The PaaX Repressor, a Link between Penicillin G Acylase and the Phenylacetyl-Coenzyme A Catabolon of *Escherichia coli* W

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The *pac* gene, encoding the penicillin G acylase from *Escherichia coli* W, is regulated by the PaaX repressor of the phenylacetate catabolic pathway. *pac* expression depends on the synthesis of phenylacetyl-coenzyme A. PaaX and the cyclic AMP receptor protein (CRP) bind in vitro to the *Ppac* promoter region. A palindromic sequence proposed as the PaaX operator is located upstream of the −35 box overlapping a CRP binding site, an unusual position that suggests a novel regulatory mechanism.

Penicillin G acylase (PAC) (penicillin G aminohydrolase, EC 3.5.1.11) is a member of a large enzyme family conventionally known as β-lactam acylases because they are used for the semisynthesis of β-lactam antibiotics (30). PAC has a broad substrate range and is able to hydrolyze different esters and amides of phenylacetic acid (PA) and other aromatic and aliphatic acids, which has made it one of the most important enzymes used at the industrial scale worldwide (1, 5, 6, 11, 33). Although many PAC enzymes have been identified and characterized in different microorganisms, the PAC from *Escherichia coli* W is by far the best-studied enzyme of this family (30, 33).

Despite the ability of PAC to hydrolyze penicillin G, it does not have a function in bacterial antibiotic resistance, and its physiological role still remains unclear. Nevertheless, its broad substrate range together with the fact that its synthesis is activated by PA has favored the proposal of PAC as a scavenger enzyme for natural compounds containing a phenylacetate or hydroxyphenylacetate residue in ester or amide linkage (10, 21). Supporting this hypothesis, we have demonstrated that the *pac* gene encoding the PAC is located in the vicinity of the *hpa* cluster responsible for the degradation of 3- and 4-hydroxyphenylacetate in the chromosome of *E. coli* W (Fig. 1) (10, 23, 24). This observation suggested the implication of evolutionary selective forces which favored the clustering of physiologically interdependent genes, like *pac* and *hpa*. However, the recently identified *paa* cluster responsible for the catabolism of PA was located very far from the *pac* gene (Fig. 1) (13). Therefore, new data are needed to solve this apparent paradox.

It is well known that the synthesis of PAC is probably one of the most complex processes for bacterial proteins described so far since it is subjected to sophisticated regulatory controls at both the transcriptional and translational levels (Fig. 1) (16, 18, 21, 22, 27, 29, 31). Despite the fact that this system was deeply studied, the regulatory proteins involved in PA induction still remained unclear. Although a *pacR* regulatory gene located inside the *pac* gene has recently been identified (7), several

data obtained by using *Ppac*::*lacZ* fusions suggested that other factors might be involved in the induction process (21).

Using different genetic and biochemical approaches, both in vivo and in vitro, we have demonstrated that the PaaX regulator that controls the expression of the PA-coenzyme A (CoA) catabolon is also involved in the transcriptional regulation of the *pac* gene. This work not only settles the basis for clarifying the puzzling data about this complex regulatory system obtained so far but definitively supports the implication of PAC in the PA-CoA catabolon and provides interesting evolutionary evidence that helps to explain how the cell is able to integrate and tune the regulation of genes that are involved in the same catabolic processes.

Localization of a PaaX binding site in the *Ppac* promoter region. It has been reported that PaaX negatively controls the expression of the *paa* catabolic cluster and that the PA-CoA generated by the PA-CoA ligase (PaaK) acts as the true inducer of the system (12, 13). Since PAC synthesis is induced by PA, we analyzed the possibility that PaaX might directly or indirectly control the transcription of the *pac* gene. A detailed analysis of the *Ppac* promoter region showed a potential PaaX binding site located between positions −125 and −111 with respect to the transcriptional start site described for the *pac* gene (27) (Fig. 2). This putative operator has a palindromic sequence with a pseudodyad axis through a central T base, in perfect agreement with the consensus sequence for the PaaX operators described so far (12) (Fig. 2). The inverted sequences of the consensus PaaX binding region are well conserved in the *Ppac* promoter since only one and two nucleotide changes are detected on the right and left half of the binding site, respectively (Fig. 2).

To test the ability of PaaX to bind a *Ppac* promoter, we performed gel retardation assays (15) using a DNA fragment of 269 bp (PPAC) that covers the entire *Ppac* promoter as a probe (Fig. 2). The PPAC fragment was generated by PCR with 10 ng of the plasmid pPGA1 (17) and the primers PAC5′ (5′-CGGAATTCTTTACATACAGATAATGACCTGAGC-3′) and PAC3′ (5′-CGGGATCCTCTATTTTTCATTGTATC CTCTGGC-3′). Cell extracts from *E. coli* W14(pAFX), which expresses the *paaX* gene, and from *E. coli* W14(pUC18), which does not produce PaaX (12), were used for the assays. The strain *E. coli* W14 (14) is a derivative of *E. coli* W (9), which

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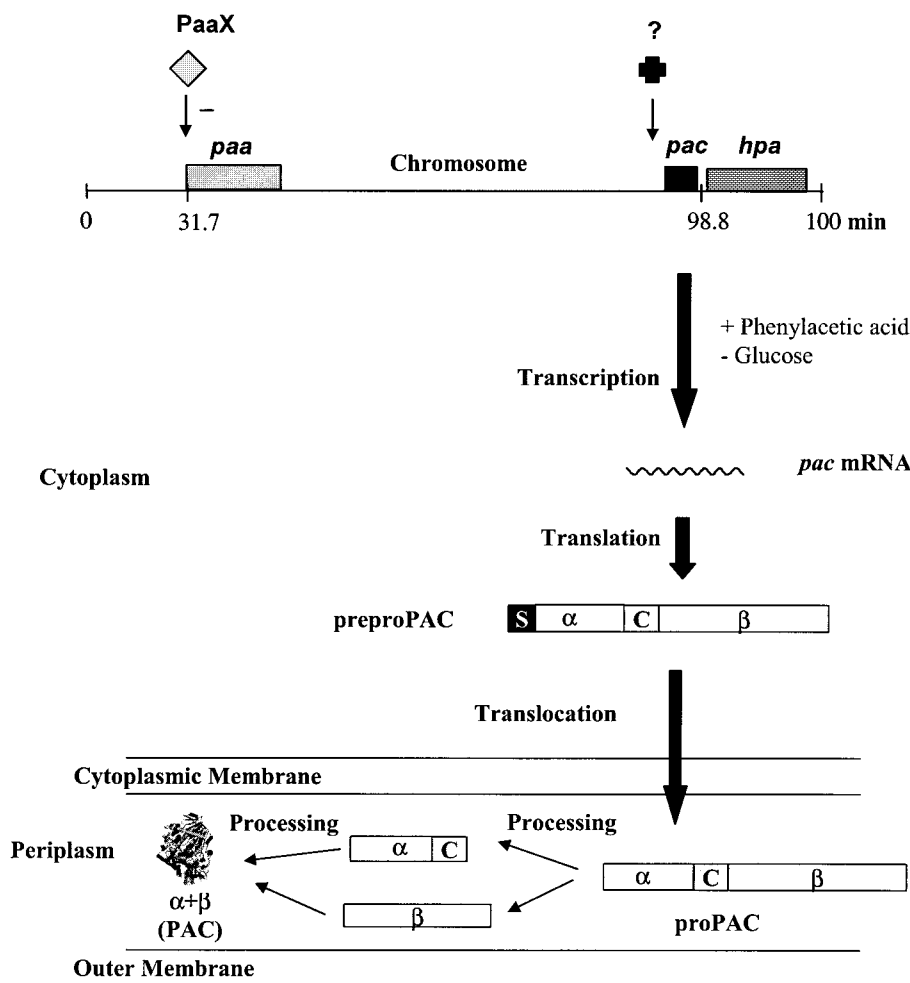
FIG. 1. General scheme of the PAC regulatory system. The top of the figure shows the relative localizations of the *pac* gene (black block), the *paa* cluster (gray block) for the degradation of PA, and the *hpa* cluster (cross-hatched block) for the degradation of 4-hydroxyphenylacetate in the *E. coli* chromosome, referred to as the *E. coli* K-12 linkage map (10). The regulatory protein PaaX is represented by a gray diamond. The − indicates transcriptional repression. The black cross represents an undetermined factor that controls the dependent PA expression of the *pac* gene. The synthesis and maturation processes of the PAC protein are shown. The *pac* gene encodes a polypeptide precursor (preproPAC) which has a molecular mass of 92 kDa and is composed of, in the direction of N to C, a signal peptide (S), an α subunit (α), a connecting peptide (C), and a β subunit (β). The signal peptide directs the export of a polypeptide precursor into the periplasm and is removed to form another precursor (proPAC) after the translocation. Periplasmic processing steps consist of autoproteolysis (α+β) of the precursor and folding of the mature PAC.

lacks the whole *paa* cluster, including the *paaX* repressor and the *paaK* ligase coding genes. As expected, whereas the cell extract containing PaaX was able to retard the migration of the PPAC probe in a protein concentration-dependent manner, no protein-DNA complexes were detected when the control extract was tested (Fig. 2). Furthermore, we proved that the interaction of PaaX with *Ppac* was specific, since a 100-fold excess of unlabeled PPAC fragment prevented the formation of the PaaX-DNA complex, whereas an excess of salmon DNA did not (data not shown). More important, PA-CoA inhibited the binding of PaaX to the *Ppac* promoter, suggesting that it should be the true inducer of the system (Fig. 2).

Demonstration that PaaX regulates in vivo the expression of the *pac* gene. To establish in vivo the role of PaaX in *pac* expression, we first compared the PAC activity (2) produced by the mutant *E. coli* W14 (Δ*paa*) (14) with that of the wild-type

E. coli W (Fig. 3). Remarkably, the *paa* mutant W14 produced a large amount of PAC in the absence of PA, strongly suggesting that the *paa* cluster is involved in the regulation of the *Ppac* promoter and that, most likely, PaaX behaves as a repressor. More interesting, when *E. coli* W14 was transformed with the low-copy-number plasmid pAAD, which harbors the *paa* cluster (13), the expression of the *Ppac* promoter recovered its normal PA dependence (Fig. 3), confirming that the *Ppac* promoter was regulated by a gene of the *paa* cluster. To unequivocally ascribe a function to PaaX in *Ppac* regulation, we compared the PAC activities of three strains: *E. coli* W14(pAAD46), carrying a pAAD derivative plasmid lacking the PaaK ligase-coding gene (13); *E. coli* W14(pAFX2), harboring a plasmid that expresses in *trans* the *paaX* gene under the control of the *Plac* promoter (13); and the wild type, *E. coli* W (Fig. 3). The PAC activity was strongly reduced in the

A

B

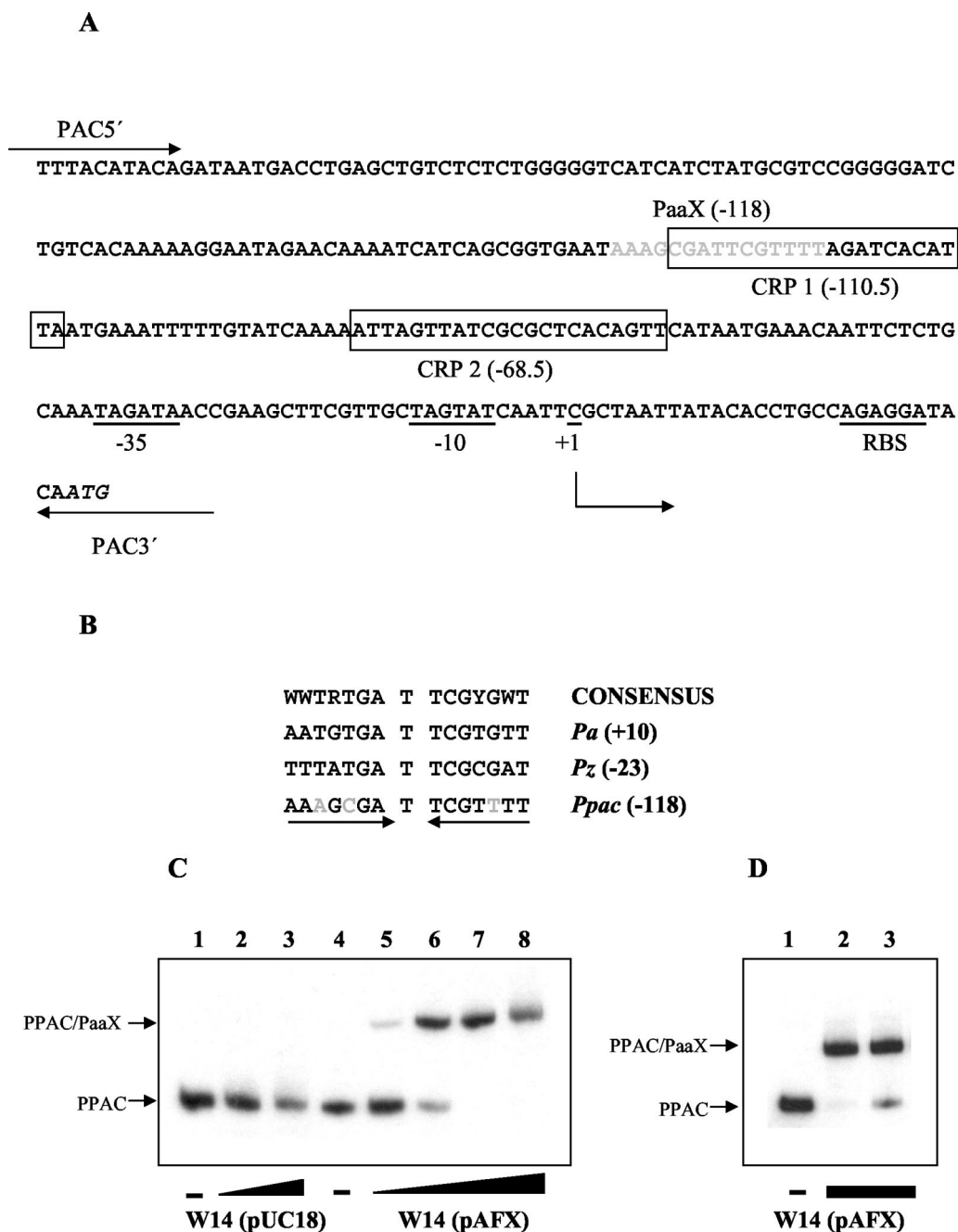**C**   **D**

FIG. 2. In vitro demonstration of the PaaX function on the *Ppac* promoter. (A) Nucleotide sequence of the *Ppac* promoter of *E. coli* W. The putative −35 and −10 boxes, the ribosome binding site (RBS), and the transcription start site (+1) are underlined. The ATG start codon of *pac* is shown in italics. The PaaX putative operator located at −118 relative to the transcription start site is indicated by gray letters. Putative CRP binding motifs (CRP 1 [−110.5] and CRP 2 [−68.5]) are boxed. The locations of the primers PAC5′ and PAC3′ used to amplify the PPAC DNA fragment are shown. (B) Alignment of the nucleotide sequences of the described operators for PaaX. The *Pz* promoter drives the expression of the *paaZ* gene, while *Pa* controls the expression of the operon *paaABCDEFGHIJK*; both promoters control expression from the *paa* cluster of the PA degradation pathway in *E. coli*. The promoters are numbered with respect to the transcription start site (+1) of each respective promoter. Nucleotides that are not conserved relative to the consensus site (12) are indicated by gray letters. (C) In vitro binding of PaaX to the *Ppac* promoter by gel retardation assay. Lanes 2 and 3 contain 31 and 630 ng, respectively, of total protein of PaaX-free extracts from strain W14(pUC18). Lanes 5 to 8 contain 6.3, 12.6, 31, and 630 ng, respectively, of total protein of extracts obtained from *E. coli* W14(pAFX). A − indicates that no extract was added to the reaction mixture. (D) PaaX released by PA-CoA. Lanes 2 and 3 contain 31 ng of total protein extracts from the strain *E. coli* W14(pAFX). PA-CoA (125 μM) was added to the reaction mixture in lane 3. The − indicates that no extract was added to the reaction mixture. The DNA probe used for the experiments whose results are shown in panels C and D was PPAC at 1 nM.
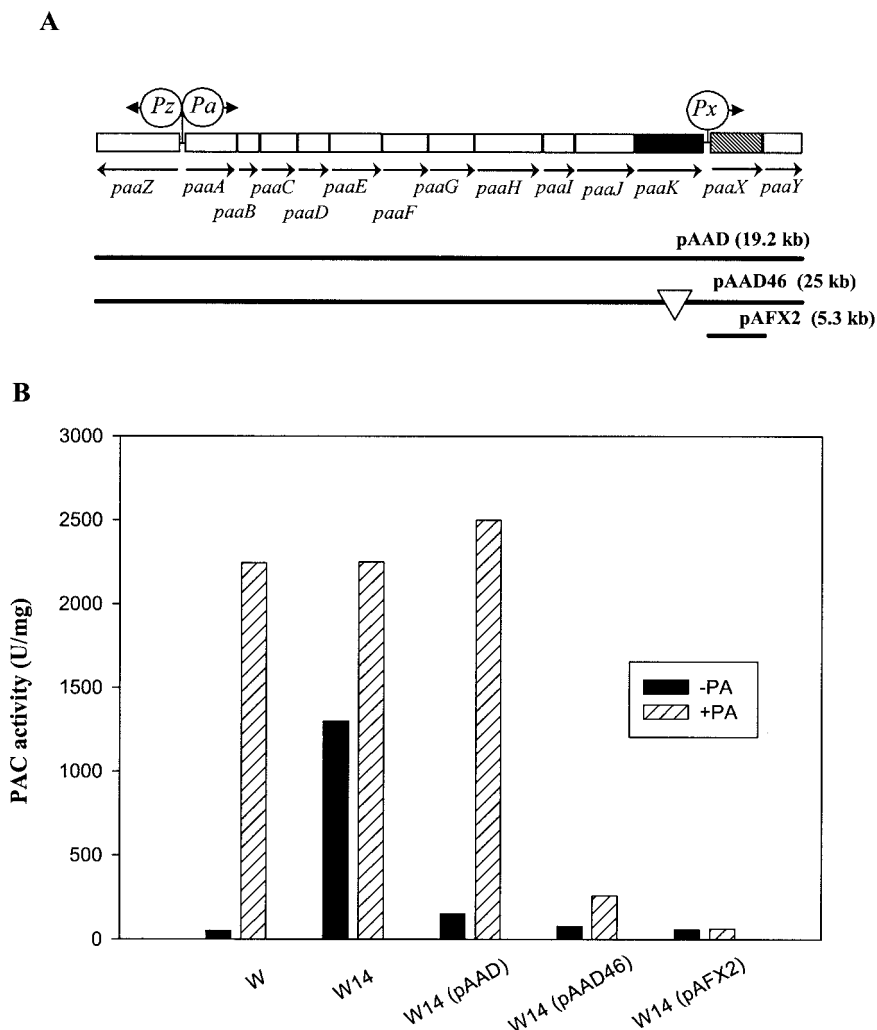
A

B

FIG. 3. In vivo demonstration of the role of PaaX in the expression driven by the *Ppac* promoter. (A) Genetic organization of the *paa* pathway for the catabolism of PA in *E. coli*. Relevant genes are indicated by blocks. The regulatory gene *paaX* and the PA-CoA ligase-coding gene *paaK* are represented by hatched and black blocks, respectively. The arrows show the direction of gene transcription. Circles indicate *Pa*, *Pz*, and *Px* promoters. Plasmids containing different subcloned DNA fragments are indicated with continuous lines. The location of the insertion of the Tn*1000* transposon in plasmid pAAD46 is indicated by a white triangle. (B) Diagram showing the PAC activity produced in *E. coli* W and W14 cells containing different plasmids. Cells were grown overnight in Luria broth at 28°C in the absence (black columns) or in the presence (hatched columns) of 1 mM PA. The crude protein extracts were obtained as described previously (12). PAC activity was measured as described previously (2), with 1 U of PAC activity being defined as the amount of enzyme required to form 1 nmol of 6-aminopenicillanic acid per min at 37°C. Protein concentrations were determined by the method of Bradford (4).

absence of PaaK ligase, suggesting that PA-CoA is the true inducer of PAC synthesis. In addition, the overexpression of *paaX* produced a hyperrepression of the *Ppac* promoter.

In vitro analysis of CRP and IHF binding to the *Ppac* promoter. The implication of the presence of the global regulators cyclic AMP receptor protein (CRP) and integration host factor (IHF) in the *pac* regulatory apparatus has been demonstrated by in vivo experiments (27, 29, 32), but direct evidence of the binding of these proteins to the *Ppac* promoter has not been obtained. Using the PPAC fragment as a probe, we tested the binding of purified CRP (kindly provided by A. Kolb) and IHF (kindly provided by F. Boccard) to the *Ppac* region by use of a gel retardation assay (Fig. 4). These analyses have demonstrated that CRP interacts directly with the *Ppac* promoter.

Remarkably, inasmuch as the amount of the CRP increased, two distinct CRP-PPAC complexes were detected, a result that is in perfect agreement with those for the two putative CRP binding sites predicted in the *Ppac* region (Fig. 2). These results also suggest that CRP binds with different affinities to both sites.

The role of IHF as a positive transcriptional regulator of the *pac* gene has been demonstrated by using an IHF⁻ strain (29). Furthermore, two putative sites for IHF binding in the *Ppac* promoter between the positions −76 and −88 and positions −137 and −149 have been proposed (29). This finding appears to be in agreement with that of the *paa* regulatory system, where CRP, IHF, and PaaX coregulate the catabolic operons (12). However, our assay showed that IHF was not able to
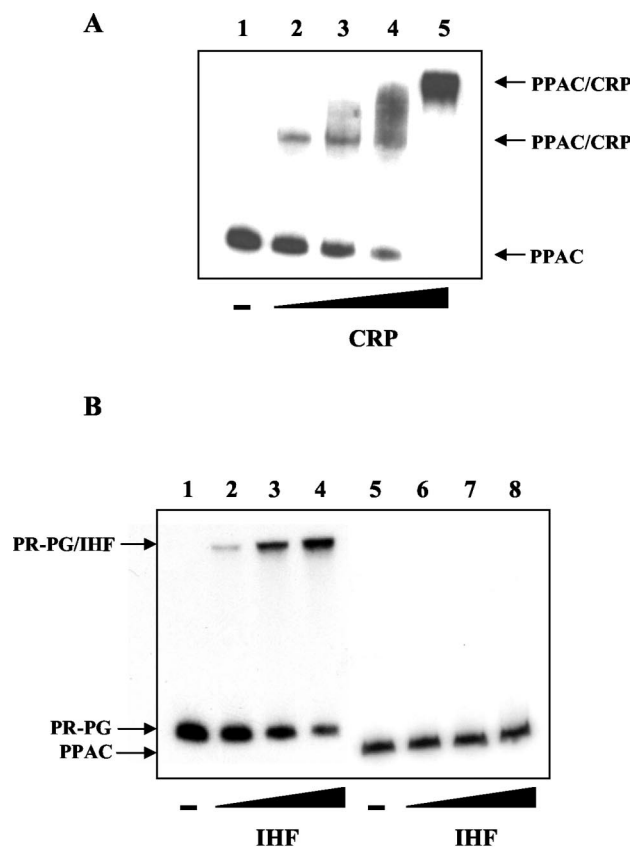
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FIG. 4. In vitro binding of purified global regulators CRP and IHF to the *Ppac* promoter. (A) CRP binding to the *Ppac* promoter. A gel retardation assay was carried out in the presence of 200 μM cyclic AMP in an electrophoresis buffer. The DNA probe used was PPAC at 1 nM. The − indicates the absence of purified CRP. Lanes 2 to 5 contain 50, 100, 150, and 300 nM of purified CRP, respectively. (B) IHF binding to the *Ppac* promoter. The DNA probes used were PR-PG at 1 nM and PPAC at 1 nM. Lanes 2 and 6 contain 100 nM protein, lanes 3 and 7 contain 200 nM protein, and lanes 4 and 8 contain 300 nM protein.
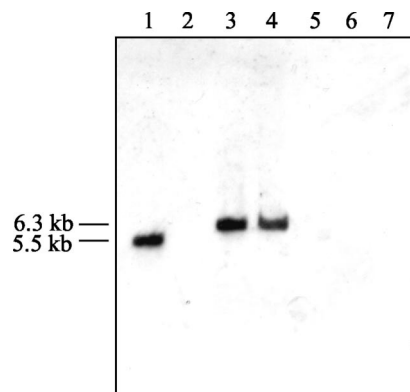

FIG. 5. Presence of *paaX*-homologous genes in heterologous microorganisms. Results of Southern blot analysis of chromosomal DNAs from *E. coli* W, *E. coli* W14, *E. coli* HB101 (28), *E. coli* MC4100 (25), *K. citrophila*, *B. megaterium*, and *P. rettgeri* are shown. Total DNAs were digested with EcoRI and were probed with fragment PAAX. The sizes of the bands corresponding to the EcoRI fragments that contain sequences homologous to the *paaX* gene are indicated.

retard the migration of the PPAC probe, whereas it was able to retard our control IHF probe, PR-PG (15). This result suggests that IHF does not bind to the *Ppac* promoter region, and therefore, the response observed in vivo is most likely due to an indirect effect on other global regulatory systems.

Occurrence of *paaX*-homologous genes in several strains used for the industrial production of PAC. The PA induction of PAC synthesis is not restricted to *E. coli* W, since other PAC producer bacteria, like *Bacillus megaterium*, have shown similar responses to PA (34, 35). Taking into account that many other bacteria mineralize PA by homologous *paa* clusters (10), we have investigated by Southern blot analysis the presence of *paaX*-homologous genes in several PAC producer microorganisms (19). The *paaX* DNA fragment (950 bp) used as a probe containing the complete *paaX* gene was generated by PCR with primers X5-Bam (5′-TCGGATCCGTAAACTTGTTAC TTTTATCC-3′) and X3-Sac (5′-CCGGAGCTCGACCATCT ATCTG-3′) with plasmid pAAD as a template. The PAAX probe was labeled by the random primer method using the digoxigenin system (Boehringer Mannheim). The results

shown in Fig. 5 suggest that the three PAC producer strains tested, *Kluyvera citrophila* ATCC 21285, a close relative of *E. coli* (3); *B. megaterium* ATCC 14945 (20); and *Providencia rettgeri* ATCC 31052 (8), did not contain DNA sequences homologous to *paaX*.

The finding of the lack of a *paaX*-homologous gene in *K. citrophila* is in agreement with the fact that it is not able to mineralize PA (unpublished data) and that PAC production is constitutive in this strain (17). Nevertheless, a detailed analysis of the *Ppac* promoter from *K. citrophila* revealed a nucleotide sequence (5′-GAAATGATTCGCTTT-3′) located between positions −64 and −78 of its corresponding transcriptional start site (26), which shows only three nucleotide mismatches compared with the consensus PaaX operator binding site (Fig. 2). This finding might explain the unexpected PA-dependent expression of the *pac* gene from *K. citrophila* when it was cloned and expressed in *E. coli* K-12 strains (26). Moreover, as shown in Fig. 5, some *E. coli* K-12 strains harbor the *paaX* gene, and therefore, we assumed that we could ascribe to this gene a role in the expression of the cloned heterologous *pac* gene. To determine if PaaX might also control the expression of the *pac* gene from *K. citrophila*, we transformed this strain with the plasmid pAFX2 expressing in *trans* the *paaX* gene from *E. coli* W. Remarkably, the PAC activity of *K. citrophila* (pAFX2) was 4.5-fold lower than that of the wild-type strain (data not shown), strongly suggesting that PaaX is also able to recognize the homologous operator sequence found in *K. citrophila* and repress *pac* expression. This result poses the intriguing question of why a *pac* gene that can be regulated by PaaX still survives in a strain which does not have PaaX and which does not use PA as a carbon source. Perhaps this is because PAC may not be exclusively dedicated to the generation of PA, and it may fulfill other missions not yet defined. In summary, the results presented above allow us to unequivocally establish that the *pac* gene of *E. coli* W is regulated by the PaaX repressor of the *paa* catabolic cluster. The induction by PA of PAC activity is mediated by the synthesis of the PA-CoA derivative, the first intermediate of the PA catabolic pathway. Moreover,

it is worth mentioning the finding that the palindromic sequence proposed as the PaaX operator for the *Ppac* promoter is located upstream of the −35 box, overlapping one of the CRP binding sites. This location is certainly very unusual and suggests a novel mechanism of repression that will require more-sophisticated analyses. Finally, our results reveal a quite complex evolutionary scheme for the PA-CoA catabolon. Taking into account both the role of PAC as a PA scavenger enzyme and the fact that both systems, the *pac* and *paa* genes, are controlled by the same regulator (PaaX), one might expect that these genes evolved in a coordinated way. However, other data suggest that these genes have followed independent evolutionary tracks, since *pac* is located far from the *paa* cluster in *E. coli* W, it is absent in *E. coli* K-12 but contains the *paa* cluster, and it is alone in *K. citrophila*. These observations suggest that the *pac* gene has been acquired by *E. coli* W as a peripheral pathway to funnel the PA esters and amides to a resident PA central pathway. Thereafter, it was further subjugated to the discipline of PaaX repression by introducing the PaaX palindromic sequences within the *Ppac* promoter.

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