INTRODUCTION

The hpa cluster of *Escherichia coli* W was the first aromatic catabolic pathway described at a molecular level in this enterobacterium and codes for a group of proteins involved in the degradation of 4-hydroxyphenylacetate (4HPA) and 3-hydroxyphenylacetate (3HPA) (Prieto *et al.*, 1996) (Fig. 1). The catabolic genes are organized in two operons: the *hpa*BC operon encoding the paradigmatic two-component 4HPA monooxygenase which transforms 4HPA or 3HPA into 3,4-dihydroxyphenylacetic acid (HPC) (Galán *et al.*, 2000; Prieto & García, 1994; Prieto *et al.*, 1993), and the *meta* operon (*hpaGEDFHI*), which codes for the enzymes cleaving the aromatic ring of HPC and allowing its complete mineralization (Prieto *et al.*, 1996) (Fig. 1). The transcription of the *hpa* operon is controlled by the products of the *hpa*A and *hpa*R genes (Fig. 1) (Prieto & García, 1997). HpaA is an activator belonging to the XylS/AraC family of regulators, which controls the expression of the *upper* operon (*Pbc* promoter) (Prieto & García, 1997), whereas HpaR is a member of the MarR family (Prieto & García, 1997; Galán *et al.*, 2003) which represses both the expression of the *meta* operon (*Pg* promoter) (Galán *et al.*, 2001; Roper *et al.*, 1993) and its own expression (*Pr* promoter) (Galán *et al.*, 2003). While 4HPA was able to induce *Pbc*, *Pr* and *Pg* promoters, HPC only induces the *Pg* and *Pr* promoters (Fig. 1). In addition to this specific regulatory system, we have previously demonstrated that the expression of the *Pg* promoter is controlled by a very unusual strict catabolite repression mechanism mediated by the common global regulators, the catabolite repression protein (CRP) and the integration host factor (IHF), connecting the physiological status of the cell to the specific induction of the *hpa* genes (Galán *et al.*, 2001). Although *Pg* is not a $\sigma^{38}$-dependent promoter, it is activated in the stationary phase when cells are cultured with glucose as the sole carbon and energy source. In the current study, we present *in vivo* and *in vitro* data concerning the performance of the *Pg* promoter in rich medium. By using a *Pg::lacZ* translational fusion and quantitative RT-PCR we have shown that the *hpa* regulatory system is under repression control and that this effect is cAMP-CRP dosage-independent, indicating that the *hpa* regulatory system is still more complex than previously envisioned. Our results reveal that the FIS regulator plays an important role in this effect.

The role of FIS protein in the physiological control of the expression of the *Escherichia coli* meta-hpa operon

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Expression from the *Escherichia coli* W meta-hpa operon promoter (*Pg*) is under a strict catabolic repression control mediated by the cAMP-catabolite repression protein (CRP) complex in a glucose-containing medium. The *Pg* promoter is also activated by the integration host factor (IHF) and repressed by the specific transcriptional regulator HpaR when 4-hydroxyphenylacetate (4HPA) is not present in the medium. Expression from the *hpa* promoter is also repressed in undefined rich medium such as LB, but the molecular basis of this mechanism is not understood. We present *in vitro* and *in vivo* studies to demonstrate the involvement of FIS protein in this catabolic repression. DNase I footprinting experiments show that FIS binds to multiple sites within the *Pg* promoter. FIS-site I overlaps the CRP-binding site. By using an electromobility shift assay, we demonstrated that FIS efficiently competes with CRP for binding to the *Pg* promoter, suggesting an antagonist/competitive mechanism. RT-PCR showed that the *Pg* repression effect is relieved in a FIS deleted strain. The repression role of FIS at *Pg* was further demonstrated by *in vitro* transcription assays. These results suggest that FIS contributes to silencing the *Pg* promoter in the exponential phase of growth in an undefined rich medium when FIS is predominantly expressed.
METHODS

Bacterial strains, plasmids and culture conditions. The strains and plasmids used throughout this study are listed in Table 1. To overexpress the crp gene, the 702 bp EcoR1–HindIII fragment from plasmid pDCRP (Bell et al., 1990), carrying the structural crp gene without the promoter region, was cloned into pUC19 treated with the same endonucleases, to yield the plasmid pBeBe. Strains carrying the Pg::lacZ fusion were constructed and validated previously (Galán et al., 2001). Reporter fusions were inserted into the chromosome of the target strains by the pUT-Kn miniTn5 delivery system (Herrero et al., 1990), which allows the generation of reporter strains carrying translational fusions with the lacZ gene that are stably inserted into their chromosome. The selection of each reporter strain was made among three different candidates with similar expression levels to avoid Pg-unrelated lacZ expression. Cells were grown aerobically in Luria–Bertani medium (LB) at 30 °C, to be consistent with data related to aromatic compound metabolism in E. coli (Prieto et al., 1996; Galán et al., 2001, 2004). When needed, antibiotics were added at the following concentrations: ampicillin, 100 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; rifampicin, 50 µg ml⁻¹; and streptomycin, 50 µg ml⁻¹. General procedures for DNA manipulations were performed as indicated elsewhere (Sambrook & Russell, 2001).

Construction of a FIS null mutant of E. coli W. The fis gene was inactivated by marker exchange as described by Kaniga et al. (1991), using the mobilizable suicide plasmid pKNGΔfis (Table 1). Deletion of the fis gene was engineered with the DNA fragments fis5 and fis3 of 313 and 280 bp respectively, generated by PCR using the primer pairs FISMUT3/FISMcoI for fis5 and FISMUT3/FISAlIII for fis3 (Table 1). These two fragments were digested with the appropriate restriction enzymes and ligated using T4 ligase, resulting in a single 593 bp fragment carrying a 332 bp deletion in the fis gene, which was cloned into the unique BanII and SmaI sites of pKNG101 to yield pKNGΔfis. Plasmid pKNGΔfis was used to deliver the fis mutation to the host chromosome via homologous recombination. Biplateral mating was performed following protocols described by de Lorenzo & Timmis (1994) using E. coli SM10::pir (pKNGΔfis) as donor and E. coli W as recipient. For conjugation, 100 µl of overnight cultures of donor and recipient strains were mixed in 5 ml 10 mM MgSO4 and collected on a Millipore filter, which was subsequently placed on an LB agar plate and incubated overnight at 30 °C. After incubation, the second crossover event was confirmed by PCR using primers FISMUT5 and FISMUT3 (Table 1). The resultant mutant strain was denoted W16 (Table 1).

Assay for β-galactosidase. An overnight culture of cells harbouring the Pg::lacZ translational fusion grown in LB medium was diluted 1:10 into fresh medium and incubated at 30 °C. If required, the inducer 4HPA was added at 1 mM. Aliquots were collected at different cell densities for the β-galactosidase activity determination. For Western blot analysis, these gels were subsequently blotted and probed with a 1:1000 dilution of a mouse polyclonal antiserum against IHF protein of Pseudomonas putida kindly provided by V. de Lorenzo (Centro Nacional de Biotecnologia-CSIC, Spain). The anti-IHF antiserum was pre-absorbed with a 1:10 dilution of E. coli crude extract. The band corresponding to this protein was developed with the ECL Western Blotting Detection kit (Amersham Biosciences) according to the protocol described by the manufacturer.

Protein techniques. SDS-PAGE was by standard methods (Sambrook & Russell, 2001). Aliquots containing E. coli cell suspensions at similar ODmax values were lyzed in phosphate buffer pH 7.5 with 2% SDS and 5% β-mercaptoethanol, and run in denaturing 17.5% polyacrylamide gels. For Western blot analysis, these gels were subsequently blotted and probed with a 1:1000 dilution of a mouse polyclonal antiserum against IHF protein of Pseudomonas putida kindly provided by V. de Lorenzo (Centro Nacional de Biotecnologia-CSIC, Spain). The anti-IHF antiserum was pre-absorbed with a 1:10 dilution of E. coli crude extract. The band corresponding to this protein was developed with the ECL Western Blotting Detection kit (Amersham Biosciences) according to the protocol described by the manufacturer.

DNase I protection experiments. For DNase I footprinting experiments, the 314 bp hpaG–hpaR DNA fragment that was used as a probe was amplified by PCR with primers PG5 and PG3 using 10 ng

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Fig. 1. (a) Regulation of the hpa cluster. The catabolic (hpaBCDEFGHI), transport (hpaX) and regulatory genes (hpaA and hpaR) of the hpa cluster are represented. The block arrows indicate the directions of gene transcription. Pr, Pg, Px, Pa and Pbc are promoter regions. □, Active form of HpaR repressor; ◻, inactive form of HpaA activator; ○, inducer. Transcriptional repression (−) and activation (+) are indicated. (b) Sequence of the hpaR-hpaG intergenic region. Pg and Pr promoter boxes −35 and −10 and the ribosome-binding sites (RBS) are indicated as white boxes. The transcription start site of Pg and Pr (+1), CRP and IHF sites are underlined with a continuous line. ATG start codons of hpaR and hpaG are shown in italics. An asterisk has been placed every 10 nt from +1 of Pg. The ORP1 and ORP2 operators for HpaR are boxed in grey. FIS sites (I–V) detected by in vitro binding are indicated in upper case and dotted underlined.
Table 1. Bacterial strains, plasmids and oligonucleotides with relevant genotype and phenotype

<table>
<thead>
<tr>
<th>Strain, plasmid or oligonucleotide</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli K-12</strong></td>
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<tr>
<td>MC100</td>
<td>F− araD319 Δ(arg-F-loc)U169 relA1 ilvB3501 deoC1 ptsF25 rbsR</td>
<td>Prieto &amp; Garcia (1997)</td>
</tr>
<tr>
<td>MCG11</td>
<td>MC100 with chromosomal insertion of mini-Tn5 KmPg::lacZ, Km' Rif'</td>
<td>Galán et al. (2001)</td>
</tr>
<tr>
<td>CC118 (pir)</td>
<td>Host for pUT-derived plasmids, Rif'</td>
<td>Herrero et al. (1990)</td>
</tr>
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<td>SBPG1</td>
<td>MC100Δcpp with chromosomal insertion of mini-Tn5 KmPg::lacZ, Km' Rif'</td>
<td>Galán et al. (2001)</td>
</tr>
<tr>
<td>S90CPlrif</td>
<td>(Δlac, pro) rpsL, Sm' Rif'</td>
<td>Ferrández et al. (2000)</td>
</tr>
<tr>
<td>S90GPlrif</td>
<td>S90C HimD51:: mini-tet, Rif' Tc' Sm'</td>
<td>Ferrández et al. (2000)</td>
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<tr>
<td>DBP101Rif</td>
<td>DBP101Rif with chromosomal insertion of mini-Tn5 KmPg::lacZ, Km' Rif'</td>
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<td><strong>E. coli W</strong></td>
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<td>WPG11</td>
<td>W14 derivative, Δpaa ΔlacZ with chromosomal insertion of mini-Tn5 KmPg::lacZ, Km' Rif'</td>
<td>Galán et al. (2001)</td>
</tr>
<tr>
<td>W16</td>
<td>W derivative Δfis</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC19</td>
<td>Cloning vector</td>
<td>Sambrook &amp; Russell (2001)</td>
</tr>
<tr>
<td>pBR322</td>
<td>Cloning vector</td>
<td>Sambrook &amp; Russell (2001)</td>
</tr>
<tr>
<td>pDCRP</td>
<td>pBR322 derivative carrying the structural crp gene</td>
<td>Bell et al. (1990)</td>
</tr>
<tr>
<td>pBeBe</td>
<td>pUC19 with 702 bp EcoRI/HindIII fragment from pDCRP carrying the crp gene</td>
<td>This work</td>
</tr>
<tr>
<td>pBM1</td>
<td>pU9J derivative, Pg::lacZ, Ap'</td>
<td>Galán et al. (2001)</td>
</tr>
<tr>
<td>pBF1</td>
<td>hpaR-hpaG region flanked by transcriptional terminators</td>
<td>Galán et al. (2003)</td>
</tr>
<tr>
<td>pKNG71</td>
<td>Sm' Mob+, sacBR, R6K replicon</td>
<td>Kaniga et al. (1991)</td>
</tr>
<tr>
<td>pKNGAfis</td>
<td>pKNG71 derivative carrying the 3' end of gene yhdG and 5' end of gene yhdI from E. coli</td>
<td>This work</td>
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<td>PG5</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>PGDE</td>
<td>ccggattgctgaattcgtagttgtaattag</td>
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plasmid pAJ40 (Table 1) as a template. Both strands were alternately labelled using a combination of one unlabelled primer and a second primer end-labelled with phage T4 polynucleotide kinase [α-32P]ATP (111 TBq mmol−1). Then the PCR fragment was purified using the High Pure PCR Product Purification kit from Roche Diagnostics as described by Galán et al. (2001). Complexes with the labelled promoter region (1 nM full concentration of DNA) were formed for 20 min at room temperature in 15 μl of a glutamate buffer solution (40 mM HEPES pH 8.0, 10 mM MgCl2, 2 mM DTT, 100 mM potassium glutamate) containing 500 μM BSA ml−1 and FIS protein (kindly provided by G. Muskheli Mtshilvili, International University Bremen, Germany). Then 3 μl of DNease I solution (0.15 units in 10 mM Tris/HCl pH 8, 10 mM MgCl2, 125 mM KCl) was added 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, 2.5 mM EDTA, 50 μg tRNA ml−1 and 5 μg salmon sperm DNA ml−1. The samples were extracted with phenol and precipitated with ethanol before analysis on a 7% (v/v) denaturing polyacrylamide gel. Protected bands were identified by comparison with the migration of the same fragment treated for the A+G sequencing reaction (Maxam & Gilbert, 1977).

**Competition gel retardation assays.** For the labelling of the probe in competition gel retardation assays, a DNA fragment of 147 bp containing the Pg promoter was amplified by PCR using 10 ng of plasmid pAJ40 (Table 1) as template and a combination of one unlabelled primer and the second primer 5’ end-labelled with phage T4 polynucleotide kinase [α-32P]ATP (111 TBq mmol−1). The primers used were PG3 and the labelled primer PGDE (Table 1). The reaction mixtures (final volume 20 μl) contained 9 μl glutamate buffer solution with 500 μg BSA ml−1, 0.5 nM DNA probe and purified CRP and FIS proteins. After incubation for 20 min at room temperature, mixtures were fractionated by electrophoresis in 7.5% polyacrylamide gels buffered with 1× TBE (45 mM Tris/borate, 1 mM EDTA). The gels were dried onto Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Pharmacia Biotech).

**Run-off transcription assays.** Single-round transcription by E. coli RNA polymerase (RNAP) was carried out under standard conditions (Marschall et al., 1998), using buffer B (40 mM Tris/HCl pH 8.0, 10 mM MgCl2, 100 mM KCl, 200 μM cAMP and 500 μg BSA ml−1) and supercoiled DNA plasmid pBF1 (Table 1). The final volume of the reaction mixture was 9 μl, containing the plasmid DNA (5 nM).
with either CRP (100 nM) and FIS (100–1000 nM) or buffer. This mixture was incubated at room temperature for 20 min. Then, 3 µl 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 µl. Elongation was started by the addition of 3 µl pre-warmed mixture (1 mM ATP, 1 mM GTP, 1 mM CTP, 50 µM UTP, 37 kBq of [α-32P]UTP and 500 µg heparin ml⁻¹ in buffer B) to the template-polymerase mix. The reactions were allowed to proceed for 5 min at 37 °C and were stopped by the addition of 12 µl 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).

**Real-time RT-PCR assay.** Total RNA was extracted from E. coli W and E. coli W16 cells that were inoculated at OD₆₀₀ of 0.05 in LB medium containing 1 mM 4HPA. Cells were harvested throughout the growth curve (OD₆₀₀ values of 0.05, 0.2, 0.9, 1.8, 3 and 6) and stored at -20 °C. Pellets were thawed and cells lysed in TE buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA) containing 5 mg lysozyme ml⁻¹ by a series of freeze/thaw cycles. RNA was extracted using the RNeasy mini kit (Qiagen), including a DNase I treatment according to the manufacturer’s instructions, precipitated with ethanol, washed and resuspended in 1 µl RNAse-free water. The concentration and purity of the RNA samples were measured by using a ND1000 spectrophotometer (Nanodrop Technologies). Synthesis of total cDNA was carried out with 20 µl reverse transcription reactions containing 1 µg RNA, 0.5 mM dNTPs, 200 U SuperScript II Reverse Transcriptase (Invitrogen) and 5 µM of random hexamers as primers, in the buffer recommended by the manufacturer. Samples were initially heated at 65 °C for 5 min and then incubated at 42 °C for 1 h, terminated by incubation at 70 °C for 15 min. The cDNA obtained was purified using GeneClean Turbo kit (MP Biomedicals) and the concentration was measured using a ND100 Spectrophotometer (Nanodrop Technologies). For the analysis of the transcript levels from the Pg promoter, target cDNAs (0.5, 2 and 5 ng) and reference samples were amplified three times in separate PCR with 0.2 µM each of HpaG5 and HpaG3 primers by using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Real-time PCR was performed using SYBR Green technology in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Samples were initially denatured by heating at 95 °C for 4 min, followed by 30 cycles of amplification (95 °C, 1 min; test annealing temperature, 65 °C, 1 min; elongation and signal acquisition, 72 °C, 30 s). For relative quantification of the fluorescent values, a calibration curve was made using dilution series from 200–0.2 ng of E. coli W genomic DNA sample.

**RESULTS**

**Monitoring the Pg expression profile in rich medium**

To investigate the expression profile of the Pg promoter in undefined rich medium, we have exploited the E. coli reporter strain WPG11 (Pg::lacZ, hpa⁺) as a reliable system (Galán et al., 2001). E. coli WPG11 cells were cultured in LB at 10 °C in the presence and absence of 4HPA as inductor, and the β-galactosidase activity was determined along the growth curve (Fig. 2a). As expected, Pg expression was negligible both in the exponential and in the stationary phases of growth when 4HPA was not added to the medium. β-Galactosidase levels were also low (40–60 Miller units) during the exponential phase of growth in the presence of 4HPA and they increased 2.8-fold at the onset of the stationary phase, i. e. after approximately 5–6 h of growth. These results strongly suggested that the promoter of this aromatic catabolic pathway was under strong repression when cells were growing in rich medium. It was initially assumed that a putative mechanism that could explain the observed Pg repression in the exponential phase may be mediated by an overexpression of the HpaR repressor, which in turn might produce an over-repression effect. To analyse this possibility, we monitored the Pg activity in an E. coli MCG11 that naturally lacks the hpa cluster (hpa⁺) (Galán et al., 2001) (Fig. 2b). As expected, the levels of β-galactosidase were fourfold higher in the absence of repressor (Fig. 2). Additionally, Fig. 2(b) shows that the repression at exponential phase of Pg activity can be reproduced in the reporter E. coli K-12 strain MCG11 (hpa⁺, Pg::lacZ) (Table 1). Since this strain does not contain the HpaR repressor or any other hpa gene product, we can conclude that the factors involved in the physiological control of Pg promoter at exponential phase

![Fig. 2. Induction of the Pg promoter in rich medium. E. coli WPG11 (a) and E. coli MCG11 (b) harbouring a Pg::lacZ fusion integrated into the chromosome were grown for 10 h in LB medium. Aliquots were taken at different times, and β-galactosidase (Miller units) was measured. Growth is indicated as optical density at 600 nm (OD₆₀₀).](image-url)
in *E. coli* W (hpa<sup>+</sup>) are also present in *E. coli* K-12 (hpa<sup>-</sup>). Therefore, this effect may be due to global regulators of the host cell rather than to specific hpa genes.

**Influence of the global regulators IHF and CRP on the Pg repression effect in rich medium**

As mentioned above, the superimposed regulation of the *Pg* promoter in a glucose-containing medium is mediated by a very severe regulatory system driven by the global regulators IHF and CRP (Galán *et al.*, 2001). Thus, we investigated the involvement of these regulators in the repression effect observed in rich medium. We first analysed the putative role of IHF in the activation of *Pg* when cells were growing in LB medium, by measuring the β-galactosidase activity in the two isogenic strains of *E. coli* DPBG11 (IHF<sup>-</sup>, *Pg*::lacZ) and *E. coli* S90G11 (IHF<sup>+</sup>, *Pg*::lacZ) (Table 1). Fig. 3(a) reveals that the absence of IHF strongly reduces *Pg* expression during stationary phase. It is well known that in the transition from the exponential growth phase to the stationary phase, IHF becomes the second-most-abundant protein of the nucleoid, reaching a maximum of 55 000 monomers per cell early in stationary phase (Azam & Ishihama, 1999). Thus, it could be presumed that a reduction of the IHF concentration below a certain threshold would decrease the activity of the *Pg* promoter. To investigate the correlation between the concentration of IHF and the downregulation of *Pg* promoter in the exponential phase, *E. coli* MCG11 (*Pg*::lacZ, hpa<sup>-</sup>) was transformed with plasmid *pP*<sub>H</sub>*hiP*<sub>H</sub>*himA*-5 (Table 1) to generate *E. coli* MCG11(*pP*<sub>H</sub>*hiP*<sub>H</sub>*himA*-5), which overproduces IHF (Nash *et al.*, 1987). The IHF production of this strain was followed along the growth curve by Western blot vs the control MCG11(*pBR322*) (data not shown). The results shown in Fig. 3(b) demonstrated that the repression of *Pg* during exponential phase of cells growing in LB medium was not overcome by the overproduction of IHF. However, as expected, high IHF levels led to an increase in the *Pg* activity in the stationary phase. Therefore, we can conclude that the exponential repressing effect observed in rich medium is not caused by a limiting concentration of IHF during this phase of growth. IHF was necessary to activate *Pg* in the stationary phase.

Furthermore, we checked the influence of CRP on the *Pg* activity when cells were cultured in LB by monitoring the *Pg* activity in the isogenic *E. coli* strains MCG11(*pUC19*) (CRP<sup>+</sup>, *Pg*::lacZ), SBPG11 (CRP<sup>-</sup>, *Pg*::lacZ) and MCG11(*pBeBe*), which overproduces CRP. Overproduction of the CRP protein in MCG11(*pBeBe*) was followed by SDS-PAGE (data not shown). The lack of CRP in strain SBPG11 led to a complete repression of the *Pg* promoter throughout the growth curve in LB, demonstrating that this global regulator is essential for *Pg* transcription. Overexpression of CRP did not alter *Pg* expression. Moreover, the external addition of 5 mM cAMP to a culture of the overproducing strain did not have any effect on *Pg* activity, excluding the involvement of cAMP in this effect (data not shown). Our results indicated that the exponential repressing effect observed in rich medium is not caused by a limiting concentration of CRP during this phase of growth (Fig. 3).

**Pg repression is relieved in a FIS mutant when cultured in LB medium**

*In silico* analysis of the *Pg* promoter revealed the presence of a putative factor for inversion stimulation (FIS) binding site located within the *Pg* promoter (see below). FIS is a nucleoid-binding protein involved in global regulatory circuits related to repression of genes that are not required
under conditions of optimal growth (González-Gil, et al., 1996). It is also well known that FIS acts as a positive regulator of the transcription of the genes that encode stable RNA species, such as rRNA and tRNA (Ross et al., 1990; Bosch et al., 1990; Nilsson & Emilson, 1994; Gralla, 2005). To investigate the putative role of FIS in the hpa regulatory system, we generated a FIS mutant of the E. coli strain W, named W16 (Table 1). The expression profiles of hpaG, the first gene of the hpa-meta operon (Fig. 1), were monitored and compared throughout the growth curve in strains W and W16. Initially, we studied the Pg expression profile in a FIS mutant using the lacZ reporter system. Using this method, the data presented related to CRP and IHF involvement in rich medium were reproducible and reliable. However our statistics were not reproducible in the early exponential growth phase for the FIS mutant carrying Pg:: lacZ fusion, resulting in a very high deviation of the data; this was very likely to have been generated by FIS-dependent regulation effects related to the synthesis of the translational machinery. To avoid misinterpretations, real-time RT-PCR was the method selected for these assays. Transcription of hpaG was detected in the wild-type from mid-exponential to stationary phase of growth, reaching a maximum at early stationary phase (Fig. 4). These results are in agreement with those obtained from the reporter strain WPG11 (Fig. 2a) concerning the growth-phase-dependent Pg expression. However, in the absence of FIS protein, Pg expression is detected at an earlier stage of growth, reaching its maximum at the mid-exponential phase. Remarkably, the transcription of hpaG was higher in strain W. In both strains, the hpaG mRNA levels decreased considerably at the end of the stationary phase. However, in the FIS mutant the activity of Pg decreased earlier than in the wild-type strain. An explanation for this will require further experimentation. These results strongly suggest that FIS is involved in the Pg repression effect observed in rich medium.

**FIS binds to the Pg promoter**

The ability of purified FIS protein to bind the hpaR-hpaG intergenic DNA region was analysed by DNase I footprinting experiments using the whole hpaR-hpaG DNA region. These experiments showed that FIS bound to this promoter region and revealed the existence of five FIS-binding sites (FIS-site I to V) (Figs 1 and 5a, b). FIS-site I, which is centred at position −52 relative to the Pg +1 site, overlaps the CRP binding site at position −61.5, suggesting that the two proteins compete for binding to the Pg promoter (Fig. 1). FIS-site II (centred at position −28) and FIS-site III (centred at position −11) overlap the −35 and −10 boxes of the Pg promoter, respectively, suggesting competition between FIS and the RNAP for Pg binding. Finally, FIS-sites IV and V, centred at positions −119 and −188, respectively (Fig. 5a, b) could also be involved in the regulation of the divergent Pr promoter. The binding of FIS to these motifs produces the characteristic DNase I hypersensitive sites, suggesting that it changes the DNA topology. The concentration of FIS needed to protect site I is 3 nM, in contrast to that needed for FIS-sites II to V, which is 10-fold higher (Fig. 5a, b). Nucleotide sequence comparison of the protected sites with the consensus sequence for the FIS-binding motif (Finkel & Johnson, 1992; Hengen et al., 1997) allowed the unambiguous definition of the core binding sites as shown in Fig. 5(c). Each binding site differs by one nucleotide to the consensus sequence, except for FIS-site IV, which shows the best match to this consensus.

**FIS represses transcription at the Pg promoter by a coordinate mechanism involving CRP**

FIS-site I overlaps the CRP-binding site on the Pg promoter, suggesting that FIS and CRP compete for binding to this DNA region. To investigate this hypothesis we performed a competition gel retardation assay with purified CRP and FIS (Fig. 6a). The addition of FIS alone up to 30 nM generated two different complexes of retarded bands: PG-FIS (1) and PG-FIS (2) (Fig. 6a, lanes 6–8). When FIS was added after CRP binding, the CRP-specific shift decreased as the concentration of FIS increased, and the specific bands for FIS–DNA complexes were detected predominantly. The effect of FIS on transcription initiation at the Pg promoter was checked by a single-round in vitro transcription assay using plasmid pBF1 as template (Table 1), incubated with CRP, RNAP and increasing amounts of FIS (Fig. 6b). When FIS was supplied to the reaction mixture, the Pg activity was repressed and only the RNAI control transcript was detected, demonstrating that this protein was able to act as a repressor in vitro. These results suggested a coordinate regulatory mechanism of repression involving at least both CRP and FIS global regulators.
DISCUSSION

Bacterial genes encoding carbon-catabolic enzymes of peripheral metabolism are often regulated in response to the availability of the respective substrate through specific regulatory systems/proteins (Díaz & Prieto, 2000). However, if a rapidly metabolizable carbon source is additionally present in the growth medium, the production of the peripheral catabolic enzymes can be downregulated until they are required. This general regulatory phenomenon, known as carbon catabolite repression, forms part of the superimposed regulatory systems of catabolic pathways that connect the expression of a peripheral metabolic pathway with the metabolic status of the cell (Cases & de Lorenzo, 1998; Galán et al., 2001; Prieto et al., 2004). In this study we have further investigated the methods utilized by a paradigmatic micro-organism, E. coli, to control the expression of genes associated with metabolism of aromatic compounds when it detects the presence of preferential carbon sources (Prieto et al., 2004). As a model system, we have used the meta operon of the hpa cluster for the degradation of 4HPA, 3HPA and HPC, whose expression is driven by the Pg promoter. The results presented here illustrate that the expression of the hpa genes is controlled by a non-conventional catabolic repression regulatory system when the cells are cultured in an undefined rich medium such as LB, which only favours hpa transcription at the onset of the stationary phase of growth. The classical catabolite repression has been well documented for E. coli, where glucose is the most preferred carbon source, and the phosphotransferase system and the CRP–cAMP complex direct this phenomenon (Busby & Ebright, 1999; Kolb et al., 1993). Our results suggest that the Pg repression effect

Fig. 5. FIS binding to the hpar-hpaG intergenic region: DNase I footprinting analysis of the interaction of the FIS protein with the Pg promoter region. The reaction mixture was treated as described in Methods, using the 5′-end-labelled non-coding strand (a), and the 5′-end-labelled coding strand (b) of the hpar-hpaG intergenic region as probes. DNase I hypersensitivity bands are indicated by arrows. The A+G sequencing ladder is indicated with an asterisk. Lane 1, naked DNA; lane 2, 3 nM FIS; lane 3, 10 nM FIS; lane 4, 30 nM FIS; lane 5, 100 nM FIS. (c) Alignment of the FIS-binding sites and comparison with the consensus FIS-binding motif (Finkel & Johnson, 1992; Hengen et al., 1997). The more conserved bases are indicated by capital letters, and the less conserved by lower-case letters. The positions that match the consensus sequence are shaded.

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Fig. 6. Repressor role of FIS on the Pg promoter. (a) Competition analysis between FIS and CRP in promoter binding. Gel retardation analysis was performed as described in Methods. The presence (+) or absence (–) of 100 nM purified CRP in the reaction mixture is indicated. FIS concentration was 3 nM (lanes 3 and 6), 10 nM (lanes 4 and 7) and 30 nM (lanes 5 and 8). The detected DNA–protein complexes, PG-FIS (1) and PG-FIS (2), PG-CRP, and the position of the corresponding free DNA band, PG, are indicated by arrows. (b) Effect of FIS on Pg-mediated transcription. In vitro transcription analysis was performed as indicated. CRP (lanes 2–5) and RNAP (lanes 1–5) concentrations were 100 nM. FIS concentration was 100 nM (lane 3), 300 nM (lane 4), and 1 μM (lane 5). The detected transcripts are indicated by arrows. RNAI is an internal control of pBF1.
observed when the bacteria are growing in rich medium is mediated by a more complex regulation system and that other global regulators, rather than the ordinary CRP-cAMP or even IHF as described previously for the hpa cluster in defined media, are involved in its control (Galañ et al., 2001; Prieto et al., 2004). This physiological control is independent of the specific regulatory system of the hpa pathway directed by the HpaR regulator, because several engineered E. coli hpa− reporter strains showed similar Pg expression profiles to that of the wild-type hpa+ strain (Fig. 2). Our results show that FIS binds to multiple sites in the Pg promoter and negatively regulates the transcription of hpa genes (Fig. 5a, b). FIS interacts with the Pg promoter at five FIS sites (I–V) showing different affinities for the hpaG-hpaR intergenic region. FIS-sites II and III are centred at positions −28 and −11 with respect to the Pg transcription initiation site overlapping with the RNAP-binding region at Pg. FIS-site I overlaps the CRP-binding site in the Pg promoter and shows the highest affinity (about 10-fold higher than the other four sites). Finally, FIS-site IV overlaps the IHF binding site at Pg. FIS is a small nucleoid binding protein that accumulates dramatically in the cell during growth in rich medium, and becomes one of the most abundant DNA-binding proteins in early middle exponential phase; it varies in amount with the growth phase, from fewer than 100 copies in stationary phase to over 50 000 copies per cell in exponential phase. However, as cells approach late exponential phase, FIS synthesis ceases, resulting in undetectable levels in the stationary phase (Ball et al., 1992). FIS is known to play a variety of roles at different promoters in E. coli. It is involved in site-specific DNA recombination and participates in the regulation of growth-related genes, assuring the synthesis of the translational machinery in rapid growth (González-Gil et al., 1996; Gralla, 2005). In accordance with its expression profile, FIS is found to directly repress genes that are not required under conditions of optimal growth, such as those whose products are involved in the utilization of alternative carbon sources and nitrate (Xu & Johnson, 1995; González-Gil et al., 1996; Browning et al., 2004b, 2005; Kelly et al., 2004; Bradley et al., 2007). In agreement with this global regulatory function, FIS appears to switch off expression of hpa genes in rich medium when the enzymes for the catabolism of 4HPA are not required. According to the localization of the FIS binding sites in the Pg promoter (Fig. 5), a repression mechanism involving interferences between FIS-RNAP (sites II and III), FIS-IHF (FIS IV) and/or FIS-CRP (FIS I) binding sites at Pg has to be considered. Competitive EMSA indicates that FIS can displace CRP from the FIS I site. In addition, in vitro transcription assays indicate that FIS can inhibit the CRP-dependent transcription activation at the Pg promoter. A similar mechanism has been previously described for several promoters, including the aldB, acsP2, bgl, hupB and crp promoters from E. coli, driving, respectively, the transcription of genes encoding aldehyde dehydrogenase, the acetyl-coenzyme A synthetase, the β-glucoside operon, the histone-like protein HU-B and the CRP protein itself (Xu & Johnson, 1995; Browning et al., 2004a; Caramel & Schnetz, 2000; Claret & Rouviere-Yaniv, 1996; González-Gil et al., 1998). In each case, a number of FIS-binding sites which may contribute to repression are located in the promoter region but one of the high-affinity FIS-binding sites overlaps the CRP-binding site, directly occluding the binding of the CRP activator. Interestingly, CRP expression is regulated by this anti-activation mechanism mediated by FIS. FIS represses crp transcription (González-Gil et al., 1998) and precludes transcription of the target promoters indirectly by restricting the availability of CRP. FIS also represses its own transcription. Thus, after its initial burst of synthesis, FIS levels drop, derepressing crp transcription. In view of our findings, the repression mechanism could consist of an antagonist/competitive mechanism inhibiting the binding of the CRP activator at the Pg promoter. However, a possibility that cannot be excluded is that a FIS multi-protein complex binds throughout the Pg region (sites I–IV) to drive the promoter into a more solid inhibitory structure, also inhibiting the interaction of RNAP and IHF.

Repression in rich medium has also been described in other micro-organisms, e.g. Bacillus (Saier et al., 1995, 1996) and Pseudomonas (Cases & de Lorenzo, 1998; Dinamarca et al., 2002; Holtel et al., 1994; Yuste & Rojo, 2001; Petruschka et al., 2001; Sze et al., 1996; Sze & Shingler, 1999), but the molecular mechanisms involved in repression in these micro-organisms are quite different (Cases & de Lorenzo, 2000; Cases et al., 1996, 1999; Dinamarca et al., 2002; Moreno et al., 2007).

The metabolic abilities of E. coli to cope with aromatic compounds make this bacterium a very useful model system to decipher biochemical, genetic, evolutionary and ecological aspects of this particular type of less-preferred carbon source metabolism (Prieto et al., 2004). The highly strict repression mechanism modulated by FIS, CRP and IHF on the Pg promoter of the hpa-meta cluster is quite unusual, since this repression control does not require the presence of specific regulators and it constitutes a fascinating model system to unravel a regulatory network governing the metabolism of less-preferred carbon sources.

ACKNOWLEDGEMENTS

We thank D. Díaz and M. Carmona for helpful comments and critical reading of the manuscript. We thank Begoña Torres for the help in the construction of plasmid pBeBe. We are indebted to Victor de Lorenzo (Centro Nacional de Biotecnología-CSIC, Spain) for the kind gift of the antibodies against IHF, and to G. Muskeshlishvili (International University Bremen, Germany), for the FIS purified protein. This work was supported by Comunidad Autónoma de Madrid Grant AMB-259-0505, by Comisión Interministerial de Ciencia y Tecnología Grants BIO2007-67304-C02 and CTM2006-04007, by European Union Grants GEN 2006-27750-C5-3-E and NMP2-CT-2007-026515, and by the Programme de Recherche Fondamentale en Microbiologie, Maladies Infectieuses et Parasitaires.
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Edited by: J. Green