The regulation of the *Pg* promoter, which controls the expression of the *meta* operon of the 4-hydroxyphenylacetic acid (4-HPA) catabolic pathway of *Escherichia coli* W, has been examined through *in vivo* and *in vitro* experiments. By using *Pg-lacZ* fusions we have demonstrated that *Pg* is a promoter only inducible in the stationary phase when cells are grown on glucose as the sole carbon and energy source. This strict catabolite repression control is mediated by the cAMP receptor protein (CRP). This event does not require the presence of the specific *HpaR* repressor or the 4-HPA permease (*HpaX*), excluding the involvement of a typical inducer exclusion mechanism. However, the acetic acid excreted in the stationary phase by the cells growing in glucose acts as an overflow metabolite, which can provide the energy to produce cAMP and to adapt the cells rapidly to the utilization of a new less preferred carbon source such as the aromatic compounds. Although *Pg* is not a σ^38^-dependent promoter, it is activated by the global regulator integration host factor (IHF) in the stationary phase of growth. Gel retardation assays have demonstrated that both CRP and IHF simultaneously bind to the promoter region. DNase I footprint experiments showed that cAMP-CRP and IHF binding sites are centered at −61.3 and −103, respectively, with respect to the transcription start site +1 of the *Pg* promoter.

Specific regulatory proteins and regulated promoters are the key elements that allow catabolic operons to be transcribed only when required and at levels sufficient to guarantee an adequate metabolic return when the particular substrate is abundant and can serve as a nutrient source (1, 2). However, very often additional regulatory circuits are found superimposed onto the gene-specific effects. Together they allow a coordinated response to the catabolic status of the bacteria (3). The classic example of this phenomenon is the repression of the synthesis of many catabolic enzymes in enteric bacteria by the presence of glucose in the culture medium (4). This phenomenon, termed “glucose effect,” regulates the transcription of catabolic operons by modulating transcription factor availability. The prototype system, which has been well characterized, is the glucose-lactose diauxie in the lactose operon of *Escherichia coli* (5, 6). First, glucose prevents the entry of lactose into the cell, resulting in an increase in the concentration of the inducer-free lac repressor (LacI). This process, called “inducer exclusion,” requires a functional phosphoenolpyruvate-sugar phosphotransferase system (PTS).

The phosphorylatable PTS protein IIA_Glc (glucose-specific IIA protein) controls the activity of the sugar-specific targets, e.g. it controls the permease LacY of the lac operon (6). A slight variant on the strategy for inducer exclusion is found in the *glp* operon for glycerol utilization, which involves facilitated diffusion. The target of IIA_Glc protein in this case is the first catabolic enzyme (7). Second, glucose lowers the level of the CRP-cAMP complex by reducing cAMP levels due to a decrease in the phosphorylated form of enzyme IIA_Glc (8, 9). It also decreases the CRP concentration by diminishing the rate of transcription initiation at the *crp* promoter (9). Hence, in the case of the *lac* operon, the disruption of the *lacI* gene or the use of isopropyl β-D-thiogalactoside as inducer only partially abolished the glucose effect (6–10), indicating that catabolite repression due to changes in cAMP/CRP levels does partially contribute to the glucose effect in this system (11). However, although CRP and cAMP provide the principal means of affecting catabolite repression (4), cAMP-independent mechanisms mediating catabolite repression in *E. coli* have been also described (12).

Although it is well known that *E. coli* controls the expression of the catabolic pathways of less preferred substrates, such as lactose, by a catabolite repression mechanism, very few data are available concerning the influence of this mechanism on the regulation of the catabolism of aromatic compounds (13–16), and a detailed study of the glucose effect on the mineralization of these compounds has not been reported. Moreover, since *E. coli* contains specific transport proteins for some of these substrates (17), it seemed possible that the glucose effect could be mediated by a conventional inducer exclusion process.

The *hpa* cluster of *E. coli* W codes for a group of proteins involved in the catabolism of 4-hydroxyphenylacetic acid (4-HPA) (14) (Fig. 1). The catabolic genes are organized in two operons: the *hpaBC* operon, encoding the two-component 4-HPA monooxygenase, which transforms 4-HPA into 3,4-dihydroxyphenylacetic acid (HPC) (18, 19), and the so-called *meta* operon (*hpaGEDFHI*), which codes for the enzymes that cleave the aromatic ring of HPC and allows its complete mineralization (14). The *hpaX* gene codes for a member of the superfamily of transmembrane facilitators involved in 4-HPA uptake (17). The transcription of the *hpa* cluster is controlled by the prod...
ucts of the HpaA and hpaR genes. HpaA is an activator belonging to the XylS/AraC family of regulatory proteins that regulates the expression of the upper pathway operon (hpaBC) but does not seem to be involved in the regulation of the meta operon (13). Carbon catabolite repression control has also been described for the hpaBC operon (13). For the meta operon, Roper et al. (20) have suggested that HpcR (the HpaR homologue in E. coli C) represses its expression and that 4-HPA and HPC are the inducers of the system. HpaR has been identified through amino acid sequence comparisons as a member of the MarR family, a group of regulatory factors whose activity is modulated in response to environmental signals such as those of phenolic compounds derived from plants (2, 13). Moreover, sequence analyses revealed a putative CRP binding site localized upstream the −35 promoter region of the Pg promoter driving the expression of the meta operon (Fig. 1) (14, 20).

In this work, we have used different genetic and biochemical approaches to demonstrate that the expression of the meta operon of the 4-HPA pathway of E. coli W is repressed by a very severe glucose effect, and we provide evidence that the integration host factor (IHF) acts in collaboration with the CRP-cAMP system, designing a novel complex mechanism to regulate tightly the catabolism of this aromatic substrate.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Media, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table I. Unless otherwise stated, bacteria were grown in Luria-Bertani (LB) medium (26) at 37 °C. Growth in M63 minimal medium (27) was achieved at 30 °C using the corresponding necessary nutritional supplements and 30 mM acetate, 20 mM glycerol, or 10 mM glucose as carbon source. When required, 1 mM 4-HPA was added to the M63 minimal medium (27) at 30 °C using the corresponding necessary nutritional supplements and 30 mM acetate, 20 mM glycerol, or 10 mM glucose as carbon source. When required, 1 mM 4-HPA was added to the M63 minimal medium (27) at 30 °C using the corresponding necessary nutritional supplements and 30 mM acetate, 20 mM glycerol, or 10 mM glucose as carbon source.

DNA and RNA Manipulations—DNA and RNA manipulations and other molecular biology techniques were essentially performed as described (26). Transformation of E. coli cells was carried out by using the RhClI method or by electroporation (Gene Pulser; Bio-Rad) (28). RNA dot blot analyses were performed as previously described (26). To construct the Lac probe containing the lacZ gene, plasmid pU9 was digested with SacI and BamHI endonucleases, and the 1.7-kilobase pair DNA fragment was isolated and labeled with digoxigenin by using a Digo DNA Labeling kit (Roche Molecular Biochemicals). Nucleotide sequences were determined directly using plasmid pBM1 (Table I). Oligonucleotides were synthesized on a DNA synthesizer (Beckman Instruments). Standard protocols of the manufacturer for Taq DNA polymerase-initiated cycle sequencing reactions with fluorescently labeled deoxyxynucleotide terminators (Applied Biosystems Inc.) were used. The sequencing reactions were analyzed using an ABI Prism 377 automated DNA sequencer (Applied Biosystems Inc.). DNA fragments were purified by standard procedures using Gene Clean (BIO 101, Inc., Vista, CA).

Construction of Strains Harboring a Translational Pg::lacZ Fusion in The Chromosome—To construct a translational fusion of the Pg promoter region of hpaG 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 I) as template and the following primers: PG5 (5'-AACGCAAGATTCTGAGGCTGATTATCCCTGCC-3'; an engineered EcoRI site is underlined) and PG3 (5'-GATAGTGGGATCATGTTACACTCTCGAGGATCC-3'; an engineered BamHI site is underlined). To create plasmid pEM1 (Table I), the PCR-amplified fragment was cut with EcoRI and BamHI endonucleases and ligated to the EcoRI and BamHI double-digested promoterless lacZ vector pLacP (Table I). The correct fusion was verified by sequence analysis. Plasmid pG11 was constructed by subcloning the Ncol cassette of pEM1 into the mini-Tn5 delivery plasmid pUT-Km (Table I), and it was used for the insertion of the Pg::lacZ fusion into the chromosome of E. coli AF15 (lacZ mutant of E. coli W), generating strain WPG11. It should be noted that the WPG11 strain, like the parental W strain, contains the complete hpa cluster in the chromosome (Fig. 1). When WPG11 cells were grown in M63 minimal medium containing acetate, glycerol, or glucose as carbon sources and then exposed to the presence or absence of 1 mM 4-HPA, we observed that the lacZ gene was only expressed in the presence of the 4-HPA inducer (Fig. 2). With glycerol or acetate, the maximum lacZ expression was detected during the exponential phase of growth and decreased at the onset of the stationary phase (Fig. 2). However, in the case of glucose, the production

RESULTS

Pg Is a Stationary Phase Inducible Promoter in the Presence of Glucose—We have analyzed in vivo the influence of the carbon source on expression driven by the Pg promoter (Fig. 1) by constructing a translational fusion with the reporter lacZ gene. The Pg::lacZ fusion was first inserted into the chromosome of E. coli AF15 (lacZ mutant of E. coli W), generating strain WPG11. It should be noted that the WPG11 strain, like the parental W strain, contains the complete hpa cluster in the chromosome (Fig. 1). When WPG11 cells were grown in M63 minimal medium containing acetate, glycerol, or glucose as carbon sources and then exposed to the presence or absence of 1 mM 4-HPA, we observed that the lacZ gene was only expressed in the presence of the 4-HPA inducer (Fig. 2). With glycerol or acetate, the maximum lacZ expression was detected during the exponential phase of growth and decreased at the onset of the stationary phase (Fig. 2). However, in the case of glucose, the production
of β-galactosidase was only detected in the late stationary phase. These results suggested that the system was under catabolite repression control and that, when the cells were cultured in a glucose-containing medium, the Pg promoter was not induced and behaved as a stationary phase promoter even in the presence of the 4-HPA inducer.

Down-regulation of Pg by Glucose Is Not Mediated by the hpa Genes—As already mentioned, the LacI repressor, the reduction in cAMP and CRP levels, and the inducer exclusion mechanism mediated by the PTS system all contribute to prevent the expression of the lac operon when E. coli cells are grown on glucose and lactose is used as inducer. This multivalent mechanism of control can be considerably bypassed when isopropyl β-D-thiogalactoside is added to the medium (6–10). This means that a typical catabolic repression mechanism such as that controlling the expression of the lac operon would be insufficient to control the expression of an operon if the inducer can enter the cell by passive diffusion. Previously, we have shown that when 4-HPA is present in the medium at concentrations lower than 10 μM, E. coli W is able to use an active uptake system mediated by the HpaX permease, whereas when 4-HPA is present at 1 mM, E. coli W takes up this compound by passive diffusion (17). Therefore, the strong repression of the Pg promoter observed in the presence of glucose, when 4-HPA was added to the medium at high concentrations (1 mM), cannot be ascribed to a typical inducer exclusion mechanism. However, the inhibition of other catabolic enzymes, as in the case of the glp operon (7), could contribute to the observed glucose effect and restrict the expression of the Pg promoter in the stationary phase. Other possibilities were that the HpaR repressor could be insensitive to the effect of the inducer during growth on glucose or could be turned into an activator only during stationary phase. To determine if the presence of the HpaR regulator or other genes of the hpa cluster were necessary for Pg repression during exponential phase, we analyzed the expression of the Pg-lacZ fusion in E. coli K12, which is devoid of the complete hpa cluster (14). E. coli AFMC, an MC4100 derivative, was selected as host for the Pg::lacZ fusion, generating the strain MCG11 (Pg::lacZ hpa−) (Table I). In glucose-containing M63 medium, the MCG11 strain yields higher β-galactosidase levels during the exponential phase of growth than the strain WPG11, due probably to the lack of hpaR. However, we observed the highest expression of the reporter gene in the late stationary phase as previously found in the WPG11 strain (Fig. 3). These results demonstrate that the activation of the Pg promoter in the stationary phase is independent of the presence of the HpaR protein or other proteins encoded by the hpa cluster. They also strongly suggested that the repression during exponential phase of growth was mediated by an extreme catabolic repression mechanism that switches off the Pg promoter when the cells are using glucose as carbon source, even in the presence of 4-HPA that can enter the cell by passive diffusion.

Carbon Starvation Response of the Pg Promoter—To test if the depletion of glucose was the key factor for the activation of the Pg promoter, WPG11 cells previously grown in glucose, without 4-HPA, were harvested, washed, and resuspended in different media. These cells were incubated for 2 h in fresh M63 salt medium containing only 1 mM 4-HPA or in spent M63-glucose medium (medium that had already supported cell growth during 10 h, filtered and sterilized after adjustment of the pH to 7.0) plus 1 mM 4-HPA (Fig. 4A). Although we could not detect a significant increase in cell density, the cells exposed to spent M63-glucose medium produced 4.7-fold more β-galactosidase than cells diluted in fresh M63 medium (Fig.
cells were incubated for 2 h in the presence of 5 mM 4-HPA (not the only inducing factor. Fig. 4A). These results suggest that the spent medium contains a compound that facilitates the rapid response of the expression system. Interestingly, the addition of 10 mM glucose to the spent M63-glucose medium inhibited the production of cAMP to fresh M63 salt medium with 4-HPA did not increase the production of cAMP (Fig. 4A). Therefore, although we cannot exclude the possibility that the extracellular cAMP, produced at the end of exponential growth on glucose, could contribute to activate Pg, this finding demonstrates that it is not the only inducing factor. Fig. 4B shows that when WPG11 cells were incubated for 2 h in the presence of 5 mM 4-HPA (i.e., the standard concentration utilized to grow E. coli using 4-HPA as sole carbon and energy sources), the Pg activation is more efficient than when the cells are exposed to 1 mM 4-HPA. However, after 4 h of incubation, the levels of β-galactosidase are identical with both substrate concentrations (data not shown). This result suggests that the utilization of 4-HPA as carbon and energy source requires an adaptive process to fully stimulate the 4-HPA metabolism when the concentration is lower than 5 mM. Taking into account these observations, we considered the possibility that the spent medium might contain an alternative energy source to overcome the slower Pg activation.

Acetic Acid Accumulates in Spent M63-glucose Medium—To monitor glucose consumption and the accumulation of an alternative carbon and energy source in the culture medium in the stationary phase of growth of E. coli WPG11 cells cultured in M63-glucose medium, the spent medium was analyzed by HPLC. We observed that when glucose was exhausted in the spent M63-glucose medium after 10 h of culture, two new products with retention times of 12.4 and 15.6 min were detected in the HPLC chromatogram. The latter product was identified as acetic acid, and its concentration was determined to be about 3 mM. The other compound could not be identified, although other putative metabolites tested as standards like D-lactate (11.3 min), formate (12.0 min), and succinate (11.9 min) did not correspond to this unknown substance.

To determine if acetic acid could be the metabolite responsible for the activation effect observed with the spent M63-glucose medium, washed WPG11 cells previously grown in glucose, in the absence of the inducer, were incubated for 2 h in fresh M63 minimal medium containing 1 mM 4-HPA and 3 mM acetic acid (Fig. 4A). Under these incubation conditions, the Pg promoter was induced at a similar level to that observed with the spent M63-glucose medium, without a significant increase of the cell density (Fig. 4A). To determine if this activation effect was specific for acetic acid, other substances were tested at similar concentrations. Thus, we observed that, in contrast to glucose, D-lactate, formate, and succinate were also able to activate the Pg promoter in the presence of 4-HPA (Fig. 4A).

It is well known that the extracellular concentration of cAMP increases upon glucose exhaustion (52); thus, cAMP could be a putative candidate as inducer. However, the addition of 5 mM cAMP to fresh M63 salt medium with 4-HPA did not increase the production of β-galactosidase (Fig. 4A). Therefore, although we cannot exclude the possibility that the extracellular cAMP, produced at the end of exponential growth on glucose, could contribute to activate Pg, this finding demonstrates that it is not the only inducing factor. Fig. 4B shows that when WPG11 cells were incubated for 2 h in the presence of 5 mM 4-HPA (i.e., the standard concentration utilized to grow E. coli using 4-HPA as sole carbon and energy sources), the Pg activation is more

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**TABLE I**

**Bacterial strains and plasmids with relevant genotype and phenotype**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli K-12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17 (ApE)</td>
<td>Host for pUT-derived plasmids</td>
<td>Ref. 21</td>
</tr>
<tr>
<td>CC118 (ApE)</td>
<td>Host for pUT-derived plasmids, Rif&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 22</td>
</tr>
<tr>
<td>AFMC</td>
<td>MC4100 Rif&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 15</td>
</tr>
<tr>
<td>AF98</td>
<td>MC4100 rpoS Rif&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 15</td>
</tr>
<tr>
<td>RH90</td>
<td>MC4100 rpoS::Tn5, Te&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ref. 23</td>
</tr>
<tr>
<td>S90CRif</td>
<td>(Δlac, pro) rpsL Sm&lt;sup&gt;+&lt;/sup&gt;, Rif&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 15</td>
</tr>
<tr>
<td>DPB101Rif</td>
<td>S90C himD451:: mini-tet, Rif&lt;sup&gt;+&lt;/sup&gt;, Te&lt;sup&gt;c&lt;/sup&gt;, Sm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 15</td>
</tr>
<tr>
<td>MCG11</td>
<td>AFMC with chromosomal insertion of mini-Tn5 Km Pg:: lacZ, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>SBSG11</td>
<td>AFSB with chromosomal insertion of mini-Tn5 Km Pg:: lacZ, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>RHG11</td>
<td>RH90 with chromosomal insertion of mini-Tn5 Km Pg:: lacZ, Km&lt;sup&gt;+&lt;/sup&gt;, Te&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>S90G11</td>
<td>S90CRif with chromosomal insertion of mini-Tn5 Km Pg:: lacZ, Km&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>DPB1G11</td>
<td>DPB101Rif with chromosomal insertion of mini-Tn5 Km Pg:: lacZ, Km&lt;sup&gt;+&lt;/sup&gt;, Te&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td><strong>E. coli W</strong></td>
<td></td>
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<tr>
<td>W14</td>
<td>W derivative (Δpaa)</td>
<td>Ref. 24</td>
</tr>
<tr>
<td>AF15</td>
<td>W14 derivative, (ΔlacZ), Rif&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 15</td>
</tr>
<tr>
<td>WPG11</td>
<td>W14 derivative, (ΔlacZ, Pg:: lacZ), Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>pUT9</td>
<td>Promoterless lacZ vector, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 25</td>
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<td>pUT-Km</td>
<td>Mini-Tn5 delivery plasmid, Km&lt;sup&gt;+&lt;/sup&gt;, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 25</td>
</tr>
<tr>
<td>pBM1</td>
<td>pUJ9 derivative, Pg:: lacZ, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pPG11</td>
<td>pUT-Km derivative, Pg:: lacZ, Km&lt;sup&gt;+&lt;/sup&gt;, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pAD140</td>
<td>pUC18 derivative containing hpa cluster, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ref. 14</td>
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**Fig. 3.** β-Galactosidase activity of E. coli MCG11, SBSG11 (erg<sup>+</sup>), and RHG11 (rpoS<sup>+</sup>) cells growing on glucose. Cells were grown for 10 h in M63 minimal medium containing 10 mM glucose. β-Galactosidase activities of K12 strains MCG11 wild type (circles), RHG11 rpoS<sup>+</sup> (triangles), and SBSG11 erg<sup>+</sup> (diamonds) are indicated. Each assay was repeated four times.
kinase pathway, which is secreted into the medium. This pathway is reversible but works only at high concentrations of acetate, above 10 mM. During the transition to stationary phase, after the exhaustion of glucose, cells reabsorb the acetate and activate it to acetyl coenzyme A by means of the acetyl-CoA synthetase pathway and generate energy and biosynthetic components via the tricarboxylic acid cycle and the acetyl-CoA synthetase pathway. It is interesting to note that when acetate is replaced by succinate, the Pg stimulation is independent of succinate concentration.

The Pg::lacZ Translational Fusion Is Not Regulated by σ^54—To ascertain whether the alternative σ^54 factor of the RNA polymerase, which is synthesized in response to organic acids such as acetic acid (35), is involved in the activation of the Pg promoter at the stationary phase of growth, we constructed the E. coli K12 strain RHG11, an MC4100 derivative harboring the Pg::lacZ fusion in the chromosome and lacking the transcriptional factor σ^54 (Table I). RHG11 cultured in M63-glucose medium also showed maximum β-galactosidase production in the stationary phase of growth. When lacZ mRNA levels were analyzed in MCG11 and RHG11 cells exposed to fresh M63 medium or to spent M63-glucose medium, we observed that the β-galactosidase data correlated with the transcriptional state of the Pg promoter (Fig. 5). These results strongly suggest that σ^54 is not contributing to the expression from the Pg promoter under these conditions.

Strict Requirement of CRP for the Expression Driven by Pg—Since a potential CRP binding site is located upstream of the Pg promoter (14, 20) (Fig. 1), we used different genetic and biochemical approaches to determine whether CRP could be involved in the catabolite repression control of this system. The E. coli strain SBSPG11 (Table I), a MC4100 crp^- derivative harboring Pg::lacZ in the chromosome, showed that the Pg expression was strongly dependent on the CRP protein (Fig. 3). Thus, the expression levels of the reporter lacZ gene in strain SBSPG11 were constant during the entire growth curve and approximately 8-fold lower than those observed in the CRP^+ MCG11 and RHG11 strains in the stationary phase of growth (Fig. 3). Moreover, the inhibition of Pg promoter observed with the WPG11 cells growing in glucose could be partially reverted by the external addition of 5 mM cAMP (Fig. 4A). Although the activation observed is not complete, these results suggest that the Pg promoter can be activated by the cAMP-CRP complex. All of these data taken together suggest that the activation of the Pg promoter is markedly dependent on the cAMP-CRP complex, as a global activator essential to switch on the transcription of the meta operon of the hpa cluster.

In Vitro CRP Binding to the Pg Promoter—To confirm that Pg is a CRP-dependent promoter, we tested by gel retardation
assays (EMSA) the ability of purified CRP to bind to the Pg promoter region (Fig. 6). EMSA were performed in the presence of cAMP, and three different DNA fragments, PR-PG, PR, and PG, were used as probes. The PR-PG fragment covers the entire DNA region located between the genes hpaR and the meta operon, while the PR and PG fragments contain the 179-bp region upstream of hpaR and the 147-bp region upstream of the meta operon, respectively. As shown in Fig. 6, a CRP-DNA complex was detected with the PR-PG and PG probes, but no binding was observed with the PR probe (Fig. 6A). To localize the CRP binding site precisely, DNase I footprint experiments were performed using the PR-PG fragment as a probe. cAMP-CRP protected a region extending from −72 to −51 with respect to the transcription start site +1 of Pg promoter (Fig. 6B). These results were in agreement with the observation that a putative CRP binding site was located just in the sequence contained within the PG fragment centered at position −61.5 (Fig. 1). The CRP binding site in the Pg promoter might be a low affinity site, since it contains a T instead of a G at position 7 of the consensus sequence for the CRP binding site (Fig. 7C). A similar substitution in the lac promoter decreases the affinity of the CRP-cAMP complex about 30-fold (36) and 50-fold in the CRP-cAMP consensus binding site (37). A low binding affinity implies that this site might only be occupied by CRP at the high CRP concentrations present after glucose depletion, which might explain the silencing of Pg during exponential growth when the cells are cultured in the presence of glucose. To compare the dissociation constant \( K_d \) of the Pg CRP binding site versus Plac, we carried out a direct competition EMSA between the PR-PG fragment and a fragment containing the Plac promoter (see “Experimental Procedures”) (Fig. 7B). Surprisingly, the calculated \( K_d \) values for the Plac and Pg promoter regions were very similar, 0.7 and 1.3 nM, respectively (Fig. 7A). This result excludes the hypothesis that the silencing of Pg during exponential growth might be due to the presence of a low affinity CRP-binding site.

**IHF Activates the Expression Driven by the Pg Promoter**—Inspection of the hpaR-hpaG intergenic region revealed the existence of two sequences centered at positions −186 and −103, the latter in the noncoding strand, relative to the +1 position of Pg (Fig. 1) that closely match the consensus sequence \( W_C N_T W T G T G A - 3' \) (where W is A or T, R is A or G, and N is any of the four bases) for binding to the IHF global regulator (38, 39). To analyze the influence of IHF on the expression of the meta operon, the Pg::lacZ fusion was transferred into the chromosome of the isogenic E. coli strains S90Rif (IHF−) and DPB101Rif (IHF−). The resulting strains, S90G11 (Pg::lacZ, IHF−) and DPB1G11 (Pg::lacZ, IHF−) (Table I), were cultured in M63 medium containing 10 mM glucose, and \( \beta \)-galactosidase activities were determined in three different states of growth, i.e. exponential phase, early stationary phase, and late stationary phase (3.5, 8, and 24 h of incubation, respectively). The results shown in Fig. 8A show that IHF does affect expression especially during late stationary phase. EMSA studies were carried out to determine if the IHF effect was due to a specific IHF binding to the hpaR-hpaG intergenic region. We found that IHF was able to bind to the PR-PG and
PR fragments but not to the PG fragment (Fig. 8B). Furthermore, the interaction of IHF with the hpaR-hpaG intergenic region was specific, since a 100-fold excess of unlabeled PR-PG fragment prevented the formation of the IHF-DNA complex. DNase I footprinting analysis demonstrated that IHF bound to a single site in the region extending from −121 to −98 with respect to the transcription start site +1 of the Pg promoter on the noncoding strand (−121 to −85 on the coding strand). Therefore, these results were in agreement with the identification of an IHF consensus binding site in the hpaR-hpaG region centered at −103 (Fig. 1). In addition, we have tested the simultaneous binding of CRP and IHF global regulators to the hpaR-hpaG region (Fig. 8D), demonstrating that both proteins concomitantly bind to this DNA region. Competitive EMSA experiments suggested that both IHF and CRP bound independently to their two sites. Hence, there was neither strong competition nor synergy in the binding (Fig. 8D).

**DISCUSSION**

Free living bacteria have to cope with considerable fluctuations in the availability of nutrients; therefore, the promoters of genes involved in the catabolism of carbon sources are subject to various types of physiological controls that adjust their transcriptional rates to the environmental conditions (3). We focused our interest on the ability of *E. coli* to respond to less preferred carbon sources such as aromatic compounds, and we have used 4-HPA as a model system. Our first aim was to characterize the regulatory system that controls the expression of the meta operon (Pg promoter) of the hpa cluster of *E. coli* W. Our results demonstrate that this regulatory system is under a severe catabolite repression control. Thus, when the cells are grown in glucose plus 4-HPA-containing medium, Pg expression is repressed until the cells enter the late stationary phase of growth. However, when acetate or glycerol are used as carbon sources, Pg behaves as a typical exponential phase promoter that is inducible by 4-HPA (Fig. 2).

Although growth phase-dependent catabolite repression has been described for other promoters such as the lac promoter, which is considered as the prototype of promoters regulated by the “glucose effect” phenomenon (5, 6), the behavior of the Pg promoter cannot be adequately explained by the same mechanisms, suggesting that other regulatory elements could be involved in this process. Since 4-HPA can be taken up by passive diffusion, the repression observed in the Pg promoter during exponential growth cannot be ascribed to the well-known inducer exclusion effect of glucose. Furthermore, by analyzing the Pg::lacZ fusion in an *E. coli* K12 strain, which lacks the hpa cluster, we have also demonstrated that the observed glucose effect in the expression driven by the Pg promoter is not due to an effect dependent upon a 4-HPA catabolic enzyme, as observed in the case of the glp operon (7), or to a direct effect on the conformation of the HpaR repressor (Fig. 3), ruling out a
putative dual function for HpaR as a repressor during exponential growth and as an activator in stationary phase. In addition, we have demonstrated that the rapid activation of the Pg promoter in the stationary phase of growth of E. coli W is not solely dependent on the depletion of glucose in the culture medium, since we have shown that the acetate excreted and accumulated in the culture medium concomitantly with the consumption of glucose is important to switch on the Pg promoter. E. coli excretes acetate as a major by-product of its aerobic metabolism (40). In fact, acetate is regarded as an overflow metabolite when the respiration capacity is saturated partially (33, 40). We suggest that the low concentration of acetate (3 mM) present in the spent culture medium will provide the energy required to allow the cells to adapt rapidly to a new, less preferred carbon source such as 4-HPA, through a cAMP-CRP-mediated mechanism. This explanation is consistent with the fact that other metabolites that can provide energy for the cell can mimic the acetate effect (Fig. 4). It should be recalled that the intracellular concentration of cAMP of E. coli is higher when glycerol, lactate, or succinate is used as a carbon source (41). Furthermore, our results are consistent with the hypothesis that the acetyl-CoA synthetase pathway is involved in the rapid Pg activation via acetate catabolism. Although it is evident that after glucose depletion, the cells can use 4-HPA as the sole carbon and energy source, this adaptive process may require a long period of time when 4-HPA concentration is lower than 5 mM (Fig. 4B). Therefore, to reduce the length of the adaptive process, the cells can reuse the secreted acetate via the acetyl-CoA synthetase route to obtain the energy necessary to adapt rapidly to the catabolism of other less preferred substrates. Under these critical environmental circumstances, these cells can survive and compete for their specific ecological niches by taking advantage of less efficient catabolic pathways, such as hpa, that have been acquired during evolution for starvation emergencies. It appears logical that the faster the adaptive response to the new substrate is, the greater is the chance of surviving.

Several stationary phase or starvation induced genes that are controlled by alternative σ factors are also subject to glucose catabolite repression (42). In fact, the induction of σ^32, the master regulator of the general stress response in E. coli (42), has been observed under conditions of glucose exhaustion. In the presence of another less preferred carbon source, this induction is only transient and stops as soon as the new carbon source starts to be metabolized (43). Although the nucleotide sequences of the −35 and −10 promoter regions of Pg are similar to those of σ^70-dependent promoters, it has been demonstrated that σ^32 RNA polymerase holoenzyme is also able to transcribe a number of promoters recognized by σ^70 (44). However, the results presented here (Fig. 3) demonstrate that the activation of the Pg promoter in the late stationary phase of growth is not dependent of the σ^32 subunit of the RNA polymerase. These results contrast with those found for the regulatory system of other aromatic catabolic pathways in several bacteria (3, 45). For instance, in the well-characterized Pm promoter of the TOL pathway of Pseudomonas putida mt-2, σ^32 and σ^38 are markedly preferred in the early exponential and late exponential/stationary phases, respectively. Furthermore, it has been proposed that the activation of the σ^32-dependent heat shock Pm promoter is an indication of the induction of a heat shock response by the inducer 3-methylbenzoate (45). Experiments carried out with the Pg::lacZ translational fusion in E. coli σ^32 mutants suggest that σ^32 is not involved in transcription of the Pg promoter.2

Although many genes use alternative σ factors to be expressed preferentially during the stationary phase, some stationary phase-induced genes in E. coli are σ^70-dependent. Moreover, whereas some of them are CRP-independent, such as the Pmcb promoter for the synthesis of the antibiotic microcin B17 (46), others strongly require the CRP protein, for example the promoter of the csta gene that is involved in the uptake of peptides under carbon starvation conditions (47). However, our results suggest that Pg cannot be considered as an exclusively stationary phase promoter (48). In contrast to these promoters, the Pg promoter can be induced during the exponential phase of growth when the inducer 4-HPA and low catabolite sugars like glycerol or acetate are present in the culture medium. Moreover, when the cells are growing exponentially in a glucose-containing medium, the addition of exogenous cAMP produces a positive effect. The other σ^70-dependent stationary phase promoters described so far, including that of csta, cannot be induced during exponential growth under any circumstance including the addition of cAMP. Therefore, the Pg promoter represents a special type of σ^70-dependent promoter inducible in stationary phase via a highly efficient catabolite repression control mediated by the CRP-cAMP complex. A remarkable conclusion of this work is that E. coli has developed such an extreme control to prevent a wasteful loss in energy when a less preferred carbon source like 4-HPA is present in the medium. This tightly regulated system is necessary because 4-HPA, like many other aromatic compounds, can passively diffuse into the cytoplasm. Thus, the cell cannot use the repression of an uptake mechanism (inducer exclusion) as an additional control to prevent a possible leakage in the expression of hpa genes. Both the inducer exclusion mechanism and catabolite repression mediated by the cAMP-CRP complex are required for the efficient repression caused by glucose on the lac operon. Furthermore, we have observed that the expression of the Pg::lacZ translational fusion in a CRP E. coli strain is practically negligible, indicating that CRP acts as an obligatory activator of the expression driven by Pg. Gel retardation assays confirmed the binding of the cAMP-CRP complex to the Pg promoter region (Fig. 6). A high affinity CRP binding site was identified at position −61.5 with respect to the transcription start site of Pg (20) (Figs. 1 and 7). This location is optimal for CRP-dependent activation of class I promoters (4). In addition, an IHF binding site has been determined around position −103. Moreover, IHF and CRP can bind simultaneously to the Pg promoter. These findings suggest that IHF also acts as an activator of the system and might, synergistically with CRP, promote the transcription driven by Pg involving a class III promoter mechanism (4). In fact, CRP and IHF binding sites are separated by 42 bp, i.e., within a distance of 4 B-DNA turns, assuming a DNA helix repeat of 10.5 bp. This positioning places the IHF- and CRP-induced bends in phase and might be responsible for the synergistic activation by CRP and IHF. The A-tracts located between the IHF and CRP binding sites in the region extending from −73 to −83 could act as an UP element facilitating the interaction of the RNA polymerase aC-terminal domain (aCTD) with the DNA at this promoter region (49).

This superimposed system of catabolic regulation in addition to the specific HpaR regulation permits the expression of the hpa catabolic genes only when the preferred carbon source glucose is not available and when the presence of 4-HPA is sensed by the specific regulatory system. Since the IHF concentration is higher in the stationary phase of cells (50), this fact might also contribute to the down-regulation of the Pg promoter in the exponential phase. However, our results do not exclude the implication of other global regulators in the Pg

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and S. Carbajo with sequencing; and the technical assistance of Az, G. Porras, S. Marque

F. Boccard for the kind gift of purified IHF protein. We thank discussions and critical reading of the manuscript. We are indebted to E. coli phenylacetic acid in direct the glucose repression effect in the CRP complex and IHF (15), suggesting that a regulatory mech-
paa regulatory system. It is worth noting that the transcriptional effect of IHF and CRP (15, 51) and that, as stated above, other factors may add further complexity to the regulation of the

Acknowledgments—We thank E. Díaz and J. Plumbridge for helpful discussions and critical reading of the manuscript. We are indebted to F. Bocard for the kind gift of purified IHF protein. We thank S. Marqués for the strain RH90. We gratefully acknowledge the help of E. Aporta with oligonucleotide synthesis; A. Díaz, G. Porras, and S. Carboja with sequencing; and the technical assistance of E. Cano, M. Carrasco, and F. Morante.

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Superimposed Levels of Regulation of the 4-Hydroxyphenylacetate Catabolic Pathway in Escherichia coli
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doi: 10.1074/jbc.M103033200 originally published online July 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103033200

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