, 100 mM KCl, 200  $\mu$ M cAMP and 500  $\mu$ g/ml acetylated bovine serum albumin) and supercoiled DNA plasmid pBF1 (Table 1). To construct pBF1, the oligonucleotides PG3' and PG5' (see above) were used for PCR amplification of the *hpaR-hpaG* intergenic region using plasmid pAJ40 as DNA template. The final volume of the run-off reaction mixture was 9  $\mu$ l, containing the plasmid DNA (5 nM) with CRP (100 nM) and HpaR (100 nM) or buffer. This mixture was incubated at room temperature for 20 min. Then, 3  $\mu$ l of RNAP at 375 nM in buffer B was added and the mixture was incubated at 37°C for 5 min in a final volume of 12  $\mu$ l. Elongation was started by the addition of 3  $\mu$ l of a prewarmed mixture containing 1 mM ATP, 1 mM GTP, 1 mM CTP, 50  $\mu$ M UTP, 1  $\mu$ Ci [ $\alpha$ -<sup>32</sup>]UTP and 500  $\mu$ g/ml heparin in buffer B to the template-polymerase mix and allowed to proceed for 5 min at 37°C. Reactions were stopped by the addition of 12  $\mu$ l of loading buffer (see above) containing 1% SDS. After heating to 70°C, samples were subjected to electrophoresis on 7% sequencing gels. Run-off products were quantified using a phosphorimager (Molecular Dynamics).

### Assay for $\beta$ -galactosidase activity

Activities of the *Pg* and *Pr* promoters were monitored by assaying  $\beta$ -galactosidase accumulation in cells harboring either *Pg::lacZ* or *Pr::lacZ* fusions. Cells were grown in M63 minimal medium and, when indicated, *Pg* and *Pr* expression was induced with different aromatic compounds.  $\beta$ -Galactosidase activity was measured as described by Miller (10) and expressed in Miller units.

### Primer extension

Primer extension analysis was used to determine the start site of the transcription of *hpaR*. The reverse transcriptase AMV primer extension system was used according to the protocol supplied by the manufacturer (Promega). The primer used to map the *hpaR* start site was R<sub>2</sub>+1 (5'-GCAACGCAATGGTTAGTGAG-3'). Primer extension products were analyzed on a 6% polyacrylamide gel next to a sequence ladder generated with the same primer. Sequencing reactions were performed with Deaza G/A T7 Sequencing Mixes (Pharmacia). RNA was prepared with a RNeasy Mini Kit from *E. coli* MC4100 (pBM2) according to the protocol supplied by the manufacturer (Qiagen).

### Homology modeling of HpaR

The 3-dimensional structure of HpaR was modeled using Swiss PDB Viewer 3.7 (18). The template used was MarR (8) as a ligand-bound model. Raw structures obtained from fitting were subjected to steepest descent energy minimization. The salicylate molecules were removed from the MarR-based structure and substituted by 4HPA molecules by manual docking using RASTOP 2.0.2. (<http://www.geneinfinity.org/rastop/>).



## RESULTS

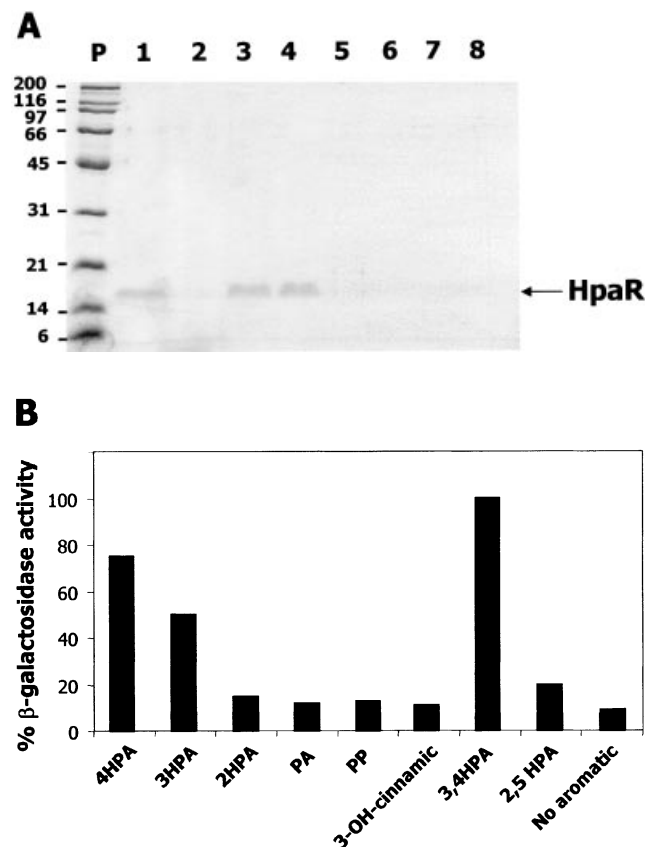
### *In vitro* and *in vivo* analysis of the HpaR ligands

At the time we were testing different chromatographic supports to purify HpaR, we discovered that this protein could be strongly and specifically retained on phenyl-Sepharose due to its ability to interact with aromatic compounds. This property allowed us to determine *in vitro* the aromatic compounds that might act as ligands of HpaR. Figure 3 shows that HpaR can be eluted from phenyl-Sepharose by 4HPA, 3HPA and 3,4HPA. However, other structurally related aromatic compounds, such as PP, PA, 2HPA, 2,5HPA and 3-hydroxycinnamic acid, were unable to release HpaR from the phenyl-Sepharose support. These results suggested that the interaction of HpaR with the phenyl group of the matrix most likely takes place through the effector recognition site of the protein, rather than by a non-specific hydrophobic interaction. Moreover, they are in perfect agreement with the observation that repression of the *hpc-meta* operon of *E.coli* C caused by the homologous HpcR regulator can be released *in vivo* by 4HPA, 3HPA or 3,4HPA (4).

To determine *in vivo* the range of HpaR inducers on the *Pg* promoter of *E.coli* W, we used the reporter strain WPG11 (*Pg::lacZ*, *hpa*<sup>+</sup>) (Table 1). WPG11 was cultured for 2 h in M63 minimal medium containing 20 mM glycerol and different aromatic acids (1 mM) as inducers. Figure 3B shows that the  $\beta$ -galactosidase activity was only increased in the presence of 4HPA, 3HPA and 3,4HPA, corroborating the hypothesis that these compounds are the prevalent and primary inducers of the HpaR repressor. It is worth noting that since WPG11 is a *hpa*<sup>+</sup> strain, we could not at this stage rule out the possibility that the effect of 3HPA and 4HPA might be due to their transformation into 3,4HPA or into other metabolites of the pathway. However, the observation that these compounds specifically interact with HpaR *in vitro* as well as the *in vivo* experiments performed on the homologous HpcR regulator (4) support the idea that these compounds could be true HpaR effectors (see also *in vitro* transcription experiments below).

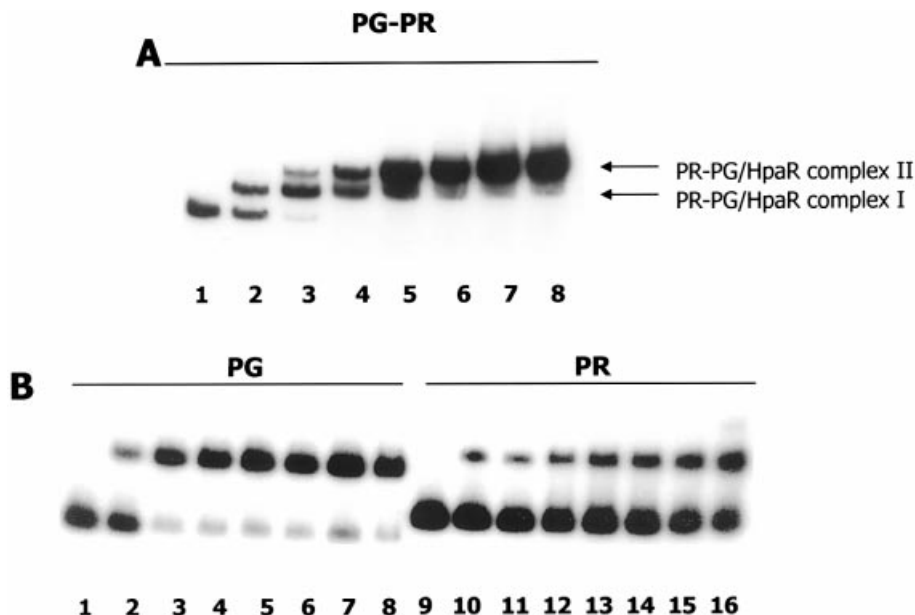
### *In vitro* HpaR binding to the *hpaG-hpaR* intergenic region

The ability of HpaR to bind to the *hpaR-hpaG* intergenic region containing the *Pg* and *Pr* promoters (Fig. 1) was tested *in vitro* by gel retardation assays (EMSA) (Fig. 4) using three different DNA fragments as probes. While the PR-PG (*Pg* and *Pr* promoters) fragment covers the entire DNA region located between the *hpaR* genes and the *hpa-meta* operon, the PR (*Pr* promoter) and PG (*Pg* promoter) fragments contain the 179 bp region upstream of *hpaR* and the 147 bp region upstream of the *hpa-meta* operon, respectively (Fig. 1). Remarkably, whereas two HpaR–DNA complexes were detected with the PR-PG probe (complexes I and II), only one was visible with each of the PG and PR probes (Fig. 4). To localize the HpaR binding sites more precisely in the *hpaR-hpaG* intergenic region, DNase I footprinting experiments were performed using the PR-PG fragment as probe. These experiments revealed that HpaR protects two operators, named OPR1 (centered at position +2) and OPR2 (centered



**Figure 3.** Inducers of HpaR protein. (A) SDS-PAGE analysis of the phenyl-Sepharose chromatography of HpaR. Lane P, molecular mass markers shown in kDa; lanes 1–8, fractions eluted with 4HPA (lane 1), 2HPA (lane 2), 3HPA (lane 3), 3,4HPA (lane 4), PP (lane 5), PA (lane 6), 3-hydroxycinnamic acid (lane 7) and 2,5HPA (lane 8). (B) Effect of several aromatic compounds on the expression of *Pg* promoter. Cells of *E.coli* WPG11 (*Pg::lacZ*) were grown in glycerol-containing minimal medium in the absence (no aromatic) or in the presence of 1 mM different aromatics (4HPA, PA, 2HPA, 3HPA, PP, 2,5HPA, 3,4HPA and 3-hydroxycinnamic acid) until the cultures reached an OD<sub>600</sub> of 0.8.  $\beta$ -Galactosidase activities were measured with permeabilized cells as described in Materials and Methods.

at position –199, relative to the transcription start site +1 of the *Pg* promoter) (Figs 1 and 5). OPR1 and OPR2 both consist of a 27 bp region containing two inverted half-sites of 9 bp separated by 4 bp (Fig. 5). These results are in agreement with those presented above (Fig. 4) showing that one HpaR binding site was located in the PG fragment (corresponding to OPR1) and the other within the PR fragment (corresponding to OPR2). In addition, EMSA experiments indicated that the OPR1 site had an affinity about 10-fold higher than the OPR2 site on the PR-PG fragment (Fig. 4A). Interestingly, when the two operator sites are located on separate fragments, the difference in their affinities appeared much greater, reaching a factor of 1000-fold, indicating that binding of HpaR to OPR2 on the PR-PG fragment is clearly cooperative with the binding to OPR1 (Fig 4). Other experiments performed in the presence of 4HPA on the PR-PG fragment revealed that the affinity of the *Pg* operator was only slightly decreased by the presence of this compound (less than 2-fold) (data not shown).



**Figure 4.** HpaR binding to the *hpaR-hpaG* intergenic region. EMSAs were performed as indicated in Materials and Methods. The DNA probes used were (A) PR-PG and (B) PG and PR. Increasing concentrations of purified HpaR were used. (A) Lane 1, 0 nM; lane 2, 3 nM; lane 3, 10 nM; lane 4, 30 nM; lane 5, 50 nM; lane 6, 75 nM; lane 7, 150 nM; lane 8, 300 nM. (B) Lanes 1 and 9, 0 nM; lane 2, 3 nM; lane 3, 10 nM; lane 4, 30 nM; lane 5, 50 nM; lane 6, 75 nM; lane 7, 100 nM; lane 8, 200 nM; lane 10, 10 nM, lane 11, 30 nM; lane 12, 50 nM; lane 13, 75 nM; lane 14, 100 nM; lane 15, 200 nM; lane 16, 1000 nM.

### *In vitro* transcription from the *Pg* promoter

Although we have demonstrated that 4HPA interacts with HpaR *in vitro* and induces the *hpa-meta* operon *in vivo*, it was only able to very slightly reduce the HpaR–DNA binding affinity *in vitro* according to the footprinting analysis (see above). Thus, to ascertain the role of 4HPA in the induction of HpaR-repressed *Pg* promoter, we developed an *in vitro* transcription assay using plasmid pBF1 as DNA template. Plasmid pBF1 contains the *hpaR-hpaG* intergenic region flanked by transcriptional terminators (Table 1). Since, as we showed previously, the *Pg* promoter is CRP dependent (6), it was not active when RNAP was the unique protein added to the reaction mixture and, thus, only the RNA1 control transcript is detected in the assay (Fig. 6). However, when CRP was supplied to the previous reaction mixture, the *Pg* activity was stimulated and a 139 nt transcript generated from *Pg* was detected (Fig. 6). As expected, the addition of HpaR clearly decreased the *Pg* transcription rate, demonstrating that this protein was able to work *in vitro* as a repressor. Moreover, the addition of 4HPA (Fig. 6), but not that of PA or 2HPA (data not shown), restored the transcription activity of the promoter. These results demonstrate, for the first time, repression by HpaR *in vitro*. But more importantly, it should be noted that in these *in vitro* experiments 4HPA cannot be transformed into other intermediates of the *hpa* catabolic pathway, which could occur in the *in vivo* experiments shown above and, therefore, these results confirm unequivocally that 4HPA is a true inducer of the *hpa-meta* operon.

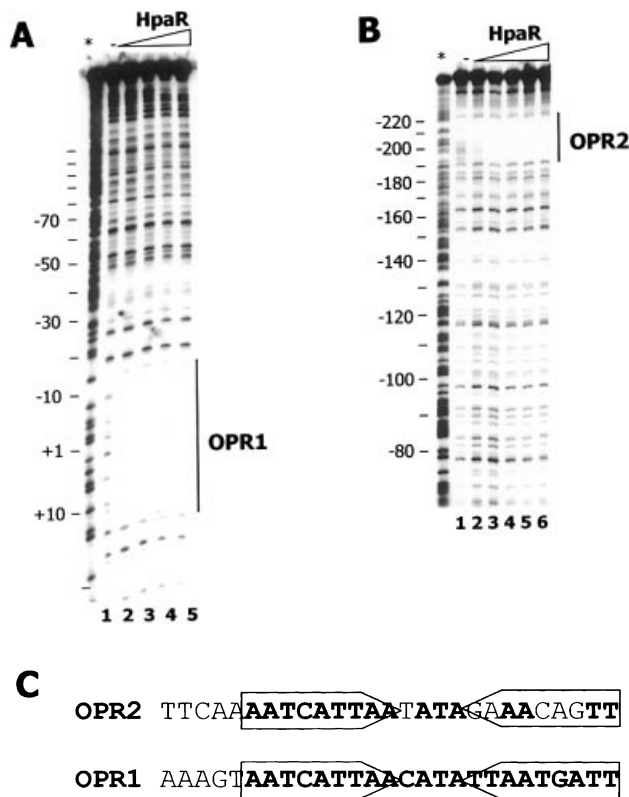
### Influence of HpaR on open complex formation

To ascertain whether the repression mechanism mediated by HpaR involves inhibition of the formation of the open complex required for transcription initiation, we performed a

potassium permanganate footprinting assay using the PR-PG fragment labeled at its *Pg* 5'-end. Figure 7 shows that RNAP alone induced only a weak reactivity of the thymine residues from +2 to –13, confirming the fact that the *Pg* promoter is inefficient in the absence of its cognate activators, CRP and IHF (6). Indeed, addition of CRP alone or a combination of CRP and IHF (data not shown) significantly increased open complex formation at the *Pg* promoter. The presence of HpaR abolished the permanganate reactivity of the thymine residues characteristic of the *Pg* promoter, in agreement with its inhibitory effect on *Pg* transcription. Although HpaR alone did not induce any pattern of reactivity to permanganate or open complex formation (Fig. 7), a new pattern of reactivity to permanganate was detected from position –22 to –32, between the –10 and –35 promoter boxes of the *Pg* promoter, just upstream of the HpaR binding site, when HpaR, CRP and RNAP were present in the same reaction mixture. This suggests that HpaR does not prevent RNAP from binding to the *Pg* promoter region but effectively blocks promoter escape by displacing RNAP from its usual location (Fig. 7).

### Regulation of the *Pr* promoter

To check whether expression of the divergently transcribed *Pr* promoter, which drives expression of the *hpaR* gene (Fig. 1), was also regulated by HpaR, we have engineered a reporter *Pr::lacZ* translational fusion within plasmid pBM2 (Table 1). Firstly, the transcriptional start site of *hpaR* was determined in plasmid pBM2 by primer extension (Fig. 8). This experiment revealed that the transcription of *hpaR* starts at a G residue located at position –153 relative to the transcriptional start site of the *Pg* promoter (Figs 1 and 8). A putative –10 box (TAAAAT) located 9 bp upstream of the *Pr* +1 site and a putative –35 box (TTAATA) with a spacer of

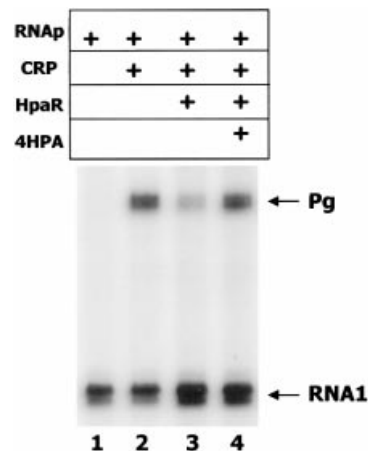


**Figure 5.** DNase I footprinting analysis of the interaction of HpaR with the *Pr-Pg* promoter region. The reaction mixture was treated as described in Materials and Methods using as probe the 5'-end-labeled non-coding strand of the *Pr-Pg* region of the *hpaGEDFHI* operon showing (A) the OPR1 and (B) the OPR2 protected regions. HpaR concentrations were as follows. (A) Lane 1, 0 nM; lane 2, 10 nM; lane 3, 30 nM; lane 4, 100 nM; lane 5, 200 nM. (B) Lane 1, 0 nM; lane 2, 30 nM; lane 3, 100 nM; lane 4, 200 nM; lane 5, 400 nM; lane 6, 1000 nM. An A+G sequencing ladder is indicated with an asterisk. (C) OPR1 and OPR2 operators. Operators are indicated and conserved nucleotides are shown in bold. Arrows represent the inverted repeat sequences.

18 bp were identified. As expected, these *Pr* promoter boxes are similar to the typical boxes of  $\sigma^{70}$ -dependent promoters.

The *Pr::lacZ* translational fusion was subcloned in a mini-Tn5 vector resulting in plasmids pPR13 and pPR14 (Table 1). These plasmids were used to deliver, by transposition, the *Pr::lacZ* fusion into the chromosomes of *E. coli* W AF15 (*hpa*<sup>+</sup>,  $\Delta$ *lacZ*) and *E. coli* K12 AFMC (*hpa*<sup>-</sup>,  $\Delta$ *lacZ*), generating the reporter *E. coli* strains WPR13 (*hpa*<sup>+</sup>, *Pr::lacZ*) and MCR14 (*hpa*<sup>-</sup>, *Pr::lacZ*), respectively.  $\beta$ -Galactosidase assays performed with permeabilized *E. coli* WPR13 and *E. coli* MCR14 cells cultured in the presence or absence of 1 mM 4HPA revealed a significant effect of 4HPA on *Pr* expression in the WPR13 strain (2.6-fold increase in  $\beta$ -galactosidase activity), whereas *Pr* expression in the MCR14 strain was constitutive (Table 2). These results showed that the *Pr* promoter was regulated by a regulator of the *hpa* cluster, but since we have demonstrated that this cluster contains two regulators, HpaR and HpaA, that respond to 4HPA (5), they did not allow us to determine if *Pr* expression was regulated by HpaR or HpaA.

To establish the role of HpaR in *Pr* expression, the *E. coli* strains S17-1 $\lambda$ *pir* (pAJ402) and S17-1 $\lambda$ *pir* (pBA2) (Fig. 9 and



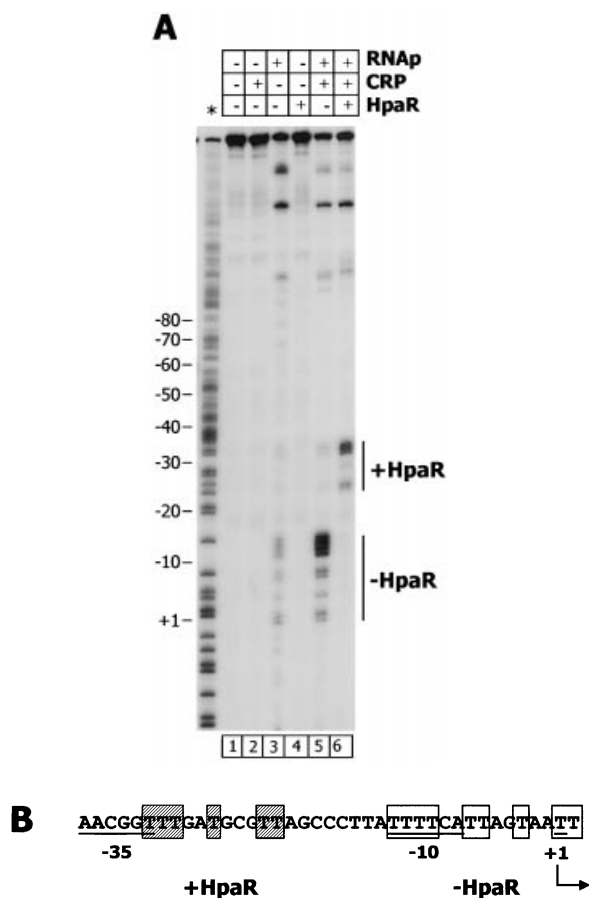
**Figure 6.** *In vitro* transcription assay of the *Pg* promoter. Run-off transcription from *Pg*. Single round *in vitro* transcription was carried out by using the plasmid pBF1 as template. RNAP, CRP and HpaR were added at final concentrations of 100 nM each and the final concentration of the inducer 4HPA was 10 mM. The *Pg*-derived mRNA (139 nt) and the vector-derived RNA1 are indicated by arrows.

Table 1) were used to transfer the *hpaR* gene into the chromosome of *E. coli* MCR14, generating the exconjugants MCR141 and MCR142, respectively. Table 2 shows that in *E. coli* MCR141 and *E. coli* MCR142 *Pr* transcription decreased 18-fold in the presence of the *hpaR* gene, strongly suggesting that HpaR represses the *Pr* promoter. The presence of 4HPA produced only a slight increase in  $\beta$ -galactosidase activity in both strains. This result could be attributed to more efficient synthesis of the HpaR protein in the heterologous K12 strain than in the wild-type W strain.

Finally, the strain WPR13 was also used to determine *in vivo* the range of HpaR inducers which act on its own *Pr* promoter. As expected,  $\beta$ -galactosidase activity was only increased in the presence of 4HPA, 3HPA and 3,4HPA, confirming that these compounds are also inducers of the HpaR repressor when it regulates its own expression (Fig. 9).

### Homology modeling of ligand-bound HpaR

Taking into account the sequence homology of HpaR with the MarR regulator (19% identity, 45% similarity; Fig. 10A), we have modeled the 3-dimensional structure of ligand-bound HpaR using the crystal coordinates of this regulator (8) (Fig. 10B and C). MarR is a dimer that contains a typical winged-helix DNA binding motif in each monomer. The two recognition helices are supposed to bind two adjacent major grooves of DNA, whereas the wings might well be positioned to make minor groove or phosphate backbone contacts to the distal parts of the inverted repeat. However, up to now, no structure of a DNA-protein complex is available for a protein of the MarR family. Based on the MarR crystal, HpaR might bind two molecules of 4HPA per monomer, one of them between the DNA recognition helix and the 'wing' structure (corresponding to the SAL-A site in MarR) and the other between the recognition helix and the  $\alpha$ 2 helix (corresponding to the SAL-B site in MarR) (Fig. 10B and C). In both cases, the ligands would be solvent exposed. A comparison of the modeled 4HPA binding sites in HpaR with those of salicylate in MarR reveals that their overall configuration is retained in

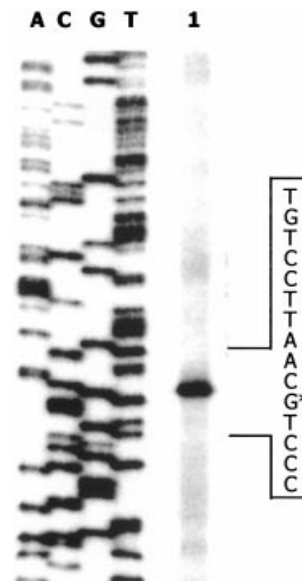


**Figure 7.**  $\text{KMnO}_4$  footprinting of the *Pg* promoter. (A) The reaction mixture was treated as described in Materials and Methods using as probe the 5'-end-labeled non-coding strand of the *Pr-Pg* region. RNAP, CRP and HpaR were added at final concentrations of 100 nM. The positions sensitive to  $\text{KMnO}_4$  are indicated. An A+G sequencing ladder is indicated with an asterisk. (B) Nucleotide sequence of the *Pg* region showing the -35 and -10 promoter boxes and +1 site. The positions sensitive to  $\text{KMnO}_4$  corresponding to the open complex (empty boxes) and to the HpaR-dependent DNA bubble (hatched boxes) are indicated.

HpaR, which is not surprising considering the similarity of the salicylate and 4HPA structures. Moreover, the non-conservative changes between the two sequences can be explained in terms of the accommodation of the hydroxyl group located in a different position in the aromatic ring of the ligand. This, and the fact that no notable steric clashes are produced in the model, support the reliability of the latter.

## DISCUSSION

Based on sequence comparison analysis, the HpaR protein of the *hpa* catabolic cluster from *E.coli* W had been proposed to be a transcriptional regulator belonging to the MarR family, but until now, there was no experimental evidence concerning its function and properties (5,6). The activity of the MarR-like proteins found in bacteria and archaea are modulated in response to environmental signals and they play important roles in global health problems caused by bacterial pathogens since they control genes implicated in antibiotic resistance and



**Figure 8.** Mapping of the *hpaR* transcriptional start site by primer extension. Total RNA was isolated from LB grown *E.coli* MC4100 (pBM2) cells (lane 1). A sequence ladder generated with the same primer ( $R_2+1$ ) is shown (lanes A, C, G and T). The position of the primer extension product is indicated by the asterisk.

hemolysis (7,19). Some members of this family are involved in specific responses to aromatic compounds, e.g. MarR (7,20), CinR (21), HpcR (4,5), BadR (22) and EmrR (23). Most of the regulators belonging to this family are transcriptional repressors with the exception of BadR (22), CbaR (24), SlyA (25) and NhhD (26), which function as activators. Moreover, MexR can act as either a transcriptional repressor or activator (27). Recently, the crystal structures of MarR and MexR have been described (8,28) and these findings have increased the interest in these regulators since they have paved the way for structural studies on other members of the family.

Remarkably, although many positive regulators have been shown to control the aromatic catabolic pathways in bacteria, only a few repressors have been described in these pathways (19). The known repressors include the CymR regulator in the catabolism of *p*-cimene in *Pseudomonas putida* F1 (29), the AphS repressor of phenol catabolism in *Comamonas testosteroni* TA441 (30,31) and the PaaX and PaaN regulators of the PA catabolic pathway from *E.coli* (12) and *P.putida* U (32), respectively. In this context it appeared very interesting to perform the first rigorous analysis of the HpaR repressor. In this work, we have demonstrated that HpaR negatively regulates not only expression of the *hpa-meta* operon but also its own expression. The fact that HpaR can regulate its own expression agrees with observations for other MarR-like proteins (23,33) and may represent a common feature of this family of regulators (21). In addition, we have illustrated experimentally that HpaR binds specifically to both the *Pg* and *Pr* promoter regions. The intrinsic binding affinity for the *Pg* operator (OPR1) was 100-fold higher than that for the *Pr* operator (OPR2) when the operators were carried on different fragments (Fig. 4B). Although HpaR bound cooperatively to both operators on the native PR-PG template, OPR1 remains the first operator to be occupied by the HpaR repressor. These data indicate that as soon as the inducers are



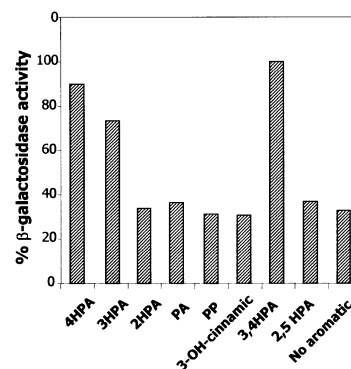
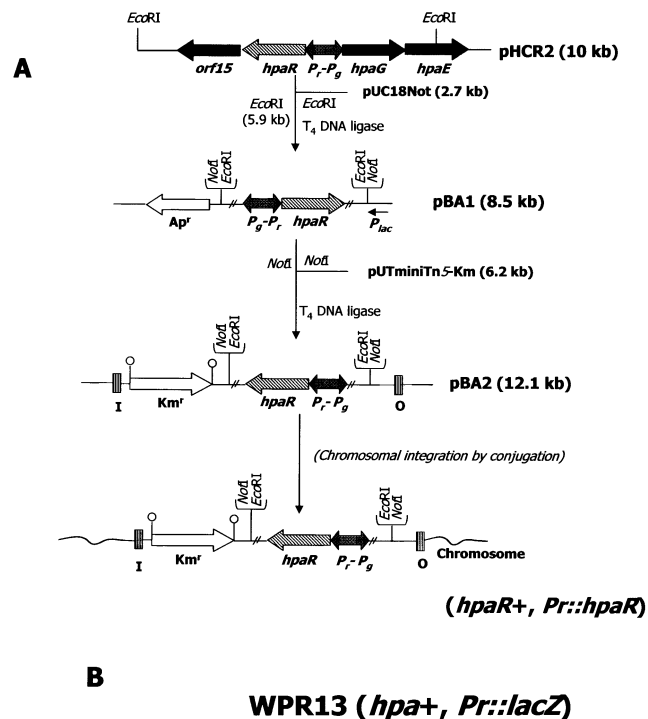
**Table 2.** Repression of *Pr* promoter by HpaR

Strain	$\beta$ -galactosidase activity (Miller units)	
	-4HPA	+4HPA
MCR14 (-HpaR, -HpaA)	1544	1495
MCR141 (+HpaR, +HpaA)	87	119
MCR142 (+HpaR, -HpaA)	72	109
WPR13 (+HpaR, +HpaA)	510	1350

depleted from the medium, HpaR binds to OPR1 to shut off expression of the catabolic enzymes before HpaR starts to inhibit its own expression by binding to OPR2. DNase I footprinting (Fig. 5) revealed that OPR1 comprises a 27 bp region containing a palindromic sequence of 9 bp on each side separated by 4 bp (Fig. 5). A similar structural design has been described for the operators of other promoters controlled by MarR-like regulators (21,33). The DNA binding site of MarR consists of a 21 bp sequence organized into an inverted repeat (33) and CinR protects a DNA region of 16 bp showing similar characteristics (21). All these findings are consistent with the dimeric structure proposed for MarR-like regulators where each subunit binds to one of the two inverted half-sites of the operator (8), and suggest an architecture conserved through evolution.

The fact that OPR1 is centered at position +2 of *P<sub>g</sub>*, i.e. overlapping the transcriptional start site, suggests that HpaR should repress transcription from *P<sub>g</sub>* by a mechanism based on steric hindrance, by inhibiting binding of RNAP to the promoter. However, in the presence of both RNAP and HpaR a new pattern of thymine residues reactive to permanganate appears ~20 bp upstream of the cognate transcription bubble. This result suggests that RNAP and the repressor are bound simultaneously to the *P<sub>g</sub>* promoter forming a ternary complex that inhibits formation of the open complex around the transcriptional start site. Taking into account the results of the *in vitro* transcription assay (Fig. 6), HpaR might repress transcription from *P<sub>g</sub>* by generating an upstream displacement of RNAP from its functional promoter binding site and hence preventing transcription initiation by a road-block mechanism. Although this sort of repression is not very frequent, there are, in the literature, some examples of regulators that remain bound to the promoter in the presence of RNAP, e.g. protein  $\omega$  of the broad host range plasmid pSM19035 (34), the factor for inversion stimulation of *E. coli* (Fis) at the *bgl* promoter (35), TyrR in the *aroP2* promoter of *E. coli* (36), Spo0A at the *abrB* promoter of *Bacillus subtilis* (37) and Arc at the *Pant* promoter of phage P22 (38). Therefore, HpaR could be considered as a new representative of this type of regulator.

Footprinting analyses have demonstrated that a second HpaR operator, OPR2, is centered at position +47 relative to the *Pr* transcriptional start site (Fig. 5). The location of OPR2 with respect to the +1 site of *Pr* suggests that the repression effect might be produced by inhibiting the transcription elongation process. OPR2 comprises a 27 bp region with a similar organization to OPR1. One of the half-sites of the operator is identical to that of OPR1 but the other consists of an imperfect repeat with four nucleotide changes (Fig. 5). These differences could explain the lower binding affinity of

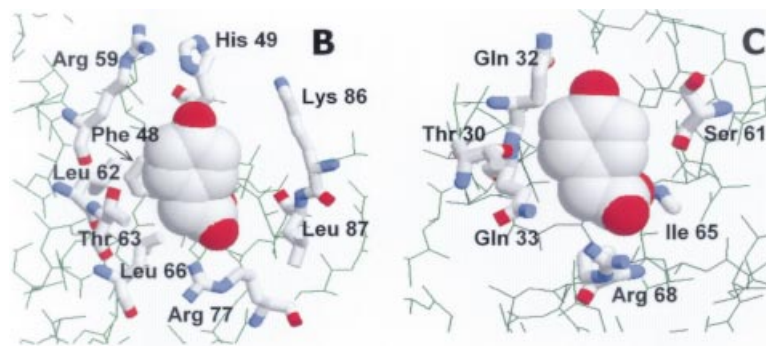


**Figure 9.** Regulation of the *Pr* promoter. (A) Schematic construction of *E. coli* MCR142 (*Pr::hpaR*, *Pr::lacZ*). (B) Effect of several aromatic compounds on expression of the *Pr* promoter. Cells of *E. coli* WPR13 (*Pr::lacZ*) were grown in glycerol-containing minimal medium in the absence (no aromatic) or in the presence of 1 mM different aromatics (4HPA, PA, 2HPA, 3HPA, PP, 2,5HPA, 3,4HPA and 3-hydroxycinnamic acid) until the cultures reached an OD<sub>600</sub> of 0.8.  $\beta$ -Galactosidase activities were measured with permeabilized cells as described in Materials and Methods.

HpaR to OPR2 when compared to OPR1. On the other hand, we have observed that the binding to OPR2 is clearly cooperative with the binding to OPR1. Since HpaR binding experiments were performed in the absence of other DNA-binding proteins, such as CRP or IHF, this cooperative effect indicates that the binding of HpaR to OPR1 stabilizes its binding to OPR2 by protein-protein interactions. Preliminary experiments performed by analytical ultracentrifugation suggest that HpaR is a dimer in the absence of effector (data not shown). Nevertheless, we cannot eliminate the hypothesis of

## A

HpaR	---MHDSLTIALLQAREAAMS-----YFRPIVKRHNltEqqWRIVRILAESPSMDFHDL	51
MarR	MKSTSDLFNEI I PLGRLIHMVNQKKDRLLNEYLSPLDItAaqFKVLCSIRCAACITPVEL	60
	* .. : .* * .. .. : :* *:::: : :... : *	
HpaR	AYRACILRPs <b>LTGiLTrMERDGLVLR</b> IKPINDQR <b>KLY</b> ISLTKEGQALYNRAQTQIEEAYR	111
MarR	KKVLSVDL <b>GaLTrmLDrLVCKGWVERLP</b> PNPNDKR <b>GVL</b> VKLTTGGAAICEQCHQLVGVQDLH	120
	.. : ** :* * : .* * ** **:* : :.*. * * : :... : : :	
HpaR	QIEAQ-FTA <b>EKM</b> QQLTHLLEEFIALGNSRQEDIPGDNE	148
MarR	QELTKNLT <b>ADEV</b> ATLEYLLK <b>KVLP</b> -----	144
	* .. : **::: * : **:::..	



**Figure 10.** Model of ligand-bound HpaR structure. (A) Amino acid sequence alignment of the HpaR and MarR proteins. Residues corresponding to the SAL-A type binding site are shown in bold. Residues corresponding to the SAL-B type binding site are represented in lower case. The putative HpaR DNA recognition helix is boxed. Alignment was done with the BESTFIT program. (B) Detail of the 'SAL-A' type 4HPA binding site. The ligand is depicted in spacefill representation and interacting residues are shown in thick wireframe format. Note that 4HPA could also bind in an alternative orientation, rotated 180° around the z-axis. (C) Detail of the 'SAL-B' type 4HPA binding site. The scheme representation is as indicated for (B).

dimerization (or oligomerization) of the HpaR dimers to explain the striking cooperativity observed in HpaR binding to OPR1 and OPR2. Two dimers of HpaR bound at OPR1 and OPR2 could interact together leading to the formation of an oligomer which could generate a repression loop. The distance between the two operators is 200 bp, which corresponds to 19 turns of B-DNA helix (assuming a pitch of 10.5 bp per turn). Hence, the two dimers of HpaR should be positioned on the same face of the DNA helix. It is also likely that CRP and IHF help to stabilize the repression loop by bending DNA, whereas 4HPA might disturb the interactions between two HpaR dimers and HpaR–DNA complexes. A similar mechanism has been proposed in the case of the *gal* promoter region where both the presence of histone-like unstable nucleoid protein and the supercoiled state of the template are required to close the repression loop formed by dimerization of the *gal* repressor dimers bound to each operator (39,40). Validation of this hypothetical mechanism for HpaR repression requires further research.

The *in vivo* experiments performed with *E. coli* WPR13 (*Pr::lacZ*, *hpa*<sup>+</sup>) and *E. coli* WPG11 (*Pg::lacZ*, *hpa*<sup>+</sup>) have demonstrated that activation of the *Pg* and *Pr* promoters requires the presence of 4HPA, 3HPA or 3,4HPA as inducer. These results correlate with the observations obtained by

phenyl-Sepharose chromatography where HpaR appears to be adsorbed to the matrix by a pseudo-affinity mechanism. The finding that only the compounds 4HPA, 3HPA and 3,4HPA were able to desorb the protein suggests that most likely the interaction of HpaR with this matrix takes place through the effector binding site of HpaR. However, it was surprising that although these results clearly demonstrate that the inducers interact directly with the protein, the presence of inducer even at high concentration (10 mM) (Fig. 4) and in different ionic strength conditions had little effect on HpaR binding. It decreased HpaR affinity for OPR1 by only 2-fold in DNase I footprinting assays and had hardly any effect on HpaR binding to DNA in EMSAs (data not shown). In this respect it has been previously documented that under *in vitro* conditions some repressors are not released from the DNA in the presence of the effector (39). It could be argued that a correct response of the HpaR–DNA interaction to the presence of the effector requires the presence of RNAP located in close contact with the OPR1–HpaR–4HPA complex in order to destabilize the repressor complex by protein–protein interactions. Moreover, the supercoiled structure of DNA might be necessary to observe derepression. The latter argument correlates with our observation that 4HPA was able to induce derepression *in vitro* in the run-off transcription experiments when the template was

a supercoiled plasmid (Fig. 6B) and not a linear fragment (data not shown).

Analysis of the 3-dimensional structures of MexR and MarR regulators suggests that despite their similarities in sequence and structure their regulatory mechanisms are different (28). MexR recognizes an inverted repeat separated by 5 bp, which facilitates binding of the two subunits to the same face of the DNA, i.e. a single dimeric molecule can interact with the operator. It has been proposed that MexR regulates its DNA binding activity by shifting the distance between the DNA binding helices of each monomer as a result of a conformational change induced by interaction of the effector with the C-terminal region of MexR (28). The case of MarR protein is more puzzling since the two inverted repeat half-sites on the DNA are separated by only 2 bp, a distance unlikely to be able to accommodate the two recognition helices in two adjacent major grooves of DNA. Moreover, in the liganded protein complex, two arginine side chains known to be involved in specific DNA binding (41) are predicted to point away from the bases in the major groove. MarR binding to the operator site as a single dimer would only take place after a considerable rearrangement in both the spacing and the orientation of the recognition helices that could require the binding of a new effector. However, it still cannot be excluded that two dimers of the MarR protein bind on different faces of the DNA to each half-site. The recent structure of SlyA, a member of the MarR family in the unliganded state at 1.6 Å resolution, does not shed more light on the mode of DNA binding of these proteins (41).

The above considerations indicate that the members of the MarR family of regulators may be diverse both in the types of effector molecules recognized and in the ways their DNA binding activity is regulated, and it appeared interesting to analyze the case of HpaR. Taking into account that the two inverted repeats in the HpaR operator are separated by 4 bp, HpaR repression could likely be explained by the binding of a single dimer to both repeats of the operator. The fact that HpaR might bind to DNA both in the absence and in the presence of 4HPA suggests that small changes in the quaternary structure of the repressor should be the key event for regulation, rather than binding itself. According to our 3-dimensional model (data not shown), binding of 4HPA, especially in the 'SAL-B' type site, may substantially affect the dimerization interface between the two monomers, leading to a different positioning of the recognition helices.

The analysis of HpaR presented here represents the most detailed characterization of a regulatory protein of an aromatic catabolic pathway from *E.coli* and provides clear evidence that the complex *hpa* regulatory system will provide new insights in the field of the regulation of secondary metabolic pathways of this model microorganism.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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