Escherichia coli mhpR gene expression is regulated by catabolite repression mediated by the cAMP–CRP complex

I. Manso, J. L. García and B. Galán

The expression of the mhp genes involved in the degradation of the aromatic compound 3-(3-hydroxyphenyl)propionic acid (3HPP) in Escherichia coli is dependent on the MhpR transcriptional activator at the Pa promoter. This catabolic promoter is also subject to catabolic repression in the presence of glucose mediated by the cAMP–CRP complex. The Pr promoter drives the MhpR-independent expression of the regulatory gene. In vivo and in vitro experiments have shown that transcription from the Pr promoter is downregulated by the addition of glucose and this catabolic repression is also mediated by the cAMP–CRP complex. The activation role of the cAMP–CRP regulatory system was further investigated by DNase I footprinting assays, which showed that the cAMP–CRP complex binds to the Pr promoter sequence, protecting a region centred at position −40.5, which allowed the classification of Pr as a class II CRP-dependent promoter. Open complex formation at the Pr promoter is observed only when RNA polymerase and cAMP–CRP are present. Finally, by in vitro transcription assays we have demonstrated the absolute requirement of the cAMP–CRP complex for the activation of the Pr promoter.

INTRODUCTION

The mhp gene cluster of Escherichia coli, which encodes the 3-hydroxyphenylpropionate (3HPP) catabolic pathway, constitutes a model system for studying bacterial degradation because of its distinctive regulation. The mhp regulatory region contains the Pr and Pa promoters, which control the expression of the divergently transcribed mhpR regulatory gene and mhp catabolic genes, respectively (Ferrández et al., 1997). Expression of the mhp catabolic genes depends on the transcriptional activator MhpR, which belongs to the IclR family of transcriptional regulators. MhpR behaves as a 3HPP-dependent activator of the Pa promoter by binding to its specific operator sequence centred at position −58 with respect to the transcription start site in the Pa promoter (Fig. 1a). (Ferrández et al., 1997; Torres et al., 2003). MhpR displays a distinctive regulation mechanism in which phenylpropionic acid activates the MhpR regulator synergistically with the true inducers, representing the first case of such an unusual synergistic effect described for a bacterial regulator (Manso et al., 2009). Gene expression from the Pa promoter is also under a strict global control system mediated by the cAMP–CRP complex that allows expression of the mhp catabolic genes when glucose is not available and 3HPP is present in the medium (Torres et al., 2003). The Pa promoter contains a potential CRP-binding site centred at position −95.5 relative to the transcription start point of Pa (Fig. 1a). In vitro experiments revealed that the specific activator, MhpR, is essential for the recruitment of CRP at the Pa promoter (Torres et al., 2003). The synergistic transcription activation by the cAMP–CRP complex and the MhpR activator allowed the Pa promoter of the mhp cluster to be classified as a class III CRP-dependent promoter (Torres et al., 2003).

Although the regulation of the mhp catabolic operon has been extensively studied, few data have been published concerning mhpR gene regulation. It has been proposed that the expression from the Pr promoter is MhpR-independent, and the expression driven by the Pr promoter is slightly affected when cells are growing on glucose (Torres et al., 2003). In this work we have demonstrated by using different in vivo and in vitro experimental approaches that transcription from the Pr promoter is not constitutive but dependent on the cAMP–CRP complex, according to a class II mechanism of CRP-dependent activation.

METHODS

Bacterial strains, plasmids and growth conditions. The E. coli strains and plasmids used in this work are listed in Table 1. Unless otherwise stated, bacteria were grown in Luria–Bertani (LB) medium (Sambrook & Russell, 2001) at 37 °C. Growth in M63 minimal medium (Miller, 1972) was achieved at 30 °C using the correspond-
ing necessary nutritional supplements and 20 mM glucose or 10 mM glucose as carbon source. When required, 1 mM 3HPP (Lancaster Synthesis) was added to the M63 minimal medium. Where appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg ml⁻¹; rifampicin, 50 μg ml⁻¹; and kanamycin, 50 μg ml⁻¹.

**Molecular biology techniques.** General procedures for DNA manipulation were performed as indicated elsewhere (Sambrook & Russell, 2001). Plasmid DNA was prepared with a High Pure plasmid purification kit (Roche Applied Science). DNA fragments were purified with Gene-Clean Turbo (Q-BioGene). Oligonucleotides were supplied by Sigma. All cloned inserts and DNA fragments were confirmed by DNA sequencing with an ABI Prism 377 automated sequencer (Applied Biosystems). Transformation of *E. coli* cells was carried out by using the RbCl method or by electroporation (Gene Pulser, Bio-Rad) (Sambrook & Russell, 2001). Proteins were prepared with a High Pure plasmid purification kit (Roche). All cloned inserts and DNA fragments were confirmed by DNA sequencing with an ABI Prism 377 automated sequencer (Applied Biosystems). Transformation of *E. coli* cells was carried out by using the RbCl method or by electroporation (Gene Pulser, Bio-Rad) (Sambrook & Russell, 2001). Proteins were analysed by SDS-PAGE (Laemmli, 1970).

**β-Galactosidase assays.** The activity of the Pr promoter was monitored by assaying β-galactosidase accumulation in *E. coli* AFMCRL and AFSBRL (CRP⁺) cells (Table 1). β-Galactosidase assays were performed according to the method described by Miller (1972). At least three independent assays were performed in each case.

**Purification of CRP.** The CRP protein was purified from *E. coli* MCG11 (pBeBe) (Table 1) as described by Ghosalidhi et al. (1988). The purified protein was dialysed at 4 °C in SB buffer (10 mM NaH₂PO₄, pH 6.8, 100 mM NaCl and 50% glycerol) and stored at −80 °C.

**Primer extension.** *E. coli* MC4100 and SBS688 cells harbouring plasmid pRAL (Table 1) were grown in LB medium until the cultures reached an OD₆₀₀ of 0.6. Total RNA was isolated by using the RNeasy Mini kit (Qiagen) according to the instructions of the supplier. Primer extension reactions were carried out with the avian myeloblastosis virus reverse transcriptase (Promega) and 15 μg total RNA as described previously (Martin et al., 1996), using oligonucleotide PP6 (5’-CCGTCTGCTCATGTTCTCTG-3’) labelled at its 5’-end with phage T₄ polymerase holoenzyme and [α-³²P]ATP (3000 Ci mmol⁻¹) using T₄ polymerase holoenzyme. To perform the PCR, 5 μl labelled and unlabelled (PP6) primers, with 10 ng plasmid pRAL as a template, were used. The 5’-end-labelled PCR product was purified using the High Pure PCR product purification kit from Roche. Complexes with the labelled promoter region (1 nM final concentration of DNA) were allowed to form for 20 min at 37 °C in 12 μl of a solution containing 0.4 M sodium acetate, 2.5 mM EDTA and 0.3 M sodium chloride. The samples were extracted with phenol and precipitated with ethanol. DNA fragments were sequenced and [α-³²P]dCTP (Amersham Biosciences) as indicated by the supplier. Products were analysed on a 6% polyacrylamide-urea gel. The gel was dried onto Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences).

**DNase I footprinting assays.** The Pa-Pr DNA fragment (274 bp) to be used as probe was 5’-end-labelled on the Pa non-coding strand by using a labelled primer during the PCR amplification reaction. The LAC-57 primer (5’-CGATATTGTTGAAGCAGAG-3’) (5 pmol) was 5’-end-labelled with [α-³²P]dATP (3000 Ci mmol⁻¹) using T₄ polymerase holoenzyme. To perform the PCR, 5 μl labelled and unlabelled (PP6) primers, with 10 ng plasmid pRAL as a template, were used. The 5’-end-labelled PCR product was purified using the High Pure PCR product purification kit from Roche. Complexes with the labelled promoter region (1 nM final concentration of DNA) were allowed to form for 20 min at 37 °C in 12 μl of a solution containing 0.4 M sodium acetate, 2.5 mM EDTA and 0.3 M sodium chloride. The samples were extracted with phenol and precipitated with ethanol before analysis on a 6% polyacrylamide-urea gel. Protected bands were identified by comparison with the migration of the same fragment treated for the A+G sequencing reaction (Maxam & Gilbert, 1977). The gels were dried onto Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Pharmacia Biotech).

**KMnO₄ footprinting.** This technique was performed according to the method described by Sasse-Dwight & Gralla (1989). CRP (200 nM) and RNAP (100 or 200 nM) were allowed to form complexes with the radioactively labelled Pa–Pr fragment for 20 min at 37 °C in 15 μl buffer (40 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂, 125 mM KCl) and incubated at 37 °C for 20 s. The reaction was stopped by the addition of 180 μl of a solution containing 0.4 M sodium acetate, 50 μg salmon sperm DNA ml⁻¹, 2.5 mM EDTA and 0.3 μg glycogen ml⁻¹. The samples were extracted with phenol and precipitated with ethanol before analysis on a 6% polyacrylamide-urea gel. Protected bands were identified by comparison with the migration of the same fragment treated for the A+G sequencing reaction (Maxam & Gilbert, 1977). The gels were dried onto Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Pharmacia Biotech).

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**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>MC4100</td>
<td>araD319 Δ(argF–lac)U169 rpsL150 (Smr⁺) relA1 flbB5301 deoC1 ptsF25 rbsR</td>
<td>Prieto &amp; García (1997)</td>
</tr>
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<td>AFMC</td>
<td>MC4100 spontaneous rifampicin-resistant mutant (Rif')</td>
<td>Ferrández et al. (2000)</td>
</tr>
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<td>Torres et al. (2003)</td>
</tr>
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<td>SBS688</td>
<td>MC4100 Δcrp</td>
<td>Prieto &amp; García (1997)</td>
</tr>
<tr>
<td>AFSB</td>
<td>SBS688 spontaneous rifampicin-resistant mutant (Rif')</td>
<td>Ferrández et al. (2000)</td>
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<td>AFSB with chromosomal insertion mini-Tn.Km Pr–lacZ</td>
<td>Torres et al. (2003)</td>
</tr>
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<td>MCG11</td>
<td>MC4100 with chromosomal insertion of mini-Tn.Km Pg–lacZ, Km'r Rif’</td>
<td>Galán et al. (2001)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector</td>
<td>Galán et al. (2008)</td>
</tr>
<tr>
<td>pBeBe</td>
<td>Ap’, pUC19 derivative vector used to produce CRP</td>
<td>Sambrook &amp; Russell (2001)</td>
</tr>
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<td>pRAL</td>
<td>Ap’, pSF3 containing a 1.1 kb DNA fragment to produce the mhpR/Pa–lacZ fusion</td>
<td>Torres et al. (2003)</td>
</tr>
<tr>
<td>pPADR2</td>
<td>Ap’, pSF3 derivative containing the complete mhpABCDEForfT region</td>
<td>Ferrández et al. (1998)</td>
</tr>
<tr>
<td>pICDR</td>
<td>pICD01 derivative containing Pr promoter</td>
<td>This work</td>
</tr>
</tbody>
</table>

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**Strains**

- DH5α
- MC4100
- AFMC
- AFMCRL
- SBS688
- AFSB
- AFSBRL
- MCG11

**Plasmids**

- pUC19
- pBeBe
- pRAL
- pPADR2
- pICD01
- pICDR
resuspended in 150 μl piperidine (1 M), heated at 90 °C for 30 min and evaporated until dryness. Then 20 μl water was added and evaporated (twice). Finally, samples were resuspended in 5 μl loading buffer (20 mM EDTA in 80%, v/v, formamide containing xylene cyanol blue and bromophenol blue) and loaded on a 6% polyacrylamide-urea gel.

**In vitro transcription assays.** Multiple-round transcription by E. coli RNAP was carried out under standard conditions (Marschall et al., 1998), using the reaction buffer 40 mM Tris/HCl pH 7.5 containing 10 mM MgCl₂, 100 mM KCl, 200 μM RNAP was carried out under standard conditions (Marschall et al., 1998), using the reaction buffer 40 mM Tris/HCl pH 7.5 containing 10 mM MgCl₂, 100 mM KCl, 200 μM RNAP, 500 μg acetylated BSA ml⁻¹, and supercoiled DNA plasmid pCDR (Table 1). To construct this plasmid, the oligonucleotides PrBam (5'-CCGGAATTCAGCTTTGCGCATCTACGCAGCC-3'; an engineered BamHI site is underlined) and PrEco (5'-CCGGAATTCAGCTTTTAAACAAATTATA-3'; an engineered EcoRI site is underlined) were used for the PCR amplification of the Pr promoter using plasmid pPADR2 (Table 1) as DNA template. The resulting PCR product was cloned into plasmid pUCD01, which contains the pUC19 polylinker between the EcoRI and PstI sites flanked by the divergent terminators rocCT and rnsHTI2, respectively (Table 1). The final volume of the reaction mixture was 9 μl, containing the plasmid DNA (6.7 nM) with CRP (42 nM) or buffer. This mixture was incubated at 25 °C for 20 min. Then 3 μl RNAP (Epigenet) at 500 nM was added and the mixture was incubated at 37 °C for 5 min in a final volume of 12 μl. Elongation was started by the addition of 3 μl of a pre-warmed mixture containing 1 mM ATP, 1 mM GTP, 1 mM CTP, 50 μM UTP and 1 μCi [³²P]UTP in reaction buffer to the template/polymerase mix and allowed to proceed for 15 min at 37 °C. Reactions were stopped by the addition of 10 μl loading buffer containing 1% SDS. After heating to 70 °C, samples were subjected to electrophoresis on a 6% polyacrylamide-urea gel.

**RESULTS**

**Influence of glucose on mhpR expression**

As mentioned above, it has been demonstrated that the transcription of the mhp cluster from the Pa promoter is activated by the cAMP–CRP complex (Torres et al., 2003). To check whether the Pr promoter is also regulated by this global control system, we tested the expression of a Pr–lacZ fusion in E. coli AFMCRL cells (Table 1) with glycerol or glucose as carbon source in the presence or absence of 1 mM 3HP. As shown in Fig. 1(b), the β-galactosidase levels in E. coli AFMCRL cells grown in glycerol, in either the absence or presence of 3HP, were sixfold higher than those in cells growing in glucose as carbon source. Because the glucose repression effect is usually mediated by the cAMP–CRP complex in enteric bacteria (Kolb et al., 1993; Saier et al., 1996), we checked whether CRP was required for mhpR expression by using an E. coli strain lacking CRP protein. When E. coli AFSBRL (CRP−) (Table 1) cells were grown in either glycerol- or glucose-containing minimal medium in the absence or presence of 3HP, only basal β-galactosidase activities were observed (Fig. 1b). Therefore, these results indicate that the cAMP–CRP complex is mediating the glucose effect at the Pr promoter.

The strict requirement of CRP for the expression of the Pr promoter was checked by primer extension using total RNA isolated from LB-grown E. coli MC4100 or SBS688 cells containing the pRAL plasmid (Table 1). We observed that the major transcription initiation site in the Pr promoter starts with an A residue located 115 nt upstream of the ATG translation initiation codon of the mhpR gene (Fig. 1c). A putative −10 box (TAAAAA) located 5 bp upstream of the Pr +1 site and a putative −35 box (ACAGTA) similar to the typical boxes of σ70-dependent promoters with a spacer of 18 bp were identified (Fig. 1a). Although other transcription initiation sites in Pr could be located a few nucleotides downstream of the major initiation site (Fig. 1c) we cannot rule out that such start sites could correspond to 5′-end processed transcripts initiated at the major start site. Significantly, the transcripts generated by the Pr promoter were not detected in the strain lacking CRP, indicating that the cAMP–CRP complex is required to activate mhpR expression.

**In vitro CRP binding to the Pr promoter**

To confirm the direct binding of the cAMP–CRP complex in vitro and to localize its operator at the Pr promoter, DNase I footprint experiments were performed using the Pa–Pr fragment as a DNA probe that covers the entire DNA intergenic region between the mhpR and mhpABCDEF genes. In the presence of cAMP–CRP a weak but detectable protected region was observed around position −30 to −51 (Fig. 2a, lane 2; Fig. 1a) with respect to the transcription start site of Pr promoter, and a DNase I-hypersensitivity site was observed at position −34 (indicated by an arrow in Fig. 2a, lane 2, and by a black box in Fig. 1a). Within this region, a binding site for the cAMP–CRP complex similar to the consensus sequence (Busby & Ebright, 1999) was identified (Fig. 2b), centred at position −40.5 with respect to the transcription start point, which suggests that CRP promotes transcription from the Pr promoter by a class II promoter mechanism (Busby & Ebright, 1997).

RNAP alone was able to bind at the Pr promoter, showing a DNase I-hypersensitivity site at position −49 (Fig. 2a). It is worth noting that in this case we were able also to observe that RNAP binds to the Pa promoter (Fig. 2a, lane 3).

In the presence of both cAMP–CRP and RNAP we were able to detect additional changes in the footprint of Pr showing new DNase I-hypersensitivity sites [A (−22), T (−43), G (−44) and A (−49)] (Fig. 2a, lane 4; Fig. 1a). In these conditions, we observed the disappearance of the hyper-reactive C (−34) that is formed in the presence of the cAMP–CRP complex alone.

**In vitro transcription at the Pr promoter**

To confirm the CRP-dependent induction of the Pr promoter, we developed an in vitro transcription assay using the pCDR plasmid as a template (Table 1). Plasmid pCDR contains the Pr promoter flanked by transcriptional terminators. As expected, when RNAP was the single protein added to the reaction mixture, only the RNA I
control transcript (107–108 nt) (Bordes et al., 2000) was detected (Fig. 3, lane 1). However, when cAMP–CRP complex was added to the reaction mixture, the Pr activity was stimulated and a 132 nt transcript generated from the Pr promoter was detected (Fig. 3, lane 2). This result demonstrates that CRP has the ability to induce the expression at the Pr promoter in vitro and this can occur independently of the presence of the Pa promoter region.

Moreover, the size of the in vitro transcript from the Pr promoter agrees with the result obtained from the primer extension experiments, in which the transcription initiation site at the Pr promoter can be mapped 115 nt upstream of the ATG translation initiation codon of the mhpR gene.

**Open complex formation at the Pr promoter**

To ascertain whether the activation mechanism mediated by the cAMP–CRP complex involves induction of the open complex formation required for transcription initiation, we performed a potassium permanganate footprinting assay using the Pa–Pr fragment labelled at its 5'–end. The hyperreactivity of single-stranded thymines to potassium permanganate provides a sensitive assay to monitor DNA duplex distortion and unwinding (Savery et al., 1996). The Pa–Pr probe was treated with permanganate after incubation with different combinations of RNAP and cAMP–CRP. The results revealed that a minimal attack by permanganate occurs in the absence of added proteins (Fig. 4, lane 1) and that RNAP alone (Fig. 4, lanes 2 and 3) had little effect on the reactivity pattern. However, the presence of cAMP–CRP and RNAP induces a strong reactivity of the thymine residues from position –11 to –12 with respect to the transcription start site due to open complex formation at the Pr promoter (Fig. 4a, lanes 4 and 5; Fig. 1a). This result confirmed that CRP interacts with the Pr promoter and demonstrates that CRP is required for open complex formation at the Pr promoter, and therefore to promote transcription initiation.
The enzymes involved in the catabolism of 3HPP by *E. coli* are encoded by the *mhp*ABCDE operon. MhpR is an activator that belongs to the IclR family of transcriptional regulators and controls positively the expression of the *mhp* operon in response to 3HPP (Ferrández et al., 1997; Torres et al., 2003). Unlike MhpR, other IclR regulators such as PcaR from *Pseudomonas putida* and PobR and PcaU from *Acinetobacter* sp. ADP1 regulate their own expression, acting as repressors (DiMarco & Ornston, 1994; Guo & Houghton, 1999; Trautwein & Gerischer, 2001). It is thought that the mechanism of auto-repression is different among IclR-type regulators, since not all of them bind at the same position on the promoter region (DiMarco & Ornston, 1994; Gerischer et al., 1998; Guo & Houghton, 1999). The small but significant increase in *mhpR* expression observed in the presence of 3HPP (Fig. 1b) could be explained as a result of a stress response due to the presence of an aromatic compound in the culture medium (Marqués et al., 1999).

In this report, we show that transcriptional activation of the *mhpR* gene is mediated by the global regulator CRP. The *pcaU* promoter, which controls the expression of the *pcaU* regulatory gene belonging to the IclR family, is also subject to catabolic repression, but the molecular mechanism remains unknown (Dal et al., 2002; Siehler et al., 2007).

CRP activates transcription from over 150 different promoters in *E. coli* (Kolb et al., 1993; Zheng et al., 2004) and this CRP regulon includes several operons mainly involved in catabolism of carbon sources other than glucose (Kolb et al., 1993). As observed for the MhpR regulator, CRP also regulates expression of other transcription factors in *E. coli* such as MelR (Webster et al., 1988), MalT (Chapon & Kolb, 1983), RhaS (Wickstrum et al., 2005), MaoB (Yamashita et al., 1996), HcaR (Turlin et al., 2001), GalS (Weickert & Adhya, 1993), RpoH (Kallipolitis & Valentin-Hansen, 1998), BlgG (Gulati & Mahadevan, 2000), PrpR (Lee et al., 2005) and Fis (Nasser et al., 2001).

Taking our results together with previous data (Torres et al., 2003) we have demonstrated that the glucose effect on *mhp* expression is exerted at the level of both catabolic and regulatory promoters. This simultaneous CRP control is not unique to the *mhp* pathway, since it has been also described for the genes for propionate catabolism in *E. coli* and *Salmonella enterica* (Lee et al., 2005) and for some regulons from *E. coli* such as those for L-rhamnose (Holcroft & Egan, 2000; Wickstrum et al., 2005), melibiose (Webster et al., 1988; Belyaeva et al., 2000) and maltooligosaccharides (Chapon & Kolb, 1983; Richet & Sogaard-Andersen, 1994; Richet, 2000). While the expression of the *mhpR*, *melR* and *malT* regulators depends only on the presence of CRP (Webster et al., 1988; Chapon & Kolb, 1983), the expression of *rhaSR* and *prpR* depends on the presence of CRP and the transcription activators RhaR and PrpR, respectively (Tobin & Schleif, 1990a, b; Wickstrum et al., 2005; Lee et al., 2005). The mechanism of CRP activation of these promoters is different, the Pr promoter of *mhpR* and the promoter of *melR* being class II CRP-dependent promoters (Webster et al., 1988; Samarasinghe et al., 2008) whereas the promoter of *malT*, and the *PSR* and *PprpR* promoters behave as class I CRP-dependent promoters (Chapon & Kolb, 1983; Wickstrum et al., 2005; Lee et al., 2005).

**DISCUSSION**

The enzymes involved in the catabolism of 3HPP by *E. coli* are encoded by the *mhpABCDFE* operon. MhpR is an activator that belongs to the IclR family of transcriptional regulators and controls positively the expression of the *mhp* operon in response to 3HPP (Ferrández et al., 1997; Torres et al., 2003). Unlike MhpR, other IclR regulators such as PcaR from *Pseudomonas putida* and PobR and PcaU from *Acinetobacter* sp. ADP1 regulate their own expression, acting as repressors (DiMarco & Ornston, 1994; Guo & Houghton, 1999; Trautwein & Gerischer, 2001). It is thought that the mechanism of auto-repression is different among IclR-type regulators, since not all of them bind at the same position on the promoter region (DiMarco & Ornston, 1994; Gerischer et al., 1998; Guo & Houghton, 1999). The small but significant increase in *mhpR* expression observed in the presence of 3HPP (Fig. 1b) could be explained as a result of a stress response due to the presence of an aromatic compound in the culture medium (Marqués et al., 1999).

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Although it was not possible to detect the binding of purified CRP to the Pr promoter by electrophoretic mobility shift assay (Torres et al., 2003), we have shown a weak but detectable binding of CRP to the Pr promoter by DNase I footprinting assays. However, when RNAP was added, the binding became stronger. Similarly, Webster et al. (1988) were unable to detect the binding of cAMP–CRP complex alone to the melR promoter. However, when these authors analysed the binding of CRP and RNAP together to the melR promoter by DNase I footprinting, they observed a clear stronger footprint from around +20 to −70 with respect to the transcription initiation. The fact that the promoter of mhpR does not contain the consensus sequence 5′-TGTGA-3′, which facilitates the strongest contact with CRP (Weber & Steitz, 1984), might explain why we were unable to detect the binding of cAMP–CRP alone to the Pr promoter. This is also the case for the promoter of melR, which does not have this consensus sequence (Webster et al., 1988). It is known that at CRP-dependent promoters CRP activates transcription by making direct protein–protein contacts with the RNAP. Therefore we suggest that the cooperative interaction between RNAP and the cAMP–CRP complex could explain the improvement in the binding of both proteins to the Pr promoter.

In the case of class II CRP-dependent promoters the activation is mainly due to CRP binding to a 22 bp target site centred at or near position −41.5 from the transcription start site, overlapping and replacing the −35 determinant for binding of RNAP (Zheng et al., 2004; Busby & Ebright, 1999). A total of 104 operons from E. coli have been predicted to contain class II CRP-binding sites (Zheng et al., 2004). As shown in Fig. 2(b), the nucleotide sequence from −51 to −30 upstream of the mhpR transcription start point shares only 13 of 22 positions with the consensus CRP-binding site (5′-AAATGTAAGCTAGATCACATTT-3′; Busby & Ebright, 1999). Interestingly, the CRP sites at the galP1 and melR promoters (de Crombrugghe et al., 1984; Webster et al., 1988) are located between positions −52 and −31 from the transcription start site and also share 14 and 12 positions, respectively, with the consensus sequence (Fig. 2b).

In the case of the Pr promoter the CRP-binding site is centred at −40.5 from the mhpR transcription start point. This unusual location of the CRP-binding site at the Pr promoter has been described for the promoter of the manX gene, which also showed a strong dependence on the presence of the cAMP–CRP complex (Plumbridge, 1998). Nevertheless, the mechanism of activation of manX expression is not identical to that of mhpR since there are two binding sites for the NagC repressor of manX (Plumbridge, 1998). In the model system of the galP1 promoter, the shift of the sequence elements by 1 bp downward from the consensus −41.5 made CRP only a moderate activator on the promoter (Lavigne et al., 1992).
Therefore, the location of CRP binding at -40.5 in the Pr promoter does not appear to be optimal, and this could explain the weak activity of this promoter. To ensure appropriate expression of catabolic operons acting on low-preference nutrients, such as aromatic compounds, it is important to tightly control the expression of their corresponding regulatory genes. In the case of the CRP-dependent Pr promoter, which shows a weak binding affinity for CRP, the efficient expression of the MhpR activator will require a high level of cAMP. In this way the activation of the mhp cluster will take place not only in the presence of the 3HPP inducer but also when the availability of catabolite repressor (best preferred substrate) is very low.

Summarizing, the CAMP–CRP complex activates transcription at both the Pa and Pr promoters of the 3HPP-catabolic operon by two different mechanisms. In the case of Pr, when glucose is not available, the cAMP–CRP complex induces mhpR expression as occurs in typical class II promoters. In the case of Pa (Manso et al., 2009), MhpR binds at the operator sequence and in the presence of 3HPP the MhpR–HPP interaction facilitates the recruitment of the cAMP–CRP complex, which synergistically activates transcription of the mhp catabolic genes as occurs in typical class III promoters.

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