Transcriptional Regulation of the Divergent paa Catabolic Operons for Phenylacetic Acid Degradation in Escherichia coli*

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The expression of the divergently transcribed paaZ and paaABCDEFGHJK catabolic operons, which are responsible for phenylacetic acid (PA) degradation in Escherichia coli, is driven by the Pz and Pa promoters, respectively. To study the transcriptional regulation of the inducible paa catabolic genes, genetic and biochemical approaches were used. Gel retardation assays showing that the PaaX repressor binds specifically to the Pa and Pz promoters were complemented with in vivo experiments that indicated a PaaX-mediated repression effect on the expression of paa-lacZ and Pz-lacZ reporter fusions. The region within the Pa and Pz promoters that is protected by the PaaX repressor in DNase I footprinting assays contains a conserved 15-base pair imperfect palindromic sequence motif that was shown, through mutational analysis, to be dispensable for PaaX binding and repression. Pa-coenzyme A (PA-CoA), but not PA, specifically inhibited binding of PaaX to the target sequences, thus confirming the first intermediate of the pathway as the true inducer and PaaX as the only bacterial regulatory protein described so far that responds to an aryl-CoA compound. Superimposed in the specific PaaX-mediated regulation is transcriptional activation by the cAMP receptor protein and the integration host factor protein. These global regulators may adjust the transcriptional output from Pa and Pz promoters to the overall growth status of the cell.

Phenylacetic acid (PA) is a central compound to which pollutants, such as styrene and trans-styrylacetic acid, as well as other aromatic compounds, such as 2-phenylethylamine, phenylacetaldehyde, and several phenylalkanoic acids with an even number of carbon atoms, converge through different peripheral catabolic pathways (1, 2). Aerobic PA catabolism in Pseudomonas putida U and Escherichia coli W has been reported to represent a novel aerobic hybrid pathway whose first step is the activation of PA to phenylacetyl-coenzyme A (PA-CoA) by the action of a PA-CoA ligase (1, 2). In E. coli K-12 and E. coli W, the paa genes responsible for PA catabolism are clustered in the chromosome at min 31.0 according to the E. coli K-12 linkage map (2). The 14 paa genes are organized in three transcription units as follows: two divergently transcribed operons, paaZ and paaABCDEFGHJK, encoding the catabolic genes and whose expression is driven by the Pz and Pa promoters, respectively, and the paaXY operon expressing the paaX regulatory gene from the Px promoter (2). The absence of the paa genes in E. coli W14, an E. coli W mutant strain (3), leads to a PA− phenotype (2).

Previous work involving Pa-lacZ translational fusions revealed that the Pa promoter is negatively regulated by the paaX gene product since the absence of the latter caused a constitutive expression of the reporter fusion (2). The PaaX repressor is a 316-amino acid protein that shows 41.4% amino acid sequence identity with its ortholog PhaN (recently renamed as PaaN (4)) from the PA degradation pathway of P. putida U (1) and contains a stretch of 25 residues at amino acids 39–64 that shares similarity with the helix-turn-helix motif for DNA recognition and binding of transcriptional repressors from the GntR family such as FadR (5) and GntR (6). It is worth mentioning that whereas activator proteins are very common in transcriptional regulation of aromatic catabolic operons, only a few repressors have been described so far in biodegradation of aromatic compounds, namely HpaR (HpcR) for the catabolism of homoprotocatechic acid in E. coli (7, 8), CymR for the catabolism of p-cymene in P. putida F1 (9), and ApbS for the catabolism of phenol in Comamonas testosteroni TA441 (10).

The repres sor effect of PaaX on the Pa promoter in E. coli W14(lacZ) cells, containing the Pa-lacZ fusion into the chromosome and the paaX gene in a plasmid, could not be alleviated by growing the cells in the presence of PA, suggesting that this aromatic compound is not the inducer of the pathway (2). However, the simultaneous expression of genes paaX and paaK, the latter encoding the PA-CoA ligase that catalyzes the activation of PA to PA-CoA, allowed activity of the Pa promoter when the cells were grown in the presence of PA, suggesting that PA-CoA rather than PA is the true inducer of the pathway (2). Due to the unusual catabolic and regulatory features mentioned above, the PA biodegradation pathway becomes a very interesting model of an aerobic hybrid route for the catabolism of aromatic compounds. In this work, we have performed both in vivo and in vitro experiments to investigate the regulation of gene expression of the paaZ and paaABCDEFGHJK catabolic operons from E. coli. Superimposed in the specific PaaX-mediated repression, two global regulators, the cAMP receptor protein (CRP) and the integration host factor protein (IHF), act as activators of the gene expression driven by Pa and Pz promoters.
The bacteria and plasmids used in this study are listed in Table I. The E. coli strains and plasmids used in this work are listed in Table I. The Plasmids were prepared by the rapid alkaline lysis method (11). Transformation of the DNA was carried out using the RbCl method (11). DNA manipulations and other molecular biology techniques were essentially as described (11). DNA fragments were purified using the Geneclean Kit (Bio 101, Inc.). Oligonucleotides were synthesized on an Oligo-1000 M nucleotide synthesizer (Beckman Instruments). Nucleotide sequences were determined directly from plasmids by using the dideoxy chain termination method (17). 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 a model 377 automated DNA sequencer (Applied Biosystems Inc.).

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Growth Conditions**—The E. coli strains and plasmids used in this work are listed in Table I. The E. coli DH5α strain was obtained by transferring the (argF-lac)U169 deletion of E. coli SH210 to E. coli W14Rif through biparental mating (12) and selecting for a clone resistant to tetracycline and rifampicin. Unless otherwise stated, bacteria were grown in Luria-Bertani (LB) medium (11) at 37°C. Growth in M63 minimal medium (16) was achieved at 30°C using the corresponding necessary nutritional supplements and 20 mM glucose or 10 mM glycerol as carbon source. When required, 1 mM PA (paa) was added to the M63 minimal medium. Where appropriate, antibiotics were added at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (35 μg/ml), tetracycline (10 μg/ml), kanamycin (50 μg/ml), and rifampicin (50 μg/ml).

**DNA Manipulations and Sequencing**—Plasmid DNA was prepared by the rapid alkaline lysis method (11). Transformation of plasmids by using the dideoxy chain termination method (11). DNA manipulations and other molecular biology techniques were essentially as described (11). DNA fragments were purified using the Geneclean Kit (Bio 101, Inc.). Oligonucleotides were synthesized on an Oligo-1000 M nucleotide synthesizer (Beckman Instruments). Nucleotide sequences were determined directly from plasmids by using the dideoxy chain termination method (17). 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 a model 377 automated DNA sequencer (Applied Biosystems Inc.).

**Mutagenesis of the PaaX Binding Motif**—Base substitutions in the PaaX binding motif were introduced through recombinant PCR using the plasmid pAAD as template. To generate mutations in the left-half of the PaaX binding motif, a first PCR reaction was performed using two pairs of primers as follows: PA5–1 (5'-CATTCTCGGAATTCT-3') and PA5–2 (5'-GGTCTAGAAGTTATCAAAATAGAGTGCG-3'), which encode amino acids 38–44 of PaaX and hybridizes with the non-coding strand of the paaX gene between nucleotides 2885 and 2867 of the paaX gene cluster (2), underlined are the bases that reconstruct a BsrI restriction site and the degenerated MUT-PA3 (5'-GGTCTAGAAGTTATCAAAATAGAGTGCG-3'), which hybridizes between nucleotides 2885 and 2872 enclosing the transcription start sites of Pa (2), see also Fig. 4, where K is G or T, M is A or C and W is A or T; and PAP (5'-GGTCTAGAAGTTATCAAAATAGAGTGCG-3'), which hybridizes between nucleotides 2885 and 2872 enclosing the transcription start sites of Pa (2), see also Fig. 4, where the engineered XbaI site is underlined and the degenerated MUT-PA3 (5'-CATTCTCGGAATTCT-3'), which is complementary to the MUT-PA5 oligonucleotide). The resulting two PCR products were mixed and subjected to a further PCR reaction using PAP and PA5–1 as

### Table I

| Strain or plasmid | Relevant genotype and characteristic(s) | Ref. or origin |
|-------------------|----------------------------------------|---------------|
| E. coli K-12      | endA1 hisdR7 supE44 thi-1 recA1 gyrA(Nal') relA1 (argF-lac)U169 deoR B80lacΔlacZΔM15 | 11            |
| S17–1apir         | Tp' Sm' recA thi hisdR M' RP4-2.2 Cc: M a Km7 αpir phage lysogen | 12            |
| CC118apir         | (ara-leu) araD ΔlacX74 galE galK phoA thi-1 rpsE (Sp') rpoB(Rif') argE(am) recA1 αpir phage lysogen | 12            |
| S90C              | Δ(lac, pro) rpsL (Sma'I) | 13            |
| DBP101            | S90C himD451::mini-tet | 13            |
| DPB102            | S90C himD452::mini-tet | 13            |
| MC4100            | araD319 Δ(argF-lac)U169 rpsL150 (Sma'I) relA1 ffB5301 deoC1 pTa57 bsrB | 14            |
| SBS688            | MC4100 ΔpA | 14            |
| SH210             | Hfr (PO2A), Δ(argF-lac)169, zai-736::Tn10(T'C) | 15            |
| AFMC              | MC4100 spontaneous rifampicin-resistant mutant (Rif') | This work |
| AFMB              | SBS688 spontaneous rifampicin-resistant mutant (Rif') | This work |
| S90C-Rif          | S90C spontaneous rifampicin-resistant mutant (Rif') | This work |
| DPB101-Rif        | DPB101 spontaneous rifampicin-resistant mutant (Rif') | This work |
| DPB102-Rif        | DPB102 spontaneous rifampicin-resistant mutant (Rif') | This work |
| S90C-PA           | S90C-Rif with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| S90C-PZ           | S90C-Rif with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| DPB101-PA         | DPB101-Rif with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| DPB101-PZ         | DPB101-Rif with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| DPB102-PZ         | DPB102-Rif with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| AFMC-PZ           | AFMC with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| AFMCZ             | AFMC with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| AFSP-PA           | AFSPB with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| AFSP-PZ           | AFSPB with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| E. coli W         | W Δpaa | 3            |
| W14Rif            | W14 spontaneous rifampicin-resistant mutant (Rif') | 2            |
| AF15              | W14Rif Δlac | This work |
| AF15-PZ           | AF15 with chromosomal insertion mini-Tn5Km2 Pa-lacZ | This work |
| Plasmids          | pUTmini-Tn5Km2 | 12            |
|                   | pS3J | 2            |
|                   | pAFF | 2            |
|                   | pAFFZ | 2            |
|                   | pAFFZT | 2            |
|                   | pAFFA1T | 2            |
|                   | pCK101 | 2            |
|                   | pAD | 2            |
|                   | pAKF5 | 2            |
|                   | pUC18 | 11           |
|                   | pAFX | 11           |
|                   | pFX | 2            |
|                   | pAFFA2 | 2            |
|                   | pAFFA-2M | 2            |
|                   | pAFFA-2M5 | 2            |
|                   | pAFFA-2M9 | 2            |
|                   | pAFFA-2M16 | 2            |
|                   | pAFFA-2M18 | 2            |
|                   | pAFFA-2M22 | 2            |
|                   | pAFFA-2M25 | 2            |
|                   | pAFFA-2M26 | 2            |

*kb, kilobase pair.*
The gels were dried onto Whatman 3MM paper and exposed to Hyperfilm MP (Amersham Pharmacia Biotech). For quantitative definition of the PaaX-DNA interactions, autoradiograms were scanned in a computing densitometer, and the intensity of the bound and unbound DNA bands was measured using the ImageQuant software (Molecular Dynamics). The apparent dissociation constants (Table II) were determined using unbound DNA. Two values for the probe DNA were defined as the amount of total protein per ml of reaction mixture at which half of the labeled DNA remained unbound.

**RESULTS**

**The PaaX Repressor Regulates the Pz Promoter**—We have previously shown that the Pa promoter, which drives the expression of the paaABCD, is induced by phenylacetic acid. In addition, we have shown that the PaaX protein is a repressor of the paaABCD genes, which are constitutively expressed in the absence of PA. To determine the role of PaaX in the regulation of the Pz promoter, we performed DNase I footprinting assays using the Pz promoter as a probe. The footprinting assay revealed that PaaX binds to the Pz promoter, but not to the Pa promoter, indicating that PaaX is a specific repressor of the Pz promoter.

**Binding of PaaX to the Pa and Pz Promoter Regions**—Cells were grown on PA-containing media to allow the expression of the paaX gene. The cells were harvested and the expression of the Pz promoter was determined using a reporter assay. The results showed that PaaX binds to the Pz promoter, but not to the Pa promoter, indicating that PaaX is a specific repressor of the Pz promoter.
Fig. 1, whereas extracts containing PaaX were able to retard the migration of the Pz-Pa probe in a protein concentration-dependent manner, control extracts prepared from E. coli W14 (pUC18) cells, did not. Moreover, whereas a 6000-fold excess amount of an unrelated DNA such as that of salmon sperm did not affect the binding of PaaX to the target DNA, migration of the labeled fragment was not retarded when a 10-fold excess amount of non-labeled Pz-Pa fragment was added to the assay (data not shown), indicating that binding of the PaaX repressor to the Pz-Pa promoter region was specific. As the amount of PaaX protein increased, two distinct complexes, complex 1 (higher mobility) and 2 (lower mobility), were observed (Fig. 1), a result that might suggest that PaaX binds with different affinities to two sites in the fragment used as probe. To confirm the existence of two different PaaX-binding sites, gel retardation assays were performed using the 242-bp Pz fragment (from position −146 to +97 relative to the transcription start site of Pz promoter) and the 274-bp Pa fragment (from position −87 to +187 relative to the major transcription start site of Pa promoter), as probes. As shown in Fig. 1, one shifted band was found with each individual probe fragment. Since the deduced 
K_{\text{d(app)}} of PaaX (see under “Experimental Procedures”) for the Pa and Pz probes, 7 and 150 μg protein/ml, respectively, were similar to that for complex 1 and complex 2 with the Pz-Pa probe, there is no cooperativity in the binding of PaaX to the Pa and Pz promoters. Therefore, these data taken together revealed the specific and independent binding of PaaX to the Pz and Pa promoters.

**PA-CoA Specifically Inhibits Binding of PaaX to Its Cognate Promoter Region**—We have shown that the lacZ expression driven by the Pa (Pz) and Pa (see above) promoters in cells containing the paaX gene in a plasmid requires the simultaneous expression of the paaK gene encoding the PA-CoA ligase activity, as well as the presence of PA in the culture medium. This has led to the suggestion that the true inducer of the paa catabolic cluster is PA-CoA rather than PA. Nevertheless, since acyl-CoA ligases have been shown to play a role in the cellular uptake of some substrates such as long chain fatty acids (20), we could not exclude the possibility that E. coli cells lacking the paaK gene could have some deficiency in PA uptake that would lead to noninducible paa cluster expression. We therefore tested by gel retardation assays the ability of PA and PA-CoA to inhibit binding of PaaX to the Pa promoter region in vitro (Fig. 2). Whereas 30 μM PA-CoA reduced the amount of bound DNA (Pa/PaaX complex) to 50%, PA did not affect the PaaX-DNA binding (Fig. 2), even when this compound was added up to a final concentration of 2.5 mM (data not shown). A close analogue of PA-CoA such as benzoyl-CoA had no effect on PaaX-DNA binding when added to the gel retardation assay up to a final concentration of 2.5 mM (data not shown). Other CoA derivatives such as acetyl-CoA and free CoA were also unable to abolish binding of PaaX to the Pa promoter (data not shown), thus confirming PA-CoA as the specific inducer molecule of the paa catabolic genes.

### Table II

**Regulation of expression from the Pz promoter by PaaX**

| Plasmid(s) | Relevant gene(s) | β-Galactosidase activity (Miller units) | β-Galactosidase activity (Miller units) |
|------------|-----------------|---------------------------------------|---------------------------------------|
| pUC19      |                 | Uninduced 940                        | Induced (1 mM PA) 950                  |
| pAFK5      | paaK            | BD                                    | BD                                    |
| pAFX2      | paaX            | BD                                    | 500                                   |
| pAFK5/pAFX2| paaK/paaX       | BD                                    | >500                                  |

*BD, below detection limits.

*Fig. 1.* Gel retardation analyses of PaaX binding to the paaZ-paaA promoter region. The probe DNAs used (Pz-Pa, Pa, and Pz) are shown schematically on the right, with the Pa and Pz promoters marked with open arrowheads and the first 44 and 23 amino acids from the paaA and paaZ gene products represented by striped and black blocks, respectively. Cell extract and gel retardation analyses were performed as described under “Experimental Procedures.” Lanes 2–5, contained 0.3, 1.0, 2.0, and 4.0 μg of total protein of PaaX+ extracts obtained from cells bearing plasmid pAFX (paaX), respectively. Lanes 1 and 6 contained no extract and 4.0 μg of total protein of PaaX− extracts obtained from cells bearing control plasmid pUC18, respectively. The DNA-PaaX complexes are indicated.

*Identification of the PaaX Binding Regions*—To localize the PaaX binding region within the Pa and Pz promoters, DNase I footprinting experiments were performed using the same target DNA fragments as those used in the gel retardation assays reported above. As shown in Fig. 3, an extended binding region was detected in each promoter. The footprinting assay with the non-coding strand of the Pa fragment (Fig. 3B) revealed a protected region, operator region A (OR A), between positions −1 and +51 with respect to the major transcription start site of paaA that partially overlaps the ribosome-binding site of the gene (Fig. 4). On the other hand, the non-coding strand of the Pz fragment was protected by PaaX from DNase I digestion between positions −30 and +17, operator region Z (OR Z), spanning the potential −10 box of the promoter (Figs. 3C and 4). Similar patterns of DNase I digestion for each of the coding strands were observed (Fig. 3, A and D). The DNase I hypersensitive sites observed in OR A and OR Z were spaced at approximately 10 nucleotide intervals, corresponding to about one helix turn (Figs. 3 and 4), which suggests binding of PaaX to one side of the double helix.
Characterization of a Conserved PaaX Binding Motif—Alignment of the PaaX binding sequences identified by DNase I footprinting analyses revealed that each of the operator regions, ORA in promoter Pa and ORZ in promoter Pz, contained a conserved 15-bp imperfect palindromic motif (Fig. 4). Whereas the conserved motif in ORA, AATGTGATTCGTGTT, is located between positions +3 and +17, that of ORZ, TTATTGACGGCGTAT, is located between positions −30 and −16 of the non-coding strands (Fig. 4). A consensus palindromic sequence was deduced to be WWTIETTCGYGWT (R is A or G; W is A or T; and Y is C or T), with its pseudo-dyad axis through the central T base (underlined) which defines a left-half (LH) and a right-half (RH) (in italics) region. Since sequences with dyad symmetry within the operator regions are usually the binding sites of bacterial transcriptional regulators (21), we predicted that the described conserved motifs might be the binding sites of PaaX within ORA and ORZ.

To check the involvement of the conserved motif in the recognition and binding of PaaX to ORA, base substitutions in the target motif were generated by recombinant PCR (Fig. 5A), and the amplified wild-type and mutant Pa fragments were ligated to the lacZ gene of the promoter-probe vector pSJ3 as indicated under “Experimental Procedures.” Since the β-galactosidase levels of permeabilized E. coli AF15 cells expressing the wild-type Pa-lacZ translational fusion were similar to that of cells expressing the different mutant Pa-lacZ fusions (Table III), the possibility that mutations in ORA could negatively influence the maximum activity of the Pa promoter can be ruled out. The binding of PaaX to the different promoter fragments was checked in vivo by assaying β-galactosidase activity in E. coli AF15 cells expressing simultaneously paaX and the different lacZ translational fusions. As shown in Table III, base substitutions in the LH region of the conserved motif, i.e. mutant promoters PaM1, PaM5, PaM9, and PaM16 (Fig. 5A), severely impaired the PaaX-mediated repression of the corresponding lacZ fusions as compared with the wild-type Pa-lacZ fusion. As expected, the reduction in repression was higher with mutant promoters PaM1 and PaM9, both containing two base substitutions, than with mutant promoters PaM5 and PaM16 bearing only one base substitution (Table III). Interestingly, mutations in the RH region of the conserved motif, i.e. mutant promoters PaM18, PaM25, and PaM26 (Fig. 5A), did not cause a reduction in PaaX-mediated repression as high as that observed with base substitutions in the LH region. Thus, deletion of the conserved guanine at position 11 of the mutant promoter PaM18 (Fig. 5A) led to a 3-fold reduction in repression compared with that observed with the wild-type Pa promoter, and the two base substitutions in mutant promoters PaM25 and PaM26 (Fig. 5A) maintained the strong repression effect of PaaX on the corresponding lacZ translational fusions (Table III).

The effects of the base substitutions on binding of PaaX to ORA were also tested in vitro by using the wild-type and the different mutant Pa promoter fragments as probes in gel retardation assays. Whereas mutations in the LH region of the
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Fig. 4. Nucleotide sequence of the paaZ-paaA intergenic region. The translation initiation codon for the paaZ and paaA genes (represented by arrows) is shown by lowercase type in the corresponding non-coding strands. M and V, is the standard one-letter code abbreviation for the amino acid of PaaZ and PaaA, respectively. The ribosome-binding site (RBS) is shown in italics. The transcription start sites in the Pz and Pa promoters (B) are double-underlined, and the positions of transcription is indicated by broken arrows. The major transcription start site in Pz is shown in boldface type. The inferred −10 and −35 regions of each promoter are underlined. The PaaX-mediated protection of the coding and non-coding strands from digestion by DNase I is indicated with boldface letters.

Fig. 5. Base substitution analyses within the PaaX binding motif. A, alignment of the sequences of wild-type and different mutant Pa promoters. The PaaX binding motif is boxed, with its LH and RH (in italics) regions indicated by convergent arrows. Numbers indicate the position of the nucleotides within the 15-bp motif. Underlines and double-underlines indicate the minor and major transcription start sites, respectively. Base substitutions are shown in lowercase letters. Asterisks indicate deleted nucleotides. B, binding affinity of PaaX to wild-type and mutant Pa promoters. Gel retardation assays were performed as described under “Experimental Procedures.” – and + indicate that no extract (lane 1) and 0.4 μg of total protein of PaaX extract (lanes 2-10) were added to the reaction mixture, respectively. The 217-bp probe DNAs used were generated as reported under “Experimental Procedures,” and they are as follows: wild-type Pa (lanes 1 and 2), mutant PaM1 (lane 3), mutant PaM5 (lane 4), mutant PaM9 (lane 5), mutant PaM16 (lane 6), mutant PaM18 (lane 7), mutant PaM22 (lane 8), mutant PaM25 (lane 9), and mutant PaM26 (lane 10). The unbound probe and the DNA-PaaX complexes are indicated by arrows.

Conserved motif (mutant promoters PaM1, PaM5, PaM9, and PaM16) significantly reduced the binding affinity of PaaX to the mutant promoters as compared with that for the wild-type Pa promoter. Mutations in the RH region (Fig. 5B). These in vitro assays, therefore, are in agreement with the in vivo results reported above using the translatable lacZ fusions. Taken together, these data suggest that the conserved motif present in the Pa and Pz promoters is critical for PaaX repressor binding, with the LH region of the motif being much more important for repressor binding than the RH region.

The mutant promoter PaM22, which in addition to a single base substitution at position 15 harbors the deletion of a nucleotide located six bases downstream of the RH region (Fig. 5A), led to a 6.8-fold reduction in repression (Table III) and to a significant decrease in PaaX binding affinity (Fig. 5B) as compared with the wild-type Pa promoter. This suggests that sequences outside the conserved motif may also influence the repressor effect of PaaX on the expression of the paa catabolic genes.

Table III

| Plasmid | Relevant genotype | β-Galactosidase activity (Miller units) |
|---------|-------------------|----------------------------------------|
|         | Pa-lacZ          | −PaaX + PaaX | −Fold repression |
| pAFPA2  | Pa-lacZ          | 9133        | 300            | 3.01 |
| pAFPA2-M1 | PaM1-lacZ       | 9715        | 8205           | 8.48 |
| pAFPA2-M5 | PaM5-lacZ       | 9625        | 2225           | 4.38 |
| pAFPA2-M9 | PaM9-lacZ       | 8500        | 4511           | 1.88 |
| pAFPA2-M16 | PaM16-lacZ      | 10,000      | 2116           | 4.73 |
| pAFPA2-M18 | PaM18-lacZ      | 9892        | 923            | 1.07 |
| pAFPA2-M22 | PaM22-lacZ      | 10,000       | 1454           | 6.83 |
| pAFPA2-M25 | PaM25-lacZ      | 9766        | 474            | 20.6 |
| pAFPA2-M26 | PaM26-lacZ      | 9077        | 449            | 20.2 |

**IH and CRP Behave as Transcriptional Activators of the Pa and Pz Promoters**—Analysis of the paaZ-paaA intergenic region revealed the existence of two sequences, TATCACTACTGTCATCAGTT, from positions −125 to −113 and −73 to −52 relative to the major transcription start site of paaA (PaA), that significantly match the consensus sequences (where W is A or T, R is A or G, and N is any of the four bases) and AANTGT-
GANNTNNNTCAantan for binding to the IHF (22) and CRP (23) global regulators (24), respectively. To determine the putative role of CRP and IHF in paa regulation, we have used both genetic and biochemical approaches.

Permeabilized cells of E. coli AFMC PA and AFMC PZ, two E. coli AFMC derivatives which respectively contain a lac Z translational fusion with the Pa and Pz promoters stably inserted into their chromosome, showed β-galactosidase activities of 2300 and 980 Miller units, respectively, when they were grown in glycerol-containing minimal medium in the presence of PA. However, when these strains were grown in glucose-containing minimal medium in the presence of PA, no β-galactosidase activity was detected, thus suggesting a repression effect on Pa and Pz by glucose. To determine whether the observed regulation by glucose was mediated by CRP, the Pa-lacZ promoters was checked by β-galactosidase assays. As shown in Fig. 6, whereas a CRP-DNA complex was observed with the Pz-Pa and the Pa probes, no binding of CRP to the promoters was due to the binding of this regulator to such promoters, gel retardation assays were carried out. Whereas purified IHF was able to bind to the Pz-Pa fragment, no binding of CRP to the Pa probe was detected. These results are also in agreement with the observation that a putative CRP-binding site is located in the paaZ-paaA intergenic region but closer to the Pa than to the Pz transcriptional start site (Fig. 4).

To study the IHF influence on the expression of the paaA operons, the Pa-lacZ and Pz-lacZ translational fusions were stably inserted into the chromosome of isogenic E. coli S90CRif (himA himD), DB101Rif (himA himD), and DBP102Rif (himA himD) strains (himA and himD encode the two subunits of the IHF heterodimer) as described under “Experimental Procedures.” The resulting strains were grown in glycerol-containing minimal medium in the presence of PA. Therefore, both the Pz-Pa and Pa promoters were to be activated by the cAMP-CRP complex, which explains their catabolite repression by glucose reported above. To confirm these genetic experiments, gel retardation assays were performed with purified CRP in the presence of cAMP and three different DNA fragments (Pz-Pa, Pz, and Pa) used as probes. As shown in Fig. 6, whereas a CRP-DNA complex was observed with the Pz-Pa and the Pa probes, no binding of CRP to the Pa probe was detected. These results are also in agreement with the observation that a putative CRP-binding site is located in the paaZ-paaA intergenic region but closer to the Pa than to the Pz transcriptional start site (Fig. 4).

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In this work, we have used both genetic and biochemical approaches to study the regulation of the expression of the divergently transcribed paaZ and paaABCDEFGHIJK catabolic operons responsible for PA catabolism in E. coli. We had shown previously that the PaaX protein behaves as a transcriptional repressor of the Pa promoter (2). Here, a genetic analysis based on lacZ translational fusions has shown that PaaX acts...
is indicated by a (Plac)) (25), the LacI repressor (26), the GntR repressor (6), and the DNase I-hypersensitive sites in the RH region of ORA and ORZ the interactions with the LacI repressor (28). The observed makes rather different sets of contacts with the two halves of research.

Although it is likely that the effect of the PaaX protein on the transcription start sites within the 50-bp that is located immediately downstream of the transcriptional operator sequences.

PaaX was 21-fold higher than that for Pz sequences of the binding sites for the PaaX repressor in Pa (PaaX(Pa)) and Pz (PaaX(Pz)) promoters, the CRP activator in lac promoter (CRP-(Plac)) (25), the LacI repressor (26), the GntR repressor (6), and the PhdR repressor (27). The (pseudo) dyad symmetry axis of each sequence is indicated by a vertical arrow at the top. Nucleotides of the RH region are shown in italics, and numbers refer to the position of the nucleotides within the 15-bp PaaX binding motif. Convergent arrows indicate the LH and RH regions of the PaaX binding motif. The nucleotides identical to that of PaaX(Pa) are shown in boldface type.

also as a repressor of the Pz promoter (Table II). The results of the gel retardation assays (Fig. 1) indicated that the PaaX repressor binds specifically and independently to each of the Pa and Pz promoters, although the binding affinity of PaaX for Pa was 21-fold higher than that for Pz. DNase I footprinting (Fig. 3) revealed that PaaX protected an extended region of about 50-bp that is located immediately downstream of the transcription start sites within the Pa promoter (OR_A) and that spans the +1 and the −10 region in the Pz promoter (OR_Z) (Fig. 4). Although it is likely that the effect of the PaaX protein on the Pa and Pz promoters will involve different mechanisms of repression, validation of this assumption requires further research.

Sequence comparison analysis between OR_A and OR_Z revealed a 15-bp consensus motif, WWRTGATTCGGYWT, located at the 5′-end of the non-coding strands (Fig. 4), which was shown by point mutation analyses to be indispensable for binding of PaaX to each promoter. The dyad symmetry of the PaaX-binding site and the probability that the repressor-DNA interaction is mediated by a putative helix-turn-helix motif, amino acid residues 39−64 of PaaX (2), that shares similarity to that of transcriptional repressors of the GntR family (5, 6) suggest that the regulator is in an oligomeric form when bound to DNA. The two sequence repeats of the PaaX binding motif are related by a (pseudo) dyad axis through the central T base at position 8 (Fig. 8). Whereas the LH region of the PaaX binding motif is rather conserved in the operator sequences of other bacterial regulators such as GntR (6) and PdrR (27) (which belong to the GntR family) and, surprisingly, CRP (23), 25 and LacI (26) (which belong to the GalR-LacI family), the RH region differs from that of other operators (Fig. 8). Interestingly, the TGTTGA subsequence of the RH region in OR_A is also present in the LH region of the LacI- and CRP-binding sites (Fig. 8), where it has been shown to play a critical role in binding to the cognate regulators (25, 28). In this sense, we have shown here that mutations in the LH region of OR_A are more deleterious for PaaX binding and repression in vitro than mutations in the RH region, suggesting that the repressor makes rather different sets of contacts with the two halves of the binding site and the key interactions are those with the LH region. Similar non-identical half-site interactions have been observed also within the lac operator; again the LH region (the one containing the TGTTGA subsequence) is the most critical for the interactions with the LacI repressor (28). The observed DNase I-hypersensitive sites in the RH region of OR_A and OR_Z could also reflect a more complete protection of the LH regions by the PaaX repressor, a situation that has already re-ported for other operator sequences (28). Although the conserved PaaX binding motif is essential for PaaX binding, point mutations outside this motif, such as that of the mutant promoter Pm22, seem to influence the intrinsic affinity of the repressor for the promoter. Similar data showing that regions distal to the regulator binding site contribute slightly to the interactions with the cognate proteins have been previously reported for other regulators (25, 26, 29).

Since induction of the gene expression driven by the Pa (2) and Pz (Table II) promoters requires both PA and a functional PA-CoA ligase, it was reasonable to anticipate that the true inducer of the paa catabolic cluster was the first intermediate of the pathway, i.e. PA-CoA, rather than the initial substrate, i.e. PA. This was confirmed in vitro through gel retardation assays which revealed that binding of PaaX to the Pa and Pz promoters is specifically prevented by PA-CoA and not by PA (Fig. 2), benzoyl-CoA, acetyl-CoA, or free CoA. To date, the E. coli FadR protein, which controls the expression of many genes involved in fatty acid synthesis and degradation, was the only regulator for which there is convincing evidence that interaction of alkyl-CoA compounds with the protein prevents DNA binding (30). The PaaX repressor constitutes, therefore, the first example of a regulator protein that responds specifically to an aryl-CoA compound. It is likely that other regulatory proteins showing a significant amino acid sequence similarity with PaaX, e.g. the equivalent PaaN repressor (41.4% identity) of the PA degradation pathway from P. putida U (1), the putative regulator, ORF13 (31.5% identity), of a predicted PA catabolic pathway from Bacillus halodurans (GenBank™ accession number AB011837), and the putative repressor (GenBank™ accession number AF042490) (24.1% identity) of the 4-chloro-benzoate dehalogenation pathway involving formation of CoA derivatives in Arthrobacter sp. TM1 (31), also interact with aryl-CoA compounds.

In addition to the operon-specific regulation mediated by the PaaX repressor, the paa catabolic cluster of E. coli is subjected to control by the two global regulators, CRP (23) and IHF (22). We have observed that CRP− E. coli strains failed to express the Pa-lacZ and Pz-lacZ translational fusions, indicating that CRP acts as an activator of the gene expression driven by the Pa and Pz promoters. Gel retardation assays confirmed the binding of the cAMP-CRP complex to the Pa promoter region (Fig. 6). Since a potential CRP-binding site was identified at position −61.5 with respect to the major transcription start site of Pa (Fig. 4), this promoter might follow a CRP-dependent mechanism similar to that described for class I promoters (32). Although CRP is also necessary for activity of the Pz promoter, no binding of CRP to the Pz fragment was observed in gel retardation assays (Fig. 6), suggesting that CRP bound to Pa is able to activate the divergent Pz promoter. Since the putative CRP-binding site is at position −138.5 with respect to the transcription start site of Pz and, therefore, too far upstream to activate directly transcription from this promoter (33), additional transcription factors may be involved. We have observed that IHF binds to the paaZ-paaA intergenic region and stimulates transcription from the Pa and Pz promoters (Fig. 7). Nucleotide sequence analysis revealed a putative IHF-binding site within the Pz fragment at position −115 with respect to the major transcription start site of Pa (Fig. 4). Whether IHF-induced binding of the paaZ-paaA intergenic region can bring together RNA polymerase bound to Pz and CRP bound to Pa could be an interesting model that remains to be tested.

Carbon catabolite repression has been extensively described for the degradation of aromatic compounds in different bacteria (34). Here we report that when E. coli cells are grown in
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PA-containing minimal medium in the presence of their preferred carbon source, i.e. glucose, gene expression from the Pa and Pz promoters is subject to carbon catabolite repression. In contrast to the situation in Pseudomonas and other Gram-negative bacteria where the mechanism of catabolite repression is not yet understood (34, 35), the effect of glucose in E. coli is mediated through the action of CRP (23, 25). A similar repression effect of glucose mediated by the cAMP-CRP complex was also observed in E. coli for the catabolism of other aromatic compounds such as 2-phenylethylamine (36) and 4-hydroxyphenylacetic acid (14), and it is likely to occur also for homoprotocatechuic acid degradation (7, 8). Interestingly, the catabolism of PA in P. putida U is also under catabolic repression by glucose (1). It is well known that promoters are subjected to various types of physiological controls which adjust their transcriptional output to the general environmental conditions of the cells (34). We have shown in this work that the specific PaaX-mediated control of the Pa and Pz promoters for PA degradation in E. coli is, in turn, subordinated to a superimposed regulation mediated by global regulators such as CRP and IHF, which connect the expression of the paa catabolic genes to the metabolic and energetic status of the cell.

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