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Superimposed Levels of Regulation of the 4-Hydroxyphenylacetate Catabolic Pathway in *Escherichia coli**

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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 *Pg* upstream region. DNase I footprint experiments showed that cAMP-CRP and IHF binding sites are centered at −61.5 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).[1] 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-

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[1] The abbreviations used are: PTS, phosphoenolpyruvate-sugar phosphotransferase system; CRP, cAMP receptor protein; IHF, integration host factor; 4-HPA, 4-hydroxyphenylacetic acid; HPC, 3,4-dihydroxyphenylacetic acid; PCR, polymerase chain reaction; bp, base pair(s); EMSA, gel retardation assay(s); HPLC, high pressure liquid chromatography.

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. Overnight cells grown in M63 minimal medium with acetate, glycerol, or glucose were diluted 1:10 in identical medium and incubated. The appropriate selection antibiotics, kanamycin (50 μg/ml), tetracycline (3 μg/ml), ampicillin (100 μg/ml), or rifampicin (50 μg/ml) were added when needed.

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 RbCl 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 pUJ9 was digested with *Sac*I and *Bam*HI endonucleases, and the 1.7-kilobase pair DNA fragment was isolated and labeled with digoxygenin by using a Dig DNA Labeling kit (Roche Molecular Biochemicals). Nucleotide sequences were determined directly using plasmid pBM1 (Table I). Oligonucleotides were synthesized on an Oligo-1000M nucleotide synthesizer (Beckman Instruments). Standard protocols of the manufacturer for *Taq* DNA polymerase-initiated cycle sequencing reactions with fluorescently labeled dideoxynucleotide 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′-AACGCAAGAATTCGTGAGTCGTGCATTATCTTTCCCC-3′; an engineered *Eco*RI site is underlined) and PG3 (5′-GATAGTGGGATC-**CAT**GGTACC**ACTCCT**CGGATTCGATC-3′; the start codon and the original RBS of the *hpaG* gene are indicated in boldface letters, and an engineered *Bam*HI site is underlined). To create plasmid pBM1 (Table I), the PCR-amplified fragment was cut with *Eco*RI and *Bam*HI endonucleases and ligated to the *Eco*RI and *Bam*HI double-digested promoterless *lacZ* vector pUJ9 (Table I). The correct fusion was verified by sequence analysis. Plasmid pPG11 was constructed by subcloning the *Not*I cassette of pBM1 into the mini-Tn*5* 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, AFMC, AFSB, RH90, S90CRif, and DPB101Rif strains (Table I) by the filter-mating technique (22). The generated exconjugants containing the *lacZ* translational fusions stably inserted into their chromosome were selected for the transposon marker, kanamycin, on rifampicin-containing LB medium to give the strains WPG11, MCG11, SBSG11, S90G11, and DPBG11 and selected on tetracycline-containing LB medium for RHG11. In each case, the final strain was selected among three different exconjugants with similar expression levels and expression profile of the reporter gene. The relevant genotypes of the resulting strains are indicated in Table I.

Gel Retardation Assays—The DNA fragments PR-PG, PG, and PR, of 314, 147, and 179 bp, respectively, used as probes were amplified by PCR using 10 ng of plasmid pAJ40 (Table I) as template and the following primers: PG5 (see above) and PG3 (see above) for PR-PG fragment; PG3 and PGDE (5′-CCGGAATTCTGTAAATAGTTTGTTA-ATTAG-3′) for PG fragment; and PG5 (see above) and PRDE (5′-CCG-GAATTCGATAAGAATATATTAAATATC-3′) for PR. The DNA fragments were labeled at their 5′-end with phage T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol) (Amersham Pharmacia Biotech). A 220-bp *lac* fragment was isolated by PCR using plasmid pBR *lac* as a template together with γ-^{32}P-labeled primer 5′-GGCGTATCACGAG-GCCCTTTCG-3′ and primer 5′-GCTGGCACGACAGGTTTCCCGA-3′ (29). The reaction mixtures contained 20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM β-mercaptoethanol, 50 mM KCl, 0.1 nM DNA probe, 50 μg/ml bovine serum albumin, 50 μg/ml salmon sperm (competitor) DNA, and purified IHF (kindly provided by F. Boccard) or CRP prepared as described (30) in a 20-μl final volume. After incubation for 20 min at 30 °C, mixtures were fractionated by electrophoresis in 4% polyacrylamide gels buffered with 0.5× TBE (45 mM Tris borate, 1 mM EDTA). The gels were dried onto Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Pharmacia Biotech).

DNase I Protection Experiments—For DNase footprinting experiments, the PR-PG DNA fragment was synthesized by PCR with the primers PG5 and PG3 (see above) using a combination of one unlabeled primer and the second primer end-labeled with phage T4 polynucleotide kinase [γ-^{32}P]ATP (3000 Ci/mmol). The PCR fragment was purified using the High Pure PCR Product Purification Kit from Roche Molecular Biochemicals. Complexes with the labeled promoter region (at 1 nM final concentration) were formed for 20 min at room temperature in 15 μl of a glutamate buffer solution (40 mM HEPES, pH 8.0, 10 mM magnesium chloride, 4 mM dithiothreitol, 100 mM potassium glutamate) containing 200 μM cAMP and 500 μg/ml bovine serum albumin using purified CRP and IHF. Then 3 μl of DNase I solution (1 μg/ml in 10 mM Tris-HCl, 10 mM magnesium chloride, 125 mM potassium chloride) 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 of tRNA/ml, 5 μg of DNA/ml. 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 (31).

Analytical Procedures—The metabolites accumulated in culture supernatants were analyzed with Gilson HPLC equipment using an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad) at 30 °C and a flow of H_2SO_4 125 μM mobile phase pumped at a flow rate of 0.6 ml/min. Peaks with retention times of 15.6 and 9.3 min, corresponding to those of authentic standard acetic acid and glucose, respectively, were monitored in a Refractive Index Detector 132 (Gilson). β-Galactosidase activities were measured with permeabilized cells as described by Miller (27).

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 in 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

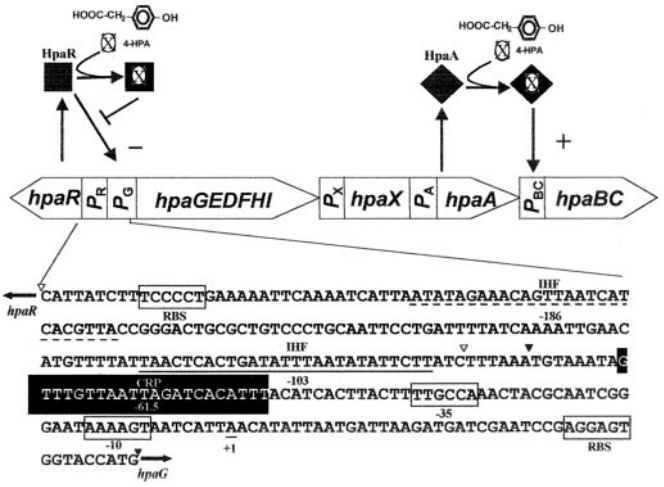

FIG. 1. **Regulation of the *hpa* cluster and sequence of the *hpaR-hpaG* intergenic region.** *R*, *G*, *E*, *D*, *F*, *H*, *I*, *X*, *A*, *B*, and *C* represent the names of the *hpa* genes. The *arrows* indicate the directions of gene transcription. P_R, P_G, P_X, P_A, and P_{BC} are promoter regions. ■, active form of HpaR repressor; ♦, inactive form of HpaA activator; ⊗, inducer 4-HPA. The complete nucleotide sequence shown from −216 to +46 corresponds to PR-PG probe. DNA regions located between *triangles* are the fragments corresponding to PG probe from −80 to +46 (▼) and PR probe from −216 to −87 (▽). Promoter boxes −35 and −10 of *Pg*, the ribosome binding site (*RBS*), the transcription start site of *Pg* (+1), and the putative CRP and IHF sites are indicated. The *continuous line* indicates a binding site demonstrated *in vitro* in this study. The *dotted line* means a potential IHF binding site that has not been investigated in this work.

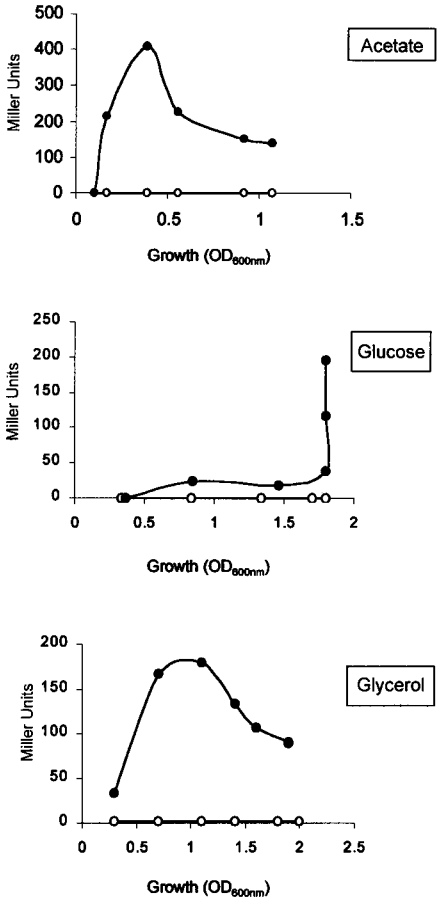

FIG. 2. **β-Galactosidase activity of *E. coli* WPG11 cells growing on acetate, glucose, or glycerol as carbon sources.** Cells were grown for 10 h in M63 minimal medium containing 30 mM acetate, 10 mM glucose, or 20 mM glycerol. *Filled* and *opened circles* mean the presence and absence of 1 mM 4-HPA in the culture medium, respectively. Each assay was repeated four times.

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. 4*A*). 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.

TABLE I
Bacterial strains and plasmids with relevant genotype and phenotype

Strain or plasmid	Relevant genotype or phenotype	Source or reference
E. coli K-12		
S17–1(λpir)	Host for pUT-derived plasmids	Ref. 21
CC118(λpir)	Host for pUT-derived plasmids, Rif^r	Ref. 22
AFMC	MC4100 Rif^r	Ref. 15
AFSB	MC4100 Δ*crp* Rif^r	Ref. 15
RH90	MC4100 *rpoS*59::Tn*10*, Tc^r	Ref. 23
S90CRif	(Δ*lac, pro*) *rpsL* Sm^r, Rif^r	Ref. 15
DPB101Rif	S90C *himD451*:: mini-*tet*, Rif^r, Tc^r, Sm^r	Ref. 15
MCG11	AFMC with chromosomal insertion of mini-Tn5 Km *Pg*::*lacZ*, Km^r	This work
SBSPG11	AFSB with chromosomal insertion of mini-Tn5 Km *Pg*::*lacZ*, Km^r	This work
RHG11	RH90 with chromosomal insertion of mini-Tn*5* Km *Pg*::*lacZ*, Km^r, Tc^r	This work
S90G11	S90CRif with chromosomal insertion of mini-Tn*5* Km *Pg*::*lacZ*, Km^r, Sm^r	This work
DPBG11	DPB101Rif with chromosomal insertion of mini-Tn5 Km *Pg*::*lacZ*, Km^r, Tc^r	This work
E. coli W		
W14	W derivative (Δ*paa*)	Ref. 24
AF15	W14 derivative, (Δ*lacZ*), Rif^r	Ref. 15
WPG11	W14 derivative, (Δ*lacZ*, *Pg*::*lacZ*), Km^r	This work
Plasmids		
pUJ9	Promoterless *lacZ* vector, Ap^r	Ref. 25
pUT-Km	Mini-Tn5 delivery plasmid, Km^r, Ap^r	Ref. 25
pBM1	pUJ9 derivate, *Pg*::*lacZ*, Ap^r	This work
pPG11	pUT-Km derivative, *Pg*::*lacZ*, Km^r, Ap^r	This work
pAJ40	pUC18 derivative containing *hpa* cluster, Ap^r	Ref. 14

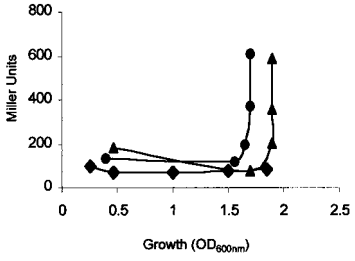

FIG. 3. **β-Galactosidase activity of *E. coli* MCG11, SBSG11 (*crp*$^-$), and RHG11 (*rpoS*$^-$) cells growing on glucose.** Cells were grown for 10 h in M63 minimal medium containing 10 mM glucose. β-Galactosidase activities of K12 strains MCG11 wild type (*circles*), RHG11 *rpoS*$^-$ (*triangles*), and SBSPG11 *crp*$^-$ (*diamonds*) are indicated. Each assay was repeated four times.

4*A*). 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 β-galactosidase (data not shown), implying that the activation effect of the spent medium is abolished by catabolite repression. To ascertain whether the β-galactosidase levels determined in strain WPG11 reflected the transcriptional state of the *Pg* promoter, the *lacZ* mRNA levels were analyzed. Fig. 5 shows that *lacZ* mRNA levels detected in cells exposed to spent M63-glucose medium were significantly higher than those observed in cells incubated in fresh M63 medium. Therefore, taken together, these findings show that the induction of the *Pg* promoter is due not only to the depletion of glucose but also to the presence of another compound in the spent culture medium.

It is well known that the extracellular concentration of cAMP increases upon glucose exhaustion (32); 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. 4*A*). 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. 4*B* 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. 4*A*). 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. 4*A*). 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. 4*A*). It is well known that cells growing on sugars that result in catabolite repression or on amino acids that feed into glycolysis undergo a metabolic switch associated with the production and utilization of acetate (33). When cells grow rapidly on glucose, they produce acetate via the phosphotransacetylase-acetate

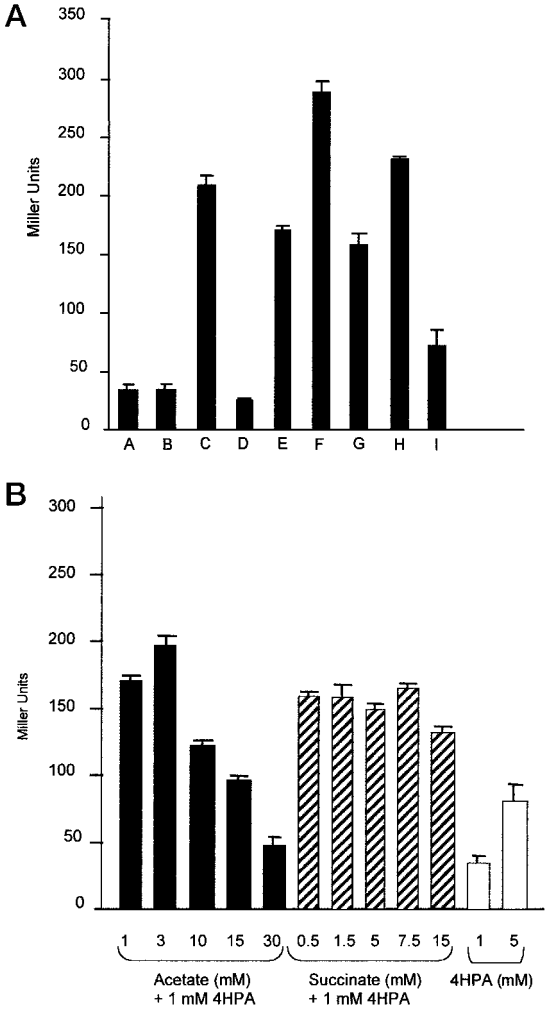

FIG. 4. **Inducibility of the *Pg* promoter in the presence of different carbon sources.** *Panel A*, WPG11 cells grown overnight in M63 medium containing 10 mM glucose but no inducer were washed and diluted 1:10 in the following: M63 medium without carbon source (*A*); M63 medium without carbon source with 5 mM cAMP (*B*); spent M63-glucose medium (*C*); M63 medium with 1 mM glucose (*D*); M63 medium plus 3 mM acetate (*E*); M63 medium plus 2 mM lactate (*F*); M63 medium plus 1.5 mM succinate (*G*); M63 medium plus 2 mM glycerol (H); and M63 medium plus 10 mM glucose and 5 mM cAMP (*I*). In every case, 1 mM 4-HPA was added to the culture medium to induce the *hpa* cluster. Cells were incubated with shaking for 2 h. *B*, cells have been incubated in a medium containing acetate (*black bars*) or succinate (*hatched bars*) at different concentrations using 1 mM 4-HPA as inducer. *White bars* indicate that the 4-HPA inducer was added as the only energy source. The concentrations of acetate, succinate, and 4-HPA are indicated at the *bottom*. Cells were incubated with shaking for 2 h.

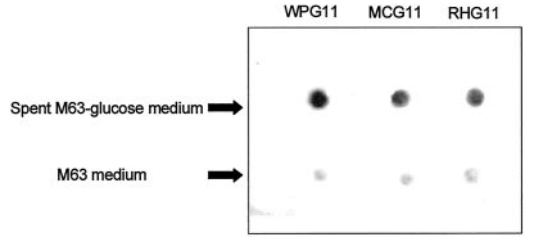

FIG. 5. **Transcriptional analysis of the expression of the *Pg::lacZ* translational fusion in spent M63-glucose medium and in fresh M63 medium.** *E. coli* W strain WPG11 (wild type) and K12 strains MCG11 (wild type) and RHG11 (*crp*$^-$) were grown as indicated in the legend to Fig. 4. Total RNAs were isolated from cells exposed for 2 h to spent M63-glucose medium (*top*) and fresh M63 medium (*bottom*) in the presence of 1 mM 4-HPA as inducer. 10 μg of total RNA were loaded on the membrane and hybridized with Lac probe (see "Experimental Procedures").

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 glyoxylate shunt, respectively. The acetyl-CoA synthetase pathway is an irreversible high affinity pathway to scavenge for small concentrations of acetate (34), and it is inducible by acetate and the cAMP-CRP complex. Since these two pathways, phosphotransacetylase-acetate kinase and acetyl-CoA synthetase, have different affinities for acetate, we have analyzed the β-galactosidase production of WPG11 as a function of acetate concentrations using 1 mM 4-HPA as inducer. Fig. 4*B* shows that during the first 2 h of incubation, the *Pg* activation level was inversely proportional to acetate concentration. However, after 4 h of incubation, the levels of β-galactosidase were identical in all conditions tested (data not shown). These results strongly suggest that the rapid activation of *Pg* observed in the presence of 3 mM acetate is mediated by 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^{38}—To ascertain whether the alternative σ^{38} 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^{38} (Table I). RHG11 cultured in M63-glucose medium also showed maximum β-galactosidase production in the stationary phase of growth (Fig. 3). Furthermore, the *lacZ* expression profiles of both MCG11 (*rpoS*$^+$) and RHG11 (*rpoS*$^-$) strains were identical throughout the growth curve. When *lacZ* mRNA levels were analyzed in MCG11 and RHG11 cells exposed to fresh M63 medium or to spent M63-glucose medium, we also observed that the β-galactosidase data correlated with the transcriptional state of the *Pg* promoter (Fig. 5). These results strongly suggest that σ^{38} 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. 4*A*). 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

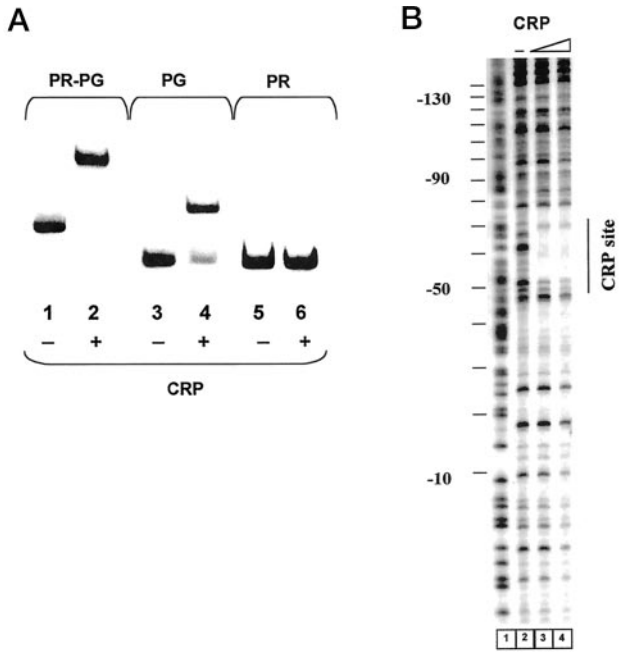

FIG. 6. **CRP binding to the *hpaR-hpaG* intergenic region.** *A*, gel retardation analyses were performed as indicated under "Experimental Procedures" but adding 200 μM cAMP to the reaction mixture and to the electrophoresis buffer. The DNA probes used were PR-PG (*lanes 1* and *2*), PG (*lanes 3* and *4*), and PR (*lanes 5* and *6*). − and +, the absence and the presence of 250 nM purified CRP, respectively. *B*, DNase I footprinting analysis of the interaction of the cAMP-CRP complex with the *Pg* promoter region. The reaction mixture was treated as described under "Experimental Procedures," using as probe the 5′-end-labeled noncoding strand of the *Pg*. *Lane 1* corresponds to the A + G sequencing ladder. CRP concentrations were as follows: 0 nM (*lane 2*), 30 nM (*lane 3*), and 100 nM (*lane 4*).

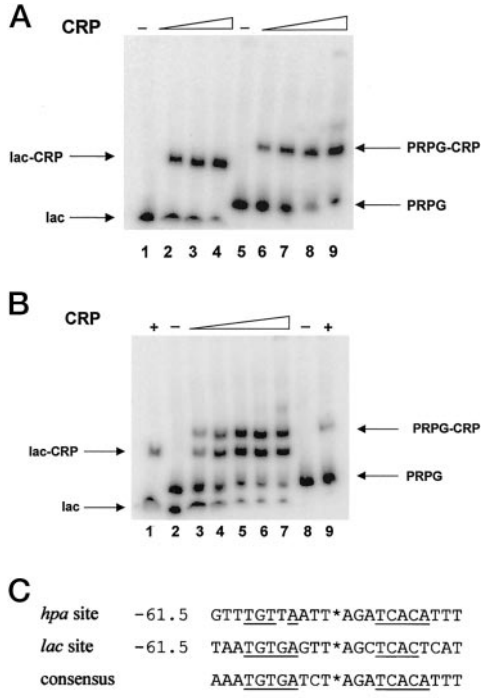

FIG. 7. **The cAMP-CRP binds the PR-PG regulatory region and the *lac* promoter with similar affinity.** The DNA-CRP complexes were analyzed by gel retardation assays as indicated under "Experimental Procedures" with the following modifications. CRP binding to the labeled DNA was performed in HEPES-Mg-glutamate buffer (40 mM HEPES, pH 8, 10 mM magnesium chloride, 100 mM potassium glutamate) containing 0.5 mg ml^{-1} bovine serum albumin without any competitor DNA. *A*, CRP titration with the 220 bp *lac* DNA fragment (*lanes 1–4*) and with the PR-PG fragment (*lanes 5–9*). *Lanes 1* and *5*, no CRP; *lanes 2* and *6*, 0.3 nM CRP; *lanes 3* and *7*, 1 nM CRP; *lanes 4* and *8*, 3 nM CRP; *lane 9*, 30 nM CRP. *B*, competition for cAMP-CRP binding between the *lac* and the PR-PG fragments at different CRP concentrations (0, 0.3, 1, 3, 10, and 30 nM; *lanes 2–7*). *Lanes 1* and *9* show 0.3 nM CRP binding to the *lac* and PR-PG fragments, respectively. *Lane 8*, PR-PG with no CRP. *C*, alignment of the sequences of the *hpa* and *lac* CRP binding sites with the consensus site for CRP. The centers of the CRP binding sites indicated by *asterisks* are numbered with respect to the transcription start sites. Bases identical with the conserved 5′-TGTGA-3′ motif of the CRP consensus site are *underlined*.

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. 6*A*). To localize the CRP binding site precisely, DNase I footprint experiments were performed using the PR-PG fragment as a probe. cAMP-CRP protects a region extending from −72 to −51 with respect to the transcription start site +1 of *Pg* promoter (Fig. 6*B*). 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. 7*C*). 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 cAMP 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. 7*B*). Surprisingly, the calculated K_d values for the *Plac* and *Pg* promoter regions were very similar, 0.7 and 1.3 nM, respectively (Fig. 7*A*). 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 $W_6N_8WATCAAN_4TTR$ (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 S90CRif (IHF^+) and DPB101Rif (IHF^-). The resulting strains, S90G11 (*Pg::lacZ*, IHF^+) and DPBG11 (*Pg::lacZ*, IHF^-) (Table I), were cultured in M63 medium containing 10 mM glucose, and β-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. 8*A* 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

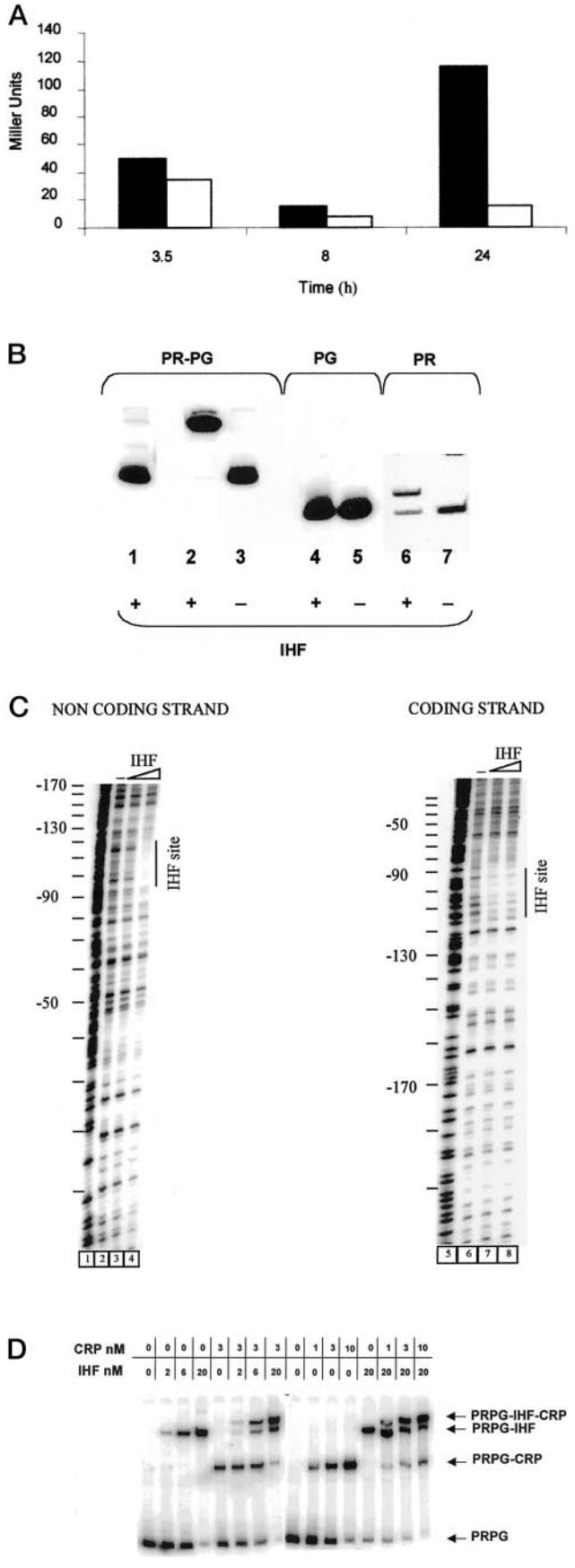

FIG. 8. **IHF mediated expression of *Pg* and IHF binding to the *hpaR-hpaG* intergenic region.** *A*, IHF *in vivo* effect. K12 cells S90G11 wild type (*black blocks*) and DPBG11 IHF (*white blocks*) were incubated in M63 medium supplemented with 10 mM glucose and 0.1 mg/ml L-proline. The three points analyzed correspond to exponential phase (3.5 h of culture and A_{600} between 0.5 and 0.6), early stationary phase (8 h of incubation and A_{600} between 1.5 and 2), and late stationary phase (24 h of growth and A_{600} between 1.7 and 1.8). *B*, gel retardation analyses were performed as indicated under "Experimental Procedures." The DNA probes used were the PG-PR (*lanes 1*, *2*, and *3*), PG (*lanes 4* and *5*), and PR (*lanes 6* and *7*). − and +, the absence and the presence of 150 ng of purified IHF, respectively. Unlabeled PR-PG fragment was added at 10 nM to the reaction mixture of *lane 1*. *C*, DNase I footprinting analysis of the complexes formed at the *Pg* promoter region with IHF. The reaction mixture was treated as described under "Experimental Procedures" using as probes the 5′-end-labeled noncoding and coding strand of the *Pg*. *Lanes 1* and *5* correspond to the A + G sequencing ladder. IHF concentrations were as follows: 0 nM (*lanes 2* and *6*), 30 nM (*lanes 3* and *7*), and 100 nM (*lanes 4* and *8*). *D*, simultaneous binding of purified CRP and IHF to PR-PG fragment. Concentrations of CRP and IHF are indicated at the *top*.

PR fragments but not to the PG fragment (Fig. 8*B*). 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. 8*D*), 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. 8*D*).

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. 4*B*). 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^{38}, 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^{38} 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^{38} 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* fusions 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 αC-terminal domain (αCTD) 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*

[2] B. Galán, A. Kolb, J. L. García, and M. A. Prieto, unpublished data.

regulatory system. It is worth noting that the transcriptional regulation of the *paa* cluster involved in the degradation of phenylacetic acid in *E. coli* is likewise dependent on the cAMP-CRP complex and IHF (15), suggesting that a regulatory mechanism similar to that described for degradation of 4-HPA might direct the glucose repression effect in the *paa* system.

Taking into account that there is only a limited number of examples in the literature illustrating the combined regulatory effect of IHF and CRP (15, 51) and that, as stated above, other factors may add further complexity to the regulation of the *hpa* pathway, we believe that the *hpa* system provides an excellent model to investigate the complex regulatory mechanisms that result from the superimposition of signals from specific regulatory proteins and different elements of global regulatory systems (3).

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