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Characterization of an *Escherichia coli* Aromatic Hydroxylase with a Broad Substrate Range

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The *hpaB* gene encoding an aromatic hydroxylase of *Escherichia coli* ATCC 11105, a penicillin G acylase-producing strain, has been cloned and expressed in *E. coli* K-12. This gene was located near the *pacA* gene coding for penicillin G acylase. The hydroxylase has a molecular mass of 59,000 Da, uses NADH as a cosubstrate, and was tentatively classified as a 4-hydroxyphenylacetic acid hydroxylase, albeit it exhibited a rather broad substrate specificity acting on different monohydric and dihydric phenols. *E. coli* W, C, and B as well as *Klebsiella pneumoniae* M5a1 and *Kluyvera citrophila* ATCC 21285 (a penicillin G acylase-producing strain) but not *E. coli* K-12 contained sequences homologous to *hpaB*. Our results support the hypothesis that *hpaB* is a component of the 4-hydroxyphenylacetic acid degradative pathway of *E. coli* W.

Next to glucosyl residues, the benzene ring is the most widely distributed unit of chemical structure in nature (11, 38). The degradation of such chemicals is accomplished mainly by microorganisms (18), and in recent years, there has been a considerable interest in exploring their abilities to degrade and detoxify the increasing amounts of aromatic compounds which enter the environment as by-products of many industrial processes (14, 26).

Although a number of genera of microorganisms degrade aromatic compounds, most of the present knowledge of bacterial aromatic catabolism stems from investigations with the genus *Pseudomonas* (38). Interestingly, a substantial number of aromatic catabolic pathways are plasmid encoded, and some of them have been elucidated in detail in terms of their biochemistry, organization, and regulation of the genes (15, 23, 33, 40).

Enteric organisms have also been studied occasionally (2, 19–21, 32), but the ability of *Escherichia coli* to degrade certain aromatic compounds was not realized until Cooper and Skinner (10) grew a strain of this organism with 3- or 4-hydroxyphenylacetic acid (3-HPA and 4-HPA, respectively) and delineated the catabolic pathway used (25). In addition, Burlingame and Chapman (8) reported that many laboratory strains and clinical isolates of *E. coli* can grow with various aromatic acids. These catabolic pathways have been found to be biochemically identical to those occurring in various soil bacteria (13, 39). More recently, the unexpected ability of *E. coli* K-12 to grow in phenylacetic acid has been reported, and the genes involved in this pathway have been located in a relatively silent region of the K-12 chromosome at 30.4 min (9), although there is still uncertainty about the pathway for phenylacetic acid metabolism. It is noteworthy that *E. coli* W is able to grow on phenylacetic acid as well as on 4-HPA or 3-HPA but *E. coli* C and B are able to use only 4-HPA or 3-HPA (two substrates that cannot be metabolized by K-12) as carbon sources (8).

The aromatic catabolic pathways tend to converge on just a few key intermediates such as catechol and substituted catechols (12). These serve as substrates for cleavage of the aromatic ring and can be further metabolized by two distinct sets of enzymes, those of the *ortho* and *meta* cleavage pathways (12). The specificities of enzymes that catalyze hydroxylation are one of the factors which determine the type of compounds metabolized by the cell (12, 18, 40). When 4-HPA can be used as a growth substrate, two metabolic routes are available. Hydroxylation at C-3 of the nucleus yields the ring fission substrate homoprotocatechuate (3,4-dihydroxyphenylacetic acid [3,4-DHPA]) (1, 5, 28), which has been shown to be metabolized to carbon dioxide, pyruvate, and succinate by *Pseudomonas putida* and by an *Acinetobacter* species (39). Alternatively, it has been reported that a soil bacterium hydroxylates 4-HPA at C-1 to give homogentisic acid (2,5-dihydroxyphenylacetic acid [2,5-DHPA]) (4).

On the other hand, it is well known that some *E. coli* strains contain phenylacetyl acylases used since 1970 in the production of semisynthetic penicillins, which have been suggested to be involved in the degradation of phenylacetylated compounds (41). On the basis of this hypothesis, we have investigated the presence of aromatic catabolic genes in the vicinity of the penicillin G acylase gene (*pacA*) of *E. coli* ATCC 11105, the best characterized *pacA* gene described so far (41).

Cloning of a chromosomal DNA fragment from *E. coli* ATCC 11105 carrying an aromatic hydroxylase gene. We had previously described a procedure to isolate *pacA* genes based on the auxotrophic complementation of *E. coli* HB101 (*leuB*) in the presence of phenylacetyl-L-leucine as a sole L-leucine source (16). During the isolation of the *pacA* gene from *E. coli* ATCC 11105 (vitamin B_{12} auxotroph derivative of the W wild-type strain ATCC 9637), we detected in a *Hin*dIII gene bank constructed in pBR322 the presence of a clone presenting an unusual black phenotype on Luria-Bertani medium. This clone produced a penicillin G acylase activity and contained plasmid pAJ19 carrying the *pacA* gene in a 10-kb *Hin*dIII DNA fragment (Fig. 1). To ascertain that pAJ19 was responsible for the black phenotype, we transformed different *E. coli* strains (DH1 [36], JM101 [36], TG1 [Amersham Corp.], SE5000 [37], and HB101 [36]) with this plasmid. All clones harboring pAJ19 produced penicillin G acylase and presented a black phenotype. Interestingly, *E.*

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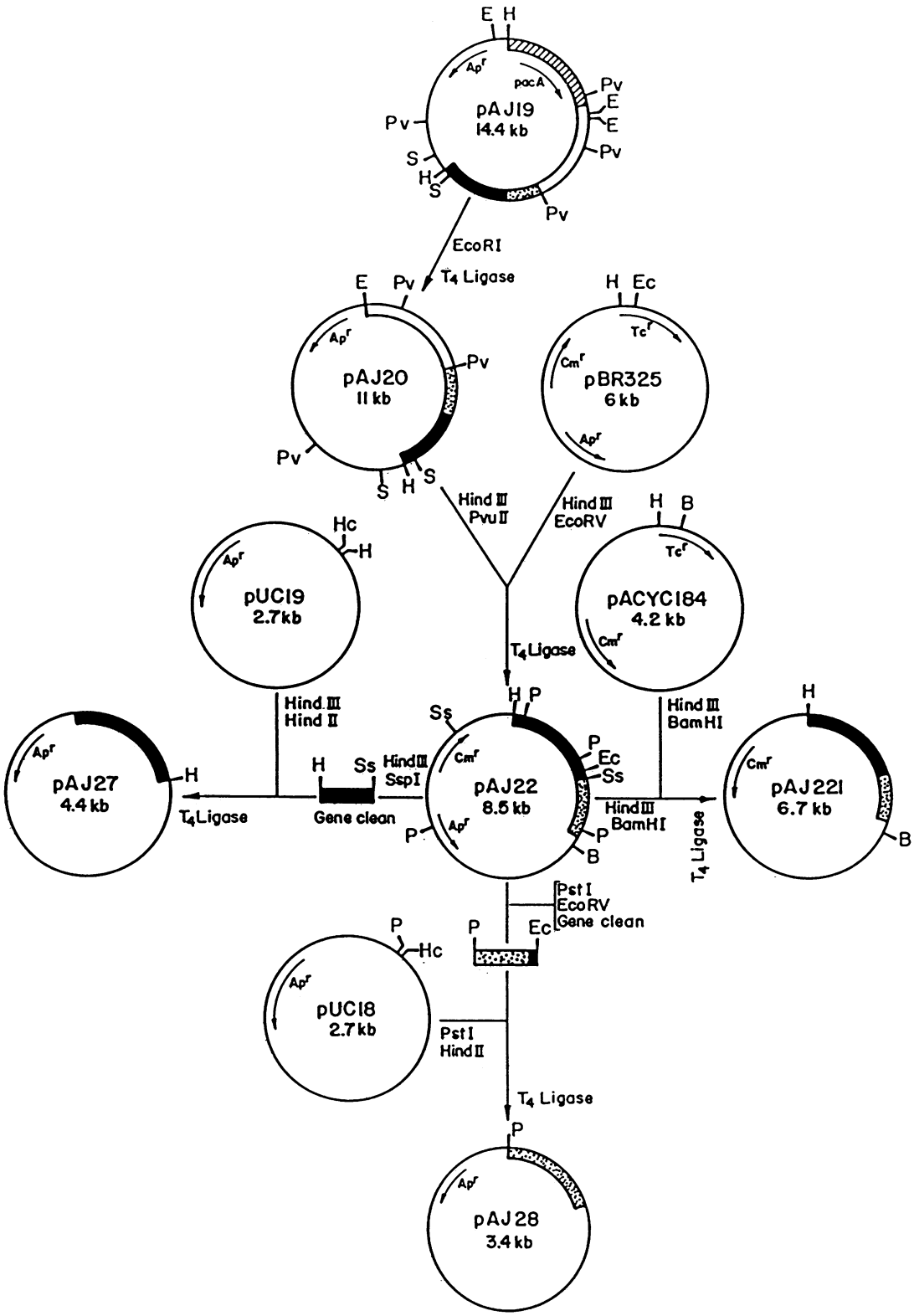

FIG. 1. Subcloning of the *hpaB* gene. Plasmids are drawn in circles; the relevant elements and restriction sites are indicated. Thin line, vector plasmid; hatched box, the *pacA* gene; black box, the *hpaB* gene; stippled box, the gene encoding protein C; white box, the gene encoding protein D. The directions of transcription of the genes are indicated by arrows. Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; B, *Bam*HI; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Ss, *Ssp*I. Plasmids: pACYC184, Cm^r Tc^r (35); pBR325, Ap^r Cm^r Tc^r (6); pUC18, Ap^r (44); and pUC19, Ap^r (44).

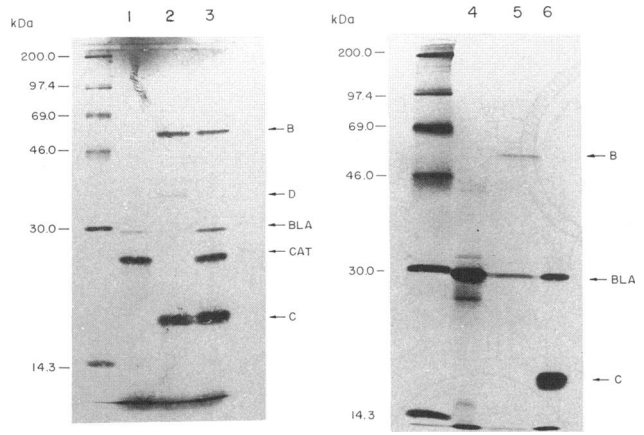

FIG. 2. Maxicell analysis of the plasmids. Autoradiography of a 12.5% sodium dodecyl sulfate-polyacrylamide gel (29) showing the [^{35}S]methionine-labeled polypeptides synthesized in *E. coli* SE5000 cells harboring plasmids (37). Lane 1, pBR325; lane 2, pAJ19; lane 3, pAJ22; lane 4, pUC18; lane 5, pAJ27; lane 6, pAJ28. Abbreviations: B, the product of the *hpaB* gene; BLA, β-lactamase; C, protein C; CAT, chloramphenicol acetyltransferase; D, protein D. The molecular sizes (in kilodaltons) of protein standards and the positions of proteins are indicated.

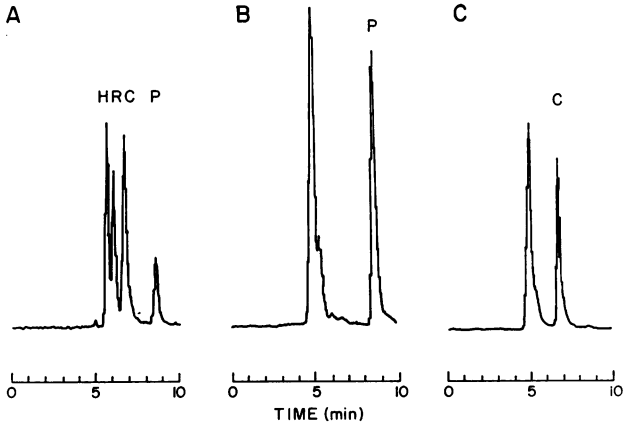

FIG. 3. Reverse-phase HPLC analysis of aromatic compounds. Cells were incubated at 37°C in M9 salts medium (36) containing 0.2% glucose and 1 mM phenol. Products contained in the culture medium were analyzed with Gilson HPLC equipment using a Nucleosil 300-5C_{18} column (250 by 4 mm) after a Nucleosil 300-5C_{18} guard column (11 by 4 mm) (mobile phase, 50 mM potassium phosphate and methanol [1:1]; flow rate, 0.5 ml/min). The detection was carried out spectrophotometrically at 280 nm. Metabolites were identified by comparison of their retention times with those of pure substances. (A) Standard mixture of hydroquinone (H), resorcinol (R), catechol (C), and phenol (P); (B) culture supernatant of *E. coli* DH1 (pBR325) cells used as a control; (C) culture supernatant of *E. coli* DH1 (pAJ221) cells.

coli clones harboring pAJ19 grown on minimal M9 glucose medium (36) produced black pigments only when supplemented with L-tyrosine. Brown pigments were also observed when L-tyrosine was replaced by other aromatic compounds such as N-acetyl-L-tyrosine, L-tyrosine-methyl ester, 4-HPA, 3-HPA, and phenol but not when it was replaced with phenylacetic acid, L-phenylalanine, or 2-HPA. Since it is well known that catechol derivatives form spontaneously black or brown oxidation products, we presumed that the cloned fragment contained an aromatic hydroxylase gene, which was named *hpaB* according to Martín et al. (30).

To localize the *hpaB* gene on plasmid pAJ19, we made a series of constructions shown in Fig. 1. The localization was facilitated by the finding that cells harboring plasmid pAJ21, constructed by a *Sal*I deletion of plasmid pAJ20, did not present the black phenotype. Plasmid pAJ27 was the smallest construction that produced a black pigment, since with further deletions and subcloning of pAJ27 this phenotype was not observed.

Maxicell analysis of the plasmids. The expression of the genes contained in the plasmids described in the previous section was analyzed by using the maxicell technique (37). Cells harboring plasmid pAJ19 showed three protein bands of 59,000, 40,000, and 19,000 Da, named B, D, and C, respectively, in addition to the band of β-lactamase (30,000 Da) (Fig. 2, lane 2). The bands corresponding to penicillin G acylase subunits (60,000 and 26,000 Da) cannot be detected on the gels, since maxicells were cultured at 37°C and without phenylacetic acid, two conditions that avoid the expression of the *pacA* gene (16). Cells containing plasmid pAJ22, which expressed proteins C and B (Fig. 2, lane 3), or plasmid pAJ27, which expressed protein B (Fig. 2, lane 5), produced hydroxylase activity and showed a black phenotype, whereas cells harboring plasmid pAJ28, which expressed protein C (Fig. 2, lane 6), did not. These results allowed us to correlate the hydroxylase activity with the expression of the 59,000-Da protein B that corresponds to the larger expected size of a protein encoded by the 1.7-kb fragment contained in pAJ27. Hence, it can be concluded that this fragment should contain only the *hpaB* gene. The molecular mass of this hydroxylase is in the same range as that for many other bacterial aromatic hydroxylases (3, 27, 31).

Coupling with the *xylE* gene. To demonstrate that the enzyme encoded by *hpaB* was able to hydroxylate phenol, we constructed pAJ221 (Fig. 1). This pACYC184 derivative plasmid allows placement of *hpaB* in *trans* with pAW31 (provided by V. de Lorenzo), a pEMBL9 derivative harboring a 1.7-kb *Sal*I fragment containing the *xylE* gene, which encodes a catechol 2,3-dioxygenase. Cells of *E. coli* JM101 (pAJ221 and pAW31) grown on minimal M9 glucose medium containing 1 mM phenol produced a characteristic yellow color due to the synthesis of 2-hydroxymuconic acid semialdehyde from catechol, which confirmed that these cells produced phenol hydroxylase activity. The presence of catechol in the culture of *E. coli* DH1 (pAJ221) grown in minimal M9 glucose medium containing phenol was also determined by high-pressure liquid chromatography (HPLC) analysis (Fig. 3). Moreover, this culture medium produced a yellow color when sprayed on plates of *E. coli* JM101 (pAW31).

Substrate specificity. Crude extracts from *E. coli* DH1 (pAJ221) were used to investigate the cofactor requirements and specificity of the cloned aromatic hydroxylase. Since we have previously determined that the hydroxylase was active on phenol, we used this compound as a substrate to determine its cofactor requirements, taking advantage of the fact that the catechol produced in the reaction can be easily and specifically detected by using extracts of *E. coli* JM101 (pAW31) containing catechol 2,3-dioxygenase. The amount of 2-hydroxymuconic acid semialdehyde produced can be determined by measuring the A_{375}. These assays allowed us to conclude that the hydroxylase activity was dependent on added NADH but not NADPH and was not stimulated by

TABLE 1. Activity of aromatic hydroxylase produced by *E. coli* DH1 (pAJ221)[a]

Compound	% Activity
4-HPA	100
3-HPA	82
2-HPA	ND[b]
3,4-DHPA	65
2,5-DHPA	155
3-Chloro-4-HPA	16
Phenylacetic acid	ND
4-Chloro-phenylacetic acid	5
o-Cresol	ND
m-Cresol	ND
p-Cresol	51
Phenol	28
2-Chlorophenol	ND
3-Chlorophenol	5
4-Chlorophenol	41
Catechol	2
Resorcinol	28
Hydroquinone	32
L-Tyrosine	5
L-Phenylalanine	ND
L-Dopa	7
D-4-Hydroxyphenylglycine	ND

[a] Cell pellets were disrupted by sonication. Hydroxylase activity was assayed by adding 10 μl of 0.1 M substrate to 0.5 ml of a solution containing 0.1 M sodium phosphate buffer [pH 8], 0.2 mM NADH, and 60 μl of clear supernatants. The initial rate of oxidation of NADH was determined on a Shimadzu UV-160 spectrophotometer from the decrease in A_{340} by using ε = 6,220 for NADH. Values were corrected for oxidation of NADH in the absence of substrate. A unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH per min. Protein was determined by the method of Bradford (7). The crude extracts contained about 5 mg of protein per ml. One hundred percent activity corresponds to about 0.2 U/mg of protein.

[b] ND, not detected.

flavin adenine dinucleotide. This behavior is similar to that showed by the 4-HPA hydroxylase from *E. coli* C (10) or the phenol hydroxylase from *Bacillus stearothermophilus* (22). However, a more precise characterization of its cofactor dependence must await purification of the enzyme, a task that has proved to be rather difficult because of the low stability of the enzyme in solution.

According to the above observation, the initial rate of oxidation of NADH determined from the decrease in A_{340} was later used to assay the hydroxylase activity. Table 1 summarizes the results obtained with different compounds as substrates. Although the hydroxylase presents a broad substrate range, it might be classified as a 4-HPA hydroxylase, since plasmid pAJ221 was able to complement the 4-HPA$^-$ (unable to grow on 4-HPA) phenotype of *E. coli* W21, which has been characterized as a 4-HPA hydroxylase-deficient mutant of *E. coli* W (17). In addition to the 4-HPA hydroxylase of *E. coli* C (10), similar enzymes from other microorganisms have been described (4, 24, 30, 42, 43); however, each enzyme has a different substrate specificity. Interestingly, 4-HPA is also capable of hydroxylating chloro- and methylaromatic compounds, which increases the potential for constructing microorganisms that can degrade environmental pollutants (34).

To demonstrate that 3,4-DHPA is the immediate degradation product of 4-HPA, *E. coli* DH1 (pAJ221) was transformed with plasmid pAG464, a pUC18 derivative carrying *hpaC* encoding the 3,4-DHPA 2,3-dioxygenase from *Klebsiella pneumoniae* M5a1 (17, 30). As expected, the characteristic yellow color of 5-carboxymethyl-2-hydroxymuconic

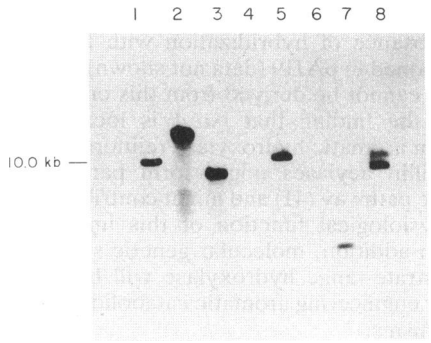

FIG. 4. Southern analysis of various chromosomal DNAs probed with the 1.6-kb *Hin*dIII-*Eco*RV fragment of pAJ22. Lanes 1 to 7, chromosomal DNAs from *E. coli* ATCC 11105, B/rK, C, DH1, W, and W3110 and *K. pneumoniae* M5a1, respectively, digested with *Hin*dIII. Lane 8, DNA from *K. citrophila* digested with *Eco*RI. The size of the band corresponding to the *Hin*dIII fragment contained in pAJ19 is indicated. The DNA fragment was labelled with [α-^{32}P]dCTP (36).

acid semialdehyde, which presents a maximum at 380 nm (10), was detected when the recombinant cells harboring both plasmids were cultured in minimal M9 salts medium containing glucose plus 4-HPA or 3-HPA but not in the presence of phenylacetic acid or phenol. These experiments allowed us to postulate that *hpaB* is a component of the 4-HPA degradative pathway of *E. coli* W.

Southern blot hybridizations of *E. coli* strains. Taking into account the fact that 4-HPA and 3-HPA were appropriate substrates for the hydroxylase (Table 1) and that the presence of a 3-HPA hydroxylase and a 4-HPA hydroxylase in *E. coli* C has been described (10), we decided to investigate by Southern blot analysis the presence of genes homologous to *hpaB* in other *E. coli* strains. The results shown in Fig. 4 indicated that *E. coli* B/rK (a UV-resistant derivative of the wild-type B strain supplied by M. Vicente), *E. coli* C (a wild-type strain supplied by M. Vicente), and *E. coli* W (a wild-type strain from R. A. Cooper laboratory stock supplied by A. Garrido-Pertierra), as well as *K. pneumoniae* M5a1 (strain UN, supplied by A. Garrido-Pertierra) (30) and *Kluyvera citrophila* ATCC 21285 (penicillin G acylase producer) (16), contained DNA sequences homologous to *hpaB*. However, the genomes of two different *E. coli* K-12 strains, DH1 and W3110 (CECT 416, ATCC 27325) (36), did not contain any homologous DNA fragment, which suggests that *hpaB* is not a gene involved in the pathway of phenylacetic acid in these strains (9). On the contrary, the hybridization observed with the other strains might indicate that *hpaB* is a gene involved in 4-HPA degradation, since all of these strains are able to metabolize 4-HPA, a characteristic not shared by K-12 strains (8). Using *hpaB* as a probe, we have isolated from an *E. coli* C genomic library a DNA fragment containing the homologous gene that expressed a similar aromatic hydroxylase activity (data not shown). The relationships between both *E. coli* genes and the homologous gene from *K. pneumoniae*, which has been recently cloned (17), will be determined after DNA sequencing of all these genes, a task that is currently in progress.

Presence of plasmids in *E. coli* ATCC 11105. Since many enzymes for aromatic catabolism are plasmid encoded, we have investigated the presence of plasmids in *E. coli* ATCC 11105. Surprisingly, we found that this strain carries a 5-kb cryptic plasmid that has not been described previously (data

not shown). However, the small size of this plasmid together with the absence of hybridization with the 10-kb *Hin*dIII fragment cloned in pAJ19 (data not shown) indicated that the *hpaB* gene cannot be derived from this cryptic plasmid.

Finally, the finding that *pacA* is located near a gene encoding an aromatic hydroxylase reinforces the hypothesis that penicillin acylases might form part of an aromatic degradative pathway (41) and might contribute to elucidation of the physiological function of this important industrial enzyme. In addition, molecular genetic studies of this new broad-substrate-range hydroxylase will be of considerable interest for engineering aromatic catabolic pathways in other microorganisms.

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