Identification and characterization of the PhhR regulon in *Pseudomonas putida*

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Summary

***Pseudomonas putida* is a soil microorganism that utilizes aromatic amino acids present in root exudates as a nitrogen source. We have previously shown that the PhhR transcriptional regulator induces *phhAB* genes encoding a phenylalanine hydroxylase. In this study we show, using microarray assays and promoter fusions, that PhhR is a global regulator responsible for the activation of genes essential for phenylalanine degradation, phenylalanine homeostasis and other genes of unknown function. Recently, it has been shown that phenylalanine catabolism occurs through more than one pathway. One of these possible pathways involves the metabolism of phenylalanine via tyrosine, *p*-hydroxyphenylpyruvate, and homogentisate. We identified two genes within this pathway that encode an acyl-CoA transferase involved in the metabolism of acetoacetate. All genes in this pathway were induced in response to phenylalanine in a PhhR-proficient background. The second potential degradative pathway involves the degradation of phenylalanine to produce phenylpyruvate, which seems to be degraded via phenylacetyl-CoA. A number of mutants in the *paa* genes encoding phenylacetyl-CoA degradation enzymes fail to grow on phenylpyruvate or phenylacetate, further supporting the existence of this second pathway. We found that the PhhR regulon also includes genes involved in the biosynthesis of aromatic amino acids that are repressed in the presence of phenylalanine, suggesting the possibility of feedback at the transcriptional level. In addition, we found that PhhR modulates the level of expression of the broad-substrate-specificity MexEF/OprN efflux pump. Expression from this pump is under the control of *mexT* gene product because phenylalanine-dependent transcription from the *mexE* promoter does not occur in a *mexT* mutant background. These results place PhhR as an important regulator in the control of bacterial responses to aromatic amino acids.**

Introduction

Amino acids are abundant in the environment and are produced by the proteolysis of peptides and proteins that occurs during the recycling of organic matter. Free amino acids are also found in soils as components of root exudates where they influence the composition of the soil microbial community in the immediate vicinity of plants, while favouring beneficial plant–microbe interactions (Rovira, 1969; Prikryl and Vancura, 1980; Bertin *et al.*, 2003).

Pseudomonas putida KT2440 is a soil bacterium that colonizes the roots of a wide variety of plants at a high cell density and is responsive to compounds present in plant root exudates (Molina *et al.*, 2000; Matilla *et al.*, 2007). This microorganism can use a number of amino acids, such as proline, glutamate and lysine, as both C- and N-sources (Letto *et al.*, 1986; Vílchez *et al.*, 2000; Revelles *et al.*, 2005; Revelles *et al.*, 2007). In *P. putida* and other species of the genus *Pseudomonas*, the use of *L*-phenylalanine as an N-source involves its conversion into *L*-tyrosine (Zhao *et al.*, 1994; Song and Jensen, 1996; Wei-Gu *et al.*, 1998) (Fig. 1), through a reaction mediated by a pterin-dependent phenylalanine hydroxylase. This catabolic step requires the products of the *phhA* and *phhB* genes, which together form an operon. PhhB serves to regenerate the pterin cofactor, whereas PhhA is the catalytic hydroxylase. It has been proposed that, in *Pseudomonas* sp. strains, *L*-tyrosine is metabolized following its deamination to produce *p*-hydroxyphenylpyruvate (Arias-Barrau *et al.*, 2004). In fact, five enzymes with tyrosine aminotransferase activity have been found in *Pseudomonas aeruginosa* (Whitaker *et al.*, 1982), whereas in *P. putida* KT2440 two open reading frames (ORFs), *tyrB1* and *tyrB2*, encoding PP1972 and PP3590, respectively, were annotated as tyrosine aminotransferases (Nelson *et al.*, 2002). *p*-Hydroxyphenylpyruvate has been shown to be subsequently converted into Krebs cycle intermediates

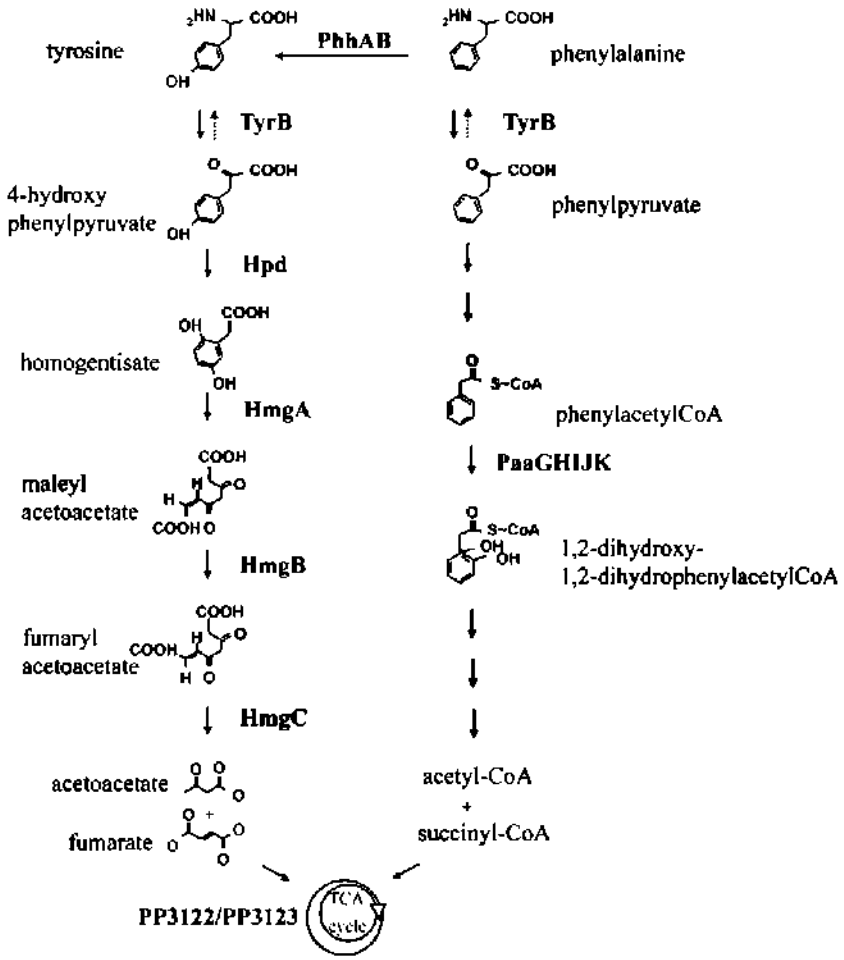

Fig. 1. Metabolic circuits for the degradation of phenylalanine. Degradation of phenylalanine via tyrosine and homogentisate was documented in previous studies in the genus *Pseudomonas*. The phenylpyruvate/phenylacetyl-CoA branch is proposed based on the results presented in this study.

through a series of steps in which homogentisate (2,5-dihydroxyphenylacetate), maleylacetoacetate and fumarylacetoacetate, which is hydrolysed to produce fumarate and acetoacetate, are produced (Arias-Barrau *et al.*, 2004) (Fig. 1).

Importantly, we found that with phenylalanine as the sole N-source *P. putida* KT2440 mutants with knockouts in *phhA* and *phhB* still grew, although at a slower rate than the parental strain (Herrera and Ramos, 2007). This suggests that the pathway originally annotated as the *phh* pathway may not be the only pathway involved in the initial catabolism of this aromatic amino acid in this strain. It should also be mentioned that for some Gram-positive and Gram-negative bacteria the production of phenylpyruvate during degradation of phenylalanine has been reported (Lee and Desmazeaud, 1986; Spaepen *et al.*, 2007). This alternative pathway may involve the transamination of the amino group of phenylalanine into a 2-keto acid leading to the production of phenylpyruvate and the corresponding amino acid (i.e. glutamate from α-ketoglutarate). Another alternative pathway has been described in *Rhodobacter sphaeroides* and certain actinomycetes, which metabolize *L*-tyrosine from *L*-phenylalanine through 2,4-dihydroxyphenylalanine (Sukumaram *et al.*, 1979; Ranjith *et al.*, 2007).

To more fully explore the metabolism of phenylalanine we used DNA microarrays to study the response of the parental *P. putida* KT2440 and its isogenic PhhR-deficient strain to phenylalanine. The results of these studies led to the present results, which include the identification of the PhhR regulon in which PhhR controls the expression of multiple genes potentially involved in phenylalanine degradation and phenylalanine homeostasis.

Results and discussion

Identification of genes induced/repressed by PhhR using microarray analysis

To study the response of *P. putida* KT2440 (Table 1) to phenylalanine we carried out a series of microarray experiments. First, *P. putida* cells were cultured in modified M9 minimal medium with glucose as a C-source until the mid-exponential phase (turbidity at 660 nm was

Table 1. Strains and plasmids used in this study.

	Relevant characteristics	Reference
Strain		
P. putida KT2440	Cm^R; Ap^R	Abril *et al.* (1989)
KT2440-MCH4 (*phhR:aphA3*)	Cm^R; Km^R	Herrera and Ramos (2007)
KT2440-MCH5 (*tyrB-1*:Tc)	Cm^R; Tc^R	This study
KT2440-MCH6 (PP3122:Km)	Cm^R; Km^R	This study
P. putida KT2440 *tyrB-2*	Cm^R; Km^R	Revelles *et al.* (2005)
P. putida KT2440 *hpd*	Cm^R; Km^R	Duque *et al.* (2007b)
P. putida KT2440 *mexT*	Cm^R; Km^R	Duque *et al.* (2007b)
P. putida KT2440 PP3458:Km	Cm^R; Km^R	Duque *et al.* (2007b)
Plasmids		
pMRS101	Suicide vector, Sm^R	Sarker and Cornelis (1997)
pMP220	'*lacZ*; IncP; Tc^R	Spaink *et al.* (1987)
pMCR1	P_{phhR}:*lacZ*; Tc^R	Herrera and Ramos (2007)
pMCA1	P_{phhA}:*lacZ*; Tc^R	Herrera and Ramos (2007)
pMChmg	P_{hmg}:*lacZ*; Tc^R	This study
pMC3122	P_{PP3122}:*lacZ*; Tc^R	This study
pMC3278	P_{PP3278}:*lacZ*; Tc^R	This study
pMC2827	P_{PP2827}:*lacZ*; Tc^R	This study
pMC3285	P_{PP3285}:*lacZ*; Tc^R	This study
pTYRB-1	pUC18NotI; *tyrB-1*; Ap^R	This study
pTYRB-1Tc	pUC18NotI; *tyrB-1:tet*; Ap^R; Tc^R	This study
pMRS101-TYRB-1Tc	pMRS101; *tyrB-1:tet*; Sm^R; Tc^R; *sacB*	This study
pCHESI	suicide vector, Km^R	Sarker and Cornelis (1997)
pCHESI:PP3122	Km^R	This study

Mutants with insertions in the *hpd, mexT and* PP3458 genes were obtained from the *Pseudomonas* Reference Culture Collection. The transposon insertion site in each of the mutants was confirmed by DNA sequencing. Cm^R, Ap^R, Km^R and Sm^R stand for resistance to chloramphenicol, ampicillin, kanamycin, tetracycline and streptomycin respectively.

around 0.6), at which point the cultures were split into two equal aliquots – one aliquot was kept as a control and to the other we added 5 mM phenylalanine. After a 15 min incubation, the total RNA was extracted, from which cDNA was synthesized and differentially labelled with fluorophores C3 (RNA from cultures without phenylalanine) or C5 (RNA from cultures with phenylalanine), and then hybridized to a KT2440 DNA microarray (Yuste *et al.*, 2006; Duque *et al.*, 2007a), as described in the *Experimental procedures*. In *P. putida* KT2440, in response to phenylalanine, we found that 21 genes were induced if we established a cut-off value of ≥ 1.8 and a *P*-value of ≤ 0.05 while considering three independent experiments (Table 2, column A). When the physical organization of the induced genes was analysed in detail we found that some of these genes form operons due to the presence of overlapping ORFs as well as extremely short distances between consecutive genes, whereas others were monocystronic units (i.e. PP3458, PP3468, PP4489). For the newly identified phenylalanine-induced genes, mRNA contiguity was confirmed between adjacent genes (for example between sets PP3122/PP3123, PP2595/PP2596, PP2608/PP2607 and PP3433/PP3434) using reverse transcriptase-polynucleotide chain reaction (RT-PCR) assays with primers that hybridized at the 3′ end of the upstream gene and at the 5′ end of the downstream gene (not shown). Taking into account previously available information about the operon structure of *phhAB*, the PP4619/PP4621 gene set, and *paaGHIJK* genes, we were able to deduce that the number of phenylalanine-dependent promoters was 11.

To obtain a more complete picture of the genes whose expression is modulated by phenylalanine and/or PhhR, we carried out a new set of microarray experiments in which we compared the global transcriptional profiles of the wild-type strain versus the PhhR mutant with cells grown in the absence (Table 2, column B) and in the presence of phenylalanine (Table 2, column C). We identified 10 more genes whose expression level did not change in the wild type regardless of the presence of phenylalanine, but did so when parental versus PhhR mutant were compared (see column A versus B and C in Table 2).

The analysis of the data in Table 2 allowed us to establish four groups of genes.

(1) Group 1 includes genes induced by PhhR in a phenylalanine-dependent manner, i.e. genes that in the parental versus mutant (PhhR null) cells only showed increased expression when phenylalanine was present. This group contains 13 genes: PP2595/2596, PP3122/PP3123, PP3433/PP3434, PP3458, PP3468, PP4490/PP4491, and PP4619/PP4620/PP4621 (genes separated by a backslash are co-transcribed). When the operon organization of this group of genes is considered, the number of putative PhhR- and phenylalanine-dependent promoters identified becomes 7. Analysis of the potential

Table 2. Global transcriptional analysis of genes under PhhR control.

	A	B	C	PhhR box
Group 1				
PP2595 ABC transporter permease/ATP-binding protein putative	**2.3**		**–2**	–
PP2596 ABC transporter permease/ATP-binding protein putative	**1.8**		**–1.8**	–
PP3122 CoA-transferase subunit A putative	**4.2**		**–4.3**	+
PP3123 CoA-transferase subunit B putative	**2.1**		**–2.1**	–
PP3433 Hpd-4-hydroxyphenylpyruvate dioxygenase	**11.5**		**–9.8**	–
PP3434 hypothetical protein	**9.5**		**–7.2**	+
PP3458 long-chain-fatty-acid-CoA ligase putative	4.8		–3.2	–
PP3468 hypothetical protein	2		–1.4	–
PP4490 phhA-phenylalanine-4-hydroxylase	**3.2**		**–3**	+
PP4491 phhB-pterin-4-alpha-carbinolamine dehydratase	**2.8**		**–2**	–
PP4619 maleylacetoacetate isomerase putative	**3.9**		**–3.4**	–
PP4620 fumarylacetoacetase	**2.1**		**–2.1**	–
PP4621 *hmgA*-homogentisate 1 2-dioxygenase	2.1		–2.7	+
Group 2				
PP0913 conserved hypothetical protein		–1.9	–2.4	–
PP2078 transcriptional regulator LysR family		–1.8	–1.8	+
PP2646 conserved hypothetical protein		–2.2	–2.1	–
PP2827 alcohol dehydrogenase zinc-containing		–2.1	–2.7	+
PP3425 *mexE*-multidrug efflux RND membrane fusion protein MexE		**–2**	**–3.7**	–
PP3427 *oprN*-multidrug efflux RND outer membrane protein OprN		**–5.1**	**–5**	–
PP4858 conserved hypothetical protein		–4	–2.1	–
Group 3				
PP2520 hypothetical protein		2.3	1.3	–
PP2607 conserved hypothetical protein	1.9	1.9	1.4	–
PP2608 shikimate dehydrogenase family protein	1.8	2.3	1.4	–
PP3067 hypothetical protein		2.1	2.1	–
PP4489 *phhR*, PhhR	2	4.1	4.4	+
PP3080 *aroF*, -2-phospho-2-dehydro-3-deoxyheptonate aldolase class I		2	2	–
PP3285 *paaY*, -PaaY protein	3.9	1.8	1.8	+
Group 4				
PP3275 *paaH, paaJ*-ring-hydroxylation complex protein 3	**16.2**			–
PP3276 *paaI*, ring-hydroxylation complex protein 2	**2.4**			–
PP3277 *paaH*, phenylacetic acid degradation protein PaaB putative	**1.9**			–
PP3278 *paaF, paaG*-ring-oxidation complex protein 1	**3.2**			–

Values shown are the average of three independent assays with standard errors below 5% of the given values.
Column A: Wild-type cells grown in the absence of phenylalanine versus cells grown in the presence of phenylalanine.
Column B: Wild-type cells versus PhhR-mutant cells grown in the absence of phenylalanine.
Column C: Wild-type cells versus PhhR-mutant cells grown in the presence of phenylalanine.
PhhR box: + and – means that upstream from the first ATG, a sequence highly similar to the PhhR box recognized by PhhR in the *phhA* promoter was present (+) or absent (–).
Grey shadows indicate potential operons and bold-face type letters show the gene and/or protein.

function of these operons based on gene annotations and reported activities derived from literature revealed that this group contains genes involved in the catabolism of *L*-phenylalanine via *L*-tyrosine (i.e. *phhAB*, PP4490/PP4491), *p*-hydroxyphenylpyruvate (*hpd*, PP3433/PP3434) and homogentisate (*hmgABC,* PP4619 through PP4621) into Krebs cycle intermediates, which is in agreement with previous findings for phenylalanine degradation in this microorganism (Whitaker *et al.*, 1982; Olivera *et al.*, 1998; Arias-Barrau *et al.*, 2004; Herrera and Ramos, 2007). Also within group 1 is a putative two-subunit CoA-transferase (PP3122/PP3123), which exhibits high identity to acetoacetyl-CoA-transferase in *Escherichia coli* (Reva *et al.*, 2006) suggesting that it may be involved in the last step of phenylalanine catabolism in *P. putida* KT2440. To provide experimental support we generated a ΔPP3122/PP3123 mutant and tested its growth with *L*-tyrosine as a C-source. We found that the PP3122 mutant was compromised in its ability to catabolize tyrosine (Table 3) and turned black due to the accumulation of homogentisate. Other genes that were induced in response to phenylalanine included an ABC transport system consisting of the co-transcribed genes PP2595/PP2596 that may be involved in the uptake of phenylalanine. PP2597 may also form a part of this ABC transport system, although it showed a lower induction level of only 1.6-fold and was therefore not included in Table 2. We also found two induced proteins of unknown function, namely PP3458 and PP3468. A mutant deficient in PP3458, a potential acyl-CoA ligase, was available from the *P. putida* mutant collection (Duque *et al.*, 2007b). This mutant was able to grow on minimal medium with phenylalanine as N-source and no insights on its potential contribution to phenylalanine metabolism are currently available.

Table 3. Growth of KT2440 and its isogenic mutants with NH_4^+, phenylalanine and tyrosine as the sole N-source.

	Doubling time (h)		
Genotype	NH_4^+	Phenylalanine	Tyrosine
Wild-type	2 ± 0.1	8 ± 0.4	1.8 ± 0.1
Δ*phhAB*	2.1 ± 0.1	21.7 ± 0.7	2.3 ± 0.1
tyrB-1::Tc	1.1 ± 0.1	8 ± 0.5	3.2 ± 1
tyrB-2::Km	1.7 ± 0.1	12 ± 0.3	3.0 ± 0.2
PP3122	1.2 ± 0.4	17.4 ± 0.6	17.1 ± 1.1
PP3458	1.1 ± 0.5	7 ± 0.9	2.1 ± 0.2

Pseudomonas putida KT2440 or its isogenic mutants were grown in M8 minimal medium with glucose as a C-source. The nitrogen source used was ammonium, phenylalanine and tyrosine. Doubling times were estimated in the exponential growth phase and the values are the mean ± standard deviation of three to six independent assays.

(2) Group 2 includes genes induced by PhhR regardless of the presence of phenylalanine. Within this set of seven genes, only PP3425 through PP3427 form an operon, whereas the other genes seem to be independent transcriptional units (note that PP3426 expression increased 1.7-fold and is not present in the table). In this group, all genes show reduced expression in the PhhR-deficient mutant versus the parental strain regardless of the presence of phenylalanine (see Table 2). Interestingly, the most dramatic reductions in expression levels were observed for the MexEF/OprN efflux pump genes. A number of proteins in this second group are of unknown function, including a putative transcriptional regulator belonging to the LysR family.

(3) The third group consists of seven genes that show higher expression in the PhhR mutant strain than in the parental strain (Table 2), suggesting that they may be under negative control by PhhR. The genes within this group are PP2520, PP2607/PP2608, PP3067, PP3080 (*aroF*), PP3285 (*paaY*) and PP4489 (*phhR*). The inclusion of PhhR within this group implies that PhhR may control its own synthesis. This result is important in that it greatly strengthens previous *phhR*:'*lacZ* fusion studies that hinted at PhhR self-regulation (Zhao *et al.*, 1994). Other genes found within this group, namely PP2607/PP2608 and PP3080 (*aroF*), encode enzymes involved in the biosynthesis of aromatic compounds, and may be indicative of a potential feedback mechanism involved in the control of phenylalanine biosynthesis. Also found within this group are PP2520, PP3067 and PP3285 which have unknown functions. The PP3285 ORF, annotated as *paaY*, is clustered with the *paa* genes involved in phenylacetyl-CoA metabolism; however, its role, if any, in the catabolism of phenylacetate is unknown.

(4) Group 4 includes an operon that consists of ORFs PP3275 through PP3278 (*paaGHIJ*). Expression of this operon is PhhR-independent but induced in the presence of phenylalanine in the wild-type strain (Table 2, column A), and encodes a set of proteins involved in the hydroxylation of phenylacetyl-CoA to 1,2-dihydroxy-1,2-dihydrophenylacetyl-CoA.

Transcriptional fusions to 'lac confirm microarray results

The above set of microarray results indicate that bacterial cells respond to phenylalanine via PhhR and probably other regulators, such as the gene encoded by ORF PP2078, which is a LysR-family transcriptional regulator found within group 2. To confirm transcriptomic results we generated fusions of the promoter regions of representative genes of the different groups to '*lacZ* in the broad-host range pMP220 vector (Spaink *et al.*, 1987) and determined β-gal activity under different genetic backgrounds with and without phenylalanine (Table 4). From group 1, the genes induced by PhhR in a phenylalanine-dependent manner, we selected the promoters of the *phhA* and *hmgA* genes, which have well-characterized roles in phenylalanine/tyrosine metabolism, as well as the promoter region of the newly identified PP3122/PP3123 operon. Plasmids containing each promoter were transformed into the parental strain and the *phhR* mutant strain. β-Galactosidase assays were carried out with cells growing in M9 medium with glucose in the absence and in the presence of phenylalanine (Table 4). In the *phhR* mutant background, expression of the different promoters occurred at a low basal level that was not influenced by

Table 4. Expression of several promoters of the PhhR regulon in the wild-type and Δ*phhR* mutant backgrounds in response to phenylalanine.

			β-Galactosidase (Miller Units)	
Promoter fusion	Group	*phhR*	–Phe	+Phe
P_{phhA}::*lacZ*	1	–	1 ± 1	5 ± 1
P_{phhA}::*lacZ*	1	+	5 ± 1	2780 ± 150
P_{hmg}::*lacZ*	1	–	45 ± 5	50 ± 4
P_{hmg}::*lacZ*	1	+	50 ± 20	240 ± 70
P_{PP3122}::*lacZ*	1	–	25 ± 2	40 ± 15
P_{PP3122}::*lacZ*	1	+	20 ± 5	1450 ± 410
P_{PP2627}::*lacZ*	2	–	800 ± 160	840 ± 130
P_{PP2627}::*lacZ*	2	+	4565 ± 150	5040 ± 200
P_{mexE}::lacZ	2	–	20 ± 2	20 ± 2
P_{mexE}::lacZ	2	+	180 ± 3	190 ± 6
P_{phhR}::*lacZ*	3	–	95 ± 10	65 ± 9
P_{phhR}::*lacZ*	3	+	40 ± 5	25 ± 2
P_{PP3285}::*lacZ*	3	–	545 ± 45	1660 ± 200
P_{PP3285}::*lacZ*	3	+	40 ± 5	355 ± 65
P_{PP3278}::*lacZ*	4	–	340 ± 40	1730 ± 170
P_{PP3278}::*lacZ*	4	+	230 ± 15	1880 ± 20

Plasmids derived from pMP220 bearing the indicated fusion were transformed into PhhR proficient (+) wild-type cells or into Δ*phhR* mutant (–) cells. Transformants were grown in M9 minimal medium with glucose in the absence (–Phe) or presence of 5 mM phenylalanine (+Phe). β-Galactosidase activity was determined in cultures in the exponential phase as described in *Experimental procedures*.

the presence of phenylalanine (Table 4). In the parental background we found that expression from P_{phhA}, P_{hmg} and P_{PP3122} promoters increased when phenylalanine was present, which confirms that these genes are under PhhR control and that their expression is phenylalanine-dependent. However, the extent of the induction varied from about fivefold for the P_{hmg} promoter to more than 500-fold in the case of the *phhA* promoter with an intermediate level of induction (36-fold) for the PP3122 promoter.

For group 2 genes, those induced by PhhR regardless of the presence of phenylalanine, we fused the promoter region of PP2827 and P_{mexE} to *'lacZ* and measured β-galactosidase activity within both genetic backgrounds. We found that the expression ratio in each strain with and without phenylalanine was close to 1, and therefore, as expected, phenylalanine did not influence expression levels (Table 2, column A). Also in agreement with the array assays, was the observation that the level of expression from P_{PP2827} and P_{mexE} was lower in the mutant than in the wild-type strain regardless of the presence of phenylalanine, which suggests a phenylalanine-independent positive effect of PhhR on the transcription of these genes. The molecular mechanisms that allow PhhR to activate promoters with and without phenylalanine is subject to further studies in our group.

In *P. aeruginosa*, it is well established that expression from the *mexE* promoter is mediated by the MexT, a member of the LysR family of transcriptional activators, although the signal to which MexT responds is unknown (Köhler *et al.*, 1999; Roca *et al.*, 2008). To see if this is also the case in *P. putida* KT2440, we then tested whether the expression from *mexE* varied in a *mexT* mutant background. To this end we introduced a *mexE:'lacZ* fusion into the parental strain and the *mexT* mutant background and tested its expression in the absence and in the presence of phenylalanine. No β-galactosidase activity was observed in the MexT-deficient background, indicating that the PhhR effect on *mexE* expression is indirect and occurs via MexT (not shown). As expression of *mexT* is not altered in the microarray of the wild type versus the PhhR mutant background, a third element could influence the expression from *mexE*, i.e. PP3765 a protein that is homologous to the DNA binding MvaT regulator and that affects the level of expression from the *mexE* promoter (Shintani *et al.*, 2010).

For the genes in group 3, those with expression levels that appeared to be repressed by PhhR, we used the previously constructed P_{phhR}:*'lacZ* fusion, and generated a *'lacZ* fusion to the *paaY* promoter region. Using the P_{phhR}:*'lacZ* construct, β-gal assays confirmed that expression of *phhR* was slightly higher in the Δ*phhR* strain than in the parental strain. These results confirm that PhhR regulates its own expression, while doing so within a twofold range (Table 4). Expression from the PP3285 (*paaY*) promoter was higher in the PhhR-deficient background than in the parental background confirming that PhhR also represses the expression of this promoter. It should be noted that expression from the P_{paaY} promoter under each background with phenylalanine was higher than in the absence of the aromatic amino acid, indicating that *paaY* gene expression is modulated in response to several signals. Taken together these results confirm the role of PhhR as a repressor of genes in group 3. The presence of the *aroF* gene and genes encoding shikimate dehydrogenase (PP2607/PP2608) within group 3, which are involved in aromatic amino acid biosynthesis, suggests that PhhR may exert feed-back control at the transcriptional level in the biosynthesis of aromatic amino acids, as previously described in other microorganisms (Pittard, 1996).

Group 4 consists of four genes that were shown in the array assays to be induced in response to phenylalanine. Because all four genes were shown to be part of a single operon, we simply fused the promoter region of PP3278 to *'lacZ*. As expected, expression from the P_{PP3278} promoter in the parental strain with phenylalanine was higher than in the absence of phenylalanine.

In silico *identification of PhhR binding sites and characterization of gene expression using transcriptional fusions*

The array results and the β-galactosidase assays reported above suggest that the effect of PhhR on gene expression could be exerted by direct or indirect regulation of the corresponding promoters by PhhR. In order to gain insight into which promoters are regulated directly and which are regulated indirectly, we decided to take a bioinformatic approach. To this end, we took into consideration that the only promoter previously characterized as directly activated by PhhR is *phhA*, for which we have reported that expression of the *phhAB* operon requires two upstream activator sequences recognized by PhhR and defined by the 5′-NNNAAAANTNTNNTTNCG-3′ consensus sequence (Herrera and Ramos, 2007). This sequence was used to search for potential PhhR boxes in the region upstream of the first ATG of the genes and operons shown in Table 2. The PhhR box was found in the upstream extragenic region of eight promoters within groups 1, 2 and 3, as defined above (Table 2), while it was absent from the rest of the induced/repressed genes (Table 2). We then hypothesized that the induction/repression of the set of genes lacking the PhhR box might be mediated by other regulators.

The sequences identified as potential PhhR box(es) are shown in Table 5 together with the consensus derived from the multialignment of these sequences. Two PhhR boxes were present upstream of *phhAB*, in agreement

Table 5. PhhR boxes present in the promoter region of cognate genes under PhhR regulation.

TIGR identifier	Sequence
PP2078 transcriptional regulator LysR family	GGTCATGGTGGCGTTACT
PP2827 alcohol dehydrogenase zinc-containing	GGTAATTAGAGGTTCACA
PP3122 CoA-transferase subunit A	TGTAAAAATTATCAATCG
	GGTGAAGATGTCTGCACA
PP3285 *paaY*	AGTGATACACGATTGACG
PP3434/PP3433 hypothetical protein	TGTCGAGGATGTGTTCGA
	TGCTGGCCTGCATTTCAC
PP4489 *phhR*	CGTAACGATAATTTTACA
	CGGAAACACAGTTTTTGT
PP4490 *phhA*-phenylalanine-4-hydroxylase	ACAAAAACTGTGTTTCCG
	TGTAAAATTATCGTTACG
PP4621 *hmgA*-homogentisate 1 2-dioxygenase	AGCAAATTACGTTATTCG
Consensus promoter box	T**GTAAA**GATA**G**T**TTTAC**A

PhhR box(es) was/were searched for within the region between the first ATG of the gene and 500 nucleotides upstream. Sequences are aligned regardless of whether PhhR functioned as an activator or as a repressor.
In bold are nucleotides conserved in more than 50% of the aligned sequenced.

with our previous experimental results (Herrera and Ramos, 2007). Two PhhR boxes were also found upstream of the PP3434/PP3433 operon promoter, which encodes *p*-hydroxyphenylpyruvate dioxygenase genes and PP3122/PP3123, the newly identified acetoacetyl CoA-transferase. A single PhhR box was present upstream of each of the four remaining promoters. One of these promoters was that of the PP2078 gene, which encodes a LysR-family transcriptional regulator that may, in turn, regulate other genes whose expression varies in response to phenylalanine (Table 2), although the gene(s) under the direct regulation of PP2078 is (are) unknown.

To provide further support to our hypothesis that the *in silico* identified sequences are direct targets for PhhR, we carried out electrophoresis mobility shift assay experiments using the target DNA sequences, and 2 μM PhhR – a concentration of PhhR known to be sufficient to bind and retard the *phhA* promoter region within these assays (Herrera and Ramos, 2007). As expected, PhhR retarded all tested target DNAs (Fig. 2). The binding was specific since addition of cold DNA resulted in release of PhhR (not shown) and no binding of PhhR to the promoters of genes in the PhhR regulon lacking the PhhR box was found.

The closest homologue to the *P. putida* PhhR protein is the *E. coli* TyrR regulator, which also functions as an activator or as a repressor (Pittard and Davidson, 1991; Yang *et al.*, 1993; Bailey *et al.*, 1996; Rojo, 1999; Pittard *et al.*, 2005) and can also recognize one or two binding sites – a feature that may be characteristic of this group of regulators (Andrews *et al.*, 1991a; 1991b). Nonetheless, we should note that the ability of PhhR to induce expression of target genes in the absence of phenylalanine contrasts with TyrR, which can only activate transcription from specific promoters in response to the presence of an aromatic amino acid in the culture medium (Sarsero and Pittard, 1991; Argaet *et al.*, 1994; Lawley *et al.*, 1995; Pittard *et al.*, 2005).

Is phenylalanine degraded in P. putida *KT2440 through more than one catabolic pathway?*

We previously generated a knockout mutant in the *phhA* gene that prevented synthesis of *L*-tyrosine from *L*-phenylalanine (Herrera and Ramos, 2007). If this were the only pathway to remove nitrogen from this aromatic amino acid, growth with *L*-phenylalanine as sole source of nitrogen would be impeded; however, our previous results showed that, with phenylalanine as the only N-source, the Δ*phhAB* mutant was still able to grow, although requiring a doubling time of nearly 22 h compared with 8 h for the wild type (Table 3). Importantly, no significant difference was

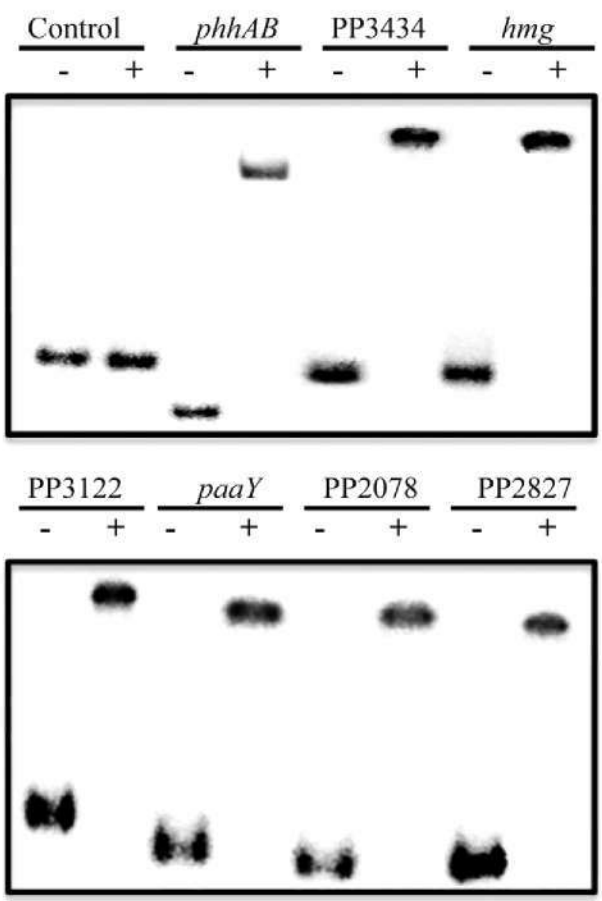

Fig. 2. Electrophoretic mobility shift assays. Specific conditions for these assays are detailed in *Experimental procedures*. DNA fragments carrying the indicated promoters were labelled with ^{32}P-ATP and incubated in the absence (–) or in the presence (+) of 2 μM PhhR.

observed between the Δ*phhAB* mutant and the parental strain when *L*-tyrosine was the sole N-source (Table 3). This suggests that in KT2440 there might be an alternative pathway to the PhhAB-mediated catabolism of phenylalanine. We then hypothesized that two potential phenylalanine degradation pathways coexist in *P. putida* KT2440; one that is mediated by PhhA/PhhB yielding tyrosine and subsequently *p*-hydroxyphenylpyruvate[13] (Fig. 1), and another involving the transamination of phenylalanine into a ketoacid to yield an amino acid and phenylpyruvate (Fig. 1). There are 15 ORFs in the genome of *P. putida* KT2440 that are annotated as amino transferases and two more specifically as aromatic amino transferases [*tyrB1* (PP1972) and *tyrB2* (PP3590)]. These amino transferases are expressed constitutively, and although they were not upregulated in response to phenylalanine, we still analysed their potential involvement in *L*-phenylalanine catabolism by generating knockout mutants. Mutants deficient in either one of these transaminases were generated as described in *Experimental procedures* or requested from the *Pseudomonas* Culture Collection (Duque *et al.*, 2007b). These mutants grew on minimal medium with phenylalanine or tyrosine as an N-source suggesting that – if they are involved in catabolism of these amino acids – they can replace each other in the metabolism of these amino acids (Table 3). We have found that most amino transferases in *P. putida* exhibit a very wide substrate specificity being able to use many different amino acids as a source of amino groups (M.A. Molina-Henares, E. Duque and J. de la Torre, unpublished).

To further test the hypothesis that a second pathway in *P. putida* for phenylalanine catabolism exists, we analysed whether phenylpyruvate could be used as a C-source by this strain. We found that KT2440 is capable of using phenylpyruvate as the sole C-source (doubling time ~10 h). The array data and β-gal assays presented above show that phenylalanine induces the *paaGHIJ* operon that contains genes (PP3278–PP3275) associated with phenylacetyl-CoA degradation, and may therefore be involved in the alternative pathway. To further explore this hypothesis, we tested whether the *paaG* promoter is induced in cells growing with phenylpyruvate by measuring β-galactosidase activity from the P_{paaG}:'*lacZ* construct under different backgrounds. In the parental strain, β-galactosidase levels with phenylpyruvate were similar to those that were observed with phenylalanine, and were about 1000 and 1500 units respectively, versus a basal level of 230 units in the absence of effector (data not shown). When testing tyrosine as an effector, we found that it did not induce expression from P_{paaG} promoter suggesting that the observed induction with phenylalanine is most probably indirect, and may be mediated by the subsequent metabolism of phenylalanine into phenylpyruvate. β-Galactosidase levels were also measured in *phhAB* and *hpd* mutant backgrounds, in which phenylalanine metabolism via *L*-tyrosine is blocked. In both mutants phenylpyruvate induced *paaG* expression, providing grounds for the notion that phenylacetyl-CoA may be an intermediate in the proposed alternative phenylalanine degradation pathway. We previously isolated mutants with knockouts in genes required for phenylacetate metabolism, namely *paaB* (PP3283) and *paaC* (PP3282) (Duque *et al.*, 2007b). These mutants failed to grow on phenylpyruvate and phenylacetate as the sole C-source (Table S1) in contrast with the parental strain. These results then suggest that phenylpyruvate is a key metabolite in the degradation of phenylalanine and that its metabolism most likely proceeds via phenylacetyl-CoA – a result that has not been described before in KT2440 or other *Pseudomonas* sp. (Evans *et al.*, 1987).

We also carried out massive mutagenesis of *P. putida* KT2440 and searched for mutants proficient in the use of phenylacetate but deficient in the use of phenylpyruvate. We recurrently isolated mutants that exhibited an insertion in PP0946. BLAST hits suggest that this protein belongs to a family of transporters and therefore PP0946 could be involved in the uptake of phenylpyruvate, although further functional analysis are necessary to determine the substrate specificity of this transporter.

In summary, our results show that phenylalanine is at a metabolic crossroad with at least two potential pathways available for the removal the amino group, thereby yielding either phenylpyruvate, or after its conversion to tyrosine yielding *p*-hydroxyphenylpyruvate. We have also shown that PhhR is able to modestly regulate its own synthesis, and that PhhR represses the biosynthesis of aromatic amino acids in response to the presence of phenylalanine. Our results revealed that in response to phenylalanine, cells induce a dedicated system that may control phenylalanine homeostasis. Therefore, metabolism of phenylalanine in *P. putida* seems to integrate a number of signals that optimize its use as a nitrogen source by the cells.

Experimental procedures

Bacterial strains, plasmids and culture media

The bacterial strains, cloning vectors and plasmids used in this study are shown in Table 1. *Pseudomonas putida* KT2440 and its mutant derivatives were grown on M9 minimal medium supplemented with a micronutrient solution and iron (Abril *et al.*, 1989) with glucose [0.5 (w/v)], citrate (16 mM), benzoate (10 mM) or phenylpyruvate (15 mM) as the carbon source. When indicated, M8 (M9 without NH_4Cl) minimal medium was used with either phenylalanine or tyrosine a concentration of 15 mM (Abril *et al.*, 1989). Cultures were incubated at 30°C and shaken on an orbital platform operating at 200 strokes per minute. *Escherichia coli* strains were grown at 37°C in Luria–Bertani medium with

shaking. *Escherichia coli* DH5α was used for gene cloning. When required, antibiotics were used at the following final concentrations (in micrograms per millilitre): ampicillin, 100; chloramphenicol, 30; kanamycin, 50; and tetracycline, 20.

RNA preparation and microarrays assays

Pseudomonas putida KT2440 and the *phhR*-deficient regulator knockout mutant were grown overnight in M9 minimal medium with 25 mM glucose as the sole carbon source (Del Castillo *et al.*, 2007). Cells were then diluted 100-fold in fresh medium until the culture reached a turbidity of about 0.6 at 660 nm and aliquots were incubated in the absence and in the presence of 5 mM phenylalanine for 15 min. Cells (10 ml) were harvested by centrifugation (9000 *g* for 15 min) in disposable plastic tubes that had been pre-cooled in liquid nitrogen, after which they were kept at −80°C until use. RNA was extracted using the TRI Reagent (Ambion, ref. 9738, Austin, TX, USA) and modified as follows: the lysis step was carried out at 60°C, and a final digestion step with RNase-free DNase was introduced at the end of the process followed by purification with RNeasy columns (Qiagen, Cat. No. 74104, Hilden). The RNA concentration was determined spectrophotometrically at 260 nm and its integrity was assessed by agarose gel electrophoresis.

RNA (30 μg) was primed with 30 μg of pd(N)6 random hexamers (Amersham, cat. No. 27-2166-01, Piscataway, NJ, USA). cDNA synthesis was performed at 42°C for 2 h in a 30 μl reaction volume containing 0.5 mM (each) dATP, dCTP and dGTP; 0.25 mM (each), dTTP and aminoallyl-dUTP (aa-dUTP; Sigma cat. No. A0410); 10 mM DTT; 40 U of RNase OUT (Invitrogen, ref. 10777-019, Carlsbad, CA, USA); and 400 U of SuperScript II reverse transcriptase (Invitrogen, ref. 18064-014) in reverse transcriptase reaction buffer. The reaction was stopped by adding 10 μl of 50 mM EDTA and the RNA template was hydrolysed with the addition of 10 μl of 1 N NaOH followed by an incubation period of 15 min at 65°C. Samples were then neutralized by adding 25 μl of 1 M Hepes (pH 7.5) and the hydrolysed RNA and residual dNTPs were removed using QIAquick PCR purification columns (Qiagen, ref. 28104) according to the manufacturer's recommendations, except that the Tris-based elution buffer supplied with the kit was replaced by a phosphate elution buffer (4 mM potassium phosphate pH 8.5) to avoid any potential interference with subsequent labelling steps. cDNA samples were dried in a Speed-Vac and the aminoallyl-labelled cDNA was resuspended in 9 μl of 0.1 M sodium carbonate buffer (pH 9.0), mixed with either Cy3 (control) or Cy5 (phenylalanine-treated) fluorescent dyes (mono-reactive NHS-esters; Amersham Biosciences, cat. No. PA23001 and PA25001 respectively), and allowed to couple for 2 h at room temperature in the dark. After coupling, the reaction was quenched with 4.5 μl of 4 M hydroxylamine for 15 min. Finally, labelled cDNA probes were again purified with QIAquick PCR purification columns. Labelling efficiency was assessed using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Pre-hybridization and hybridization were carried out as previously reported (Yuste *et al.*, 2006; Duque *et al.*, 2007a).

Images were acquired at 10 μm resolution, and the background-subtracted median spot intensities were determined using GenePix Pro 5.1 image analysis software (Axon Instruments). Spots with anomalies were discarded and excluded from further analysis. Spot signal intensities were normalized by applying the Lowess intensity-dependent normalization method, and statistically analysed using the Almazen System software (Alma Bioinformatics SL, Madrid, Spain). Three independent biological replicates were performed for each experiment. *P*-values were calculated using Student's *t*-test. An ORF was considered differentially expressed if: the fold change was at least 1.8; the *P*-value was lower than 0.05; and the average signal-to-noise (A) was at least 64. Microarray data have been deposited in the ArrayExpress database (E-MEXP).

Reverse transcriptase-polynucleotide chain reaction assays

RNA for this assay was subsequently used for microarray analysis. Reverse transcriptase-polynucleotide chain reaction was carried out using 1 μg of RNA in a final volume of 25 μl using the Titan OneTube RT-PCR system according to the manufacturer's instructions (Roche Laboratories). The annealing temperature used for the RT-PCR was 56°C. The cycling conditions were as follows: 30 cycles at 94°C for 30 s, at 56°C for 1 min and at 72°C for 1 min. Positive and negative controls were included in all assays. The primers used to test contiguity in the mRNA of the different genes are available upon request.

β-Galactosidase assays

Fusions of the promoters of different genes to a promoterless '*lacZ* gene were constructed in the low-copy-number pMP220 vector (Spaink *et al.*, 1987; Segura *et al.*, 2003). The corresponding promoter regions were amplified by PCR with primers incorporating restriction sites (EcoRI at the 3′ end and PstI at the 5′ end). Upon amplification, DNA was digested with EcoRI and PstI and ligated to EcoRI-PstI-digested pMP220. The fusion constructs were confirmed by DNA sequencing. The plasmids were electroporated into the wild-type *P. putida* KT2440 strain and the Δ*phhR* mutant strain. The corresponding transformants were grown overnight in M9 medium with glucose plus tetracycline and then diluted 100-fold in the same medium and grown until the culture reached a turbidity of about 0.6 at 660 nm. Aliquots were incubated in the absence or presence of 5 mM of the effector under scrutiny at 30°C for 2 h with shaking, and the β-galactosidase (β-gal) activity was assayed on permeabilized whole cells according to Miller's method. Assays were run in triplicate and repeated for at least three independent experiments.

Construction of tyrB-1 *mutant strain*

To construct the *tyrB-1* knockout mutant, the *P. putida tyrB-1* gene was amplified from chromosomal DNA and was cloned into pUC18Not to obtain the pTYRB-1 plasmid. This plasmid was cut with NaeI and the 2-kb SmaI fragment from pHP45Tc bearing the *tet* gene that encodes tetracycline resistance was inserted into pTYRB-1 thereby producing the pTYRB-1Tc

plasmid. A 3.2 kb NotI fragment containing the *tyrB-1::tet* mutant allele was subcloned into pMRS101 previously cut with NotI to obtain pMRS101-pTYRB-1Tc (Table 1). This plasmid was used for allelic exchange, for which it was transferred to the *P. putida* KT2440 strain via triparental mating. Merodiploids were selected in M9 minimal medium with benzoate plus tetracycline and streptomycin, and the double recombination event was selected based on sucrose toxicity, as described by Rodríguez-Herva and colleagues (1996). The correct insertion of the mutant allele of *tyrB-1* into the chromosome was checked by PCR analysis and Southern blot hybridization (not shown).

Construction of PP3122 mutant strain

To construct a mutant strain bearing an inactivated chromosomal version of the PP3122 gene, we generated the corresponding knockout using the appropriate derivative of pCHESIΩKm (Llamas *et al.*, 2003). Plasmid pCHESIΩKm is based on pUC18 and bears the origin of transfer *oriT* of RP4 and the Ω-Km interposon of plasmid pHP45ΩKm cloned as a HindIII fragment. To generate the desired mutation, an internal 500 bp fragment of the target gene was amplified by PCR with primers containing the EcoRI and BamHI sites to amplify an internal part of the PP3122 gene. The amplified fragment was subsequently cloned between the EcoRI and BamHI sites of pCHESIΩKm (pCHESIPP3122, Table 1). The recombinant plasmid was introduced into *P. putida* KT2440 by electroporation, and transformants bearing a co-integrate of the plasmid in the host chromosome were selected on M9 minimal medium with benzoate as a carbon source and Km. The correctness of the construction was confirmed by Southern blotting using the target gene as a probe (not shown).

Electrophoresis mobility shift assay

The DNA fragment containing the *phh*, *hmg*, PP3434, PP3122, *paaY*, PP2078 and PP2827 promoters was amplified by PCR from the *P. putida* chromosome, separated in agarose gels and subsequently extracted from this matrix. These fragments were end-labelled with ^{32}P as described above. Labelled DNA (about 1 nM; 10^5 c.p.m.) were incubated with 2 μM of PhhR-His_6 for 30 min at 30°C in 10 μM of binding buffer containing 20 μg of poly(dI-dC) ml^{-1}, 200 μg ml^{-1} of bovine serum albumin, 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 0.1 mM DTT, 3 mM magnesium acetate and 0.1 mM EDTA. Reaction mixtures were then electrophoresed in a non-denaturing 4.5% (w/v) polyacrylamide gel in 50 mM Tris buffer (pH 7.8), 50 mM borate, 1 mM $MgCl_2$, and 10% (v/v) glycerol. The results were analysed with a model GS525 Molecular Imager (Bio-Rad).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Doubling time of the parental strain and mutants in the phenylacetate pathway.