Identification of the 4-hydroxyphenylacetate transport gene of *Escherichia coli* W: construction of a highly sensitive cellular biosensor

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Abstract The mechanism of uptake of 4-hydroxyphenylacetate (4-HPA) by *Escherichia coli* W was investigated. The 4-HPA uptake was induced by 4-HPA, 3-hydroxyphenylacetate (3-HPA) or phenylacetate (PA) and showed saturation kinetics with apparent K_t and V_{max} values of 25 μM and 3 nmol/min per 10^9 cells, respectively. Transport of 4-HPA was resistant to *N,N'*-dimethylcarbodiimide (DCCD), but was completely inhibited by cyanide and 4-nitrophenol, and, to a lower extent, by arsenate and azide, suggesting that energy is required for the uptake process. Competition studies showed that 4-HPA uptake was inhibited by 3-HPA or 3,4-dihydroxyphenylacetate (3,4-DHPA) but not by 2-hydroxyphenylacetate (2-HPA), L-tyrosine or other structural analogues, indicating a narrow specificity of the transport system. We have demonstrated, using two experimental approaches, that the *hpaX* gene of the 4-HPA catabolic cluster, which encodes a protein of the superfamily of transmembrane facilitators, is responsible for 4-HPA transport. Aside from the aromatic amino acid transport systems, *hpaX* is the first transport gene for an aromatic compound of enteric bacteria that has been characterized. A highly sensitive cellular biosensor has been constructed by coupling the 4-HPA transport system to a regulatory circuit that controls the production of β-galactosidase. This biosensor has allowed us to demonstrate that the transport system performs efficiently at very low external concentrations of 4-HPA, similar to levels that would be expected to occur in natural environments.

Key words: Aromatic uptake; Aromatic metabolism; *Escherichia coli*

1. Introduction

Notwithstanding the fact that transport of a compound across the membrane is a requirement for its catabolism, few reports on the characterization of the processes involved in the uptake of aromatic compounds by soil bacteria have appeared in the literature. The uptake of substrates and particularly the aromatic acids, being lipophilic weak acids, is often ignored or considered to proceed by passive diffusion. However, some studies have shown that the transport of aromatic compounds can be carried out by specific permeases of soil bacteria [1–6]. Up to now, three *Pseudomonas* genes, *pcaK*, *pht1* and *todX*, have been proposed to encode membrane proteins required for the transport of 4-hydroxybenzoate [1], phthalate [7] and toluene [8], respectively, but of these, only the *pcaK* gene has been shown to encode an authentic transporter [1].

The capacity to use aromatic compounds as sources of carbon and energy has been mainly studied in soil bacteria, but recently enteric bacteria have also been shown to possess aromatic catabolic pathways [9–14]. The transport of aromatic compounds in these bacteria, with the exception of the aromatic amino acid transport in *Escherichia coli* K-12 [15], has only been investigated in *Klebsiella pneumoniae* M5a1 [16,17]. This strain has been shown to possess inducible transport systems for 4-HPA [16] and 3-hydroxybenzoate [17], but the genes responsible for these systems remain unknown. Recently, a DNA fragment of *E. coli* W encoding the complete 4-HPA catabolic pathway has been cloned and sequenced [12]. The gene cluster that encodes this pathway contains the *hpaX* gene, whose gene product is related to the superfamily of transmembrane facilitators and which might function as the 4-HPA specific transporter [12].

This paper reports on the identification of HpaX as a transport system for 4-HPA in *E. coli*. To the best of our knowledge, this represents the first example so far of a transport protein for an aromatic compound, other than amino acids, that has been identified in enteric bacteria and particularly in this important model microorganism.

2. Materials and methods

2.1. Materials

Restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were from Pharmacia Fine Chemicals. L-Amino acid oxidase, 2-HPA, 3-HPA, 4-HPA, 3,4-DHPA, PA, phenylpropionate, 3-hydroxyphenylpropionate, L-tyrosine, 2,5-dihydroxyphenylacetate (homogentisate), 4-nitrophenol, and DCCD were obtained from Sigma. L-[U-^{14}C]Tyrosine (443 mCi/mmol) was supplied by the Radiochemical Center, Amersham. Culture media were from Difco. All other chemicals were of the highest grade available and were purchased from Sigma or Merck.

2.2. Strains, plasmids, media and growth conditions

The bacterial strains used were *E. coli* W ATCC 11105, *E. coli* ET4025 [12], *E. coli* ET8000 [12] and *E. coli* MC4100 [13]. The plasmids used were pACYC184 [18], pRX1 and pRA2 [13]. Bacteria were grown at 30°C in Luria broth or M63 minimal medium [19] containing 20 mM glycerol, thiamine (1 mg/ml) and vitamin B12 (1 mg/ml). These media were supplemented, when required, with the appropriate antibiotics.

2.3. DNA manipulations and transformation

Plasmid preparation and isolation of DNA fragments were carried out by standard procedures [20]. Restriction endonucleases and T4 DNA ligase were used according to the manufacturer's instructions. Transformation of *E. coli* cells was carried out using the RbCl method [20] or by electroporation (Gene Pulser, Bio-Rad).

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Abbreviations: 2-HPA, 2-hydroxyphenylacetate; 3-HPA, 3-hydroxyphenylacetate; 4-HPA, 4-hydroxyphenylacetate; PA, phenylacetate; 3,4-DHPA, 3,4-dihydroxyphenylacetate; DCCD, *N,N'*-dicyclohexylcarbodiimide

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2.4. Preparation of 4-[U-^{14}C]HPA

Labeled 4-[U-^{14}C]HPA was prepared as previously described [16] from L-[U-^{14}C]tyrosine by using L-amino acid oxidase which oxidatively deaminates L-tyrosine to 4-hydroxyphenylpyruvate which, in the absence of catalase, is spontaneously decarboxylated to 4-HPA. The reaction was started by addition of 5 μl (1 U) of L-amino acid oxidase in 95 μl of 20 mM phosphate buffer, pH 7, containing 45 nmol (20 μCi, 443 mCi/mmol) of L-[U-^{14}C]tyrosine. After 12 h of incubation at 37°C, 10 μl of H_2O_2 was added to the mixture and vigorously vortexed for 1 min. Then, the mixture was heated at 80°C for 30 min and the clear supernatant was separated by centrifugation at 4°C for 15 min in a microcentrifuge. 4-[U-^{14}C]HPA was isolated by HPLC (Gilson) using a Nucleosil 300-5C_{18} column (4 mm×250 mm) and a Nucleosil 300-5C_{18} guard column (4 mm×11 mm) (mobile phase 8% acetonitrile, pH 3.5; flow rate, 1 ml/min). The detection was carried out spectrophotometrically at 280 nm. Samples containing 4-[U-^{14}C]HPA were pooled, concentrated in a Speedvac, and stored at −70°C.

2.5. Transport assays

Bacteria from an overnight culture were inoculated into fresh M63 medium at an A_{600} of 0.1, and cultured for 1 h before the addition of the inducer. After the induction, cells were grown for two additional hours until the culture reached an A_{600} of 0.3–0.7. Cells were harvested by centrifugation, washed twice in 50 mM sodium phosphate buffer, pH 7, and the pellet was resuspended in the same buffer to an A_{600} of 5.0 (1 $A_{600} = 4.6 \times 10^8$ cfu/ml). The uptake of radioactive 4-HPA into cells was followed in a reaction mixture containing 50 μl of prewarmed cells (at 30°C for 5 min) and 50 μl of 40 μM 4-[U-^{14}C]HPA (20 000 cpm). Samples were incubated at 30°C in a water bath, filtered at various time intervals through Millipore HA filters (0.45 μm pore diameter) and washed, under suction, with 10 ml of cold water. The filter was dissolved in 3 ml of scintillation fluid (Cocktail Ready Safe, Beckman) and counted in a LKB Wallac liquid scintillation counter. To test the effect of energy metabolic inhibitors the assays were performed according to the standard procedure but using 50 mM Tris-HCl, pH 7.0 instead of phosphate buffer to avoid the competitive effect of phosphate on the assays where arsenate is involved. Control experiments were carried out to confirm that the 4-HPA transport in the presence of Tris-HCl was similar to that observed in phosphate buffer. The inhibitors were added, to a final concentration of 1 mM, 2 min before the addition of labeled 4-HPA.

2.6. β-Galactosidase assays

β-Galactosidase levels were determined in cells permeabilized with chloroform and SDS according to Miller [19].

3. Results and discussion

3.1. Characterization of the 4-HPA transport in E. coli W

The transport of 4-HPA was firstly investigated in the wild type strain *E. coli* W by growing the cells in minimal medium containing 20 mM glycerol plus 1 mM 4-HPA. In this medium, 4-HPA uptake reached a maximum in the early exponential phase (Fig. 1). Likely, the uptake decreases as soon as the 4-HPA is metabolized. The time course of 4-HPA uptake revealed that 4-HPA is accumulated for 3 min, but after this time, the rate of uptake plateaued, possibly because the system became saturated with labeled substrate (Fig. 2). When the uptake was studied as a function of the 4-HPA concentration we obtained saturation kinetics with a K_t of 25 μM and a V_{max} of 3 nmol/min per 10^9 cells. The K_t value was similar to the K_t values found for the transport in *K. pneumoniae* M5a1 of 4-HPA (16.3 μM) [16] and 3-hydroxybenzoate (13 μM) [17]. When cells were grown in the absence of 4-HPA, uptake was not detected suggesting that it was inducible. When other potential inducers were tested we observed that, while 3-HPA induced similar transport rates to 4-HPA, PA induced the uptake at a lower rate (Table 1). Interestingly, the addition of the structural analogues 2-HPA and 3,4-DHPA did not induce 4-HPA transport suggesting some structural restrictions in the induction mechanism. These results are in good agreement with those observed for 4-HPA transport in *K. pneumoniae* M5a1 [16]. When the competitive inhibition of 4-HPA uptake by structural analogues of 4-HPA was investigated we observed that 3-HPA and 3,4-DHPA are able to reduce 4-HPA transport, whereas 2-HPA, PA and the other aromatic compounds tested were ineffective as inhibitors (Table 2). L-Tyrosine did not inhibit the 4-HPA uptake, suggesting that 4-HPA is not taken up by the transport system involved in the uptake of this aromatic amino acid. Interestingly, it has been shown that 3-HPA and 3,4-DHPA also act as competitive inhibitors of the 4-HPA transport in *K. pneumoniae* M5a1 [16]. Transport of 4-HPA was completely abolished by cyanide and the transport uncoupler 4-nitrophenol. Azide and arsenate resulted in a decrease of the transport rate up to 62% and 21%, respectively, but the ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD) did not affect the transport. These results suggest that the 4-HPA transport is controlled by an active transport system and not by a passive or facilitated mechanism. To ascertain whether the transport system uses high-energy phosphates as source of energy or is driven by an energy-rich membrane state will require further analysis.

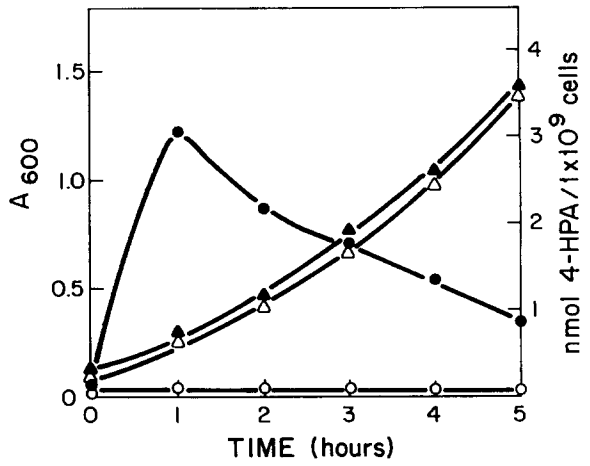

Fig. 1. Development of 4-HPA transport activity during the growth of *E. coli*. Cells of *E. coli* W were cultured in M63 minimal medium containing 20 mM glycerol in the presence (▲) or absence (△) of 1 mM 4-HPA. The uptake of 4-[U-^{14}C]HPA was determined for 1 min in the presence (●) or absence (○) of 4-HPA.

Table 1
Transport of labeled 4-HPA by *E. coli* W grown on various compounds

Inducer	4-HPA transport rate (%)[a]
None	ND[b]
4-Hydroxyphenylacetate	100
3-Hydroxyphenylacetate	80
2-Hydroxyphenylacetate	ND
Phenylacetate	25
3,4-Dihydroxyphenylacetate	ND
3-Hydroxyphenylpropionate	ND
Pheylpropionate	ND

[a]Cells were grown in M63 minimal medium containing 20 mM glycerol supplemented with 1 mM of the potential inducer until the culture reached an A_{600} of 0.5. Cells were harvested, washed and assayed for 4-HPA transport during 1 min as described in Section 2 using 40 μM 4-[U-^{14}C]HPA (20 000 cpm).
[b]ND, below the limit of detection.

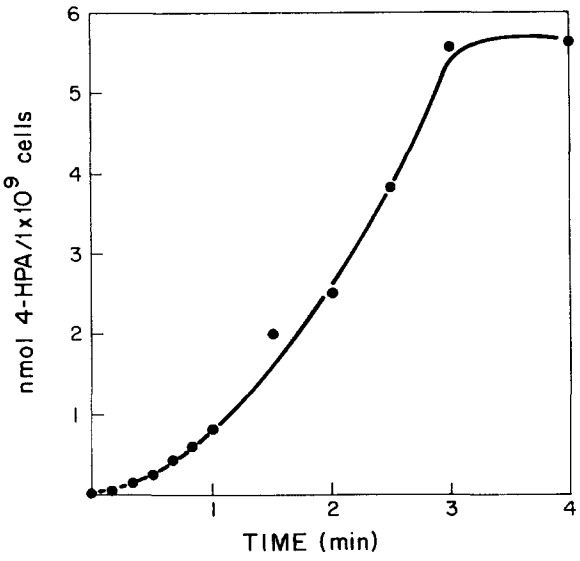

Fig. 2. Time course of the 4-[U-^{14}C]HPA uptake in *E. coli* W. The assay was carried out as described in Section 2 using *E. coli* W cells cultured in M63 minimal medium containing 20 mM glycerol and 1 mM 4-HPA.

Although it could be mandatory to first investigate the uptake rate of a catabolic substrate in the wild type strains as is shown above, it is well known that the observed accumulation of radioactive materials in these strains could arise from the contribution of two different phenomena. The uptake activity can be a direct consequence of the expression of the transport machinery, but it might be also ascribed to a simultaneous or independent expression of the catabolic cluster that rapidly metabolized the internalized labeled substrate increasing the accumulation of radioactive metabolites. Moreover, at the concentrations normally used in the induction (1 mM) or inhibition (250 μM) assays a significant amount of the tested compound could be internalized by passive diffusion, which might induce or inhibit the substrate catabolism. In this context, it is interesting to point out that the induction profile of the 4-HPA uptake is identical to that observed for the expression of the first step of the 4-HPA catabolism, i.e. the induction of 4-hydroxyphenylacetate-3-hydroxylase [13]. In addition, the two major uptake inhibitors, 3,4-DHPA and 3-HPA, can also be metabolized by the 4-HPA cluster [12], and therefore, they could reduce the mineralization of the labeled 4-HPA. Thus, to accurately determine the properties of the 4-HPA uptake system it would be necessary to isolate and test the 4-HPA transport gene in strains such as *E. coli* K-12, which are able to correctly express it, but cannot metabolize 4-HPA [12,13]. In support of this, we have observed that the insertion of a single copy of the 4-HPA catabolic cluster into the chromosome of the *E. coli* K-12 strain ET8000 transfers to the recombinant strain ET4025 the capacity to metabolize [12] and transport 4-HPA (data not shown). This result confirms that *E. coli* K-12 can be used as host to analyze the transport system and strongly suggests that the 4-HPA catabolic cluster encodes the 4-HPA transport gene.

Table 2
Competitive inhibition of 4-HPA transport by structural analogues of 4-HPA

Structural analogue	4-HPA transport rate (%)[a]
None	100
4-Hydroxyphenylacetate	17
3-Hydroxyphenylacetate	24
2-Hydroxyphenylacetate	96
Phenylacetate	87
3,4-Dihydroxyphenylacetate	24
2,5-Dihydroxyphenylacetate	96
L-Tyrosine	89

[a]Cells were grown in M63 minimal medium containing 20 mM glycerol and 1 mM of 4-HPA until the culture reached an A_{600} of 0.5. Cells were harvested, washed and assayed for 4-HPA transport during 1 min as described in Section 2 using 40 μM 4-[U-^{14}C]HPA (20000 cpm) in the presence of 250 μM of the structurally related compounds.

3.2. Identification of the 4-HPA transport gene

Analysis of the deduced amino acid sequence encoded by the *hpaX* gene of the 4-HPA degradative cluster indicated that HpaX could be a member of the large group of transport proteins termed the major facilitator superfamily [12]. This superfamily, primarily made up of prokaryotic and eukaryotic transporters of sugars and drugs, was identified on the basis of the functions and conserved secondary structures of its members. These proteins are predicted to have 12 hydrophobic or amphipathic membrane-spanning α-helices that form a channel for transport through the cytoplasmic membrane. In this sense, the hydrophilicity profile of HpaX suggested this protein as a hydrophobic polypeptide that may indeed span the membrane 12 or more times [12]. In addition, the primary structure of HpaX shows all the characteristic motifs of the superfamily [12,21] that are also present in all the characterized or putative transporters of aromatic compounds other than aromatic amino acids that have been sequenced so far [14]. Nevertheless, since the number of the primary structures that have been determined is still small, a comparative anal-

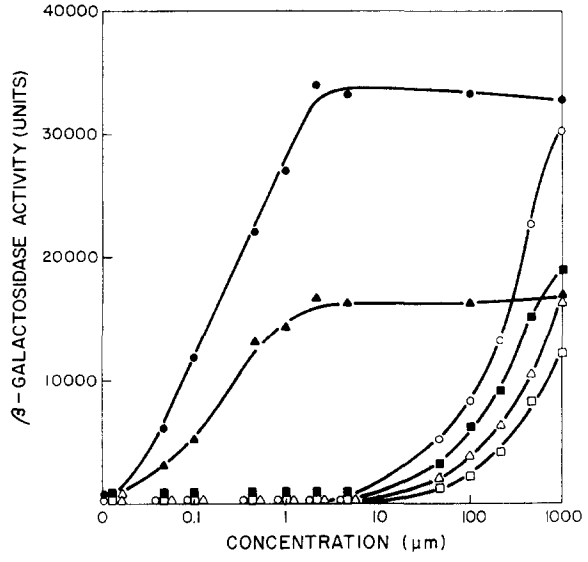

Fig. 3. Induction of the P_{BC}-*lacZ* fusion in the presence or absence of the *hpaX* gene. The β-galactosidase activity was determined in *E. coli* MC4100 (pRX1, pRA2) (solid symbols) or *E. coli* MC4100 (pACYC184, pRA2) (open symbols) cells cultured in M63 medium containing glycerol 20 mM, chloramphenicol (34 μg/ml), kanamycin (50 μg/ml) and different concentrations of 4-HPA (○, ●), 3-HPA (△, ▲), or PA (□, ■). Cells were inoculated at an A_{600} of 0.3 and the assays were performed after about 5 h of incubation at 30°C when cultures reached an A_{600} of 1.0.

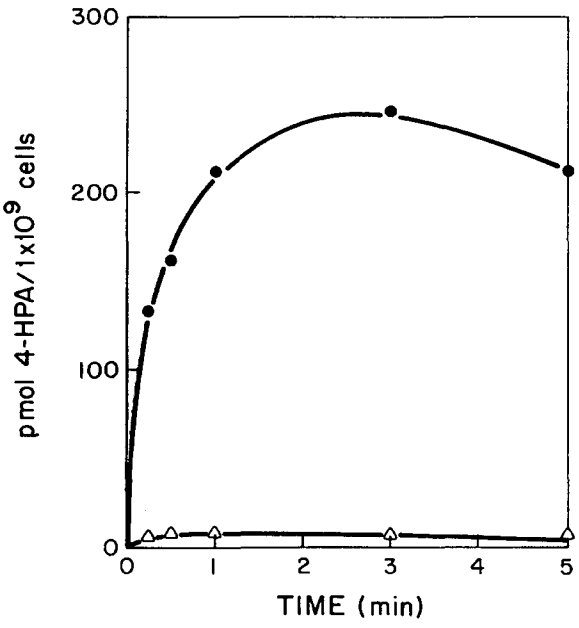

Fig. 4. Transport of 4-HPA in *E. coli* MC4100 (pRX1). 4-[U-^{14}C]-HPA uptake by *E. coli* MC4100 (pRX1) (●) was compared with that by *E. coli* MC4100 (pACYC184) cells used as control (△). Cells were grown in M63 minimal medium containing 20 mM glycerol and chloramphenicol (34 μg/ml). The assays were performed using 10 μM 4-[U-^{14}C]HPA (10^6 cpm).

ysis did not reveal any significant similarity that could account for the specific recognition of the aromatic ring [14,22]. Moreover, as pointed out above, a direct implication in transport has only been demonstrated in the case of *pcaK* [1]. Therefore, it became necessary to demonstrate that the *hpaX* gene encoded the 4-HPA specific transporter and this was carried out by different experimental approaches.

The first evidence that the *hpaX* gene was responsible for the 4-HPA uptake was obtained by constructing a cellular biosensor coupling the 4-HPA uptake to the expression of the *lacZ* gene under the control of the P_{BC} promoter. It is known that the expression of the *hpaBC* operon encoding 4-hydroxyphenylacetate-3-hydroxylase is driven by the P_{BC} promoter [13], and that this promoter is activated by 4-HPA in the presence of the positive regulator HpaA, which belongs to the AraC/XylS family of regulators [13]. The experiments were carried out using the compatible plasmids: pRA2 (P_A-hpaA-P_{BC}-lacZ) which harbors the 4-HPA inducible *lacZ* gene, and pRX1 (P_X-*hpaX*) which expresses the *hpaX* gene [13]. Thus, we tested in *E. coli* MC4100 (pRX1, pRA2) the effect of 4-HPA and other structurally related compounds on β-galactosidase production. As a negative transport control we used *E. coli* MC4100 (pACYC184, pRA2) where plasmid pRX1 was substituted by the parental vector pACYC184. Fig. 3 shows that the highest β-galactosidase production in *E. coli* MC4100 (pRX1, pRA2) was observed at 1 μM 4-HPA, whereas in the control strain the maximum activity was reached at a 1000 times higher concentration of inducer. Moreover, we were able to detect β-galactosidase production in *E. coli* MC4100 (pRX1, pRA2) at a 4-HPA concentration lower than 100 nM demonstrating that the presence of the HpaX transport system transforms this strain into a highly sensitive 4-HPA cellular biosensor. Interestingly, 3-HPA was also able to induce the production of β-galactosidase at low concentrations, whereas PA produces a significant induction effect only at high concentrations (Fig. 3). The higher level of β-galactosidase produced in the presence of 4-HPA correlates with the efficiency of this compound on HpaA induction when compared to 3-HPA or PA [13]. These results are in agreement with the observation that the 4-HPA cluster can also metabolize 3-HPA but not PA [12] and strongly suggest that *hpaX* encodes a transporter specific for 4-HPA/3-HPA. Moreover, these experiments also show that the production of HpaX is essential for the transport of 4-HPA or 3-HPA at low concentrations. Thus, most probably the effects observed at high concentrations should be ascribed to passive diffusion. In general, since aromatic acids are membrane permeable, it has been argued that specific carriers for this class of compounds are not necessary. This assumption could be the reason why the transport of aromatic acids across bacterial cytoplasmic membranes is still poorly understood and why the existence of specific transporters has mainly been inferred from physiological studies. However, our findings support the idea that an active transport system would be important for optimal growth at the very low substrate concentrations that would occur in natural environments.

Finally, the role of the HpaX protein for transporting 4-HPA was determined directly by measuring the uptake of 4-HPA in *E. coli* MC4100 (pRX1). Fig. 4 shows that *E. coli* MC4100 (pRX1) cells accumulate 4-HPA at a substantial rate. Cells harboring the control plasmid pACYC184 failed to accumulate label, ruling out the possibility that 4-HPA was simply sticking to the cell surface. Since *E. coli* MC4100 cannot metabolize 4-HPA, this result confirmed the indirect observation obtained with the *lacZ* fusions, that HpaX can transport 4-HPA independently of the presence of the catabolic machinery. In this connection, we have observed that the accumulation of 4-HPA in *E. coli* MC4100 (pRX1) was completely abolished by the transport uncoupler 4-nitrophenol but not by DCCD. This finding is in agreement with the results observed in *E. coli* W and reinforces the idea that 4-HPA is transported by an active transport and not by a passive mechanism coupled to the metabolism of 4-HPA. Interestingly, the production of HpaX in *E. coli* MC4100 (pRX1) appears to be constitutive, suggesting either that it is controlled by a promoter contained in the pACYC184 vector plasmid or that the *hpaX* gene is expressed constitutively in the absence of a regulator. In support of this, we observed with a *lacZ* reporter fused to the upstream region of the *hpaX* gene, which should contain the P_X promoter, that the β-galactosidase production was constitutive in *E. coli* MC4100 harboring plasmid pRX2 (P_X-*lacZ*) (data not shown).

Summarizing, the results presented here provide conclusive evidence that the *hpaX* gene encodes the first aromatic transporter genetically identified in enteric bacteria, excluding those involved in the uptake of aromatic amino acids. These studies open a new scenario to analyze in a model microorganism such as *E. coli* the relevance of a specific transport system in the mineralization of aromatic compounds. Moreover, the characterization of this aromatic transport system provides the opportunity of modifying its substrate specificity in order to create transposable transport cassettes. These, when introduced into a microorganism, could favor the uptake of low concentrations of aromatic xenobiotic compounds for which no transport systems have yet evolved. Finally, by coupling the modified transport proteins to different sensor

circuits, highly sensitive cellular biosensors for aromatic contaminants can be engineered.

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