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Catabolismo del aminoácido aromático fenilalanina por *Pseudomonas putida* KT2440.

Tesis doctoral

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Catabolismo del aminoácido aromático fenilalanina por *P. putida* KT2440

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A mi maravillosa familia
A Andreas

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Abreviaturas

abreviaturas	Significado
ΔG	energía libre de Gibbs
ΔH	entalpía
ΔS	entropía
Da	dalton o g/mol
dNTPs	desoxinucleótidos trifosfato
DO_{660}	turbidez medida a 660 nm
DTT	di-tiotreitol
EDTA	tetra acetato de etilendiamina
HTH	hélice-giro-hélice (del inglés, helix-turn-helix)
IHF	factor de integración del hospedador
IPTG	Isopropil ß-D-tiogalactopiranósido
ITC	calorimetría de titulación isotérmica (del inglés, isothermal titration calorimetry)
K_A	Constante de asociación al equilibrio
Kb	Kilobase(s)
K_D	constante de disociación al equilibrio
LB	Luria-Bertani
ONPG	o-nitrofenilgalactopiranósido
ORF	fase de lectura abierta (del inglés, open reading frame)
pb	pares de bases
RNAPσ^{70}	RNAP asociada a σ^{70}
RNAPσ^{54}	RNAP asociada a σ^{54}
SDS	dodecilsulfato sódico
SDS-PAGE	electroforesis en gel de poliacrilamida en presencia de SDS
v/v	volumen/volumen
X-gal	5-bromo 4-cloro 3 indolil ß-D- galactopiranósido

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II. Objetivos

La biotransformación, considerada como la tercera ola en la biotecnología y en la producción microbiana de productos de valor añadido a partir de baratas y abundantes fuentes, es de gran interés industrial. Al menos dos condiciones son imprescindibles para llevar a cabo cualquier biotransformación con células enteras, el microorganismo debe tener la capacidad biocatalítica apropiada para dar lugar al producto deseado y que el microorganismo no presente sensibilidad frente a ciertos productos tóxicos que puedan bloquear estas rutas catalíticas. En los últimos años, nuestro grupo junto a otros hemos demostrado que algunas cepas de *P. putida* son especialmente tolerantes a altas concentraciones de compuestos aromáticos tóxicos, además de ser la base de posibles catálisis para la producción de productos de valor añadido a partir de fuentes de carbono baratas (Meyer *et al*., 2005; Ramos *et al*., 1995; Ramos *et al*., 2002; Rojas *et al*., 2004 y Wierckx *et al*., 2005). Nosotros estamos interesados en la biosíntesis de fenilalanina por *P. putida* KT2440 como base de la producción de otros productos de valor añadido tales como dihidroxifenilalanina, dopamina y otros. *Pseudomonas putida* podría sintetizar fenilalanina a partir de glucosa y tolera hasta 25-30 g/l de este aminoácido aromático en el medio, sin embargo su síntesis presenta un inconveniente ya que esta cepa utiliza la fenilalanina como fuente de nitrógeno. Por lo que se requiere previamente el bloqueo del catabolismo de este aminoácido aromático y por tanto su estudio. Con este fin los objetivos específicos en esta Tesis Doctoral son los siguientes:

1. **Estudiar las rutas catabólicas mediante las que *Pseudomonas putida* KT2440 degrada la fenilalanina, caracterizar los genes que participan en ellas y estudiar la regulación de las mismas.**

2. **Definir PhhR como un regulador global capaz de activar y reprimir un conjunto de genes u operones algunos de los cuales participan en el metabolismo de la fenilalanina.**

3. **Proponer un mecanismo general de regulación transcripcional mediante el cual PhhR modula el conjunto de los genes que pertenecen al regulón PhhR.**

III. Introducción

1. El género *Pseudomonas*

El término *Pseudomonas,* cuyo significado literal es "falsa unidad", deriva del griego; *pseudo* ($\psi\varepsilon\upsilon\delta o$ "falso") y *monas* ($\mu o\nu\alpha\varsigma$ / $\mu o\nu\alpha\delta\alpha$ "una sola unidad"). El término "*monada*" se usaba en la microbiología antigüa para nombrar a los organismos unicelulares. Las bacterias del género *Pseudomonas* son bacilos Gram negativos, rectos o ligeramente curvados (0.5-0.8 μm x 1-3 μm), que se incluyen dentro de la clase de las γ-proteobacterias. Son móviles gracias a los flagelos que poseen, generalmente polares, aunque en algunas especies se han descrito flagelos laterales. La mayoría de las especies no pueden crecer bajo condiciones ácidas (pH≤4,5) y no requieren factores de crecimiento. Están descritas como oxidasa positivas, catalasa positivas y aerobias estrictas, pero también se han encontrado formadoras de biopelículas que pueden utilizar el nitrato como aceptor de electrones (Hassett *et al*., 2002). El catabolismo de los glúcidos lo realizan mediante la ruta de Entner-Doudoroff y el ciclo de los ácidos tricarboxílicos. Algunos miembros del género son psicrófilos de manera que sus temperaturas óptimas de desarrollo se encuentran entre 4º y 15ºC. Otros sintetizan sideróforos fluorescentes de color amarillo-verdoso con gran valor taxonómico. Suelen portar plásmidos. No forman esporas. Algunas especies sintetizan una cápsula de exopolisacáridos que facilita la adhesión celular, la formación de biopelículas y protege de la fagocitosis, de los anticuerpos o del complemento, aumentando así su patogenicidad (Palleroni, 1984).

Las bacterias del género *Pseudomonas* se encuentran ampliamente distribuidas en la naturaleza, particularmente en suelo, agua, alimentos y plantas. La ubicuidad de estas bacterias y la capacidad para crecer en medios de cultivo muy simples hicieron que se las considerase protagonistas en el proceso de mineralización de materia orgánica en el medioambiente, un papel que fue claramente demostrado a principios del siglo pasado por den Dooren de Jong (1926). Poseen gran versatilidad metabólica y usan distintas fuentes de carbono. Así, algunas cepas se utilizan en biodegradación de xenobióticos (Gibson *et al*., 1984; Ramos *et al*., 1994; Timmis *et al*., 1988 y Parales and Haddock, 2004). La mayoría de las cepas pueden sintetizar un buen número de

mono- y dioxigenasas que son de interés en procesos industriales de fabricación de productos de valor añadido tales como 3-metilcatecol (Hüsken *et al*., 2001 y 2002; Rojas *et al*., 2004 y Neumann *et al*., 2005) o nitroaromáticos (Schackmann and Müller, 1991). Algunas cepas de *Pseudomonas* del suelo sintetizan productos tóxicos para otros microorganismos o quelan hierro con altísima afinidad, por lo que son útiles en procesos de biocontrol (Kiely *et al*., 2006; Shoda, 2000 y Walsh *et al*., 2001).

Aunque hay más de 200 especies agrupadas en este género, sólo tres de ellas se conocen como patógenas para el hombre: *P. aeruginosa*, *P. mallei* y *P. pseudomallei*. Existen patógenas de plantas como *P. syringae* y cepas que en cambio estimulan el crecimiento de plantas como *P. fluorescens* (Lugtenberg, 1999).

La reciente secuenciación del genoma completo de 21 miembros de este género, pertenecientes a las siguientes especies: *P. aeruginosa* (Stover *et al*., 2000 y Lee *et al*., 2006), *P. putida* (Nelson *et al*., 2002), *P. syringae* (Buell *et al*., 2003; Field *et al*., 2005 y Joardar *et al*., 2005), *P. fluorescens* (Paulsen *et al*., 2006), *P. mendocina*, *P. entomophila* (Vodovar *et al*., 2006) y *P. stutzeri* (Yan *et al*., 2008), ha abierto nuevos frentes para entender la singular capacidad adaptativa de este género oportunista, con importantes repercusiones, tanto en el ámbito ecológico-agronómico como en el clínico. Sus genomas son de gran tamaño (entre 6 y 7 x 10^6 pb), con unos 5.500 genes por genoma, la mitad de los cuales tienen una función desconocida y es de destacar su alta proporción de genes reguladores. Todas estas características reflejan la gran capacidad de integración de señales y de adaptación a los cambios ambientales, así como la versatilidad metabólica de estas cepas, ambas necesarias para su singular ubicuidad medioambiental.

2. *Pseudomonas putida* KT2440

Pseudomonas putida KT2440, cepa de estudio de este trabajo, es una bacteria Gram negativa perteneciente al grupo de las *Pseudomonas* "fluorescentes", denominadas así por la producción de pigmentos que emiten

fluorescencia. Sus primeros estudios se remontan al año 1963 en Japón, donde aislada por Hosakwa (Nozaki *et al*., 1963), fue identificada como una cepa capaz de degradar *meta*-toluatos, de donde derivó el nombre mt-2 (cepa parental de KT2440). En principio fue incluida dentro de la especie *Pseudomonas arvilla* y no fue hasta 1974 (Williams and Murray, 1974) cuando se introdujo dentro de la especie *Pseudomonas putida*. En 1975 se comprobó que la cepa mt-2 contenía el plásmido TOL, el cual porta los genes que codifican las enzimas necesarias para la degradación de tolueno y xilenos (Worsey and Williams, 1975). En 1981 se describió *Pseudomonas putida* KT2440 (Bagdasarian *et al*., 1981) como un derivado de mt-2 curado del plásmido TOL y presuntamente deficiente en el mecanismo de restricción de ADN exógeno. Esto útimo hace ideal a esta cepa para la expresión de rutas catabólicas con fines degradativos (Bagdasarian and Timmis, 1982 y Ramos *et al*., 1994) y como hospedador en la clonación y expresión de genes heterólogos para su utilización en procesos de biotransformación de compuestos químicos con valor añadido (Delgado *et al*., 1992; Kraminoácidok *et al*., 1997 y Kellerhals *et al*., 1999) o de interés farmacológico (Tan *et al*., 1997).

El genoma de *P. putida* KT2440 consta de un único cromosoma de 6,18 Mb, en el cual encontramos un alto contenido medio en G+C (61,6%). Sin embargo, se han descrito 105 islas genómicas con un porcentaje distinto en G+C (Weinel *et al*., 2002). Estas islas están principalmente implicadas en la captación y degradación de compuestos orgánicos, transporte de iones, síntesis y secreción de metabolitos secundarios, siendo las principales responsables de la elevada versatilidad metabólica presente en esta cepa. Esta versatilidad metabólica se encuentra ligada en muchas ocasiones a plásmidos que portan genes cuyos productos se encargan de canalizar el sustrato hacia el metabolismo central. Por ejemplo, el plásmido TOL (Williams and Murray, 1974), el plásmido NAH7 (Dunn and Gunsalus, 1973), el plásmido CAM (Rheinwald *et al*., 1973) y el plásmido OCT (Chakrabarty, 1973) que codifican los genes implicados en el catabolismo de tolueno/xilenos, naftaleno, alcanfor y octano, respectivamente. En su cromosoma se han encontrado genes que codifican enzimas implicadas en rutas de degradación de compuestos orgánicos que en ocasiones son productos naturales como los derivados de la degradación de la

lignina (Jiménez *et al*., 2002) o xenobióticos como haloaromáticos, poliaromáticos, nitroaromáticos, etc. que constituyen una importante fuente de carbono para organismos asociados a la rizosfera. Una estrategia muy utilizada por *Pseudomonas*, que les permiten la degradación de diversos compuestos aromáticos, consiste en la modificación de estos compuestos en intermediarios que pueden ser degradados a través de las rutas del metabolismo central. Además hay que destacar la gran cantidad de transportadores de compuestos aromáticos identificados en KT2440 (dos Santos *et al*., 2004). Todas estas características contribuyen al espectro nutricional de esta especie, permitiéndole reproducirse en distintos ambientes y metabolizar una amplia gama de compuestos naturales y sintéticos.

La anotación inicial del cromosoma de la cepa KT2440 sugería 5.420 ORFs, dentro de los cuales, 80 genes aparecen truncados o alterados como consecuencia de la inserción de transposones o elementos móviles. Por otro lado, se han identificado 804 copias de un elemento específico de 35 pb conocido como REP (del inglés *Repetitive Extragenic Palindromic sequences*) (Aranda *et al*., 2002). Se trata de un palíndromo con una orientación definida cabeza-cola cuya función continúa siendo desconocida. El genoma de KT2440 codifica 184 proteínas relacionadas con elementos móviles, 82 genes que codifican transposasas, 8 intrones de grupo II, etc. Además, se han identificado en el cromosoma de KT2440 tres genomas de bacteriófagos. De los 5.420 ORFs predichos, 600 aparecen anotados como proteínas hipotéticas de función desconocida. Es posible que algunos de estos casos puedan tratarse de artefactos producto de los algoritmos utilizados en la predicción de genes (dos Santos *et al*., 2004).

P. putida KT2440 se caracteriza por presentar una gran capacidad de transporte en general. Aproximadamente el 12% del genoma está dedicado a cdificar sistemas transportadores, siendo los de tipo ABC (del inglés *ATP-Binding-Cassette*) la principal familia presente en esta cepa. Destaca la participación de estos sistemas en el transporte de aminoácidos, reflejo de la capacidad de esta cepa para colonizar la raíz de la planta. Mediante estos sistemas de transporte es además capaz de protegerse frente a compuestos tóxicos exportándolos a través de bombas de extrusión como las bombas RND

(Resistencia, Nodulación y División) y los transportadores MFS (del inglés *Major Facilitator Superfamily*) (Ramos *et al*., 2002). *Pseudomonas putida* KT2440 también posee sistemas de mono- y dioxigenasas, oxidorreductasas, ferredoxinas y citocromos, dehidrogenasas, proteínas del metabolismo del azufre y glutatión-S-transferasas, factores sigmas alternativos, reguladores y sistemas de respuesta a estrés (Nelson *et al*., 2002). Tiene 330 genes asociados a la biosíntesis de fimbrias de tipo IV, al menos cuatro operones involucrados en la biosíntesis del flagelo (Rodríguez-Herva *et al*., comunicación personal), de fibras rizadas y de pilis tipo I, que le confiere a la cepa la gran capacidad de adhesión a superficies (Espinosa-Urgel *et al*., 2002).

No se conoce ninguna cepa de *P. putida* patógena de plantas o animales, lo que facilita su aplicación en el desarrollo de numerosas técnicas biotecnológicas, tales como, el diseño de nuevas rutas catabólicas destinadas a la degradación de compuestos contaminantes (Ramos *et al*., 1986; Rojo *et al*., 1987; Ramos *et al*., 1987 y Erb *et al*., 1997), producción de intermediarios en la síntesis química de moléculas complejas (Wubbolts and Timmis, 1990) o en la mejora de la calidad de combustible fósil, por desulfurización (Galan *et al*., 2000). A su vez, es un buen colonizador de rizosfera de maiz, trigo, fresa, caña de azúcar y espinacas (Espinosa-Urgel *et al*., 2002), por lo actualmente, se emplean en el desarrollo de biopesticidas y como promotora del crecimiento de plantas.

La ubicuidad de *P. putida* refleja su habilidad para adaptarse a una enorme variedad de condiciones físico-químicas presentes en sus distintos hábitats. Esto es debido a su capacidad de integrar las señales recibidas del medio extracelular con el estado fisiológico celular, lo que le permite la activación de la red de regulación apropiada que controla el metabolismo celular (Regenhardt *et al*., 2002).

3. La rizosfera: Nicho ecológico de *P. putida*

Las bacterias del género *Pseudomonas*, y entre ellas la cepa *P. putida* KT2440, colonizan la superficie de la raíz de las plantas (Rodríguez-Herva *et al*.,

1999 y Molina *et al*., 2000) así como el suelo que rodea a la raíz, nicho ecológico conocido como rizosfera. Este término, introducido por Hilter (Hilter, 1904), se definió como la región del suelo que está en contacto directo con la raíz, estando por lo tanto bajo la influencia biológica y física de la misma. La rizosfera se divide en "rizoplana" (superficie de la raíz), "endorizosfera" (partes internas de la raíz) y "ectorizosfera" (capa fina de tierra y suelo adherida a la raíz) (Lugtenberg, 2004). Se trata de un entorno complejo, rico en nutrientes, donde aparecen aminoácidos, proteínas, ácidos grasos, flavonoides, hormonas, ácidos orgánicos, polisacáridos, compuestos organofosforados, purinas, pirimidinas, esteroles, azúcares incluyendo oligosacáridos, vitaminas y compuestos indefinidos que inhiben o estimulan el crecimiento de hongos, bacterias y nematodos (Curl and Truelove, 1986; Lugtenberg *et al*., 1999 y Lugtenberg and Dekkers, 1999). Aproximadamente un 20% del peso seco de la planta se libera a través de la raíz en forma de mucílagos, lisado y células desprendidas de la raíz entre otros, que constituyen lo que se denomina "exudados de raíz". La naturaleza de estos compuestos y su cantidad no sólo varía de unas plantas a otras, sino que, dentro de una misma planta puede variar en función de la edad, el estado fisiológico o de las condiciones ambientales (Barber and Lynch, 1977; Lugtenberg *et al*., 1999 y Rovira, 1969).

Determinados compuestos presentes en los exudados, incluso a bajas concentraciones, actúan como quimioatrayentes para las bacterias que habitan la rizosfera. Así, el benzoato a concentración de 10^{-9} a 10^{-12} M atrae a especies de *Azospirillum* (López-de-Victoria and Lovell, 1993) y la luteolina (10^{-9} M) atrae a *Agrobacterium tumefaciens* y *Rhizobium meliloti* (Caetano-Anolles *et al*., 1988; Bauer and Caetano-Anollés, 1991 y Dharmatilake and Bauer, 1992). En cambio, ciertos aminoácidos (glutamato, treonina, serina, cisteina y arginina), a elevadas concentraciones (10^{-2} a 10^{-3} M) atraen a ciertas especies de *Pseudomonas*, como por ejemplo, *P. lachrymans* (Chet *et al*., 1973) y *P. aeruginosa* (Nikata *et al*., 1992 y Craven *et al*., 1985).

El entorno rizosférico es altamente complejo y posee una elevada densidad microbiana, del orden de 10^9 células por gramo de suelo o raíz (Curl and Truelove, 1986 y Campbell and Greaves, 1990), lo que supone entre cien y mil veces más, en número de microorganismo, que en el caso de suelo no

rizosférico. Aparecen, entre otros, nematodos, hongos y bacterias, siendo, el 95% de los microorganismos no cultivables (Kowalchuk *et al*., 2002). Dentro de los microorganismos cultivables el 30-90% lo representan bacterias del género *Pseudomonas* (Vancura, 1980). Los microorganismos no se distribuyen uniformemente a lo largo de la raíz, sino que se encuentran restringidos al 6% del total de la superficie de la misma (Neman and Bowen, 1974; Foster and Rovira, 1976; Foster, 1981; Foster, 1982 y Fukui *et al*., 1994) y numerosos factores físicos, como el tipo de suelo, pH, oxígeno y agua, afectan a la población microbiana en la rizosfera.

Las interacciones plantas-microorganismos pueden ser de diferentes tipos. Entre éstas cabe destacar la importancia de los patógenos vegetales, responsables de la pérdida de hasta un 30% de las cosechas anuales. Hasta el momento, se han descrito al menos 120 géneros de hongos patógenos y 8 géneros de bacterias patógenas como *Pseudomonas*, *Erwinia* y *Xanthomonas*, entre otros. Determinados compuestos presentes en el exudado de raíz contribuyen al desarrollo de la respuesta infecciosa.

Otras bacterias, sin embargo, tienen efectos beneficiosos para la planta. Algunas promueven el crecimiento de plantas, facilitando la captación de nutrientes o produciendo fitohormonas (*Pseudomonas*, *Serratia*, *Azospirillum* y *Bacillus*), otras actúan como agentes de control biológico (Montesinos *et al*., 2002) produciendo, por ejemplo, antifúngicos (Lugtenberg *et al*., 1999). Por tanto, actualmente se propone el uso de estos microorganismos beneficiosos para la planta en el control de plagas como métodos menos agresivos que los pesticidas, ampliamente usados. No obstante, es necesario que previamente tenga lugar una efectiva colonización por parte del microorganismo en cuestión, que requiere el establecimiento del microorganismo en la raíz emergente y multiplicación del mismo para su posterior establecimiento (Kloepper *et al*., 1988; Ramos *et al*., 2009 y Segura *et al*., 2009).

Cada vez son más numerosos los estudios centrados en ampliar el conocimiento de los mecanismos moleculares que operan en la interacción planta-microorganismo. La rizosfera es un hábitat complejo donde existen fuentes de carbono y/o de nitrógeno alternativas, que conllevan que determinadas rutas del microorganismo estén funcionando, mientras que por el

contrario, otras estén silenciadas. Por ejemplo, en *P. fluorescens* induce la expresión de genes implicados en el transporte y metabolismo de azúcares, en el transporte de aminoácidos y de respuesta a estrés oxidativo (Rainey, 1999).

También en el suelo como componentes exudados de la raíz se encuentran de manera abundante aminoácidos que se producen como consecuencia de la proteolísis de péptidos y proteínas. Los aminoácidos libres influyen en la comunidad bacteriana del suelo ya que se encuentra en las inmediaciones de las plantas y generalmente favorecen interacciones beneficiosas con éstas (Bertin *et al*., 2003; Prikryl and Vancura, 1980 y Rovira, 1969). Los aminoácidos constituyen una fracción importante del total de los exudados de raíz, por ejemplo en *Brassica napus* pueden alcanzar el 60-85% del total de nutrientes (Sundin *et al*., 1990 y Svenningsson *et al*., 1990). En ensayos con mutantes afectados en el catabolismo de aminoácidos se ha demostrado que éstos son esenciales para la capacidad de la bacteria de colonizar la raíz. (Bayliss *et al*., 1997; Bhagwat and keister, 1992 y Espinosa-Urgel and Ramos, 2001).

Pseudomonas putida KT2440 tiene la capacidad de colonizar la rizosfera de plantas a una alta densidad celular (Ramos *et al*., 2000), pero necesitan reajustar y dirigir su programa genético para aprovechar así al máximo los nutrientes presentes en este hábitat (Matilla *et al*., 2007). Se ha comprobado que existe una elevada expresión de los genes catabólicos de algunos aminoácidos en presencia de exudados de raíz. Vílchez *et al*. (2000), demostraron que los genes implicados en el catabolismo de la prolina, *putA* y *putC*, se inducen en *P. putida* KT2440 cuando se encuentra en la rizosfera. De igual modo se ha puesto de manifiesto que la ruta de degradación de L-lisina se activa en esta cepa en presencia de exudados de maíz, lo que indica la participación de ésta en la adaptación de KT2440 a la rizosfera (Revelles *et al*., 2004). Además, recientemente, se ha puesto de manifiesto la utilización de aminoácidos aromáticos tales como la fenilalanina y la tirosina en la rizosfera. Se ha observado que auxótrofos de estos aminoácidos aromáticos, no pierden la capacidad de colonización en la rizosfera ya que son capaces de utilizar la fenilalanina y tirosina presentes en ella (Molina-Henares *et al*., 2008). Estos resultados demuestran el requerimiento de estos aminoácidos en la adaptación

de KT2440 a la rizosfera, motivo por el cual nos interesa estudiar su catabolismo.

4. Metabolismo de aminoácidos aromáticos

En la naturaleza existen tres aminoácidos aromáticos, la fenilalanina, la tirosina y el triptófano, cuyo nombre se debe a la presencia de cadenas laterales aromáticas. La fenilalanina es de los tres aminoácidos aromáticos el más hidrófobo, mientras que la tirosina y el triptófano poseen un carácter menos hidrofóbico, debido a el grupo hidroxilo en la posición *para* del anillo aromático. Además, la tirosina puede ionizarse a pH elevado.

4.1 Biosíntesis de aminoácidos aromáticos

La síntesis de los anillos aromáticos a partir de precursores no cíclicos implica una química compleja, por lo que no resulta extraño observar que la mayoría de estas capacidades biosintéticas se hayan conservado a lo largo de la evolución. Archeabacterias, eubacterias, plantas y hongos son capaces de sintetizar *de novo* los tres aminoácidos aromáticos, mientras que los mamíferos son capaces de sintetizar tirosina a partir de la fenilalanina, pero requieren los otros dos aminoácidos aromáticos en su dieta (Haslam, 1974).

El metabolismo intermediario de los aminoácidos aromáticos en los mamíferos afecta a un elevado número de procesos biológicos esenciales en la vida, tales como, la utilización de la tirosina en la síntesis de los pigmentos y las hormonas, y la utilización de la tirosina, el triptófano y la histidina en la síntesis de aminas biogénicas. La conversión de la fenilalanina en tirosina está catalizada por la fenilalanina hidroxilasa (Kaufman, 1987), que es oxigenasa de función mixta, que utiliza un cofactor de pteridina, la BH_4. La reacción oxida la BH_4 para dar lugar al isómero quinonoide de la dihidrobiopterin. La coenzima se regenera mediante la acción de la dihidrobiopterin reductasa que requiere NADPH. Este sistema enzimático se encuentra casi enteramente en el hígado.

Un defecto genético en esta enzima es el responsable de la enfermedad Fenilcetonuria (PKU), primera enfermedad genética humana que fue descubierta (Woo, 1989). Esta enfermedad es la principal responsable de la hiperfenilalaninemia. Cuando la fenilalanina hidroxilasa es defectuosa genéticamente una segunda vía del metabolismo de la fenilalanina, que normalmente se utiliza muy poco, entra en acción. En este proceso, la fenilalanina sufre una transaminación con el piruvato formando fenilpiruvato que es un potente neurotóxico de nuestro cerebro, sobre todo, en su desarrollo y maduración, dando lugar a la oligofrenia fenilpirúvica. Tanto la fenilalanina como el fenilpiruvato se acumulan en la sangre y otros tejidos y se excretan a través de la orina, de ahí el nombre de la enfermedad PKU. El fenilpiruvato puede ser descarboxilado formando fenilacetato o puede ser reducido formando fenilactato. El primero provoca un olor característico en la orina, lo cual es parte de la sintomatología.

Una única ruta ramificada en los microorganismos y las plantas lleva a la síntesis de fenilalanina, tirosina y triptófano. Esta ruta común, denominada ruta del ácido sikímico, conduce a la síntesis de casi todos los compuestos aromáticos, incluyendo la lignina, siendo pues una de las rutas más productivas de la biología. La casi total identificación de esta ruta común se completó a principios de los años 60 mediante el estudio de mutantes auxótrofos para la tirosina, la fenilalanina y el triptófano en *E. coli* (Davis, 1950). La ruta del sikimato consta de siete reacciones enzimáticas, codificadas por los genes *aro* (Figura 1), capaces de producir corismato a partir de eritrosa 4-fosfato y el fosfoenolpiruvato. Esta ruta parece ser invariable en todos los organismos eucariotas y procariotas estudiados hasta ahora.

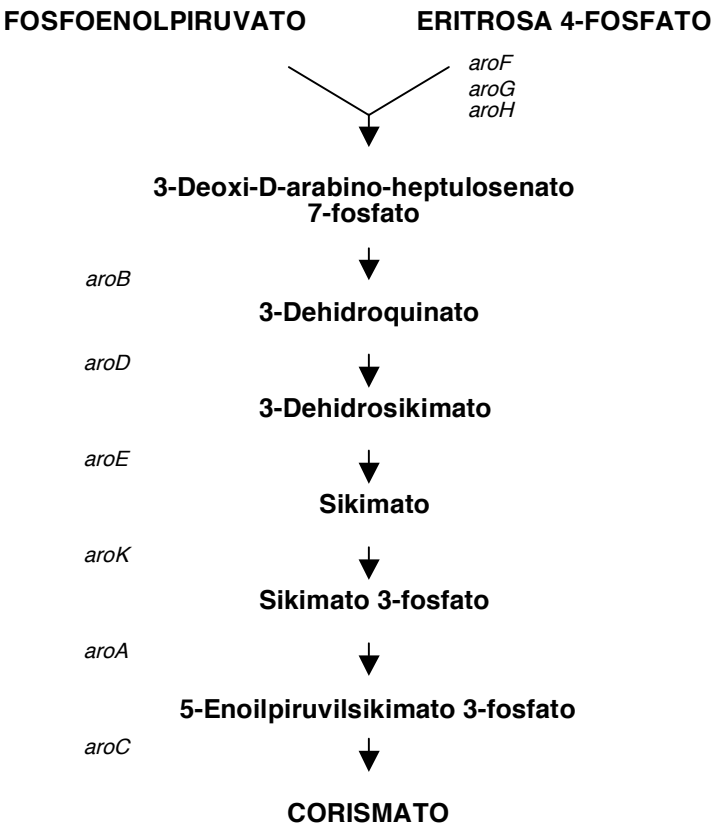

Figura 1. Ruta del Sikimato, en la que se sintetiza el corismato a partir del fosfoenolpiruvato y 4-eritrosa fosfato.

El corismato es el primer intermediario común de los tres aminoácidos aromáticos y es distribuido hacia el triptófano, la fenilalanina y la tirosina (Gibson and Gibson, 1964). Mientras que la biosíntesis de triptófano se realiza mediante cinco reacciones conservadas en todos los organismos estudiados, existen dos rutas independientes para la biosíntesis de la fenilalanina y de la tirosina a partir del corismato. Éste se isomeriza para dar lugar al prefenato, punto de ramificación hacia la fenilalanina y la tirosina (Figura 2), mediante la acción de la corismato mutasa (*pheA*). La descarboxilación y deshidratación del prefenato dan lugar al fenilpiruvato, que conduce directamente a la fenilalanina mediante transaminación (Cotton and Gibson, 1965 y Davis, 1953). Otra vía es la descarboxilación oxidativa del prefenato, produciendo *p*-hidroxifenilpiruvato, el precursor inmediato de la tirosina. La enzima que participa en la síntesis de la fenilalanina se denomina corismato mutasa-prefenato dehidratasa (*pheA*), mientras que la responsable de la síntesis de la tirosina es la corismato mutasa-

prefenato dehidrogenasa (*tyrA*). Debido a la alta similitud que presentan estas enzimas sus estudios han estado siempre muy relacionados y a veces se han desarrollado en paralelo (Cotton and Gibson, 1965 y Xia *et al*., 1993). Se ha descrito además en diferentes cepas del género *Pseudomonas*, la participación de otras enzimas en la síntesis de estos aminoácido aromáticos. El corismato puede dar lugar al arogenato, por acción de la prefenato aminotransferasa, y posteriormente a la fenilalanina, por acción de la arogenato dehidratasa (*pheA*), o a la tirosina, mediante la acción de la arogenato dehidrogenasa (*tyrA*) (Graham *et al*., 1983 y Genshi *et al*., 1991). Recientemente, Molina-Henares *et al*., (2008) han propuesto la ruta de síntesis de los aminoácidos aromáticos para *P. putida* KT2440, deducida a partir de la caracterización de diferentes mutantes auxótrofos en estos aminoácidos y análisis bioinformáticos (Figura 2).

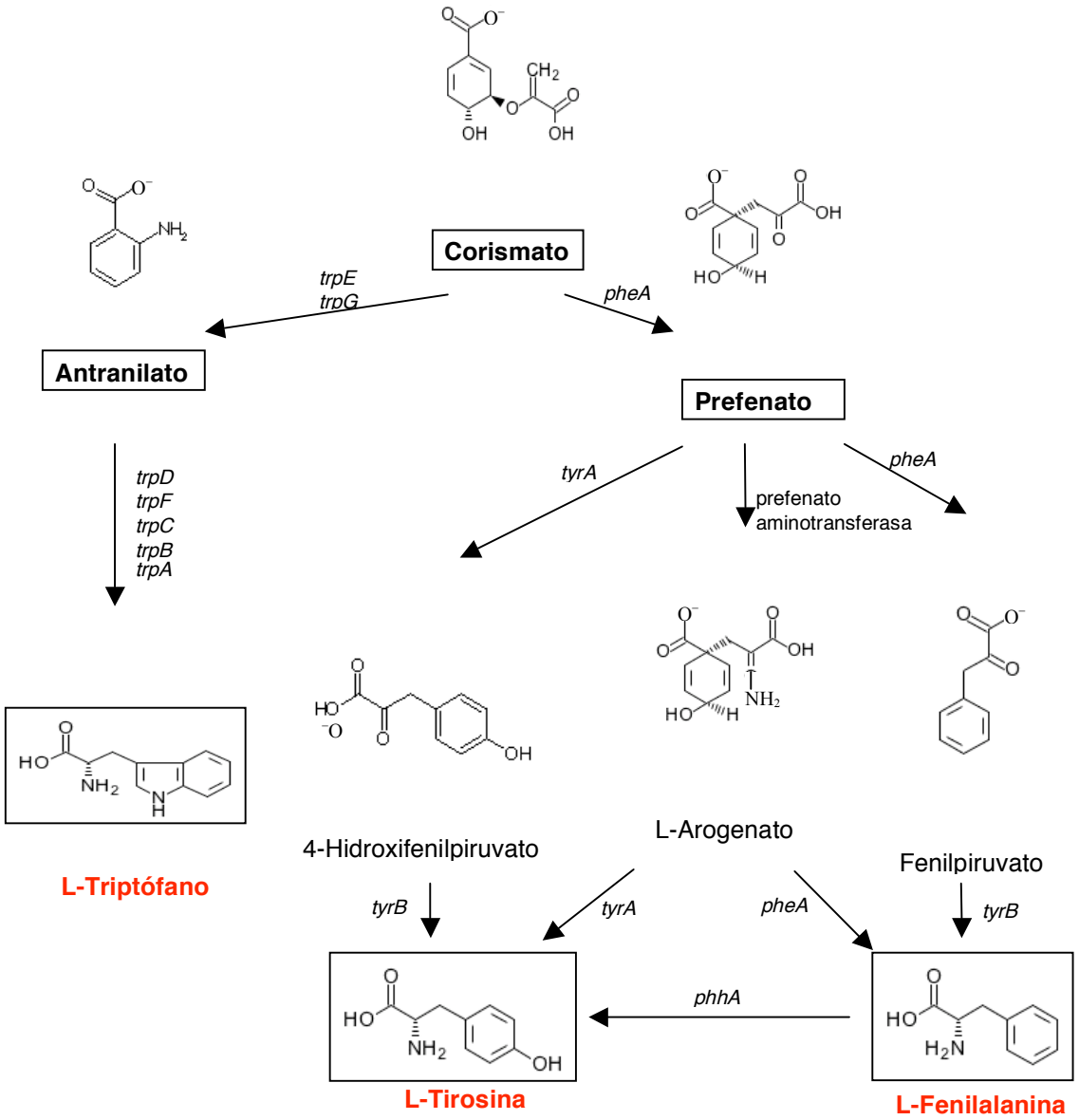

Figura 2. Ruta propuesta para la biosíntesis de aminoácidos aromáticos, fenilalanina, tirosina y triptófano, en *P. putida* KT2440.

Estas rutas biosintéticas parecen estar conservadas en otras especies de *Pseudomonas* tales como *P. aeruginosa*, *P. fluorescens*, *P. entomophila* y *P. syringae*, ya que presentan una organización génica bastante conservada y una alta similtud en las secuencias de proteínas que participan en la ruta metabólica.

4.2 Catabolismo de los aminoácidos aromáticos

a) Catabolismo de los aminoácidos aromáticos en eucariotas

Los aminoácidos aromáticos se metabolizan para rendir amoníaco y urea, dióxido de carbono y agua en organismos eucariotas. En el caso de los humanos, aparece implicado un conjunto de enzimas que pertenecen a la familia de las hidroxilasas, incluyendo la fenilalanina hidroxilasa, la tirosina hidroxilasa y la triptófano hidroxilasa. Éstas presentan alta homología entre sí, utilizan el mismo cofactor, BH_4 y poseen una extensión N-terminal de aproximadamente 200 aminoácidos, que están ausentes en las hidroxilasas microbianas. Se sabe que esta región, indispensable para la función catalítica, posee determinantes para la fosforilación y activación de la fenilalanina (Dahl *et al.*, 1986).

Como se ha mencionado anteriormente, la fenilalanina hidroxilasa en mamíferos ha sido extensamente estudiada debido a que es la responsable de la síntesis de la tirosina y cuya ausencia provoca una grave enfermedad genética, la PKU. Esta enzima también puede considerarse como el primer paso del catabolismo de la fenilalanina. Sin embargo, la principal ruta catabólica para la tirosina implica primero su transaminación mediante la tirosina aminotransferasa, una enzima del hígado cuya concentración se regula hormonalmente. El producto, el *p*-hidroxifenilpiruvato, sufre la acción de la *p*-hidroxifenilpiruvato dehidrogenasa, una enzima poco habitual que cataliza una hidroxilación de anillo, descarboxilación y migración de la cadena lateral. Esta reacción tiene lugar a través de un mecanismo denominado desplazamiento NIH, en honor de los

científicos de los National Institutes of Health de Estados Unidos, que descubrieron una hidroxilación de anillo que tiene lugar a través de la formación de un intermediario epóxido. La reacción de la fenilalanina hidroxilasa comporta también un desplazamiento NIH, con migración del hidrógeno del C-4 de la fenilalanina al C-3 de la tirosina, sin mezclarse con el disolvente.

El producto de la oxidación del *p*-hidroxifenilpiruvato, el ácido homogentísico, se oxida por una enzima que contiene hierro, la ácido gentísico dioxigenasa, que rompe el anillo para dar un compuesto de ocho carbonos de cadena lineal que se isomeriza a fumarilacetoacetato. Este último se rompe finalmente para producir fumarato y acetoacetato, sustancias ambas que se catabolizan mediante rutas estándar de producción de energía.

b) Catabolismo de aminoácidos aromáticos en procariotas

Como ya hemos mencionado, los organismos eucariotas catabolizan la fenilalanina y la tirosina a través de la ruta periférica común que conduce a la producción de homogentisato como intermediario central. Esta ruta bien caracterizada en eucariotas contrasta con la escasa información obtenida hasta ahora en procariotas. Ésto puede deberse al hecho de que *Escherichia coli*, el organismo procariota más representativo y estudiado, es incapaz de mineralizar la fenilalanina, tirosina, fenilpiruvato y *p*-hidroxifenilpiruvato (Fernández A., *et al*., 1998). A pesar de ello estudios realizados en otras bacterias indican que el catabolismo de estos aminoácidos aromáticos se lleva a cabo de forma similar a los eucariotas. Con objeto de determinar el grado de conservación evolutiva en el catabolismo de los aminoácidos aromáticos, en esta Tesis Doctoral nos hemos centrado en el catabolismo de la fenilalanina y de la tirosina en el género *Pseudomonas.*

Bacterias del género *Pseudomonas* como *P. putida* y *P. aeruginosa* entre otras (Arias-Barrau *et al*., 2004 y Song *et al*., 1999), metabolizan la fenilalanina y la tirosina a través de una ruta periférica que incluye la hidroxilación de la fenilalanina (*phhAB*) para dar lugar a la formación de la tirosina. Posteriormente una aminotransferasa (*tyrB*) cataliza la conversión de la tirosina en 4-

hidroxifenilpiruvato y éste a su vez es transformado en homogentisato por la acción de la 4-hidroxifenilpiruvato dioxigenasa (Hpd). El homogentisato se metaboliza entonces por una ruta catabólica central que incluye tres enzimas, la homogentisato dioxigenasa (HmgA), maleilacetoacetato isomerasa (Mai o HmgC) y fumarilacetoacetato hidrolasa (Fah o HmgB) encargadas de transformar el homogentisato en acetoacetato y fumarato que finalmente se metabolizan mediante el ciclo de Krebs.

En *P. aeruginosa* el gen *phhA* (que codifica para la fenilalanina hidroxilasa) es parte de un operón que también incluye el gen *phhB* (que codifica para la pterin 4a-carbinolamina dehidratasa). Aguas arriba de éste y formando parte del mismo operón, se encuentra el gen *phhC*, o también llamado *tyrB*, que codifica una aminotransferasa que da lugar a la formación del 4-hidroxifenilpiruvato (Zhao *et al*., 1994). PhhA es una enzima poco frecuente en procariotas, ya que se encuentra predominantemente en una única división filogenética de bacterias Gram negativas (Guroff and Ito, 1963; Dedicco and Umbreit, 1964; Friedrich and Schlegel, 1972; Letendre *et al*., 1974 y Berry *et al*., 1985). Se ha clonado el gen que codifica la subunidad PhhA de *Chromobacterium violaceum* y *P. aeruginosa* lo que ha permitido purificarla y caracterizarla bioquímicamente (Nakata *et al*, 1979; Zhao *et al*., 1994; Chen and Frey, 1998; Zoidakis *et al*., 2005; Erlandsen *et al*., 2002 y Ekstrom *et al*., 2003). La actividad de PhhA es dependiente de hierro y es un monómero en solución, a diferencia de lo que ocurre en eucariotas, donde el estado oligomérico predominante es el tetramérico. Song *et al*., (1999) pusieron de manifiesto la necesidad *in vivo* de PhhB para mantener el proceso de reciclaje del cofactor, previniendo así la formación de 7-biopterin, un compuesto que inhibe la acción de PhhA. En mamíferos, PhhB es bifuncional, presenta además de un papel catalítico, un papel de regulación. Sin embargo, en bacterias PhhB presenta un modesto efecto de regulación además de su ya citada función catalítica. En *P. aeruginosa* se ha observado una regulación de PhhA y PhhB semicoordinada, es decir, ambas enzimas son inducidas coordinadamente en la presencia tanto de L-fenilalanina como de L-tirosina, pero PhhB, a su vez, exhibe un nivel basal significativo de actividad del que PhhA carece. Por otro lado, se ha visto, que PhhB ejerce un efecto de regulación positivo a nivel postranscripcional sobre

PhhA mediante la formación de un complejo proteína-proteína (Song, *et al.,* 1999). El gen *phhC* de *P. aeruginosa* codifica una proteína perteneciente a la familia I de aminotransferasas que requiere la formación de homodímeros. Esta enzima resulta imprescindible en el catabolismo de la fenilalanina y la tirosina, a pesar de la presencia de otras aminotransferasas que presentan un modelo similar de amplia especificidad de sustrato *in vivo*. Esto implica una orientación espacial de PhhC que lo hace específico para el catabolismo de aminoácidos aromáticos (Gu *et al*., 1998).

En *P. aeruginosa*, el operón *phhABC* se transcribe de forma divergente al gen *phhR*, el cual codifica una proteína reguladora que pertenece a la familia de reguladores NtrC-dependientes de σ^{54}. Esta proteína presenta un dominio N-terminal que media la regulación, un dominio central, altamente conservado en la familia de reguladores NtrC, caracterizado por la presencia de dos motivos conservados responsables de la unión e hidrólisis del ATP (Morret and Segovia, 1993) y un dominio C-terminal que reconoce la secuencia promotora.

El producto final de la acción del conjunto de enzimas codificadas por el operón *phhABCR*, el 4-hidroxifenilpiruvato, es transformado por la 4-hidroxifenilpiruvato dioxigenasa (Hpd) para dar lugar al homogentisato. Esta reacción incluye una decarboxilación, una migración del sustituyente y una oxigenación aromática en un simple ciclo catalítico. Esta proteína se encuentra en casi todas las formas de vida conocidas, excepto en bacterias Gram positivas, y se asocia formando tetrámeros y dímeros en bacterias y eucariotas, respectivamente. Esta enzima fue cristalizada por primera vez a partir de la proteína de *P. fluorescens* (Serre *et al*.,1999).

Posteriormente el homogentisato se cataboliza hasta fumarato y acetoacetato que a su vez se degradan mediante el ciclo de Krebs. En *Pseudomonas* los genes *hmgABC*, que participan en la ruta central del homogentisato, son homólogos a los genes que codifican la homogentisato dioxigenasa (HmgA), maleilacetoacetato isomerasa (Mai o HmgC) y fumarilacetoacetato hidrolasa (Fah o HmgB) en *Sinorhizobium meliloti* (Milcamps and de Bruijn, 1999) y *Emericella nidulans* (Fernández-Cañón and Peñalva, 1998). El gen, *hmgR*, que se transcribe divergentemente a los genes catabólicos *hmgABC*, codifica el regulador HmgR, un represor de la familia de reguladores IclR. Mientras que en

P. putida, *P. aeruginosa* y *P. fluorescens* la organización génica del conjunto de los genes *hmg* es similar, en *P. syringae* aparece diferente. Se ha encontrado la presencia de un gen (*hmgT*) que codifica para una posible proteína transportadora de la familia MFS aguas abajo del gen *hmgC* en el genoma de *P. aeruginosa* y *P fluorescens*.

c) Catabolismo de fenilalanina y tirosina en *P. putida* KT2440

P. putida KT2440 es una bacteria del suelo que coloniza la raíz de un amplio rango de plantas a una alta densidad celular respondiendo a productos presentes en los exudados de raíz de plantas. Estos microorganismos pueden utilizar aminoácidos, como prolina, glutamato y lisina como fuentes de carbono y de nitrógeno (Revelles *et al*., 2005; Revelles *et al*., 2007 y Rodríguez-Herva *et al*., 1996). La anotación en el genoma de *P. putida* KT2440 identifica un conjunto de genes entre las posiciones 5100 y 5111 Kb, cuyos productos exhiben una identidad de alrededor del 80 % con genes capaces de degradar la fenilalanina (*phh*) en otras *Pseudomonas* (Tabla 1). De este conjunto génico, tres se encuentran transcritos en la misma dirección, mientras que el cuarto se transcribe de forma divergente. Los genes están anotados como *phhA*, *phhB*, *phhT* y *phhR*. El codón de parada de *phhA* se dispone a 41 nucleótidos del codón de inicio de *phhB*. El codón de parada de *phhB* está separado del codón de inicio de *phhT* por alrededor de 700 pb. Este último gen presenta similitud a transportadores de aminoácidos aromáticos y se encuentra próximo al gen *aroP2* que codifica una permeasa general de aminoácidos aromáticos. Aunque no hay homólogo al gen *phhC* de *P. earuginosa* en el operón *phh*, en el genoma de *P. putida* KT2440 presenta dos ORFs, PP1972 (*tyrB1*) and PP3590 (*tyrB2*) que se anotaron como genes que codifican tirosina aminotransferasas, sugiriendo su participación en la transformación de tirosina a *p*-hidroxifenilpiruvato, a pesar de que ninguno de ellos se encuentra próximo a los genes *phh* (Jiménez *et al*, 2002).

Cepa	PhhR	PhhA	PhhB	PhhT	AroP2	TyrB-1	TyrB-2	Hpd	HmgA	HmgB	HmgC
P. aeruginosa	87	85	86	73	79	43	44	55	81	79	76
P. entomophila	96	97	94	91	92	93	91	94	94	90	85
P. fluorescens	92	90	86	82	85	85	52	89	82	80	70
P. mendocina	88	85	82	41	21	77	51	54	23	27	51
P. stutzeri	84	81	83	28	72	76	51	54	24	-	-
P. syringae	85	86	83	80	85	80	66	86	74	69	68

Tabla 1. Porcentaje de identidad entre las proteínas que participan en la degradación de fenilalanina y tirosina.

El producto del gen *phhR* presenta similitud con miembros de la familia de reguladores NtrC-dependientes de σ^{54}. Este regulador posee un elevado porcentaje de identidad con PhhR de otros miembros del género *Pseudomonas* (Tabla 1). PhhR de *P. earuginosa* es un activador del operón *phhABC* que se transcribe de forma divergente (Song and Jensen, 1996). La proteína homóloga más próxima a PhhR de *Pseudomonas* es la proteína TyrR de *E. coli* y de *Haemophilus influenzae*. TyrR de *E. coli* es miembro de una familia de proteínas poco común, caracterizadas por activar la transcripción de promotores dependientes de σ^{70}, a pesar de ser homólogas a proteínas de la familia NtrC.

El gen *hpd* localizado en la posición 3890 Kb del genoma codifica una 4-hidroxifenilpiruvato dioxigenasa que da lugar al homogentisato a partir del 4-hidroxifenilpiruvato.

Arias-Barrau *et al*, (2004), identificaron y caracterizaron el conjunto de genes que participan en la ruta central del homogentisato en *P. putida* KT2440. Se encontraron tres ORFs, *hmgA*, *hmgB* y *hmgC*, formando una única unidad transcripcional, que codifican una homogentisato dioxigenasa, una fumarilacetoacetato hidrolasa y una maleilacetoacetato isomerasa respectivamente, este conjunto de enzimas transforman el homogentisato en fumarato y acetoacetato. Participa además, una proteína de la familia IclR de reguladores transcripcionales, codificada por el gen *hmgR* que se transcribe de forma divergente al operón *hmgABC*.

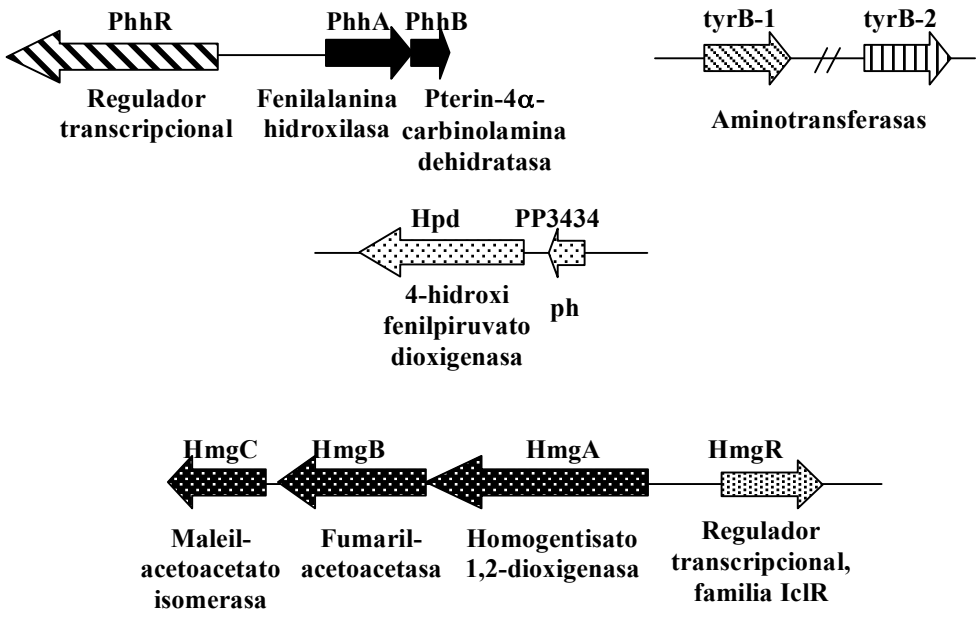

Figura 3. Organización en el cromosoma de *P. putida* KT2440 de genes involucrados en el catabolismo de la fenilalanina.

5. Transporte de los aminoácidos aromáticos en bacterias

Los sistemas de transporte de aminoácidos son ubicuos en el conjunto de seres vivos, apareciendo tanto en eucariotas como en procariotas, ya que los aminoácidos pueden ser utilizados en el catabolismo, en la síntesis proteica y/o en la traducción de señales. Debido a que existen diferentes grupos de aminoácidos, cabe esperar la existencia de diferentes sistemas de transporte específicos de cada uno de ellos. Sin embargo, se han observado en los diferentes microorganismos tanto transportadores generales de aminoácidos, como transportadores de alta afinidad específicos para un aminoácido o una familia de aminoácidos relacionados estructuralmente. Por ejemplo, en *E. coli*, *C. glutamicum* y en *P. aeruginosa,* el transporte de aminoácidos aromáticos no solapa con ningún otro aminoácido neutral, es decir, se lleva a cabo mediante sistemas de transporte que presentan especificidad para este tipo de aminoácidos (Piperno and Ofender, 1968; Ikeda and Katsumata, 1994 y Kay and Gronlund, 1971). Por el contrario, en otros organismos como *Salmonella*

typhimurium, los aminoácidos aromáticos son transportados tanto por transportadores de aminoácidos generales como de sistemas de transporte específicos para aminoácidos aromáticos (Ames, 1964).

Las propiedades de los transportadores específicos de aminoácidos aromáticos han sido ampliamente estudiados en bacterias Gram negativa. Estudios cinéticos, de competición cruzada y experimentos de intercambio han demostrado la presencia de sistemas de transporte múltiples en cada especie. *S. typhimurium* y *E. coli* K-12 poseen sistemas generales, que muestran igual actividad de transporte para cada uno de los aminoácidos aromáticos, y sistemas específicos que presentan una alta especificidad y actividad de transporte para aminoácidos aromáticos individuales. *Pseudomonas aeruginosa* tiene dos sistemas de transporte ambos funcionales para el transporte de fenilalanina, tirosina y triptófano (Kay and Gronlund, 1971), mientras que *Yersinia pestis* presenta tres sistemas de transporte de aminoácidos aromáticos: un transporte específico de triptófano, otro específico para fenilalanina siendo también activo para tirosina y triptófano cuando están presentes a elevadas concentraciones en el exterior, y un sistema general que reconoce a cada uno de los aminoácidos aromáticos a alta o baja concentración externa con cierto grado de especificidad por la tirosina (Smith and Montie, 1975). Mientras que un sistema de transporte común aparece frecuentemente en bacterias Gram negativas, ninguno ha podido ser detectado en *Brevibacterium linens* (Boyaval *et al*., 1983).

El transporte de fenilalanina y triptófano en *Y. pestis* y en *B. linens* es estereoespecífico para el isómero L. Sin embargo el transporte de tirosina no presenta este grado de especificidad, ya que el transporte de éste es inhibido por cada uno de los D-aminoácidos aromáticos (Smith and Montie, 1975; Boyaval *et al*., 1983). Esto mismo ocurre en *P. aeruginosa*, donde D-fenilalanina y D-tirosina inhiben el transporte de la tirosina (Kay and Gronlund, 1971).

En *E coli* se han descrito, al menos, cinco sistemas de transporte de aminoácidos aromáticos. Éstos incluyen un sistema para la L-tirosina (producto del gen *tyrP*), otro para la L-fenilalanina (producto del gen *pheP*), dos sistemas para el L-triptófano (productos de los genes *mtr* y *tnaB*), además del sistema de transporte general de los tres aminoácidos aromáticos (producto del gen *aroP*)

(Ames, 1964 y Brown, 1970).

Cosgriff and Pittard (1997) propusieron un modelo topológico para la permeasa de aminoácidos aromáticos general, AroP. Éste consiste en 12 regiones transmembrana conectados por un loop hidrofílico. Encontraron que tres residuos (E151, E153 y K160) altamente conservados en una gran familia de proteínas transportadoras de aminoácidos, eran esenciales para la función de transporte de esta permeasa.

La proteina responsable del transporte activo de la fenilalanina en *E. coli* es la permeasa PheP, la cual presenta una alta afinidad por fenilalanina y es incapaz de transportar tirosina y triptófano (Pi *et al*., 1991 y Whipp *et al*., 1980). A pesar de que las proteínas AroP y PheP presentan un alto porcentaje de identidad (aproximadamente del 60%), difieren en la especificidad de sustrato y afinidad. Mientras PheP muestra una actividad específica para transportar fenilalanina, AroP es capaz de transportar por igual cada uno de los aminoácidos aromáticos. Diferencias estructurales entre ambas proteínas, estudiadas por Cosgriff *et al*. (2000), proporcionaron una mayor información en la relación función-estructura en estas proteínas. Un análisis de sus respectivos perfiles de sustrato y actividades han identificado un residuo (T103) presente en AroP y ausente en PheP, responsable del transporte del triptófano.

También se ha confirmado la presencia de un transportador de fenilalanina en *Staphylococcus aureus*, PheP, bajo condiciones de bajo nivel de oxígeno (Malcolm *et al*., 2004).

En *E. coli* el sistema de transporte específico de la tirosina se lleva a cabo mediante una proteína de membrana citoplasmática codificada por *tyrP* (Wookey *et al*, 1984; Wookey and Pittard, 1988). Se sabe además que el transportador TyrP es un sistema dependiente de energía que opera mediante la fuerza protón-motriz. Wolken *et al.* (2006) han demostrado recientemente que el transportador TyrP en *Lactobacillus brevis*, cataliza con alta eficiencia un intercambio tirosina-tiramina produciendo una carga neta positiva. Los genes que codifican la tirosina decarboxilasa (*tyrDC*) han sido identificados junto a *tyrP* y a un posible transportador antiporte Na^+/H^+.

En *P. putida* KT2440, se han encontrado al menos dos genes que codifican posibles transportadores de aminoácidos aromáticos. PhhT (codificado

por el gen *phhT*), localizado aproximadamente a 700 pares de bases de los genes *phh,* presenta un porcentaje de identidad de un 73% con la proteína PhhT de *P. aeruginosa*. El otro transportador, codificado por el gen *aroP2* (PP4495), posee alta identidad con AroP2 de *P. aeruginosa* (79%) y con PheP de *E. coli* (66%), por lo que parece estar involucrado en el transporte de los aminoácido aromáticos.

6. Regulación metabólica de los aminoácidos aromáticos

6.1 Regulación de la biosíntesis

Las rutas de biosíntesis de los aminoácidos aromáticos están altamente controladas ya que presentan diferentes puntos de regulación, tanto a nivel de la ruta común, como en las rutas ramificadas de la síntesis específica de cada uno de los aminoácidos aromáticos.

Para la síntesis común de los aminoácidos aromáticos se ha encontrado regulación a nivel de casi todas las enzimas que participan, sin embargo para una gran cantidad de bacterias, como *P. aeruginosa, P. putida, P. fluorescens, D. acidovorans* y *C. testosteroni*, el control de la primera reacción, llevada a cabo sobre la 3-deoxi-D-arabinoheptulosenato-7-fosfato-sintasa por fenilalanina y tirosina, parece ser el de mayor relevancia (Olekhnovich *et al.*,1991 y Herrmann, 1995).

El control de las rutas ramificadas difieren entre microorganismos, pero en general está afectado tanto por la inhibición feedback solamente, como por una combinación de la inhibición feedback y de represión. En *E. coli* La inhibición por feedback a diferentes niveles enzimáticos se lleva a cabo por alguno de los aminoácidos aromáticos, mientras que la represión está mediada por diferentes reguladores (Herrmann, 1995). En el primer caso están incluidas las enzimas antranilato sintasa (por el triptófano), prefenato dehidrogenasa (por la tirosina) y la prefenato dehidratasa (por la fenilalanina) (Pittard, 1996). La corismato mutasa se inhibe por tirosina, por fenilalanina, o por ambas, en

algunos organismos como *E. coli* y *S. typhimurium* (Baldwin and Davidson, 1981 y Hudson *et al*., 1983). En cuanto a la regulación por represión mediada por reguladores, un análisis computacional comparativo puso de manifiesto la existencia de varios regulones de aminoácido aromáticos en γ-proteobacterias (Panina *et al*., 2001). Se han caracterizado los regulones TyrR (en la síntesis de la fenilalanina y tirosina) y TrpR (en la síntesis del triptófano) en los genomas de *Haemophilus influenzae*, *Salmonella typhimurium, E. coli* y otras bacterias (Bennett and Yanofsky, 1978; Gollub and Sprinson, 1973; Pittard and Davidson, 1991; Yanofsky, 1981 and Zhu et al., 1997).

El regulón TyrR de *E. coli*, el más estudiado hasta ahora, contiene al menos nueve unidades transcripcionales, cada una de las cuales es regulada de una manera diferente (Pittard and Davidson, 1991). TyrR actúa tanto como activador como represor de la transcripción. Puede interaccionar con los tres aminoácidos aromáticos, con ATP y bajo ciertas condiciones con la región carboxilo terminal de la subunidad α de la RNA polimerasa.

TyrR tiene tres dominios funcionales, un dominio N-terminal, un dominio central y un dominio C-terminal (Cui and Somerville, 1993a; kwok, 1998; MacPherson *et al*., 1999 y Dixon *et al*., 2002)(Figura 4). El dominio N-terminal juega un papel importante en la activación de la expresión de los genes en respuesta a los aminoácidos aromáticos, interaccionando con el αCTD de la RNA polimerasa (Cui and Somerville, 1993b,c y Yang *et al*., 1993a, 1996a). Este dominio posee un sitio de unión para los aminoácidos aromáticos referido como ACT (del inglés *aspartokinase, chorismate mutase*, TyrA) (Aravind and Koonin, 1999 y Ettema *et al*., 2002), un segundo motivo, denominado PAS (del inglés Per-Arnt-Sim) relacionado, en otros sistemas, con la traducción de señales y con la interacción con el αCTD de la RNA polimerasa (Ettema *et al*., 2002), y por último, posee un motivo de dimerización de la proteína DIM (Kwok, 1998). El dominio central muestra gran homología con reguladores de la familia NtrC. Posee un sitio de unión al ATP y otro a la tirosina dependiente de ATP que es esencial para llevar a cabo la hexamerización y represión mediada por tirosina (Pittard and Davidson, 1991 y Wilson *et al*., 1994). A diferencia de otros miembros de la familia de reguladores NtrC, TyrR no activa la expresión de promotores mediante σ^{54} ya que carece de la secuencia conservada en el

dominio central, responsable de esta interacción (Morrett and Segovia, 1993). Sin embargo esta secuencia está presente en proteínas homólogas de TyrR, como es PhhR de *P. earuginosa*, la cual se ha sugerido que activa la expresión de promotores mediante σ^{54} (Song and Jensen, 1996). El dominio central de TyrR posee actividad autokinasa y autofosfatasa, siendo esta última susceptible de inhibición por fenilalanina y tirosina (Zhao *et al*., 2000). El dominio C-terminal es responsable de la unión al ADN ya que presenta un motivo de hélice-giro-hélice, que ha sido ampliamente estudiado, por mutagénesis y mediante análisis *in vitro*, confirmando su participación en el reconocimento de las cajas TyrR en los promotores (Yang *et al*., 1993 y Hwang *et al*., 1997, 1999).

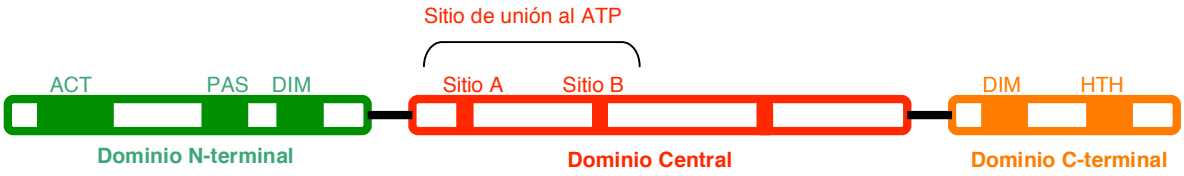

Figura 4. Organización física de dominios de la proteína TyrR de *E. coli*.

TyrR es un dímero en solución, pero en la presencia de tirosina y ATP se autoasocia para formar un hexámero. Mientras que TyrR en forma de dímero y en ausencia de aminoácidos, es capaz de reconocer ciertas secuencias referidas como “cajas TyrR fuertes”, TyrR en forma de hexámero puede reconocer con menor afinidad unas secuencias denominadas como “cajas TyrR débiles”. Experimentos de unión *in vitro* y ensayos funcionales, realizados con mutaciones en las cajas, han demostrado la existencia de 19 cajas TyrR en los nueve miembros caracterizados del regulón. Todas las secuencias son palindrómicas y mantienen concordancia con el consenso, 5´-TGTAAAN_6TTTACA-3´. Ocho miembros del regulón poseen dos o más cajas.

TyrR como activador requiere la presencia de al menos una caja TyrR fuerte localizada aguas arriba del hexámero -35 del promotor, además de la presencia de alguno de los aminoácidos aromáticos y la subunidad α de la RNA polimerasa para activar la transcripción. La expresión de cuatro miembros del regulón se activa por TyrR, a saber *mtr*, *tyrP, aroPP3* y *folA*, que codifican un transportador de triptófano, un transportador de tirosina, un transportador de aminoácidos aromáticos general y una dihidrofolato reductasa, respectivamente

(Whipp and Pittard, 1977; Andrews *et al*., 1991a,b; Heath-wole and Somerville, 1991; Yang *et al*., 1996b, 2004; Wang *et al*., 1997a,b, 1998 y Yang *et al*., 2007). La activación de cada unidad transcripcional viene determinada por la posición y naturaleza de las cajas TyrR asociadas con cada uno de los promotores. Así por ejemplo en *tyrP*, se ha establecido que la distancia entre los centros de la caja y el hexámero -35 del promotor, debe ser de 31 ó de 42 pb para obtener una activación óptima (Andrews *et al*., 1991a). Estos promotores también se caracterizan por presentar un hexámero -10 imperfecto, lo que los hace dependientes del activador TyrR (Yang *et al*., 2004).

La represión mediada por TyrR puede ser dependiente o independiente de efectores. Los promotores *aroF, aroG, aroL* (codifican las enzimas primera y quinta de la ruta de síntesis del shikimato)*, tyrP* y *tyrR* (codifica el regulador TyrR), están sujetos a diferentes grados de represión independiente de efectores (Camakaris and Pittard, 1982 y Kwok *et al*., 1995). Sin embargo, en la presencia de tirosina, la proteína TyrR también puede autoasociarse formando un hexámero, uniéndose posteriormente a las cajas como ocurre en los genes *aroF, aroL* y *aroP* (Andrews *et al*., 1991b; Pittard and Davidson, 1991 y Lawley and Pittard, 1994). En el caso del gen *tyrB* (codifica la aminotransferasa que participa en la síntesis de aminoácido aromáticos), existe una fuerte represión mediada por la presencia de tirosina, pero también por la presencia de fenilalanina (Yang *et al.,* 2002). Todas las unidades transcripcionales reprimidas por tirosina presentan dos cajas adyacentes, una fuerte y otra ligera, separadas por tan sólo una única base. La unión cooperativa entre estas cajas incluye la formación de hexámeros de la proteína en presencia de tirosina y ATP. La localización de las cajas y la afinidad con la que TyrR las reconoce, requiere la hexamerización de la proteína. Esta situación ocurre cuando una caja fuerte y otra débil están localizadas en la misma cara de la hélice adyacente, como en el caso de *tyrP*, *tyrB*, *aroF* y *aroL*, o no más de una vuelta de hélice (Andrews *et al*., 1991b).

No existe ningún mecanismo general de represión. En algunos casos incluye exclusión de la RNA polimerasa del promotor, y en otros interferencia con la capacidad de unión de la RNA polimerasa para formar el complejo abierto o para liberarse del promotor.

6.2 Regulación catabólica

En general, la regulación catabólica responde a la ausencia o presencia del compuesto que va a ser asimilado (respuesta de control específico) o responde a señales que provocan la inducción de los genes de la ruta según el estado fisiológico de la célula (respuesta de control global).

La regulación catabólica de los aminoácidos aromáticos en el género *Pseudomonas* no ha sido muy estudiada, sin embargo, en las últimas décadas, se ha llevado a cabo la identificación y caracterización de algunas proteínas involucradas en la regulación catabólica de la fenilalanina y de la tirosina (Morales *et al*., 2004 y Arias-Barrau *et al*, 2004)

Los resultados obtenidos por Morales *et al*., (2004) mostraron que el regulador global Crc participa en la represión catabólica a nivel de los genes *hpd* y *hmgA* en la ruta central del homogentisato en *P. putida*. Un análisis proteómico indicó que Crc reprime la expresión de Hpd y HmgA de la ruta catabólica de la fenilalanina y tirosina. Crc es un componente de la ruta de traducción de señales que modula el metabolismo del carbono en *Pseudomonas*, mediante un mecanismo hasta ahora desconocido. El efecto de Crc es particularmente importante en células cultivadas en medio rico en fase exponencial, al menos en *P. putida* (Hester *et al*., 2000 y Yuste and Rojo, 2001).

Por otro lado en *P. putida* U y *P. putida* KT2440, se ha estudiado el papel de un regulador que participa en la regulación catabólica de la fenilalanina y de la tirosina a nivel de la ruta central del homogentisato. La proteína HmgR actúa como represor en ausencia del inductor, el homogentisato, sobre la expresión del operón *hmgABC*. Una serie de análisis *in vivo* pusieron de manifiesto el mecanismo por el cual el represor HmgR controla la expresión del promotor de los genes *hmg.* HmgR reconoce una secuencia palindrómica que solapa con la caja -10 del promotor de forma que compite físicamente con la RNA polimerasa por el promotor. La presencia del inductor, el homogentisato, provoca un cambio en la naturaleza de la interacción del represor con el promotor permitiendo que la RNA polimerasa lleve a cabo la iniciación de la transcripción (Arias-Barrau *et*

al, 2004).

PhhR de *P. putida* parece ser el regulador que participa en la ruta catabólica de aminoácidos aromáticos tales como fenilalanina y tirosina, por lo que nosotros estamos interesados en estudiar su papel y su mecanismo de acción en el metabolismo de estos aminoácidos.

IV. Resultados

Capítulo 1: Catabolismo de la fenilalanina en *Pseudomonas putida*: El regulador PhhR, miembro de la familia NtrC, reconoce dos sitios de unión del promotor del gen *phhA* y estimula la transcripción con σ^{70}

Catabolism of phenylalanine by *Pseudomonas putida*: The NtrC-family PhhR regulator binds to two sites upstream from the *phhA* gene and stimulates transcription with σ^{70}.

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Pseudomonas putida es capaz de utilizar L-fenilalananina como única fuente de nitrógeno transformándola en tirosina, la cual actúa como donador del grupo amino. Este paso metabólico requiere la participación de los productos de los genes *phhA* y *phhB*, que se disponen formando un operón. La expression del promotor *phhA* está mediada por el producto del gen *phhR* en presencia de fenilalanina y de tirosina. La proteína PhhR pertenece a la familia de proteínas NtrC. A diferencia de lo que ocurre en muchos de los miembros de esta familia de reguladores, la transcripción del operón *phhAB* (P_{phhA}) está mediada por la RNA polimerasa con σ^{70} en lugar de hacerlo con σ^{54}. El regulador PhhR se une a dos motivos muy similares pero no idénticos (5'-TGTAAATTATCGTTACG-3' y 5'-ACAAACTGTGTTTCCG-3') que se encuentran localizados a 42 y 79 nucleótidos aguas arriba del hexámero -35 propuesto para la RNA polimerasa, respectivamente. Estas secuencias se denominaron motivos de unión distal y proximal de PhhR, en función de su posición con respecto al sitio de unión de la RNA polimerasa. La afinidad de PhhR por estas secuencias diana se determinaron mediante técnicas de calorimetría de titulación isotérmica, obteniéndose una afinidad de 30 nM para el sitio proximal y de 2 μM para el sitio distal. La estequiometría de unión obtenida fue de un dímero por sitio de unión. Ambas secuencias diana son *sine qua non* para la transcripción ya que la inactivación de cualquiera de las dos da lugar a una inactivación de la transcripción del promotor *phhA*. Un sitio de unión de IHF solapa con el motivo PhhR proximal, el cual es reconocido por IHF con una afinidad de 1.2 μM. IHF puede competir con PhhR por el sitio de unión e inhibir la expression del operón *phhAB* dependiente de PhhR.

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Catabolism of Phenylalanine by *Pseudomonas putida*: The NtrC-family PhhR Regulator Binds to Two Sites Upstream from the *phhA* Gene and Stimulates Transcription with σ^{70}

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Pseudomonas putida uses L-phenylalanine as the sole nitrogen source for growth by converting L-phenylalanine to L-tyrosine, which acts as a donor of the amino group. This metabolic step requires the products of the *phhA* and *phhB* genes, which form an operon. Expression of the *phhA* promoter is mediated by the *phhR* gene product in the presence of L-phenylalanine or L-tyrosine. The PhhR protein belongs to the NtrC family of enhancers. In contrast with most members of this family of regulators, transcription from the promoter of the *phhAB* operon (P_{phhA}) is mediated by RNA polymerase with σ^{70} rather than with σ^{54}. The PhhR regulator binds two similar but non-identical upstream PhhR motifs (5′-TGTAAAATTATCGTTACG-3′ and 5′-ACAAAAACTGTGTTTCCG-3′) that are located 39 and 97 nucleotides upstream of the proposed −35 hexamer for RNA polymerase, respectively. These motifs are called PhhR proximal and PhhR distal binding motifs because of their position with respect to the RNA polymerase binding site. Affinity of PhhR for its target sequences was determined by isothermal titration calorimetry and was found to be around 30 nM for the proximal site and 2 μM for the distal site, and the binding stoichiometry is of a dimer per binding site. Both target sequences are *sine qua non* requirements for transcription, since inactivation of either of them resulted in no transcription from the *phhA* promoter. An IHF binding site overlaps the proximal PhhR proximal motif, which is recognized by IHF with a K_D of around 1.2 μM. IHF may consequently compete with PhhR for binding and indeed inhibits PhhR-dependent *phhAB* operon expression.

Keywords: NtrC family; PhhR; IHF; ITC; DNA/protein interactions

Introduction

Biotransformations represent the third wave in biotechnology and the microbial production of added-value products from abundant and cheap sources, such as sugars, is of industrial interest. Any biotransformation needs to fulfil at least two requirements: the availability of the appropriate catalytic segments to achieve the desired product; and the need of a biocatalyst not susceptible to poisoning by substrates or products during the process. In recent years, our group and others have shown that certain strains of *Pseudomonas putida* are particularly tolerant of high concentrations of toxic aromatic compounds,[1–5] and these microorganisms are at the basis of potential catalysts for the production of added-value products from cheap carbon sources.[2–5] We are interested in the biosynthesis of phenylalanine by *P. putida* KT2440 as the basis for the production of other added-value chemicals such as dihydroxyphenylalanine, dopamine, dhurrin and others. *Pseudomonas putida* can synthesize phenylalanine from glucose and tolerates up to 25–30 g/l of phenylalanine in the culture medium; however, its synthesis by this microorgan-

Abbreviations used: EMSA, electrophoretic mobility-shift assay; IHF, integration host factor; ITC, isothermal titration calorimetry; LB, Luria Bertani culture medium; ORF, open reading frame; RT-PCR, reverse transcriptase-polymerase chain reaction.

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ism presents a serious drawback derived from the fact that the strain uses this aromatic amino acid as a nitrogen source. Therefore, the use of *P. putida* to biosynthesise phenylalanine-derived products requires the blockage of catabolism of this amino acid.

Zhao *et al*. reported that *Pseudomonas aeruginosa* possesses a tetrahydrobiopterin-dependent monooxygenase encoded by the *phhA* gene, whose gene product (PhhA) catalyses the first reaction in phenylalanine metabolism, the hydroxylation of phenylalanine to yield tyrosine (Figure 1).[6] In *P. aeruginosa* the *phhA* gene is part of an operon that includes the *phhB* gene, which encodes a homologue of mammalian 4α-carbinolamine dehydratase; a key enzyme that regenerates tetrahydrobiopterin. Downstream from *phhB* and part of the same operon is *phhC*, also called *tyrB*, which encodes an aromatic aminotransferase that yields *p*-hydroxyphenylpyruvate (Figure 1).[7–9] This metabolite is channelled *via* homogentisate to Krebs cycle intermediates.[7]

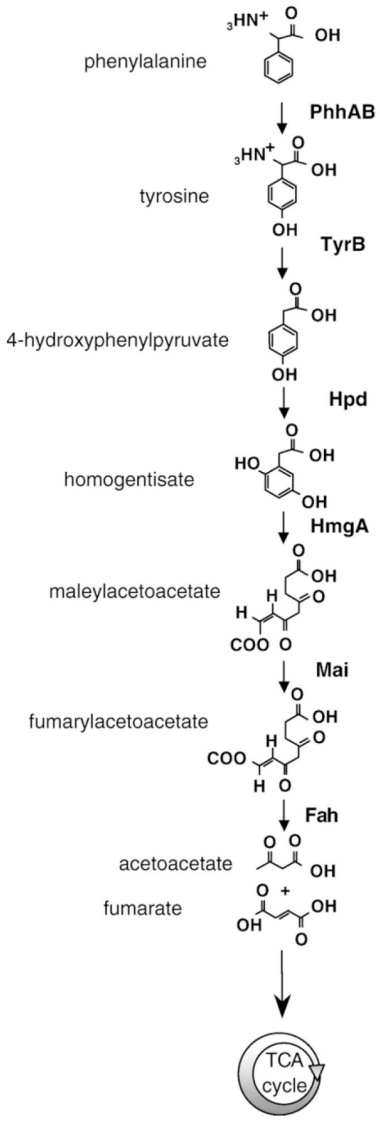

Figure 1. Proposed catabolic pathway for phenylalanine catabolism in *Pseudomonas*. Oxidation of phenylalanine by the PhhAB system yields tyrosine, which yields 4-hydroxyphenylpyruvate upon amino transfer to a ketoacid. This is oxidized by the *hpd* gene product,[7] yielding homogentisate that is metabolized to Krebs cycle intermediates.

Annotation of the *P. putida* KT2440 genome identified a cluster of genes whose translated products exhibited around 30% identity with phenylalanine (*phh*) utilization genes from other microorganisms. This cluster is made of four genes, three in the same transcriptional direction and one gene in the opposite direction (Figure 2). The genes were annotated originally as *phhA, phhB, phhT* and *phhR*.[9] The stop codon of *phhA* is 41 nucleotides from the start codon of *phhB*. The stop codon of *phhB* is separated by about 700 bp from the start of the *phhT* gene. This third gene exhibits similarity to aromatic amino acid transporters. The *phhR* gene is transcribed opposite to *phhA* (Figure 2), and its gene product exhibits similarity to members of the NtrC family of regulators. In the genome of *P. putida,* two open reading frames (ORFs) encoding PP1972 (*tyrB1*) and PP3590 (*tyrB2*) have been annotated as tyrosine aminotransferases and were suggested to be involved in the conversion of tyrosine into *p*-hydroxyphenylpyruvate, although none of these genes is linked to the *phh* gene cluster.[9]

This study was undertaken to establish the role of the different *phh* genes in phenylalanine catabolism. Our results revealed that *phhAB* forms an operon and that its corresponding gene products are needed in the first step of phenylalanine oxidation. The expression of this operon is activated by PhhR in the presence of phenylalanine or tyrosine. The promoter region of *phhA* exhibits two binding sites for PhhR that are both required for activation. The PhhR binding sites exhibit the consensus sequence: 5′-AAAANTNTNNTTNCG-3′. The closest activating motif is 39 bp from the −35 hexamer, whereas the second motif is located at 97 bp from the −35 hexamer. An integration host factor (IHF) binding site was identified overlapping the proximal PhhR binding motif, and we found that IHF binds to it and exerts an inhibitory role.

Results

Role of PhhA, PhhB and PhhR in the utilization of phenylalanine by KT2440

P. putida KT2440 is a prototrophic strain (Table 1) that grows in M9 minimal medium (NH_4^+ as a nitrogen source) with a doubling time of 0.9(±0.05) h (Table 2). When phenylalanine replaced ammonium as a nitrogen source, *P. putida* KT2440 grew with a doubling time of 7.9(±0.4) h. In contrast, growth with phenylalanine as the sole C-source was almost

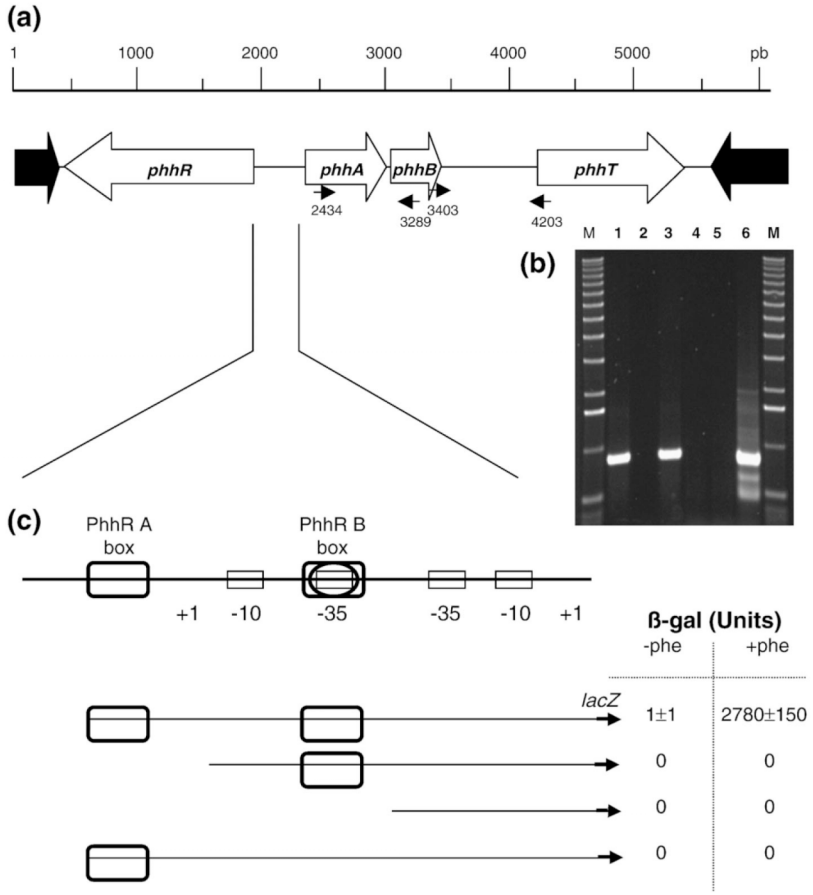

Figure 2. Physical and genetic organization of the *phh* region of KT2440. (a) Physical map of the 5341 bp region of the *P. putida* chromosome bearing the *phh* cluster. (b) RT-PCR showing that *phhA* forms an operon with *phhB*. Lane 1, RT-PCR with primers based on *phhA* and *phhB*; lane 2 is the same as the assay in lane 1, except that reverse transcriptase was not added; lane 3 is a positive control with DNA showing that PCR worked well. Lane 4, RT-PCR with primers based on *phhB* and *phhT*. Lanes 5 and 6 are the negative and positive controls, respectively. M, size markers. (c) Organization of the intergenic region between *phhR* and *phhA*, and analysis of *phhA* gene expression upon fusion of different segments of *phhA* to *'lacZ* (first three fusions) or the fusion of a *phhA* variant in which the proximal PhhR target size was mutated. The −10 and −35 hexamers are indicated by small rectangles; the PhhR binding sites are indicated by large rectangles, and the IHF site is shown by an ellipse.

negligible. Tyrosine is used efficiently by *P. putida* KT2440 as the sole nitrogen source with a doubling time of 1.8(±0.1) h. To determine whether the four ORFs annotated *in silico* as involved specifically in phenylalanine metabolism were indeed necessary for the catabolism of this amino acid, we generated mutants *via* site-directed mutagenesis (see Experimental Procedures) and tested growth of the different mutants on phenylalanine and tyrosine as the sole nitrogen source (see Table 2). We found that mutants with knockouts in *phhA*, *phhB* and in the putative regulatory *phhR* gene grew deficiently with phenylalanine as the sole nitrogen source (Table 2), whereas mutant PP4492 (*phhT*) grew at a rate similar to that of the wild-type (not shown). The wild-type strain and all of the *phh* mutants grew with ammonium or tyrosine as the sole nitrogen source with similar doubling times (Table 2).

To determine whether the three *phh* genes in the same transcriptional orientation formed an operon, we isolated total RNA from *P. putida* KT2440 growing on M9 minimal medium with glucose as a carbon source and with phenylalanine, and carried out reverse transcriptase-polymerase chain reaction (RT-PCR) assays using primers based on the 3′ end of *phhA* and the 5′ end of *phhB* on the one hand; and the 3′ end of *phhB*, and the 5′ end of *phhT* (PP4492) on the other hand (Figure 2), as detailed in Experimental Procedures. We found a band of the expected size (855 bp) with the *phhA* and *phhB* primers (Figure 2(b)), but not when primers based on *phhB* and PP4492 were used (Figure 2(b)). These results suggest that the gene encoding PP4492 is not part of the *phhAB* operon. Whether PhhT is involved in the uptake of phenylalanine and tyrosine is unknown; however, results from our laboratory

Table 1. Strains and plasmids used in this study

	Genotype	Reference
A. *Strain*		
P. putida KT2440	Cm^R	40
MCH1 (*phhA:aphA3*)	Cm^R; Km^R	7
MCH2 (*phhB:aphA3*)	Cm^R; Km^R	This study
MCH4 (*phhR:aphA3*)	Cm^R; Km^R	This study
P. putida KT2440-IHF3	*ihfA*::Km	40
E. coli DH5α	F′/*hsdR*17, *recA*1, *gyrA*	41
E. coli BL21(DE3)	F^-, *ompI*, *hsdS*$_B$ (r^-_B m^-_B) gal, dem, met	Novagen
B. *Plasmids*		
pBSaphA3	Km^R	42
pMRS101	Km^R, suicide vector	42,43
pMP220	*′lacZ*; IncP; Tc^R	45
pMCR1	P_{phhR}::*lacZ*; Tc^R	This study
pMCA1	P_{phhAI}::*lacZ*; Tc^R	This study
pMCA2	P_{phhAII}::*lacZ*; Tc^R	This study
pMCA3	$P_{phhAIII}$::*lacZ*; Tc^R	This study
pET28a (+)	Km^R protein expression vector	Novagen
pCR2.1	Amp^R; Km^R	Invitrogen
pPHHR	Amp^R, *phhR*	This study
pPHHRKm	Ap^R; Km^R	This study
pMRS101-PHHRKm	Km^R; Amp^R; *sacB*	This study
pMRS101-PHHBKm	Km^R, *sacB*	This study

support the involvement of more than one transport system in the entry of aromatic amino acids across the cell membrane of *P. putida* (E. Duque and J. de la Torre, unpublished results).

Identification of the transcription start point of *phhR* gene and the *phhAB* operon

To analyse in detail how the expression of the *phhAB* and *phhR* genes is controlled, we decided to determine their transcription start sites. Primer extension analysis using appropriate primers located the +1 point of *phhR* at a C 87 nucleotides upstream from the A of the first ATG (Figure 3(b)). Upstream from +1, we found a hexamer around −10 (TAAAAG) that resembled the –10 region of the σ^{70} factor-recognized promoters in *Pseudomonas*[10] and 18 nucleotides upstream, we identified a hexamer that exhibited a low level of similarity to the consensus

Table 2. Growth of KT2440 and its isogenic mutants with ammonium, Phe and Tyr as the sole N-source

		Doubling time (h)		
Strain	Genotype	NH_4^+	Phe	Tyr
KT2440	Wild-type	0.9±0.05	7.9±0.4	1.8±0.1
MCH1	*phhA:aph3*	2.1±0.1	21.7±0.7	2.3±0.1
MCH4	*phhR:aph3*	1.1±0.05	28.5±1.0	3.2±0.2

P. putida KT2440 and its isogenic mutants were grown overnight in M9 minimal medium with glucose as the sole carbon source. Bacterial cells were diluted 100-fold in minimal medium with 0.5% (w/v) glucose but without a nitrogen source. Bacterial cultures were split into three and NH_4Cl, phenylalanine or tyrosine was added to reach 10 mM. Cultures were incubated at 30 °C and doubling times were calculated in the exponential phase. Data are the average of three independent assays run in duplicate, with a standard deviation below 10% of the given values.

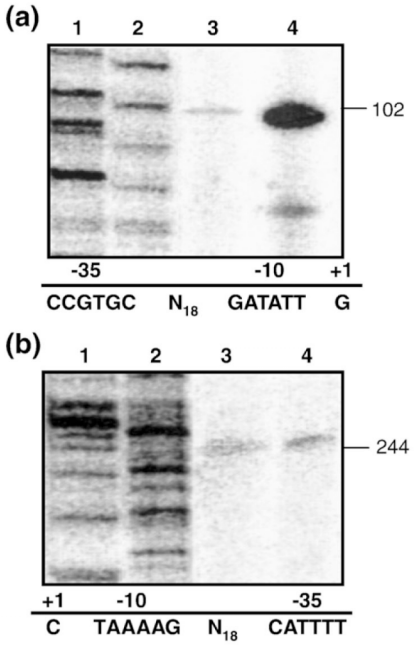

Figure 3. Transcription start point of *phhA* and *phhR*. Cells were grown in M9 minimal medium with glucose as the sole carbon source with and without 15 mM phenylalanine. RNA was prepared from cells in the mid-exponential growth phase as described in Experimental Procedures. (a) Primer extension for the identification of the *phhA* start point. (b) Primer extension for the identification of the *phhR* start point. Lanes 1 and 2 are sequencing ladders used to determine the exact site of the analysed cDNAs. Lanes 3 and 4 correspond to cultures in the absence and in the presence of the amino acid, respectively. On the right, the size in nucleotides of the cDNA is indicated. The proposed promoter sequence for *phhA* and *phhR* are given below their corresponding primer assay.

−35 hexamer. For the *phhA* gene, the +1 corresponded to a G that was located 45 nucleotides upstream from the A of the first ATG (Figure 3(a)). The −10 and −35 hexamers that fit better with *P. putida* promoters were GATATT in the −10 region and CCGTGC at the −35 region.[10] In this regard, it is worth noting that no σ^{54}-binding motifs were found upstream from +1, in spite of the fact that PhhR belongs to the NtrC family of regulators and that these proteins often stimulate transcription with RNA polymerase σ^{54}.[11,12]

PhhR is the positive transcriptional activator of the *phhAB* operon and mediates transcription activation with phenylalanine or tyrosine as the effector

Once the promoters were defined, and to study expression of the *phhR* and *phhAB* transcriptional units, fusions of the corresponding promoter regions to *′lacZ* were constructed (Table 1). We then

determined the β-galactosidase activity in PhhR-proficient and PhhR-deficient backgrounds with and without phenylalanine and tyrosine. Activity was measured in cells in the exponential phase.

Regardless of the growth conditions (absence/presence of phenylalanine or tyrosine) and the genetic background (PhhR-proficient/deficient) activity from the P_{phhR}::*lacZ* fusion was around 60 Miller units (Table 3). This suggested that PhhR does not control its own synthesis and that its expression is constitutive.

In the PhhR-proficient background and in the absence of aromatic amino acids, the level of β-galactosidase from P_{phhA}::*'lacZ* was very low (about 20 Miller units), but in the presence of the aromatic amino acid activity was 2780±150 with phenylalanine and 630±30 with tyrosine (Table 3). In the PhhR-deficient background, no expression from P_{phhA} was found, irrespective of the presence of aromatic amino acids, which indicates that PhhR is the positive transcriptional regulator of P_{phhA}. To further confirm this, the mutant strain MCH4 (PhhR-deficient) bearing P_{phhA}::*lacZ* was transformed with plasmid pMCH4, which carries the *phhR* gene. As expected, in the presence of phenylalanine or tyrosine, β-galactosidase levels were high (700–2000 Miller units), whereas in the absence of the aromatic amino acids, β-galactosidase activity was negligible.

Since tyrosine is the metabolic product of phenylalanine catabolism, the above set of assays did not allow us to distinguish between phenylalanine or tyrosine as the effector of the system. We therefore repeated the assays in the MCH1 background, in which phenylalanine is poorly metabolized. In this series of assays, we included *p*-hydroxyphenylpyruvate as a potential effector, this compound being the product resulting from deamination of tyrosine (Figure 1). The results showed that in the presence of either phenyalanine or tyrosine, *phhA* expression was enhanced so that β-galactosidase levels increased from low levels of activity (20 Miller units) in the absence of the amino acid to almost 2000 Miller units with phenylalanine and around 800 Miller units with tyrosine. *p*-Hydroxyphenyl pyruvate was not an effector.

Table 3. Expression from P_{phhR} and P_{phhA} in *phhR* proficient and deficient backgrounds

Promoter fusion	*phhR*	β-Galactosidase (Miller units)		
		−	+ Phe	+ Tyr
P_{phhR}:*'lacZ*	−	65±20	40±4	50±18
P_{phhR}:*'lacZ*	+	55±15	5±20	60±10
P_{phhA}:*'lacZ*	−	1±1	1±1	3±2
P_{phhA}:*'lacZ*	+	20±4	2780±150	630±30

Pseudomonas putida KT2440 (wild-type) and its isogenic MCH4 strain (PhhR-deficient) were transformed with pMP220 derivatives carrying the indicated promoter fusion. Bacterial cells were grown under the culture conditions described in the legend to Table 2, and β-galactosidase was determined in cells growing exponentially. The data are the average and standard deviation of three to five independent assays, each run in triplicate.

In silico analysis of the *phhA*/*phhR* intergenic region and experimental evidence for identified sites

The *phhR* gene product belongs to the NtrC family of transcriptional regulators. Members of this family usually recognize DNA-binding motifs far upstream from the downstream promoter element, work with the alternative σ^{54} factor, and often require IHF for maximal activity.[11,12] We have analysed the 256 bp sequence between the A residues of the first ATG start codon of *phhA* and *phhR* to look for three types of motifs: −12/−24 $GCN_{10}GG$ recognized by σ^{54}, IHF binding sites and inverted repeats as potential targets for PhhR.

Expression from the phhAB promoter is not σ^{54}-dependent

As mentioned above, the consensus −12/−24 $GCN_{10}GG$ sequence motif was not found upstream from the *phhA* transcription start point; however, we measured β-galactosidase activity using the P_{phhA}::*'lacZ* fusion in a *P. putida* σ^{54} mutant background. We found that in the presence of phenylalanine, expression from the *phhA* promoter was as high in the σ^{54} mutant background as in the wild-type background. These results indicated that expression of the *phhAB* operon is σ^{54}-independent, despite being mediated by PhhR, an NtrC family member. Furthermore, the σ^{54} mutant grew as fast as the wild-type strain with phenylalanine as the sole nitrogen source (not shown). Expression of the *phhR* promoter fused to *'lacZ* was also measured in the σ^{54} mutant, and levels were similar to those described above for the wild-type (not shown).

As mentioned above, PhhR belongs to the NtrC family of regulators although it seems to activate transcription from the P_{phhA} promoter by stimulating transcription with RNA polymerase/σ^{70}. Three other proteins of the NtrC family, namely TyrR in *Escherichia coli*[13,14] and NtrC and HupR in *Rhodobacter capsulatus*[15] also stimulate transcription from their target promoters with RNA polymerase/σ^{70}. The central domain of members of the NtrC family establishes productive contacts with σ^{54} and has ATPase activity that is essential for ATP hydrolysis.[16–18] Two motifs (ESELFGH and GAFTGA), as well as the Walker A and B motifs, are present in the central domain and are characteristic for ATP-binding and hydrolysis. This ATPase domain is essential for transcription activation.[18–20] A conserved GAFTGA motif in the enhancer binding proteins is critical for transcriptional activation, since this region constitutes an interaction surface that binds σ^{54}.[19,20] When we aligned the central domains of *P. putida* PhhR, NtrC of *E. coli*, DctD of *Rhizobium melilotti* and HupR of *R. capsulatus* we found that in *P. putida* PhhR neither the GAFTGA

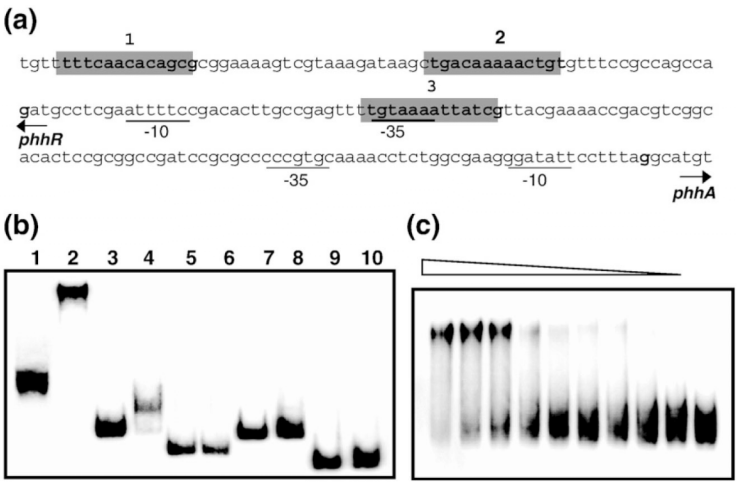

Figure 4. *In silico* analysis of the *phhA/phhR* region and identification of the IHF binding site by EMSA. (a) The nucleotide sequence covering the *phhA/phhR* intergenic region. Shaded in grey are the three potential sites for IHF binding based on similarities to the consensus IHF site. In bold are the +1 points of *phhA* and *phhR* underlined the corresponding −10/−35 hexamers proposed to be recognized by RNA polymerase. (b) Identification of IHF binding sites by EMSA in the *phhA/phhR* intergenic region. Odd-numbered lanes (1, 3, 5, 7 and 9) are controls without IHF, whereas in lanes 2, 4, 6, 8 and 10, 1.8 μM IHF was added. Lanes 1 and 2, DNA containing potential sites 1, 2 and 3. Lanes 3 and 4, DNA containing only site 3. Lanes 5 and 6, DNA containing only site 1. Lanes 7 and 8, DNA containing only site 2. Lanes 9 and 10, DNA with no IHF site. (c) EMSA of IHF with the *phhA/phhR* intergenic region. Binding reactions were carried out as with 1 nM DNA and increasing concentrations of IHF (from right to left, 0, 100, 200, 400, 600, 800, 1000, 1200, 1400, 1600 nM) for 30 min and subjected to electrophoresis as indicated in Experimental Procedures.

σ^{54} binding motif nor the catalytic ATP site is well conserved (not shown).

Identification of binding sites for IHF and the role of the bending protein in transcription from P_{phhA}

Activation of transcription by members of the NtrC family is often modulated by IHF, a protein that assists the transcriptional process by bending DNA and facilitating regulator/RNA polymerase interactions.[11] Inspection of the *phhA/phhR* intergenic region revealed up to three potential sites for IHF 5′-WATCAANNNNTTR-3′ (these sites are dubbed 1, 2 and 3 in Figure 4(a)).

To study whether IHF binds to the *phhA/phhR* region, we carried out electrophoretic gel mobility-shift assays (EMSA) with the whole intergenic region. EMSA revealed that the whole promoter region was retarded by IHF (Figure 4(b), lane 2). With this entire promoter, we carried out EMSA analyses to determine the K_D of IHF for its target sequence using increasing concentrations of protein. IHF recognized this DNA fragment with an apparent affinity of 1.2(±0.2) μM (Figure 4(c)). To establish whether IHF binds one or more of the potential IHF sites, we synthesized a number of oligonucleotides that carried site 1, site 2 or site 3, and repeated the gel mobility-shift assays (Figure 4(b)). We found that DNA was retarded only when site 3 (lane 4) was present. This was further confirmed in DNase I footprint assays with the whole intergenic region and IHF, which showed that IHF binds only at −92/−79 with respect to +1 of *phhA* (not shown). This site overlaps the −35 region of the P_{phhR} promoter (See Figure 4(a), top).

Since the affinity of IHF for its target site was low enough to have an effect *in vivo*, we measured β-galactosidase activity from the P_{phhR}::′*lacZ* and P_{phhA}::′*lacZ* fusions in IHF-proficient and IHF-deficient backgrounds. We found that the expression of P_{phhR}::′*lacZ* was similar irrespective of the IHF background, and β-galactosidase activity was of the order of 50 Miller units (Table 4). Basal levels of expression from P_{phhA} were also low irrespective of the IHF background (Table 4); however, expression levels in cells growing with phenylalanine or tyrosine were higher in the IHF-deficient background than in the IHF-proficient background (Table 4), indicating that IHF interferes with the activation of expression of the *phhA* operon.

Purification of PhhR and identification of its binding site

The *in silico* analysis revealed two poorly conserved inverted repeats (5′-AAAANTNTNNTTNCG-3′). One of these motifs was located 39 nucleotides upstream of the −35 hexamer of *phhA*. The second inverted repeat was located 97 nucleotides upstream from the −35 hexamer. We then designed assays to

Table 4. Expression from P_{phhA} and P_{phhR} in IHF-proficient and deficient backgrounds

		β-Galactosidase activity (Miller units)		
	IHF	−	+ Phe	+ Tyr
P_{phhA}::′*lacZ*	+	30±4	2780±150	630±30
P_{phhA}::′*lacZ*	−	40±10	4250±150	2430±125
P_{phhR}::′*lacZ*	+	50±4	45±5	55±5
P_{phhR}::′*lacZ*	−	50±3	55±3	50±3

Conditions are as described in the legend to Table 3 except that the wild-type or the IHF-deficient *P. putida* KT2440-IHF3 was used. Data are the average of three independent assays, each run in duplicate.

shed light on the potential role of these motifs in the transcriptional control of the expression of *phhA*. We measured β-galactosidase activity *in vivo* using different variants of the *phhA* promoter fused to *'lacZ*, and carried out *in vitro* EMSA DNase I footprint assays of PhhR in the *phhA/phhR* intergenic region, and ITC assays to establish the K_D of PhhR for these potential target sites.

For the *in vivo* assays, we amplified the upstream region of *phhA* with PCR and appropriate primers so that different regions were amplified in such a way that the 5′ start point varied (located at −376 with respect to the +1 of *phhA*; Figure 2(c)) varied, whereas the 3′ end was always at the same position (+84 in *phhA*). The amplified segments were fused to *'lacZ* and β-galactosidase activity was determined in the wild-type background in the presence of phenylalanine in cells in the exponential phase. We found that the only fusion that yielded activity was the one covering the entire intergenic region, and that the loss of the furthest inverted repeat yielded an inactive promoter.

We also generated a *phhA* variant in which the four A residues in the proximal inverted repeat (Figure 2(c)) were changed to C or to the GAGA sequence, and the corresponding DNA fragments from −376 to +84 were amplified and cloned in front of *'lacZ*. These mutant variants of the *phhA* promoter were also transformed in *P. putida* KT2440, and β-galactosidase activity was determined in cells under different growth conditions. We found that these fusions had no activity, which suggested that the proximal inverted repeat is also necessary for the activation of the *phhA* promoter (Figure 2(c)).

We expressed PhhR with a His_6 tag and purified it to homogeneity. In gel-filtration through a Sephadex column without amino acids or with phenylalanine or tyrosine, the protein behaved predominantly as a dimer (not shown). In EMSA we showed that the recombinant protein bound to and retarded the *phhR/phhA* intergenic region specifically (Figure 5). We then performed DNAse I footprint assays and found that irrespective of the protein concentration, PhhR yielded two clear protection segments that covered the proposed inverted repeats (Figure 6), which confirmed that both distal and proximal inverted repeats are necessary for the activation of *phhA* expression. We then determined the affinity of PhhR for each of the binding sites with duplex 30-mer oligonucleotides (Figure 7(a) and 7(b)). The data revealed that PhhR recognized both motifs with different affinities: whereas the proximal site was recognized with a K_D value of 30(±2) nM, the distal site was recognized with a K_D value of about 2(±0.3) μM. We also inferred that the stoichiometry of binding was one dimer of PhhR bound per target site, with each of the monomers probably recognizing half a site. EMSA and isothermal titration calorimetry (ITC) assays with an 85-mer DNA fragment containing the two PhhR binding motifs were carried out to determine whether the binding of dimers was cooperative. The results reflected that dimer binding to each site were independent events (not shown).

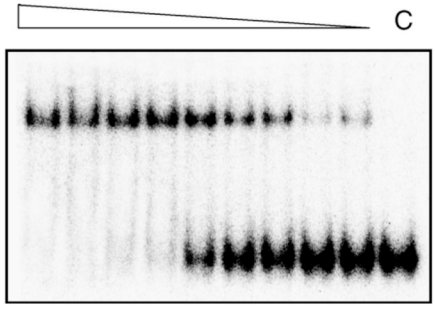

Figure 5. EMSA of PhhR with the *phhA/phhR* intergenic region. The whole intergenic region was amplified by PCR and incubated in the absence or in the presence of increasing concentrations of PhhR (from right to left 0, 100, 200, 400, 600, 800, 1000, 1200, 1400 and 1600 nM) for 30 min and subjected to electrophoresis as indicated in Experimental Procedures. Lane C shows the migration of the target DNA fragment.

We also assayed the binding of RNA polymerase to the intergenic *phhA/phhR* region. We found that in the absence of PhhR, RNA-polymerase bound preferentially to a single site that covered the region from +1 to −40 in the *phhR* promoter (Figure 6(c), lane 10), in agreement with the constitutive expression from this promoter, although no protection in the *phhA* region was found. However, when PhhR was present, protection extended to the *phhA* −10 to −35 region (Figure 6(c), lane 11).

Discussion

The established function of phenylalanine hydroxylase in eukaryotic and prokaryotic organisms is to catabolize L-phenylalanine as a carbon source; however, *P. putida* uses it only as the sole nitrogen source. This is surprising, because the first reaction in phenylalanine catabolism yields tyrosine, which can be used as the sole carbon and nitrogen source by this microorganism. In *P. putida*, phenylalanine catabolism is initiated by PhhA, a pterin-dependent hydroxylase that requires PhhB for pterin recycling. Inactivation of the *phhA* gene, for instance, in *P. putida* resulted in a marked inability to use L-phenylalanine as the sole source of nitrogen. However, the mutant strain still grew, albeit at a very low rate. It therefore seems possible that phenylalanine can also provide nitrogen for growth *via* phenylalanine aminotransferase, which may yield a metabolic intermediate that might interfere with the catabolism of phenylalanine as a carbon source. The deficient use of L-phenylalanine as a carbon source has been observed in *P. aeruginosa*, and phenylacetate was hypothesized to interfere with 4-hydroxyphenylacetate as a substrate for the monooxygenase step of L-tyrosine catabolism (Figure 1).[21] In our laboratory, phenylacetate supplied

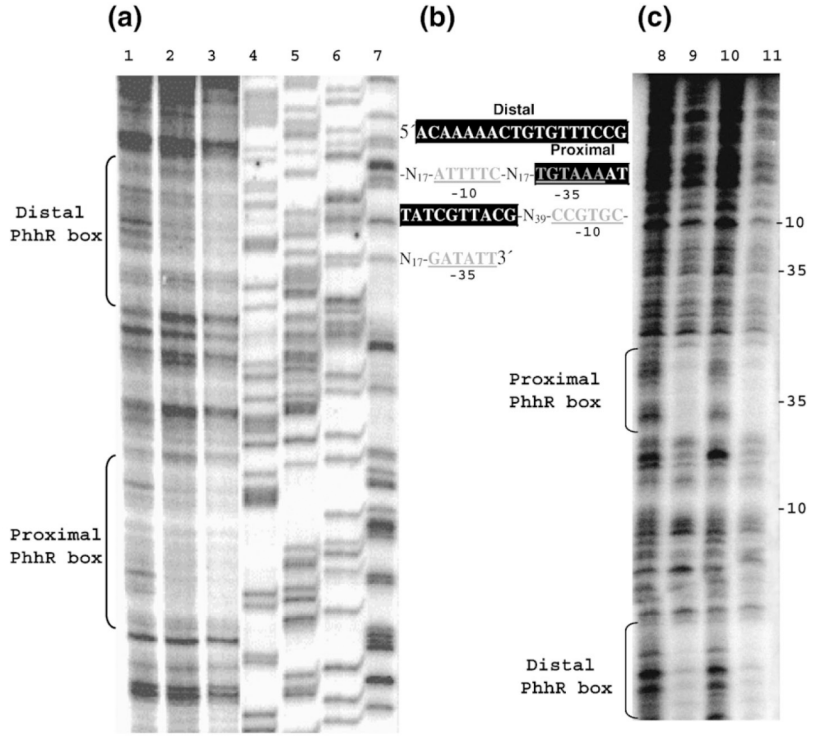

Figure 6. DNase I footprint of PhhR and RNA-polymerase/σ^{70} in the *phhA/phhR* intergenic region. (a) DNase I footprint in the lower strand with PhhR in the *phhA/pphR* intergenic region. Assays were carried out as described in Experimental Procedures in the absence (lane 1) or in the presence of 5 μM (lane 2) or 10 μM PhhR (lane 3). Lanes 4 through to 7 is a sequencing ladder. The region protected by PhhR is indicated by vertical lines. (b) Relevant motifs in the intergenic *phhA/phhR* region highlighting the distal and proximal PhhR binding motif and the −10/−35 hexamers of *phhR* (grey letters) and *phhA* (black letters). (c) DNase I footprint in the upper strand with RNA-polymerase/σ^{70} in the absence and in the presence of PhhR. Lane 8, control without protein addition; lane 9, assay in the presence of 8 μM PhhR; lane 10, assay in the presence of 200 nM RNA polymerase, and lane 11, assay with 200 nM RNA polymerase and 8 μM PhhR. The −10/−35 promoter region of *phhR* is marked in grey, whereas the −10/−35 promoter region of *phhA* is in black.

exogenously did not inhibit the use of tyrosine as a carbon source (M.C.H., unpublished results).

Downstream from the *phhAB* gene in *P. aeruginosa* is a third gene, *phhC* (also called *tyrB*), and the stop and start codons of *phhB* and *phhC* overlap. Transcription of the *phhAB* operon is mediated by PhhR.[21] In *P. putida,* two potential genes with aminotransferase motifs homologous to the *phhC* gene, and annotated as *tyrB1* (PP1972) and *tyrB2* (PP3590), were found. The latter seems to have been assigned erroneously to the metabolism of L-tyrosine, since it is in a cluster of genes involved in D-lysine catabolism, and a mutant deficient in PP3590 grew on L-tyrosine as fast as the wild-type strain (O. Revelles *et al.*, unpublished results). In other pseudomonads, such as *Pseudomonas fluorescens* PfO and *Pseudomonas syringae*, the organization of the *phhAB phhR* gene cluster is identical with that in *P. putida*, and no *phhC* is found nearby. Indeed, in *P. putida* the gene immediately downstream from *phhB* was called *phhT* because of its similarity to amino acid permeases; however, its role in L-phenylalanine or tyrosine uptake may not be relevant, since E. Duque and J. de la Torre of our laboratory recently isolated a KT2440 mutant with a knockout in PP4653, which transported phenylalanine at significantly reduced rates (personal communication).

Located divergently with respect to *phhAB* in *P. putida* is *phhR*, which encoded a member of the NtrC family of regulators. Here, we present several lines of evidence that transcription from the *phhA* promoter is subject to positive control by the PhhR protein. Inactivation of the *phhR* gene led to a negligible production of β-galactosidase from the *phhA::'lacZ* reporter system. Promoter function was restored almost completely when a plasmid able to express the *phhR* gene was introduced into this *P. putida* mutant strain.

Members of the NtrC family of regulators exhibit a modular structure, and they usually have three functional domains.[11,12] (1) One is an N-terminal domain that in some members of the family is involved in interactions with effectors, as in XylR and DmpR.[22–24] In other members of the family, this N-terminal domain is phosphorylated *via* a sensor kinase, as in the Enterobacteriaceae NtrC

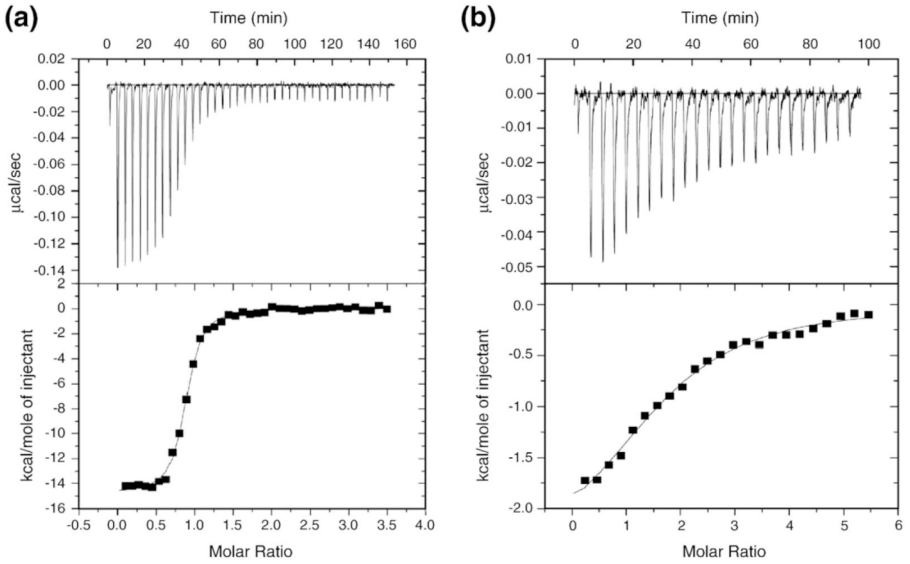

Figure 7. Isothermal titration calorimetry for the binding of PhhR to its target sites in DNA. Heat changes (upper panel) and integrated peak areas (lower panel) for the injection of a series of 1.6 µl aliquots of 6 µl PhhR in a solution of (a) 3 µM proximal and (b) distal PhhR binding sites made of 30-mer duplex DNA.

protein.[25–27] (2) Another is a central domain that is highly conserved throughout the entire family of σ^{54} enhancer-binding proteins and exhibits two established motifs for interactions with σ^{54} and a Walker motif, which reflects the binding and hydrolysis of ATP.[18,19,28] (3) The third is a C-terminal domain that contains a helix-turn-helix DNA-binding motif.[11,12] In contrast with most members of the NtrC family, but in consonance with HupR and NtrC of *Rhodobacter captulatus*[15] and TyrR of *E. coli*,[14] which are also NtrC family members, the *P. putida* PhhR protein stimulates transcription from its cognate promoter with RNA polymerase/σ^{70}. The following experimental findings support the above statement. First, when we assayed the activation of the *phhA* promoter in a σ^{54} mutant of *P. putida*, the levels of β-galactosidase were similar to those determined in the wild-type strain, which indicates that expression of the *phhAB* operon in *P. putida* is σ^{54}-independent. Second, the consensus −24/−12 $GGN_{10}GC$ target motif of RNA polymerase/σ^{54} was not found upstream from the +1 of *phhA* in *P. putida*; and thirdly, *P. putida* PhhR exhibits Gly instead of a Glu in the Walker domain involved in hydrolysis of ATP, which is needed for oligomerization of members of this family and functional interactions with σ^{54}. We noticed that the presence of a variant of GAFTGA could prevent the interaction with σ^{54}, as reported for PspF.[19]

Our footprint assays revealed that *P. putida* PhhR binds to two distinct regions in the *phhA/phhR* intergenic space that comprise nucleotides −150 to −132 and −92 to −74 with respect to the +1 in *phhA*. Footprint analysis revealed that the proximal site is protected at lower concentrations of PhhR than the distal one. This was confirmed when the K_D of PhhR for these sites was determined using ITC techniques and 30-mer oligonucleotides containing the sequence protected in the footprint. The K_D for the proximal site was around 30 nM, whereas the distal site was recognized with a K_D of 2 µM. The *phhA* promoter is not functional when one or two of the identified binding sites are deleted or altered by site-directed mutagenesis (Figure 2), which indicates that binding to both sites is *sine qua non* for transcription activation. The alignment of the two binding motifs showed a set of well-conserved A residues on the 5′ end of the sequence (Figure 8). In the case of the proximal motif, a palindromic $GTAAN_8TTAC$ sequence was found. This palindrome was less conserved in the distal site, and if these sequences turn out to be important in PhhR/DNA interactions this would account for the much higher affinity of PhhR for the proximal binding site (Figure 8).

Outside the Pseudomonadaceae, the closest homologue of the different PhhR of *Pseudomonas* is the TyrR protein from *E. coli* and *Haemophilus influenzae* (around 45% similarity).[14,29,30] TyrR is a homodimer in solution and can function as an activator or as a repressor.[14,30] The consensus sequence recognized by PhhR in the *phhA* promoter is similar to the so-called strong TyrR box ($TGTAAAN_6TTTACA$)

Proximal	5′ TGT**AAAA**T**TA**T**C**G**TT**A**CG** 3′
Distal	5′ ACA**AAAA**C**TG**T**GTT**C**CG** 3′
Consensus	5′ NNN**AAAA**N**TN**T**NN**TT**N**CG** 3′

Figure 8. Alignment of PhhR proximal and distal binding sites in *P. putida*.

identified in *E. coli* for the TyrR protein, where it functions as transcriptional activator.[14,31] However, in *P. putida* PhhR requires two target sequences upstream of the RNA polymerase binding site, whereas in the case of the *E. coli mtr* promoter, activation by TyrR requires only the binding site located 42 nucleotides upstream from −35. However, in the case of the *tyrP* promoter two TyrR binding motifs are necessary. TyrR binds as a dimer to these strong TyrR boxes in the absence of aromatic amino acids, and only mediates transcription activation in response to phenylalanine, tyrosine or tryptophan.[31–33]

In *E. coli,* TyrR can also function as a repressor. In this case, it forms hexamers in the presence of tyrosine and ATP, and it binds either to strong or to weak Tyr boxes, the latter of which exhibit less identity with the consensus sequence.[34–37] In the repression mechanism, the TyrR hexamer is proposed to bind both to a strong and a weak box with DNA looping in between,[14,38] which prevents RNA polymerase from contacting the cognate promoter. Gel-filtration studies of PhhR in the presence of Phe and Tyr with and without ATP revealed that the predominant quaternary form is a dimer, regardless of the presence of amino acids or ATP. Whether or not PhhR functions as a repressor in *P. putida* remains unknown, but if PhhR were indeed a repressor its mechanism of action would be different from that of *E. coli* TyrR since *in vitro* PhhR does not bind ATP (M.C.H., unpublished results).

In *E. coli* it has been shown that IHF inhibits transcription from the *mtr* and *tyrP+3* promoters, although the IHF site was not mapped.[39] Our results in *P. putida* suggest that IHF inhibits the expression of *phhA*: the IHF binding site was shown to overlap the PhhR site proximal to the RNA polymerase binding motif. In agreement with this observation is the increased level of β-galactosidase activity from the *phhA* promoter in an IHF-deficient *P. putida* background.

We can say therefore that in *P. putida* there are at least three critical requirements for PhhR-mediated activation of gene expression: two Phh target sequences located appropriately upstream of the RNA polymerase binding site, a functional PhhR protein, and either Phe or Tyr. Our footprint assays with RNA polymerase revealed that it protects only the *phhA* promoter in the presence of PhhR. Therefore, one of the PhhR dimers probably interacts with the α-subunit of the RNA polymerase/σ^{70} complex to allow the recognition of the *phhA* promoter; however, PhhR-dependent transcription from the *phhA* promoter occurred only if phenylalanine or tyrosine were present.

Experimental Procedures

Bacterial strains, plasmids and culture media

The bacterial strains, cloning vectors and plasmids used in this study are given in Table 1. *P. putida* KT2440[40] and its mutant derivatives were grown in M9 minimal medium with 0.55 (w/v) glucose or 10 mM benzoate as the carbon source. Cultures were incubated at 30 °C and shaken on an orbital platform operating at 200 strokes/min. When required, antibiotics were used at the following final concentrations (in micrograms per milliliter): ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 20 μg/ml. *E. coli* strains were grown at 37 °C in Luria Bertani (LB) culture medium with shaking. *E. coli* DH5α[41] was used for gene cloning, and *E. coli* BL21 (DE3) was used for protein production.

Construction of knockout mutant strains

To construct the *phhR* regulator knock-out mutant, the *P. putida phhR* gene was amplified from chromosomal DNA with primers PHHR5: 5′- TACTTGGCAGTCTTTGCGGCC-3′ (located 385 bp upstream from the *phhR* gene in the genome sequence of *P. putida*) and PHHR3: 5′-GCACCTTGTTGATCTCGCCC-3′ (located 464 bp downstream from the *phhR* gene of *P. putida*), and the amplification product was cloned into pUC18Not to obtain plasmid pPHHR. This plasmid was cut with EcoRV and the 0.8 kb SmaI fragment from pBSaphA3 bearing the *aphA3* gene,[42] which encodes for kanamycin resistance, was inserted into pPHHR to yield plasmid pPHHRKm. A 3.2 kb EcoRI fragment containing the *phhR*::*aphA3* mutant allele was blunted with Klenow and subcloned into pMRS101[42,43] previously cut with SmaI to obtain pMRS101-pHHRKm. 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 kanamycin and streptomycin, and the double recombination event was selected based on sucrose toxicity as described.[44] The correct insertion of the mutant allele of *phhR* into the chromosome was checked by PCR analysis and Southern blot hybridization (not shown).

Preparation of RNA, primer extension analysis and RT-PCR assays

P. putida KT2440 was grown overnight in M9 minimal medium with 25 mM glucose as the sole carbon source. Cells were then diluted 100-fold into fresh medium, and aliquots were incubated in the absence or in the presence of 5 mM phenylalanine until the culture reached a turbidity of about 1.0 at 660 nm. Cells (10 ml) were harvested by centrifugation (9000*g* for 15 min) in disposable plastic tubes pre-cooled in liquid N_2, and were stored at −80 °C. RNA was extracted using the TRI Reagent® from Molecular Research Center (Madrid, Spain) and modified as follows. The lysis step was carried out at 60 °C, and a final digestion step with RNase-free DNase was added at the end of the process. The concentration of RNA was determined spectrophotometrically at 260 nm. For primer extension analysis, we used as primers specific oligonucleotides complementary to either the *phhR* mRNA or the *phhA* mRNA. Primers were labelled at their 5′ ends with [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase as described.[40] About 10^5 cpm of the labelled primers was hybridized to 40 μg of total RNA, and extension was carried out using avian myeloblastosis virus reverse transcriptase as described. Electrophoresis of cDNA products was done in a urea/polyacrylamide sequencing gel to separate the reaction products and gels were exposed to a phosphor screen (Fuji

Photo Film Co, Ltd.) for 24 48 h. Phosphor screens were scanned using a phosphorimaging instrument (Molecular Imager FX, Bio-Rad).

Reverse transcriptase-PCR was done with 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 RT-PCR was 62 °C and 57 °C for the *phhA/phhB* and *phhB/phhT* genes, respectively. The cycling conditions were as follows: 94 °C for 30 s, 30 cycles at 62 °C or 57 °C for 1 min and 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 *phhA, phhB* and *phhT* genes are available from the authors upon request.

β-Galactosidase assays

We constructed fusions of the promoters of the *phhR* and *phhAB* operon to a promoterless *'lacZ* gene in the low-copy-number pMP220 vector.[45] The *phhR-phhA* intergenic region was amplified by PCR with primers incorporating restriction sites (an EcoRI site in the primer designed to meet the 5′ end and a PstI site in the primer designed to meet the 3′ end) to create a fusion of the promoters of the *phhA* operon to *'lacZ*. The same oligonucleotides, except with a PstI site in the one meeting the 5′ end and an EcoRI site in the one meeting the 3′ end, were used to create a fusion of the *phhR* operon promoter to *'lacZ*. Upon amplification, DNA was digested with EcoRI and PstI and ligated to EcoRI-PstI-digested pMP220 to produce pMCA1 (P_{phhA}::*'lacZ*) and pMCR (P_{phhR}::*lacZ*). Other transcriptional fusions were constructed by cloning different DNA segments from the *P. putida phh* operon, amplified by PCR as EcoRI-PstI fragments with appropriate primers, into the EcoRI-PstI sites of pMP220 (See Figure 2). All fusion constructs were confirmed by DNA sequencing. These plasmids were electroporated into the wild-type *P. putida* KT2440 strain and different mutant strains. The corresponding transformants were grown overnight on M9 medium with glucose plus tetracycline, and then the cultures were diluted 100-fold in the same medium. After 1 h of incubation at 30 °C with shaking, the cultures were supplemented or not with 5 mM phenylalanine or 5 mM tyrosine, and 6 h later β-galactosidase activity was assayed in permeabilized whole cells according to Miller's method. Assays were run in triplicate and were repeated for at least three independent experimental rounds.

Over-expression and purification of His-tagged PhhR and IHF

The *phhR* gene was amplified by PCR from the *P. putida* KT2440 chromosome with primers incorporating NdeI and BamHI restriction sites at their 5′ and 3′ ends, respectively, and cloned in pCR2.1 to yield pPhhR1. The 1.58 kb NdeI-BamHI fragment was subsequently subcloned in pET28a(+) to produce pPhhR2 so that the gene product, when expressed, would carry an N-terminal histidine tag. For His_6-tagged N-PhhR purification, plasmid pPhhR2 was transformed in *E. coli* BL21 (DE3). Cells were grown in 1 l batches at 30 °C in LB medium with 50 μg/ml of kanamycin to a turbidity at 660 nm of about 0.7, and induced with 0.5 mM isopropyl-β,D-thiogalactopyranoside. Cells were harvested after of induction at 18 °C overnight; resuspended in 50 mM Tris–HCl (pH 8), 300 mM NaCl, 0.1 mM DTT, 10 mM imidazole and Protease Inhibitor Cocktail (Complete; Roche); and broken in a French press after treatment with 20 μg/ml of lysozyme/ml. Following centrifugation at 18000*g* for 1 h, the protein was found predominantly (more than 80%) in the soluble fraction. His_6-tagged PhhR was purified by nickel-affinity chromatography using a Ni^+-Sepharose matrix (Amersham-Biosciences) as described by the supplier, and the bound protein was eluted with an imidazole gradient in the above-mentioned buffer. Homogenous peak fractions were pooled and dialysed against 100 mM sodium phosphate buffer (pH 7.2) supplemented with 1 mM EDTA, 10 mM β-mercaptoethanol, 100 mM NaCl to which 10% (v/v) glycerol was added, and stored at −80 °C (long-term storage) or −20 °C (short-term storage). Concentrations of protein were determined using a Bio-Rad protein assay kit. Purified IHF was a gift from Jesús Lacal.

Electrophoresis mobility-shift assay

A 374 bp DNA fragment containing the divergent *phhR* and *phhA* promoters was amplified by PCR from the *P. putida* chromosome, separated in agarose gels and subsequently extracted from this matrix. This fragment was end-labelled with ^{32}P as described above. Labelled DNA (about 1 nM; 10^5 cpm) was incubated with increasing amounts of PhhR-His_6 or IHF for 30 min at 30 °C in 10 μl of binding buffer containing 20 μg of poly (dI-dC)/ml, 200 μg/ml 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. For EMSA with IHF, the binding buffer was: 20 μg of poly(dI-dC)/ml, 50 μg/ml of bovine serum albumin, 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM DDT and 10% (v/v) glycerol. 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).

DNase I footprinting

For these assays, a 374 bp PCR fragment generated with the primers indicated below was used. For the footprint on the top strand of the *phh* operon, we amplified DNA using primers 5′-TGAATTCACCAGCAGGTTGA-3′ (end-labelled with [α-P^{32}]ATP as described above) and 5′-ATCTGCAGATAAAACCATGC-3′. For the footprint on the bottom strand, the same primers were used but the latter primer rather than the former primer was end-labelled. Each labelled probe at a concentration of about 5 nM (10^4 cpm) was incubated in 10 μl reaction mixtures with or without His_6-tagged PhhR (5 μM and 10 μM) and when indicated 200 nM RNA polymerase/σ^{70} was added to the binding buffer. Reaction mixtures were incubated for 30 min at 30 °C before being treated with 40 μl of DNase I (final concentration 1.4×10^{-4} U/μl) diluted in 10 mM Tris–HCl (pH 7) supplemented with 2.55 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA and 50 mM KCl. After 3 min at 30 °C, reactions were stopped with 2 μl of 0.5 mM EDTA, the sample was extracted with phenol, and then DNA was precipitated with two volumes of ethanol and resuspended in 5 μl of TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) and 2.5 μl of loading dye. Equal amounts of DNA (5000–6000 cpm) were heated at 90 °C for 3 min and electrophoresed through a 6.5% (w/v) denaturing polyacrylamide gel. Sequencing ladders were generated in each case with

the corresponding labelled primer, using a bacteriophage T7 DNA polymerase sequencing kit (USB-Amersham) and the pMC plasmid.

Isothermal titration calorimetry

Measurements were done on a VP-Microcalorimeter at 25 °C. The protein was dialysed thoroughly against 100 mM phosphate (pH 7.2), 100 mM NaCl, 1 mM magnesium acetate, 10 mM β-mercaptoethanol, 1 mM EDTA, 300 mM imidazole. For these DNA-binding studies, oligonucleotides corresponding to both strands of the PhhR proximal and distal boxes were synthesized. Annealing was carried out by mixing equimolar amounts (at a concentration of 60 μM) of each complementary oligonucleotide in 50 mM phosphate buffer (pH 7.0), 5 mM EDTA, 750 mM NaCl. The mixture was incubated at 95 °C for 25 min and then chilled on ice and dialysed in the buffer used for ITC studies. The titrations involved 1.6 μl injections of 6 μM PhhR into a solution of 3 μM 30 mer duplex DNA. Titration curves were fit by a non-linear least-squares method (ORIGIN software) to a function for the binding of a ligand to a macromolecule.[46]

Acknowledgements

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Capítulo 2: Dos rutas catabólicas de la fenilalanina junto con el sistema MexEF/OprN forman un regulón controlado por PhhR en *Pseudomonas putida*

PhhR controls a regulon including two phenylalanine degradation pathways and the MexEF/OprN system in *Pseudomonas putida*

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Pseudomonas putida es un microrganismo del suelo que utiliza los aminoácidos aromaticos presentes en exudados de raíz como fuente de nitrógeno. En este estudio hemos demostrado que el metabolismo de la fenilalanina en *P. putida* ocurre mediante dos rutas: una en la que los intermediarios son la tirosina, el *p*-hidroxifenilpiruvato y el homogentisato, y otra en la que la fenilalanina es deaminada para producir fenilpiruvato y fenilacetil-CoA. Basándonos en ensayos de microarrays y utilizando una amplia serie de fusiones al promotor, hemos mostrado que PhhR es un regulador global que activa genes necesarios para la degradación de fenilalanina a través de ambas rutas. Entre los genes activados aparece una acil-CoA que puede estar involucrada en la producción de acetoacetil-CoA, y el regulador transcripcional *paaY* que controla algunos genes *paa* que codifican enzimas del metabolismo del fenilacetil-CoA. El regulón PhhR incluye genes involucrados en la biosíntesis de aminoácidos aromáticos y cuya expresión está reprimida en la presencia de fenilalanina, sugiriendo la posibilidad de un efecto *feedback* a nivel transcripcional. PhhR también modula el nivel de la expresión de la bomba de eflujo MexEF/OprN y otras proteínas que pueden estar involucradas en la homeostasis de la fenilalanina.

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PhhR controls a regulon including two phenylalanine degradation pathways and the MexEF/OprN system in *Pseudomonas putida*

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Pseudomonas putida **is a soil microorganism that utilizes aromatic amino acids present in root exudates as a nitrogen source. In this study we show that the metabolism of phenylalanine by *P. putida* is likely to occur via two pathways: one in which tyrosine, *p*-hydroxyphenylpyruvate and homogentisate are intermediates, and another in which phenylalanine is deaminated to produce phenylpyruvate and phenylacetyl-CoA. Based on microarray assays and using a wide series of promoter fusions we show that PhhR is a global regulator that activates genes needed for phenylalanine degradation through both pathways. Among the genes activated is an acyl-CoA that may be involved in the production of acetoacetyl-CoA, and the *paaY* transcriptional regulator that controls a number of *paa* genes that encode enzymes for phenylacetyl-CoA metabolism. The PhhR regulon includes genes involved in the biosynthesis of aromatic amino acids and whose expression is repressed in the presence of phenylalanine suggesting the possibility of feedback at the transcriptional level. PhhR also modulates the level of expression of the MexEF/OprN efflux pump and other proteins that may be involved in phenylalanine homeostasis.**

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 (7,25,33).

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 (18,19). This microorganism can use a number of amino acids, such as proline, glutamate, and lysine, as both C- and N-sources (16,28,29,42).

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 (39,43,47; Figure 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* strains, L-tyrosine is metabolized following its deamination to produce *p*-hydroxyphenylpyruvate (5). In fact, five enzymes with tyrosine aminotransferase activity have been found in *P. aeruginosa* (44), whereas in *P. putida* KT2440 two ORFs, *tyrB1* and *tyrB2*, encoding PP1972 and PP3590 respectively, were annotated as tyrosine aminotransferases (20). *p*-Hydroxyphenylpyruvate has been shown to be subsequently converted into Krebs cycle intermediates through a series of steps in which homogentisate (2,5-dihydroxyphenylacetate), maleylacetoacetate, and fumarylacetoacetate, which is hydrolyzed to produce fumarate and acetoacetate, are produced (5, Figure 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 (12). 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 -negative bacteria the production of phenylpyruvate during degradation of phenylalanine has been reported (15,36).This alternative pathway may involve transamination of the amino group of phenylalanine to a 2-keto acid leading to the production of phenylpyruvate and the corresponding amino acid. Another alternative pathway has been described in *Rhodobacter sphaeroides* and certain actinomycetes, which metabolize L-tyrosine from L-phenylalanine

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through 2,4-dihydroxyphenylalanine (DOPA) (26,41).

To more fully explore the potential existence of multiple phenylalanine degradation pathways in *P. putida* KT2440, we used DNA microarrays to study the response of the parental *P. putida* KT2240 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 two potential phenylalanine degradation pathways, as well as an efflux pump that may be important for phenylalanine homeostasis.

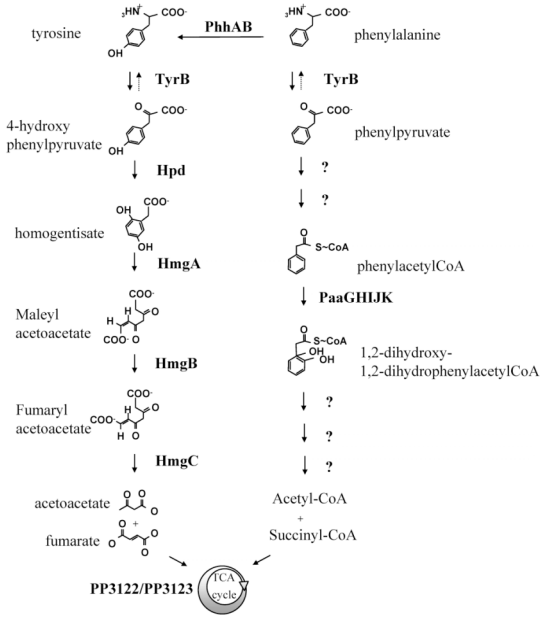

FIG. 1. Metabolic circuits for the degradation of phenylalanine. Degradation of phenylalanine via tyrosine and homogentisate was documented in previous studies in the genus Pseudomonas (34, 42). The phenylpyruvate/phenylacetyl-CoA branch is proposed based on the results presented in this sudy.

MATERIALS AND METHODS

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 (1) with glucose (0.5 [wt/vol]), 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 (1). Cultures were incubated at 30 ºC and shaken on an orbital platform operating at 200 strokes per minute. *Escherichia coli* strains were grown at 37 °C in LB medium with shaking. *Escherichia coli* DH5α was used for gene cloning. When required, antibiotics were used at the following final concentrations (in micrograms per milliliter): ampicillin, 100; chloramphenicol, 30; kanamycin, 50; and tetracycline, 20.

RNA preparation and microarrays assays. *Pseudomonas putida* KT2440 and the *phhR*-deficient regulator knock-out mutant were grown overnight in M9 minimal medium with 25 mM glucose as the sole carbon source (8). 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 hydrolyzed with the addition of 10 µl of 1 N NaOH followed by an incubation period of 15 minutes at 65°C. Samples were then neutralized by adding 25 µl of 1 M HEPES (pH 7.5) and the hydrolyzed 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-labeled 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 minutes. Finally, labelled cDNA probes were again purified with QIAquick PCR purification columns. Labelling efficiency was assessed using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Prehybridization and hybridization were carried out as previously reported (10, 46).

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, Inc.). 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 open-reading-frame 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 (RT-PCR) assays. RNA for this assay was subsequently used for microarray analysis. Reverse transcriptase-PCR 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 ºC. The cycling conditions were as follows: 30 cycles at 94 °C for 30 s, at 56 ºC for 1 minute and at 72 °C for 1 minute. 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 (40). The corresponding promoter regions were amplified by PCR with primers incorporating restriction sites (EcoRI at the 3' end and PstI at the 5' end).

Strain	Relevant characteristics	Ref
P. putida KT2440	Cm^R; Ap^R	1
KT2440-MCH4 (*phhR:aphA3*)	Cm^R; Km^R	12
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	28
P. putida KT2440 *hpd*	Cm^R; Km^R	9
P. putida KT2440 *hmg*	Cm^R; Km^R	9
P. putida KT2440 *mexE*	Cm^R; Km^R	9
P. putida KT2440 PP3458	Cm^R; Km^R	9
Plasmids		
pMRS101	suicide vector, Sm^R	35
pMP220	'*lacZ*; IncP; Tc^R	40
pMCR1	P_{phhR}::*lacZ*; Tc^R	12
pMCA1	P_{phhA}::*lacZ*; Tc^R	12
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*; Am^R	This study
pTYRB-1Tc	pUC18NotI; *tyrB-1::tet*; Am^R; Tc^R	This study
pMRS101-TYRB-1Tc	pMRS101; *tyrB-1::tet*; Sm^R; Tc^R; *sacB*	This study
pCHESI	suicide vector, Km^R	17
pCHESI::PP3122	Km^R	This study

TABLE 1. Strains and plasmids used in this study. Mutants with insertions in the *hpd, hmg*, *mexE and PP3458* genes were obtained from the *Pseudomonas* Reference Culture Collection (9). The transposon insertion site in each of the mutants was confirmed by DNA sequencing.

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 ºC for 2 h with shaking, and the ß-galactosidase (ß-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* knock-out mutant, the *Pseudomonas 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 *et al*. (31). 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 (17). 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 cointegrate 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).

RESULTS

Identification of genes induced/repressed by PhhR using microarray analysis. To study the response of *Pseudomonas putida* KT2440 to phenylalanine we carried out the following microarray experiment. *Pseudomonas 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 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 minute 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 (10), as described in Materials and Methods. In *P. putida* KT2440, in response to phenylalanine, we found that 20 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 analyzed in detail we found that some of these genes form operons due to the presence of overlapping open reading frames (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 or PP3433/PP3434) using 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

	A	B	C	PhhR box
Group 1				
PP2595 ABC transporter permease/ATP-binding protein	**2.3**		**-2**	-
PP2596 ABC transporter permease/ATP-binding protein	**1.8**		**-1.8**	-
PP3122 CoA-transferase subunit A	**4.2**		**-4.3**	+
PP3123 CoA-transferase subunit B	**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		**-2**	**-3.7**	-
PP3427 oprN-multidrug efflux RND outer membrane		**-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	1.4	-
PP2608 shikimate dehydrogenase family protein	1.8	2.3	1.4	-
PP3067 hypothetical protein		2.1	2.1	-
PP4489 phhR transcriptional regulator PhhR	2	4.1	4.4	+
PP3080 aroF-2-phospho-2-dehydro-3-deoxyheptonate aldolase			2	-
PP3285 paaY-PaaY protein	3.9	1.7	1.7	+
Group 4				
PP3275 *paaH*-ring-hydroxylation complex protein 3	**16.2**			-
PP3276 ring-hydroxylation complex protein 2	**2.4**			-
PP3277 phenylacetic acid degradation protein PaaB putative	**1.9**			-
PP3278 *paaF*-ring-oxidation complex protein 1	**3.2**			-

TABLE 2. Global transcriptional analysis of genes under PhhR control. Values shown are the average of 3 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.

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 11 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 cotranscribed). 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 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*-hydroxyphenyl-pyruvate (*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 (5,12). Also within group 1 is a putative two-subunit CoA-transferase (PP3122/PP3123), which exhibits high identity to acetoacetatyl-CoA-transferase in *E. coli* (27) suggesting that it may be involved in the last step of phenylalanine catabolism in *P. putida* KT2440 (See also below). Other genes that were induced in response to phenylalanine include an ABC transport system consisting of the cotranscribed 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 *Pseudomonas putida* mutant collection. This mutant was able to grow on minimal medium with phenlyalanine as N-source and no insights on its potential contribution to phenylalanine metabolism are currently available. Group 2 includes genes induced by PhhR regardless of the presence of phenylalanine. Within this set of 7 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. It is worth noting that a mutant in PP3425, deficient in the MexEF/OprN efflux pump, was more sensitive to phenylalanine than the parental strain. Thus KT2440 was able to grow on M9 minimal medium plates with up to 25 mM phenylalanine, while the MexEF mutant only tolerated up to 16 mM phenylalanine, suggesting a potential role of this efflux pump in tolerance to this aromatic amino acid. (3) The third group consists of 7 genes that show increased expression in the mutant strain versus the parental strain in either a phenylalanine-dependent or -independent manner (Table 2), suggesting that they may be repressed 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 (12). 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 and PP3067, which have unknown functions. The transcriptional regulator, *paaY*, which may be involved in the control of the phenylacetyl-CoA catabolism (21), also showed increased expression in a phenylalanine-dependent manner, and may therefore be repressed by PhhR.

(4) Group 4 includes an operon that consists of ORFs PP3275 through to PP3278 (*paaFGHIJK*). This operon is 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. This set of genes is known to be under the control of PaaY and therefore the observed induction seems to be the result of a regulatory cascade.

Transcriptional fusions to '*lac* confirm microarray results. The above set of results indicate that bacterial cells respond to phenylalanine via PhhR and through other regulators. In order to confirm these results we generated fusions of the promoter regions of representative genes of the different groups to '*lacZ* in the broad-host range pMP220 vector and determined β-gal activity under different genetic backgrounds with and without phenylalanine (Table 3). From group 1, the genes induced by PhhR in a phenylalanine-dependent manner, we selected the promoters of *phhA* and *hmgA* genes, which are well-characterised in phenylalanine/tyrosine metabolism, as well as the promoter region of the newly identified PP3122 gene. 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 3). In the *phhR* mutant background, expression of the different promoters occurred at a low basal level that was not influenced by the presence of phenylalanine (Table 3). In the parental background we found that expression from P_{phhA}, P_{hmg}, and PP_{3122} 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 5-fold 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 to *'lacZ* and measured β-galactosidase activity within both genetic backgrounds.

Promoter fusion	Group	*phhR*	β-galactosidase (Miller Units)	
			−Phe	+ Phe
P_{phhA}:*lacZ*	1	-	1±1	5±1
P_{phhA}:*lacZ*	1	+	4±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_{PP2827}:*lacZ*	2	-	800±160	840±130
P_{PP2827}:*lacZ*	2	+	4565±1530	5040±2000
P_{phhR}:*lacZ*	3	-	94±10	65±90
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	880±20

TABLE 3. Expression of several promoters of the PhhR regulon in the wild-type and Δ*phhR* mutant backgrounds in response phenylalanine. 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 Materials and Methods.

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 1, column A). Also in agreement with the array assays, was the observation that the level of expression from P_{PP2827} was higher in the wild-type than in the mutant strain regardless of the presence of phenylalanine, which suggests a phenylalanine-independent positive effect of PhhR on the transcription of this gene. For the genes with expression levels that appeared repressed by PhhR, those of group 3, we used the previously constructed P_{phhR}:'*lacZ* fusion (12), 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 2-fold range

(Table 3). Expression from the PP3285 (*paaY*) promoter was higher in the PhhR-deficient background than in the parental background indicating that PhhR represses the expression of this promoter. It should also be noted that expression from the P_{PP3285} 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. Group 4 consists of 4 genes that were shown in the array assays to be induced in response to phenylalanine. Because all 4 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 parental strain with phenylalanine was higher than in the absence of phenylalanine. The ratio of β-galactosidase levels between wild-type and mutant cells was, as expected, close to 1.

Evidence for the existence of two catabolic pathways for phenylalanine in *P. putida* KT2440. We previously generated a knock-out mutant in the *phhA* gene that prevented synthesis of L-tyrosine from L-phenylalanine. 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 to 8 h for the wild-type (Table 4). Importantly, no significant difference was observed between the Δ*phhAB* mutant versus the parental strain when L-tyrosine was the sole N-source (Table 4). This suggests that in KT2440 there is an alternative pathway to the PhhAB-mediated catabolism of phenylalanine. We hypothesize that two potential phenylalanine degradation pathways coexist in *P. putida* KT2440; one, that is mediated by PhhA/PhhB yielding tyrosine and subsequently *p*-hydrophenylpyruvate (5, Figure 1), and another involving the transamination of phenylalanine into a ketoacid to yield an amino acid and phenylpyruvate (Figure 1). There are two ORFs in the genome of *P. putida* KT2440 that are annotated as potential aromatic amino acid transaminases (*tyrB1* [PP1972] and *tyrB2* [PP3590]). Mutants deficient in either one of these transaminases are available and they grow on minimal medium with phenylalanine or tyrosine as a N-source suggesting that they can replace each other in the metabolism of these amino acids (Table 4). A double *tyrB1*/*tyrB2* mutant was also able to grow with either phenylalanine or tyrosine without significant delay suggesting that other aromatic amino transferases are encoded in the genome of this strain.

To further test the hypothesis that a second pathway in *P. putida* for phenylalanine catabolism exists, we tested 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 *paaGHIJK* operon that contains genes (PP3275 – PP3278) associated with phenylacetyl-CoA degradation, and may therefore be involved in the alternative pathway that we have proposed. 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. Interestingly, our reported array results (Table 2) indicated that PP3122/PP3123 and PP3458 were induced in response to phenylalanine – a set of genes that has been annotated as potential acyl-CoA transferases. To explore the possibility that these putative acyl-CoA transferases may be involved in this alternative pathway, we generated mutants that inactivated these loci and tested growth on phenylalanine as an N source and phenylpyruvate and tyrosine as a C-source. Our results show that the PP3458 mutant is able to grow under all conditions with a doubling time similar to that of the parental strain (data not shown). In contrast, the PP3122 mutant is deficient in the use of tyrosine (Table 4) and turned black because of homogentisate accumulation. These results support that PP3122 showing high identity to the *E. coli* cetoacetyl-CoA transferase (27), is involved in the late steps of phenylalanine degradation via the tyrosine branch. Importantly, the PP3122 mutant showed no defects for growth on phenylpyruvate, which rules out the role of PP3122 in the conversion of phenylpyruvate to phenylacetyl-CoA.

DISCUSSION

Our set of earlier results based on the growth characteristics in *Pseudomonas* of a *phhA* mutant indicated that phenylalanine was metabolized through more than a single pathway; one of which involves hydroxylation via phenylalanine hydroxylase to yield tyrosine; and another that may involve the direct deamination of phenylalanine yielding phenylpyruvate and another amino acid – a reaction that could be carried out by any of the TyrB enzymes. Genes in catabolic pathways can be identified through transcriptomic analyses (10,30). Analysis of global transcriptional expression profiles of the parental strain with and without phenylalanine revealed a new set of genes that may participate in the metabolism of phenylalanine, and we obtained data supporting metabolism via two parallel pathways. We found that in response to phenylalanine the set of genes for the metabolism of L-phenylalanine via L-tyrosine, *p*-hydroxyphenylpyruvate and homogentisate were induced as expected from previous studies, which validated the array assays. Transcriptomic analysis identified an acyl-CoA transferase encoded by the gene products of ORF PP3122/PP3123 as induced by PhhR and phenylalanine. The role of this protein of unknown function was determined by characterizing a mutant with a knock-out in ORF PP3122. The mutant exhibited growth defects with tyrosine, but not on phenylpyruvate (Table 4), suggesting that PP3122/PP3123 was involved in the tyrosine branch of the phenylalanine degradation pathway, and its role is most likely related to the metabolism of acetoacetyl-CoA (Figure 1).

Genotype	Doubling Time (h)		
	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±0.2
PP3122	1.2±0.4	7.4±0.6	7.1±1.1
PP3458	1.1±0.5	7±0.9	2.1±0.2

TABLE 4. Growth of KT2440 and its isogenic mutants with NH_4^+, phenylalanine and tyrosine as the sole N-source. *P. 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 3 to 6 independent assays.

The other proposed phenylalanine degradation pathway places phenylpyruvate as a key metabolite in the degradation of phenylalanine, something that had not been described before in KT2440 or other *Pseudomonas sp.* (11). In this study we have characterised the growth of KT2440 with phenylpyruvate as a C-source and found it to be a relatively poor C-source as compared to glucose or L-tyrosine. The array results with cells growing with phenylalanine suggested that phenylpyruvate is metabolized via phenylacetyl-CoA and in agreement with this is that expression from the *paaG* promoter is induced *in vivo* in response to L-phenylalanine and phenylpyruvate, but not by tyrosine.

The closest homolog to the *P. putida* PhhR protein is the *E. coli* TyrR regulator, which functions as an activator or as a repressor (6,23,24,32,45). The analysis of transcriptomic patterns enabled us to differentiate promoters that were induced or repressed by PhhR in *P. putida* KT2440 grown with and without phenylalanine. Three types of PhhR-inducible promoters were identified and clustered in groups 1, 2 and 4. The first group included promoters that are induced with PhhR in a phenylalanine-dependent manner, while those in Group 2 were induced by PhhR regardless of the presence of phenylalanine. These sets of genes, as summarised in Table 2, while deduced from global transcriptional analysis, were also confirmed using '*lacZ* fusions of representative promoters from each group (Table 3). 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. Group 4 is comprised of genes found on a single operon that are induced in the presence of phenylalanine, and are under the transcriptional control of the *paaY* gene product.

Genes in group 3 were repressed by PhhR. Included within this group is *paaY*, which was shown to be strongly repressed by PhhR in the absence of phenylalanine (Table 3). In the presence of phenylalanine, PhhR seems to be released from the *paaY* promoter allowing higher expression to take place and leading to higher levels of PaaY that stimulate expression from target genes such as P_{paaG}. In agreement with this hypothesis is the observation that in the *phhR*-deficient background basal and induced levels of *paaG* were higher than in the parental background (Table 3). In addition to *paaY,* a number of other genes were repressed in the PhhR regulon, such as *aroF* and PP2607/PP2608, which encode enzymes involved in aromatic amino acid biosynthesis. This suggests that PhhR exerts feed-back control at the transcriptional level in the biosynthesis of aromatic amino acids, as previously described in other microorganisms (22).

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

Due to the nature of the array results reported above, they cannot be used to distinguish *per se*

between direct and indirect regulation of the corresponding promoters by PhhR. The only characterized genes regulated by PhhR were *phhAB/phhR* and expression of the *phhA* operon was reported to require two upstream activator sequences recognized by PhhR and defined by the 5'-NNNAAAANTNTNNTTNCG-3' consensus sequence (12). This sequence was used to search for potential PhhR boxes in the promoter region of the genes and operons shown in Table 2. We found

TIGR identifier	Sequence
PP2078 transcriptional regulator LysR family	GGTCATGGTGGCGTTACT
PP2827 alcohol dehydrogenase zinc-containing	GGTAATTAGAGGTTCACA
PP3122 CoA-transferase subunit A	TGTTTTGTAAAAATTATC 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**G**A**T**AG**T**TTTAC**A

TABLE 5. PhhR boxes present in the promoter region of cognate genes under PhhR regulation. PhhR box(es) 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 70% of the aligned sequenced.

that the PhhR box was absent from most of the induced/repressed genes (Table 2), and therefore we hypothesized that the induction/repression of the set of genes lacking the PhhR box might be mediated by other regulators. In all we found PhhR boxes in the upstream extragenic region of 8 promoters within groups 1, 2 and 3, as defined above (Table 2). The sequences identified as potential PhhR box(es) are shown in Table 5 together with the derived consensus. Two PhhR boxes were present upstream of *phhAB*, in agreement with our previous experimental results (12). Two PhhR boxes were also found upstream of the PP3434/PP3433 operon promoter, which encodes *p*-hydroxyphenylpyruvate dioxygenase genes and PP3122/PP3123, the identified acetoacetyl CoA-transferase. A single PhhR box was identified in front of each of the 4 remaining promoters. Two of these were *paaY* and PP2078, both of which encode transcriptional regulators that may, in turn, regulate other genes whose expression varies in response to phenylalanine (Table 2). While PaaY seems to regulate expression from the P_{paaG} promoter, genes under the direct regulation of PP2078 are unknown. It should be noted that while PhhR acts as an activator of PP2078 it functions as a repressor of *paaY* expression. Therefore PhhR, in a manner similar to TyrR (2-4,13,14,37), is a dual regulator that can recognize one or more target sites to control expression from target promoters to exert its transcriptional role.

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. PhhR regulates its own synthesis and 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 potential specific uptake transport system (PP2595/PP2596), as well as a multidrug extrusion pump (MexEF/OprN), suggesting that a dedicated uptake/extrusion system may control phenylalanine homeostasis. Indeed, levels of phenylalanine above 25 mM inhibit growth, as do other aromatic compounds (38), and a *mexE*-deficient mutant tolerated lower levels of phenylalanine than the parental strain. Therefore, metabolism of phenylalanine in *Pseudomonas putida* seems to integrate a number of signals that optimize its use as a nitrogen source by the cells.

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Capítulo 3: PhhR reconoce una secuencia diana a diferentes distancias con respecto a la RNA polimerasa con objeto de activar la transcripción

PhhR binds to target sequences at different distances with respect to RNA polymerase in order to activate transcription

M. Carmen Herrera[1], Tino Krell[1], Xiadong Zhang[2] and Juan-Luis Ramos[1]

JMB (submitted)

La proteína PhhR de la familia NtrC de *Pseudomonas putida* es una proteína de regulación dual que activa la transcripción del promotor con RNA polimerasa/σ^{70} mientras que reprime la transcripción de ciertos promotores por impedimento estérico. Los sitios dianas de unión en ambos casos, se definen como una secuencia consenso 5´-TGTAAAGATAGTTTTACA-3´. PhhR se une a esta secuencia como un dímero y la afinidad está localmente definida como por la especificidad de la secuencia. Estudios de calorimetría por titulación isotérmica han revelado que el rango de afinidad se encuentra entre 0.03 y 6,6 μM. PhhR activa la transcripción de los promotores de PP2078 y PP2827 bajo cualquier condición de crecimiento, mientras que PhhR estimula la transcripción de ciertos promotores regulados positivamente (P_{phhA}, P_{PP3122}, P_{PP3434} y P_{hmg}) solamente en la presencia de fenilalanina y tirosina, o sus correspondientes cetoácidos fenilapiruvato y *p*-hidroxifenilpiruvato, respectivamente. Una característica sorprendente de estimulación en la transcripción es que PhhR puede unirse a una o dos secuencias diana en el mismo promotor y que se encuentran localizadas a diferentes distancias del sitio de unión de la RNA polimerasa, por lo que PhhR puede funcionar como un regulador de Clase I (sitios diana a -66/-83), como un regulador de Clase II (sitios diana aproximadamente a -40) y como una proteína activadora (sitios diana a > -128), en este último caso la transcripción está modulada por la proteína IHF.

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PhhR binds to target sequences at different distances with respect to RNA polymerase in order to activate transcription

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The NtrC-family PhhR protein of *Pseudomonas putida* is a dual regulatory protein that activates transcription from its cognate promoters with RNA polymerase/σ^{70} while repressing transcription from certain promoters by steric hindrance. The target binding sites in repressed and activated promoters is defined by the consensus sequence 5′-TGTAAAGATAGTTTTACA-3′. PhhR binds to target sites as a dimer, but binding affinity is locally defined by specific sequences. Isothermal Titration Calorimetry has revealed that affinity ranges from 0.03 to 6.6 μM. PhhR activates transcription from the promoter of PP2078 and PP2827 under all growth conditions, while PhhR estimulates transcription from certain positively-regulated promoters (P_{phhA}, P_{PP3122}, P_{PP3434} and P_{hmg}) only in the presence of phenylalanine and tyrosine, or their corresponding keto acids phenylpyruvate and *p*-hydroxyphenyl pyruvate. A surprising feature of transcription stimulation by PhhR is that it can bind to one or two upstream target sequences that are located at different distances from the RNA polymerase binding site, so that PhhR can function as a Class I regulator (target sites at -66/-83), a Class II regulator (target sites around -40) and as an enhancer protein (target sites > -128), in the latter case transcription is modulated by the integration Host Factor protein.

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Introduction

Transcriptional activation in prokaryotes is mediated by RNA polymerase, whose activity can be modulated by a number of transcriptional regulatory proteins known as activators and repressors. In a recent survey of regulators it was found that about 80% of described regulators are exclusively activators or repressors, with the remaining being dual regulators.[1]

The interactions between RNA polymerase and activators are strongly influenced by the location at which they bind to the promoter relative to the transcription start point (tsp), and a wide range of locations and interactions between regulators and RNA polymerase have been described.[2] The most frequently observed regulator binding sites are found close to the -35 region of the promoter.[2-4] In other promoters, known as Class I promoters, the activator binding site is around the -60 position, and often involves interactions between the regulator and the extended C-terminus end of alpha subunit of RNA polymerase.[2] Other upstream activator sequences have also been found more than 100 bp away from the tsp, a feature that is typical of regulators that stimulate transcription with RNA polymerase/σ^{54} [5-6] but that has also been reported for a few regulators with RNA polymerase/σ^{70}; for example, TyrR of *E. coli* and NtrC-like of *Rhodopseudomonas palustris*.[7-8] Less frequently occurring activator binding sites are located at

the -10/-35 regions of promoters, which are utilised by the MerR family of transcriptional regulators. Structural studies have shown that when MerR family members bind to -10/-35 motifs, which are not present on the same DNA face, they are able activate expression in these promoters by distorting the DNA so that the RNA polymerase binding sites become aligned for correct recognition.[9]

Repression of transcription occurs through various mechanisms that inhibit the key processes of transcription initiation. The most often described repression mechanism involves binding of the repressor protein to the promoter in a way that impedes the binding of RNA polymerase, although it should be mentioned that steric hindrance is just one of the several mechanisms used by repressors to achieve their function.[10]

PhhR is the ultimate regulator of phenylalanine catabolism within a number of pathogenic and non-pathogenic *Pseudomonas* strains.[11-14] Additionally, based on microarray assays and a wide series of promoter fusions, we have recently shown that PhhR is a dual regulator.[15] In fact, the PhhR regulon includes 20 gene operons whose gene products are involved in phenylalanine catabolism via two parallel pathways, efflux pumps that prevent accumulation in the cells and protein of unknown functions.[15] PhhR activates expression from certain promoters, such as *phhAB* and PP3122, in response to phenylalanine, while also being able to activate promoters, such as the gene encoding a zinc-containing alcohol dehydrogenase (PP2827) and the LysR family member PP2078 regulator, in the absence of phenylalanine.[15] As a repressor PhhR controls its own synthesis and represses the expression of some *aro* genes involved in the biosynthesis of aromatic amino acids, so that L-phenylalanine seems to exert a kind of negative feedback control at the transcriptional level on its own synthesis.[15]

Although PhhR is a critical regulator that modulates the expression of many genes, the mechanism of transcriptional activation by PhhR has not been studied in detail. The only promoter under PhhR control that has been previously analyzed is that of the *phhAB* operon in *P. putida* and *P. aeruginosa*.[11] The *phhAB* genes encode a pterin-dependent phenylalanine hydroxylase that transforms L-phenylalanine into L-tyrosine. The PhhR regulator binds to the *phhAB* promoter of *P. putida* at two similar, yet non-identical, upstream motifs (5'-TGTAAAATT-ATCGTTACG-3' and 5'-ACAAAAACT-GTGTTTCCG-3') that are centred at positions -83 and -141 upstream from the proposed +1, respectively.[11] In addition to PhhR, the maximal transcriptional activity of the *phhA* promoter is modulated by Integration Host Factor (IHF), and a single binding site for IHF was found to overlap with the proximal PhhR motif.[11]

The PhhR protein belongs to the NtrC family of enhancers; however, in contrast with most members of this family of regulators, transcription from the promoter of the *phhAB* operon (P_{phhA}) is mediated by RNA polymerase with σ^{70} rather than σ^{54} [11], suggesting that the mechanism of activation by PhhR may have characteristics that are distinct from those of other NtrC family members.

The closest homolog to PhhR is the *Escherichia coli* TyrR protein, which is also a dual regulator. This protein activates transcription from cognate promoters as a dimer in response to phenylalanine and tryptophan, and it represses transcription from certain promoters as an hexamer that is most effective with tyrosine and ATP.[7]

This study was undertaken to learn about the physical and functional organization of the different genes regulated by PhhR. To this end we mapped the transcription start sites of different promoters whose expression is directly modulated by PhhR. We have also identified PhhR target sites using *in silico* and *in vitro* footprinting techniques, having found that the promoter region of the genes activated by PhhR can exhibit one or two PhhR binding sites, whose consensus sequence is defined by the following nucleotide stretch 5′-AAAANTNTNNTTNCG-3′. The target sites were located in different promoters centred at -9, -40, -66, -83, -93, -128, -141, -226, and -277 with respect to +1. For promoters with binding sites located further than -128, transcription activation is influenced by IHF. In promoters in which PhhR acted as a repressor, the PhhR binding site overlaped with the RNA polymerase recognition sequence, supporting the hypothesis that PhhR can repress genes through the physical occlusion of RNA polymerase.

Results

The PhhR effector profile

We have previously shown that expression from the *phhA* promoter was dependent on both PhhR and phenylalanine.[11] To establish the effector profile of PhhR, we measured β-galactosidase activity using '*lacZ* fusions of a number of promoters regulated by PhhR in response to all aromatic compounds (at 5 mM) proposed to be intermediates in the two parallel pathways for L-phenylalanine degradation, namely, tyrosine, *p*-hydroxyphenylpyruvate, phenylpyruvate and homogentisate. We also included tryptophan, as the third aromatic amino

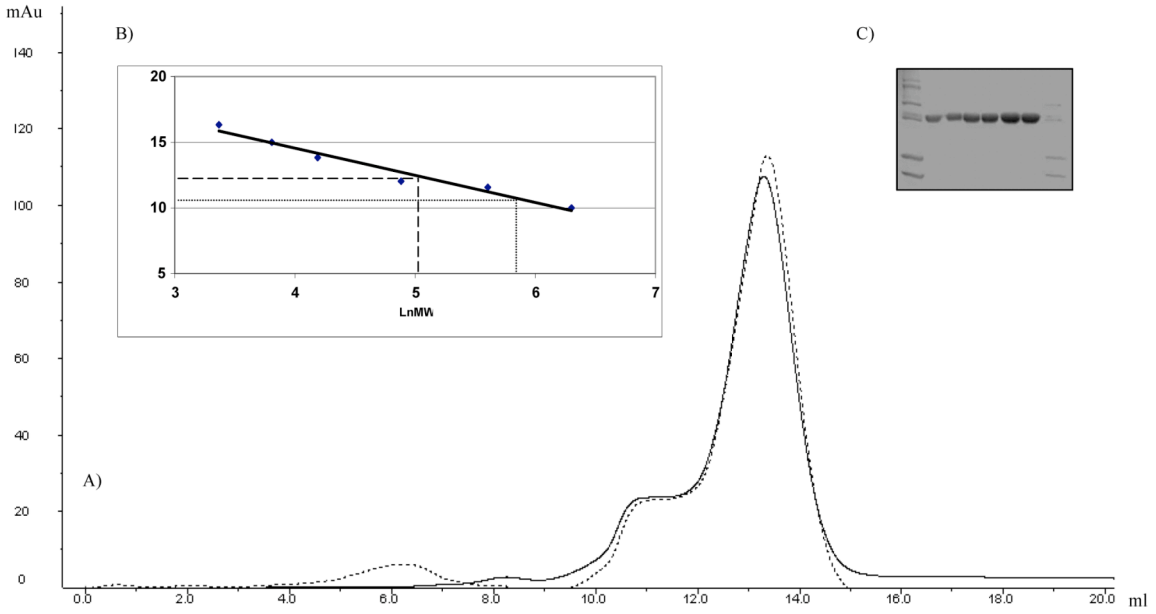

Figure 1. Determination of the oligomeric state of PhhR by analytical gel filtration**.** A) Elution profile from the analysis of 86 µM purified PhhR. Continuous track, protein eluted in the absence of effectors; dotted line, protein eluted in the presence of 1 mM tyrosine and 100 µM ATP. B) Calibration curve using protein standards: lactalbumin (A; 14 KDa), carbonic anhydrase (B; 29 KDa), chicken egg albumin (C; 45 KDa), bovine serum albumin monomer (D; 66 KDa), and bovine serum albumin dimer (E; 132 KDa). Shown is a liner regression of the resulting five elution volumes. The elution volumes and the corresponding molecular mass of PhhR are indicated.

acid to be tested as an inducer. For positively-regulated promoters we used the available P_{phhA}::'*lacZ* fusion and a fusion of the PP3122 promoter region to *'lacZ* in pMP220 too. We found that phenylalanine, tyrosine and *p*-hydroxyphenylpyruvate and phenylpyruvate were effectors. Homogentisate and tryptophan showed no effect on expression from the constructs. To test for effectors that influence repression by PhhR, we included the *paaY* promoter, which we have previously characterised as being repressed by PhhR, and measured expression from the P_{paaY}::'*lacZ* construct in response to the above effectors. We found the same effector profile as for positively regulated promoters (Table 1).

Promoter fusion	β-galactosidase (Miller Units)						
	-	Phe	Tyr	Trp	4-OHphe	phenylp	hmg
P_{phhA}::'*lacZ*	5±1	2780±150	630±30	5±1	150±25	2260±50	30±2
P_{PP3122}::'*lacZ*	21±4	1444±41	5462±145	21±4	1219±90	605±12	108±18
P_{paaY}::'*lacZ*	40±6	345±65	50±15	50±13	485±60	1650±50	45±9

Table 1. Profile of PhhR effectors measured with positively- and negatively-regulated promoters. *P. putida* KT2440, bearing the indicated promoter::*lacZ* fusion in the low-copy number wide-host range pMP220 vector[21] was grown on M9 minimal medium with glucose as a C-source and 5 mM of the indicated aromatic compound. Data are the average of 4 independent determinations done in duplicate, and values are the average of all determinations and the corresponding standard errors. Other conditions are given under Experimental Procedures. Phe, Tyr, Trp, 40H-phe, phenylpyruvate and hmg stand for phenylalanine, tyrosine, tryptophan, 4-hydroxyphenylpyruvate, phenylpyruvate and homogentisate, respectively.

Oligomeric form of PhhR

To determine the oligomeric form of PhhR we purified the protein to homogeneity, as described previously[11] and carried out gel filtration assays under different conditions. We found that close to 70% of the PhhR protein was a dimer when the assay was carried out in the absence of aromatic amino acids (Figure 1A and B), and that the remaining protein eluted as a peak corresponding to a potential hexameric form of the protein. We collected fractions corresponding to both peaks and analyzed them on SDS-PAGE and verified that the protein was, in fact, PhhR. To test whether the presence of aromatic amino acids influences the oligomeric state of PhhR we pre-incubated the protein with 5 mM phenylalanine or tyrosine and eluted the protein with buffers containing 5-10 mM of the same aromatic amino acid. The behaviour of the protein under these conditions was identical to that seen in the absence of aromatic amino acids (not shown).

A major difference between NtrC family members and TyrR with respect to PhhR is that PhhR does not hydrolise ATP. In spite of this, we tested if oligomeric state of PhhR was affected by the presence of ADP or ATP, but no change in the conformational state was observed (not shown). We found no change in the oligomeric state when incubation was performed in the presence of 1 to 10 mM of each aromatic amino acid and 100 µM-100 mM of ATP (See

Figure 1 for the case of 1 mM tyrosine and 100 μM ATP). It should be noted that the ratio dimer:hexamer was 6.7±0.2 regardless of the protein concentration and regardless of the presence of aminoacids and/or nucleotides.

Although the set of chromatographic results supported that PhhR is a dimer in both the absence and presence of phenylalanine or tyrosine, interactions with phenylalanine may have an influence on the conformational state of PhhR as determined during the establishment of optimal conditions for crystallization of this protein (data not shown). We found that the presence of the aromatic amino acids favours the crystallization of the protein and that a set of thin needles are produced only when the aromatic amino acid was present. Further optimization of the crystallisation conditions are being carried as these crystals are extremely fragile and are not suitable for the resolution of 3D structure.

We also explored the possibility that the oligomeric state of PhhR may be influenced by binding to specific DNA target sequences. Electrophoretic Mobility Shift Assays (EMSA) were carried out using homogenous PhhR protein with target DNA of the promoter region of PP3122, which is a PhhR- upregulated promoter. These EMSAs were carried out in the presence and in the absence of phenylalanine or tyrosine, in order to determine whether aromatic amino acids can influence the position of the retarded band due to changes in the oligomeric state of the protein while bound to DNA. Under these premises we incubated a 460-bp fragment of PP3122 from +100 to -360 with increasing concentrations of PhhR in the absence and in the presence of 10 mM L-phenylalanine or L-tyrosine. Figure 2 shows the results in the absence (Figure 2A) and in the presence of phenylalanine (Figure 2B). We found that the retarded band was observed at concentrations above 1 μM PhhR and that the size of the retarded band was not influenced by the presence of amino acids (Figure 2), which suggests that the oligomeric state of PhhR when bound to its target sites is not influenced by the presence of amino acids.

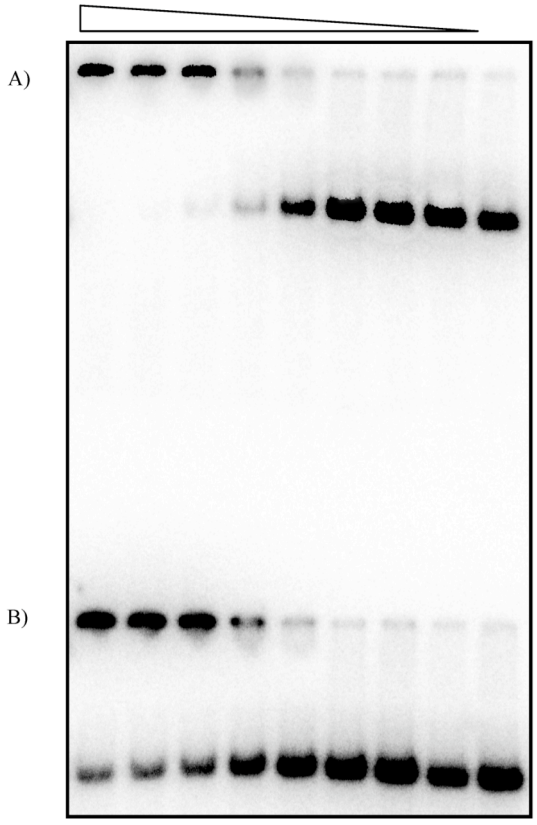

Figure 2. EMSA of PhhR with the PP3285 (up) and PP3122 (down) intergenic region. Binding reactions were carried out as with 0.5 nM DNA and increasing concentrations of PhhR (from right to left, 0-2.5 μM) for 30 min and subjected to electrophoresis as indicated in materials and methods.

We determined the molecular mass of the DNA/PhhR complex and found that it agrees with the size of one dimer of PhhR bound per target site. This data agrees with our earlier studies which showed that PhhR binds to both *phhA* promoter binding sites as a dimer.

Determination of the transcription start point of promoters activated and repressed by PhhR and identification of PhhR binding sites by foot-printing

As a first step in the determination of the potential binding sites for PhhR within regulated promoters, we decided to define the transcription start points (tsp) of these promoters by carrying out primer extension assays. In a previous study we determined the tsp of *phhA* and *phhR*. Therefore in the current study, chose the remaining known activated promoters, i.e., PP3122, PP3434, PP2078, PP2827, as well as the recently-identified PhhR-repressed promoters.[15]

The tsp of the activated promoters (PP3122, PP3434, PP2078 and PP2827) were determined using RNA prepared from cells in the exponential growth phase with phenylalanine as a N-source. For the repressed promoter, the tsp was determined using RNA from cells devoid of PhhR regulator in order to facilitate a high yield of the signal. Figure 3 shows the results for the positively-regulated PP3122 promoter and the repressed *paaY* promoter. It was found that both negatively-regulated promoters (*paaY* and *phhR*) exhibited a single tsp. The sequences of the positively-regulated promoters were aligned at the +1 position, and we found that the most conserved region (-16 to -11) corresponds to a TAWART consensus. This sequence resembles the proposed GATATT hexamer proposed to be recognized by σ^{70} in *Pseudomonas*.[16] The *paaY* –10

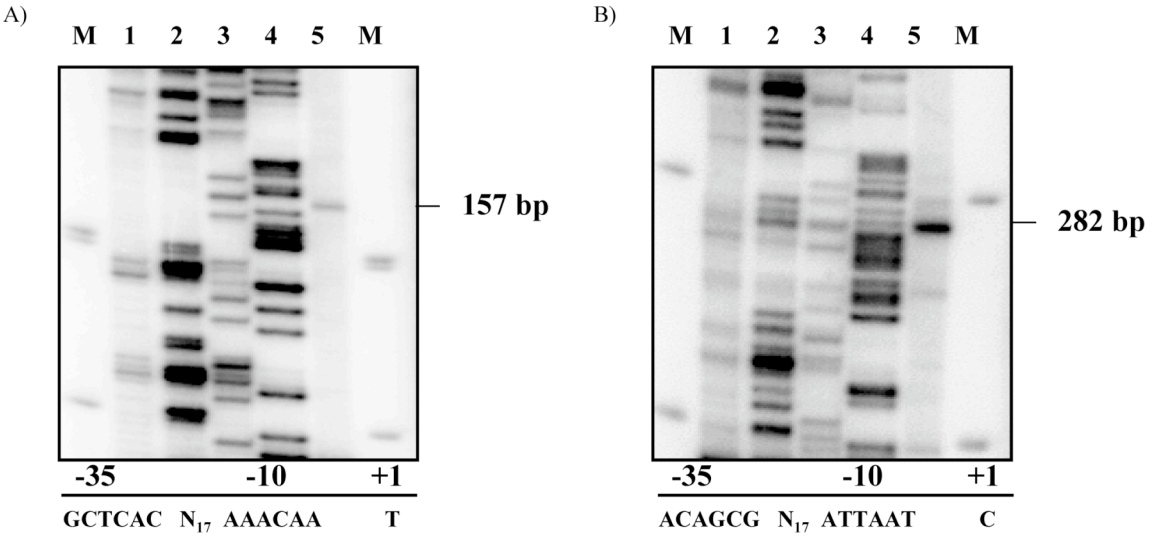

Figure 3. Transcription start point of PP3122 (A) and PP3285 (B). Cells were grown in M9 minimal medium with glucose as the sole carbon source with phenylalanine. RNA was prepared from the cells in the mid-exponential growth phase, as described in material and methods. Lanes 1-4 are sequencing ladders used to determine the exact site of the analyzed cDNAs. Lane 5 corresponds to the sample, and M is a single strand size marker. On the right is the size of the cDNA extended product, and at the bottom is the deduced hexamers at -10 and -35 for the corresponding promoters.

region, corresponding to 5'-ATTAAT-3, also shows similarity to the sequence recognized by RNA polymerase/σ^{70}. No sequence similar to the σ^{54}-recognized consensus GGN_9 TGCA in the -24/-9 stretch was found in any of the promoters.

Since PhhR recognises two target sites in *phhA*, which is the only PhhR-regulated promoter confirmed with *in vitro* and *in vivo* data, we aligned the sequences of the two sites and derived a consensus sequence (5´-NNNAAAANTNTNNTTNCG-3′) that was used to screen from positions +1 to –500 of each promoter in order to identify potential PhhR binding sites in the mapped promoters. Using the derived consensus, we were able to re-locate the two PhhR boxes present in the *phhA* promoter and centred at –141 and –83 (Figure 4). Upon screening the other promoters, two binding sites were found for PP3122 centred at –93 and –40; two were also found for PP3434 centred at –277 and –226; a single binding site was found for PP2078 at –128; and for PP2827 at –66; and, surprisingly, at –9 for the *hmgA* promoter. For the repressed promoters a single potential binding site was found centred at +10 for PP3285, and two sites at -41 and +16 were found for *phhR* (Figure 4).

The sites that we found using the derived consensus for *phhA* and *phhR* were the same as those that have been previously characterized, confirming the efficacy of the screen (Herrera and Ramos, 2007). These *in silico*-derived binding sites were then validated for the PP3122 promoter using footprint assays (Figure 5). In this series of assays increasing concentrations of PhhR (0 to 15 μM) were incubated with a 480 bp-DNA fragment spanning –360 to +100 in the PP3122 promoter. We found that two regions were protected, and that these regions corresponded to the distal and proximal sites that were identified above in the *in silico* screening. Therefore, this wet assay confirms and validates our *in silico* results.

Thermodynamic characterization of the interactions of PhhR with its target sequences

To shed further light on the binding process between PhhR and its target sequences we determined the thermodynamic parameters of interactions between a homogeneous preparation of PhhR and 26-mer double stranded oligonucleotides, corresponding to each of the PhhR targets in the different regulated promoters The PhhR titrations were carried out at a fixed DNA concentration of 3 μM. Figure 6 shows the results of the titration of the two target PhhR sites of the PP3434 promoter as a representative example (other titration curves are given in Suppl. Mat.). Binding experiments gave rise in all cases to exothermic heat changes indicative of favourable entalphy changes, which were accompanied by either favourable or unfavourable entropy changes (Table 3). In the case of the PP3434 sites, we found that enthalpy changes and affinity of PhhR was higher for the site corresponding to –226 (725 nM) than for the more distant site centred at –277 (6.5 μM). We

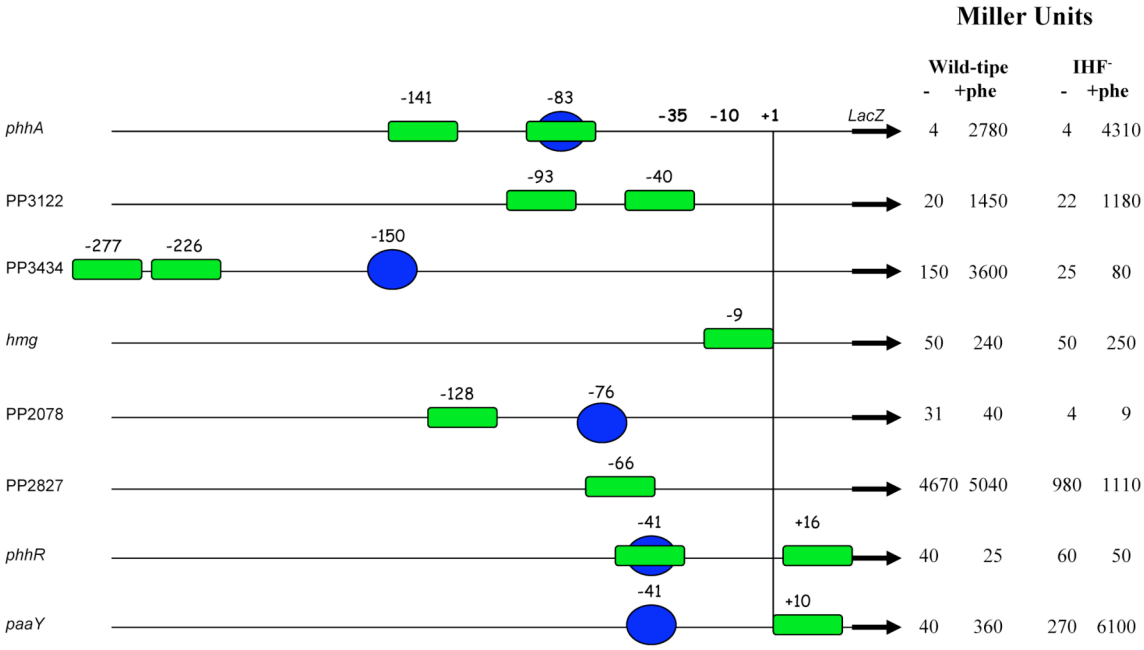

Figure 4. PhhR binding sites in the mapped promoters. Schematic representation of the fusion of different promoters to '*lacZ*. The *lacZ* gene is represented by a thick arrow. Green boxes represent PhhR binding sites and the number above indicate the center of the target site. Blue ellipse indicate IHF sites and the center of the site is indicted too. β-galactosidase activity was determined in cells in the exponential phase in the parental strain KT2440 and its isogenic IHF mutant in cells growing in the absence (-) or in the presence of phenylalanine (+ phe). Data are the average of at least 3 independent assays done in duplicate. Standard error were below 10% of the given number.

also inferred from the stoichiometry of binding that one PhhR dimer binds per site with each PhhR monomer probably recognizing half of each site. ITC data derived for the rest of the target sequences are also presented in Table 2. It should be noted that affinity of PhhR for these binding sites varies between 30 nM for the high affinity site of the *phhA* promoter, and 6.6 μM for the low affinity site of the PP3434 promoter. For most target sequences the affinities were in the range of 200 to 660 nM. The ITC data revealed no significant difference in binding affinity according to the position of the site with respect to +1 or between the the repressed and activated promoters.

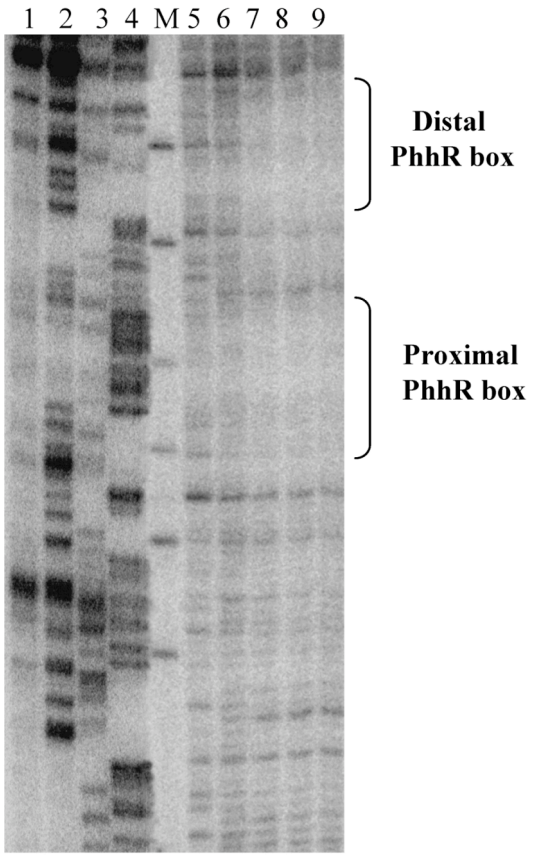

Figure 5. DNase I footprint in the lower strand with PhhR in the PP3122 intergenic region. Assays were carried out as described in Materials and Methods in the absence (lane 5) or in the presence of 5 μM (lane 6), 8 μM (lane 7), 12 μM (lane 8) or 15 μM (lane 9). PhhR. Lanes 1 through to 4 is a sequencing ladder and lane M is a size marker. The region protected by phhR is indicated by vertical lines.

IHF influences transcription from some of the PhhR-regulated promoters

In some of the positively-regulated promoters, one or more PhhR targets were found upstream from the -35 sequence. We previously showed that a single IHF site was present in the *phhA* promoter, which overlapped the proximal PhhR binding site (Figure 4). We found that in an IHF deficient background, the expression induced from *phhA* was higher than in an IHF-proficient background.

We found IHF binding sites in the promoter region of PP3434 (5'-GGGCAAGAGCGTG-3', centred at −150), PP2078 (5'-ATTCAGA-AAAATA-3', centred at −76) and the two repressed promoters *phhR* and *paaY* (centred at −41). No IHF site was found in the PP3122 or *hmg* promoters. We have tested the influence of IHF in all of the above promoters and found that the expression pattern of PP3122 and *hmg* was not influenced by the host background, as expected, (Figure 4). Similarly to what we found for *phhA*, expression from *phhR* and *paaY* in the IHF-deficient background was higher than in the parental background. This was particularly evident in the presence of phenylalanine (Figure 4). More strikingly, PP2078 and PP3434 expression was found to be completlely IHF-dependent, as they were not expressed at all under an IHF-null background. Therefore, we can conclude that IHF modulates PhhR-mediated transcription, and that its effect may be influenced by location of the IHF site relative to the tsp.

Discussion

The *Pseudomonas putida* PhhR transcriptional regulator is a dual regulator, in that it can act both as an activator and a repressor of transcription and we present data that support that PhhR carries out its regulatory function through at least three different molecular mechanisms: 1) activation in response to aromatic amino acids, 2) activation regardless of the presence of aromatic aminoacids, 3) repression by competing with RNA polymerase.
The most similar protein to PhhR is TyrR of *E. coli*. TyrR, like PhhR, is also a dual regulator, but its role as an activator or a repressor is dictated by its oligomeric state. Specifically, when TyrR is a dimer it acts as an activator and when TyrR is a hexamer it acts as a repressor, the transition from dimer to hexamer is mediated by the presence of tyrosine and ATP. Furthermore, when TyrR functions as a repressor, it does so as a hexamer that binds to high and low affinity sites, creating loops that prevent the binding of RNA polymerase to target promoters.[7] The data that we have obtained in this study suggests that PhhR is preferentially a dimer regardless of the presence of aromatic amino acids or ATP and that its oligomeric state is not influenced by the nature of the promoter to which it binds. What makes PhhR of particular interest is that its role as an activator is not strictly dependent on the presence of effector molecules. As such, while PhhR stimulates expression from certain promoters (*phhA*, PP3122, *hmg*) in response phenylalanine, tyrosine, *p*-hydroxyphenylpyruvate, and phenylpyruvate, expression of PP2078 is positively regulated by PhhR regardless of the presence of the effector molecules. This is of particular interest because PP2078 is, in turn, a regulator of the LysR family.[15] The promoters activated by PP2078 without phenylalanine belong to a group of genes that encode proteins involved in overcoming toxicity of aromatic compounds such as phenylalanine but also TNT or naphthalene.[17] The mechanism of PhhR-mediated repression at the *phhR* and *paaY* promoters seems to be that of PhhR competing against RNA polymerase for the same binding site without any changes in its oligomeric state. Modulation of these two target genes is, in addition, influenced by IHF because an IHF binding site overlaps one of the PhhR binding sites (Figure 4). In agreement with this is our observation that under an IHF-deficient background, expression from these repressed promoters is maximal in the presence of phenylalanine.

We reported previously that PhhR belongs to the NtrC family of transcriptional regulators, but that in contrast to the members of this family that work with sigma-54, PhhR transcribed the *phhA* promoter with RNA polymerase/σ^{70}.[11] At present we have determined the tsp of all promoters under PhhR control and we have searched for potential target sequences similar to those recognized by σ^{70} for σ^{54}. In all of the analysed promoters we found −10/−35 regions rather than −12/−24 regions, indicating that in contrast with most of NtrC family members, transcription is mediated by RNA polymerase/σ^{70}.

In a similar manner to TyrR, PhhR can activate promoters that contain two PhhR binding sites (*phhAB*, PP3122, PP3434) as well as those that contain a single binding site (PP2078, *hmg* and PP2827). It is important to note that for the first set of promoters – those with 2 binding sites – their expression is both PhhR- and phenylalanine-dependent (Figure 4), whereas the promoters with a single site may be phenylalanine-dependent (*hmg)* or permanently activated regardless of the presence of phenylalanine (PP2827 and PP2078).

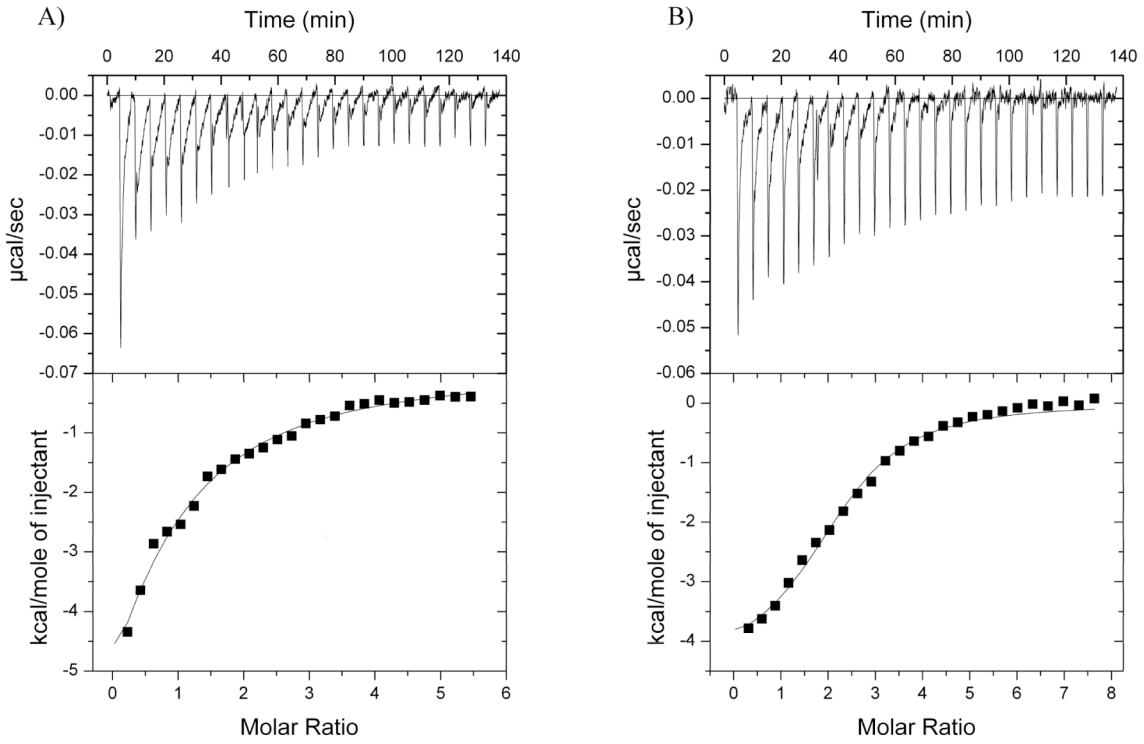

Figure 6. Isothermal titration calorimetry for the binding of PhhR to its target sites in PP3434 promotor. Heat changes (upper panel) and integrated peak areas (lower panel) or the injection of a series of 1.6 µl aliquots of 6 µl PhhR in a solution of A)3 µM proximal and B) distal PhhR binding sites of 26-mer duplex DNA.

	Boxes PhhR	K_D (µM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
Activated genes				
phhAB (PP4490)*	GCTGACAAAAACTGTGTTTCCGCCAG	2.03±0.25	-2.59	5.18
phhAB (PP4490)*	GTTTTGTAAAATTATCGTTACGAAAA	0.03±0.01	-14.7	-4.47
PP3122	GTTTTGTAAAAATTATCAATCGATGA	1.71±0.13	-9.59	-2.20
PP3122	TTGCGGTGAAGATGTCTGCACACGCT	2.2±0.2	-1.80	5.81
PP3434	AGGTTGTCGAGGATGTGTTCGACGTG	0.72±0.1	-4.43	3.81
PP3434	TGGCTGCTGGCCTGCATTTCACGTTC	6.6±1	-1.33	5.96
hmg (PP4621)	GCGGAGCAAATTACGTTATTCGTAAT	2.12±0.38	-7.51	0.2
PP2078	GTGCGGTCATGGTGGCGTTACTCGTA	0.27±0.06	-11.3	-2.29
PP2827	CAGTGGTAATTAGAGGTTCACAAAGG	2.47±0.21	-2.37	5.27
Repressed genes				
paaY (PP3285)	CACAAGTGATACACGATTGACGACCA	0.85±0.1	-5.02	3.09
phhR (PP4489)*	GTTTTGTAAAATTATCGTTACGAAAA	0.03±0.01	-14.7	-4.47
phhR (PP4489)*	GCTGACAAAAACTGTGTTTCCGCCAG	2.03±0.25	-2.59	5.18

Table 2. Affinity of PhhR for its binding sites as determined from ITC titrations

A second relevant insight gained by the current study are the locations of the PhhR binding sites with respect to the +1 tsp; which, in the promoters investigated, vary from -9 to -277. This suggests that the different interactions that occur between PhhR with its target sequences are important in determining how PhhR activity is modulated. In addition, in three of the PhhR positively-regulated promoters, IHF binding sites were found. While in *phhA* the IHF bindin site has a negative effect due to the competition of IHF/PhhR for the proximal activation site; in the other two promoters, in which PhhR binding sites are located >–128 bp (i.e., PP3438 and PP2078), we have found that stimulation of transcription is strongly influenced by IHF, which suggests that DNA bending is needed to facilitate interactions between the regulator bound at upstream activator sequences and RNA polymerase bound to the -10/-35 elements.

For the PP3122 promoter, which has a PhhR box at –40 and another at –93, we propose that it is a class II promoter and that the PhhR regulator bound to the high-affinity –40 site may establish direct interactions with σ^{70}. On the other hand, we deduce that the PP2827 is a class I promoter and permanent contacts with the alpha subunit of the RNA polymerase are established. The *hmgA* promoter requires some specific attention since this gene is the first one in the *hmgABC* operon involved in homogentisate metabolism.[12] Homogentisate catabolic genes are known to be induced when *P. putida* cells are grown on homogentisate and early studies revealed that the expression of this operon was mediated by the

hmgR gene product.[12] HmgR is known to bind the P_{hmg} promoter spanning positions –16 to +29 with respect to the P_{hmg} transcription start site, at a perfectly palindromic 17-bp motif (5′-TCGTAATCTGATTACGA-3′).[12]

An unexpected finding of our study is that the induction of the *hmgABCD* operon was also PhhR- and phenylalanine-dependent.[15] It could be argued that this is due to the synthesis of homogentisate from phenylalanine, but the fact that *hmgA* expression occurred with phenylalanine in an *hpd* mutant background suggests that the effect cannot be attributed to the internal synthesis of homogenisate from phenylalanine. The PhhR box identified in this study overlaps with the HmgR site, and spans positions –18 to –1. Given the overlapping nature of the sites we hypothesize that PhhR inhibits HmgR binding to its target site thereby facilitating the recruitment of RNA polymerase to the promoter, although further experiments are required to confirm this.

In summary, we have shown that PhhR is a dual regulator that modulates transcription activation by binding to the PhhR box(es) and establishing potential interactions with the RNA polymerase/σ^{70}. As a repressor, PhhR may prevent the entry of RNA polymerase to target sites and, as an activator it seems to work as a class I, class II and enhancer-like regulator. This is dictated by the location of the PhhR box upstream of –35, which may, in turn, lead to differential regulator/DNA polymerase σ^{70} interactions. The specifics of these interactions between PhhR and DNA polymerase require further research in order to explain the novel and interesting modes of activation and repression mediated by PhhR.

Experimental procedures

Bacterial strains, plasmids and culture media

The bacterial strains, cloning vectors and plasmids used in this study are shown in Table 3. *Pseudomonas putida* KT2440 and the Δ*phhR* mutant strain were grown in M9 minimal medium with glucose (0.5 [wt/vol]) as the carbon source. When indicated 5 mM phenylalanine was added and 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 LB medium with shaking. *Escherichia coli* DH5α was used for gene cloning and *E. coli* BL21 (DE3) was used for protein expression. When required, antibiotics were used at the following final concentrations in micrograms per millilitre: ampicillin, 100; chloramphenicol, 30; kanamycin, 50; and tetracycline, 20.

Strain	Genotype	Ref
P. putida KT2440	Cm^R; Ap^R	18
MCH4 (*phhR:aphA3*)	Cm^R; Km^R	11
P. putida KT2440-IHF3	*ihfA*::Km	19
E. coli DH5α	F'/*hsdR*17, *recA*1, *gyrA*	20
E. coli BL21 (DE3)	F^-, *ompI*, $hsdS_B$ (r^-_B m^-_B), *gal*, *dcm*, *met*	Novagen
Plasmids		
pMP220	'*lacZ*; IncP; Tc^R	21
pMCR1	P_{phhR}::*lacZ*; Tc^R	11
pMCA1	P_{phhA}::*lacZ*; Tc^R	11
pMChmg	P_{hmg}::*lacZ*; Tc^R	15
pMC3122	P_{PP3122}::*lacZ*; Tc^R	15
pMC3434	P_{PP3434}::*lacZ*; Tc^R	This study
pMC2827	P_{PP2827}::*lacZ*; Tc^R	15
pMC3285	P_{PP3285}::*lacZ*; Tc^R	15
pMC2078	P_{PP2078}::*lacZ*; Tc^R	This study
pMC3067	P_{PP3067}::*lacZ*; Tc^R	This study
pMC3002	P_{PP3002}::*lacZ*; Tc^R	This study
pMC3122P	'orfPP3122'::pGEMT, Ap^R	This study

Table 3. Plasmids and strains used in this study

Analytical gel filtration chromatography

Analytical gel filtration chromatography using an Åkta FPLC system (Amersham Bioscience) was carried out to determine the oligomeric state of PhhR in the absence or in the presence of effector. Purified PhhR protein at a concentration of 86 μM was loaded onto a Superdex-200 10/300 GL column (Amersham Bioscience) and equilibrated in buffer (100 mM phosphate, pH 7.2, supplemented with 1 mM EDTA, 10 mM β-mercaptoethanol, 100 mM NaCl, 300 mM imidazol). Protein was eluted with a constant flow rate of 1 ml/min, and the absorbance of the eluate was monitored at 280 nm. The column was calibrated with α-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), and bovine serum albumin (66 and 132 kDa) (Sigma). Aliquots of the peak fractions were analyzed by SDS-PAGE according to standard protocols. The molecular mass of PhhR was inferred by plotting the elution volume of PhhR and marker proteins against the log molecular mass of marker proteins, according to instructions provided by Amersham Bioscience.

β-Galactosidase assays

We used fusions of the PhhR target promoters of genes to a promoterless '*lacZ* gene in the low-copy-number pMP220 vector. The corresponding promoter regions were amplified by PCR with

primers incorporating restriction sites (3' end: EcoRI site for PP3434 promoter, and BamHI site for the PP2078 promoter; 5' end: PstI site for all promoters) in order to facilitate the fusion of the promoters to '*lacZ*. Upon amplification, DNA was digested with EcoRI or BamHI and PstI and ligated to EcoRI or BamHI -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*ihf* mutant strain. The corresponding transformants were grown overnight in M9 medium with glucose plus tetracycline. The cultures were then diluted 100-fold in the same medium and grown to a turbidity of about 0.6 at 660 nm. Aliquots were prepared and incubated in the absence or presence of 5 mM effector at 30 ºC for 2 h with shaking. ß-Galactosidase activity was assayed in permeabilized whole cells according to Miller's method (1972). Assays were run in triplicate and were repeated for at least three independent experiments.

Preparation of RNA and primer extension analysis

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. 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 at 30 ºC for 15 min. Cells (10 ml) were harvested by centrifugation (9000 × *g* for 15 min) in disposable plastic tubes pre-cooled in liquid nitrogen, and 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 carried out at the end of the process followed by purification using RNeasy columns (Qiagen, cat no. 74104, Hilden). The RNA concentration was determined spectrophotometrically at 260 nm and its integrity was assessed by agarose gel eletrophoresis.

For primer extension analysis, primers were labeled at their 5' ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. About 10^5 cpm of the labeled primers were hybridized to 30 µg of total RNA, and extension was carried out using avian myeloblastosis virus reverse transcriptase (RT). Electrophoresis of cDNA products was done using a urea-polyacrylamide sequencing gel to separate the reaction products and gels were exposed to a phosphor screen (Fuji Photo Film Co, Ltd.) for 24-48 h. Phosphor screens were scanned using a phosphorimaging instrument (Molecular Imager FX, Bio-Rad).

DNase I footprinting

A 466-bp PCR fragment was used in these assays. For the top strand of the PP3122/PP3123 operon, we amplified DNA using primers 5'-TGAATTCGGCAGTCAGGCTG-3' (end labeled with [αP^{32}]-ATP as described above) and 5'-CGCGCCGCTTGATTTCGCTG -3'. For the bottom strand, the same primers were used but the latter primer was end-labeled. Solutions containing 5 nM (10^4 cpm) of each labeled probe were incubated without or with His_6-tagged PhhR at 5, 8, 12 or 15 µM for 30 min at 30 °C in 10 µl reaction mixtures. The solutions were then treated with 40 µl of DNase I (final concentration 1.4×10^{-4} U/µl) diluted in 10 mM Tris-HCl (pH 7) supplemented with 2.55 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA and 50 mM KCl. After 4 min at 30 °C, reactions were stopped by adding 2 µl of 0.5 mM EDTA. The solutions were then extracted with phenol, and the DNA was precipitated using 2 volumes of ethanol and finally resuspended in 5 µl of TE (10 mM Tris-0.1 mM EDTA [pH 8.0]) and 2.5 µl of loading dye. Equal amounts of DNA (5,000 to 6,000 cpm) were heated to 90 °C for 3 min and electrophoresed through a 6.5% (wt/vol) denaturing polyacrylamide gel. Sequencing ladders were generated with the corresponding labeled primer, using a T7 DNA polymerase sequencing kit (USB-Amersham) and the pMC3122P plasmid.

Isothermal titration calorimetry

Measurements were done on a VP-Microcalorimeter at 25° C. The protein was thoroughly dialyzed against 100 mM phosphate buffer, 250 mM NaCl, 10 mM magnesium acetate, 2 mM DTT and 1 mM EDTA, pH 7.2. For these DNA binding studies, oligonucleotides corresponding to both strands of the PhhR target sequences were synthesized. Annealing was carried out by mixing equimolar amounts (at a concentration of 60 µM) of each complementary oligonucleotide in 50 mM phosphate buffer, pH 7.0, 5 mM EDTA and 750 mM NaCl. The mixture was incubated at 95 °C for 25 min and then chilled on ice and dialyzed in the buffer used for ITC studies. The titrations involved injections of 70 µM PhhR into a solution containing 3 µM of the 26 mer duplex DNA. Titration curves were fitted by a nonlinear least squares method (ORIGIN software) to a function for the binding of a ligand to a macromolecule.

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V. Discusión

Pseudomonas putida KT2440 es una bacteria del suelo que coloniza una amplia variedad de plantas a alta densidad celular y responde a compuestos presentes en exudados de raíz de plantas (Matilla *et al*., 2007 y Molina *et al*., 2000). Los aminoácidos aromáticos se encuentran en los exudados de raíz y por lo tanto influyen en la composición de la comunidad microbiana de los alrededores más inmediatos de las plantas, favoreciendo las interacciones beneficiosas planta-microorganismo (Bertin *et al*., 2003; Prinkryl and Vancura, 1980 y Rovira, 1969). Estos microorganismos utilizan distintos aminoácidos, como la prolina, la lisina y el glutamato, tanto como fuente de carbono como de nitrógeno (Revelles *et* al., 2005; Revelles *et al*., 2007 y Vilchez *et al*., 2000). Recientemente se ha comprobado que además utilizan directamente aminoácidos aromáticos presentes en exudados de raíz ya que auxótrofos para estos aminoácidos no presentan alterada su capacidad de colonización (Molina-Henares *et al*., 2008). En *P. putida* y otras especies del género *Pseudomonas*, el catabolismo de la L-fenilalanina incluye su conversión a tirosina (Arias-Barrau *et al*., 2004; Song and Jensen, 1996; Wei-Gu *et al*., 1998 y Zhao *et al*., 1994) (Figura 1, capítulo 1), a través de una reacción mediada por la fenilalanina hidroxilasa dependiente de pterin. Este proceso metabólico requiere los productos de los genes *phhA* y *phhB*, que se disponen formando un operón. PhhB es la enzima responsable de regenerar el cofactor pterin, mientras que PhhA es la hidroxilasa catalítica. Se ha comprobado que posteriormente la tirosina es deaminada para producir el *p*-hidroxifenilpiruvato (Arias-Barrau *et al*., 2004). En *P. aeruginosa* se han encontrado cinco enzimas con actividad tirosina aminotransferasa (Whitaker *et al*., 1982), mientras que en *P. putida* KT2440, se han encontrado dos ORFs, *tyrB-1* y *tyrB-2*, codicados por PP1972 y PP3590, respectivamente. El *p*-hidroxifenilpiruvato es transformado mediante un conjunto de reacciones, en homogentisato, maleilcetoacetato y fumarilacetoacetato, los cuales a su vez son hidrolizados para producir fumarato y acetoacetato (Figura 1, capítulo 1). Un conjunto de resultados obtenidos en esta tesis doctoral a partir de estudios del perfil transcriptómico, análisis mutacional y estudios de expresión *in vivo* de promotores, ha confirmado esta ruta catabólica de la fenilalanina en *P. putida* KT2440.

Además, hemos encontrado una nueva enzima implicada en esta vía que no ha sido descrita previamente. La caracterización de un mutante que inactiva el operón PP3122/PP3123, nos ha permitido relacionar la actividad de la correspondiente enzima codificada, una acetoacetil-CoA transferasa, en el último paso del catabolismo de la fenilalanina. Esta enzima incorpora un grupo CoA al acetoacetato para dar lugar acetoacetil-CoA (Figura 1, capítulo 2).

Existe una segunda ruta catabólica para la fenilalanina en *P. putida* KT2440

En este tesis doctoral se describe que los mutantes en *P. putida* que tienen una insercción que inactiva los genes *phhA* y *phhB*, que codifican las enzimas responsables de la hidroxilación de la fenilalanina para dar lugar a la tirosina, aún crecen en un medio con fenilalanina como única fuente de nitrógeno, aunque a una menor velocidad que la cepa silvestre (Tabla 2, capítulo 1). Ésto se interpretó como que la ruta hasta ahora descrita en *P. putida*, no es la única activa en el catabolismo de este aminoácido aromático. Koyama (1982, 1983 y 1984) describió y caracterizó una L-fenilalanina oxidasa en *Pseudomonas* sp P-501 que cataliza la deaminación oxidativa y decarboxilación oxigenativa de este aminoácido aromático. Sin embargo ninguna proteína homóloga se ha podido encontrar en el genoma de *P. putida* KT2440. Un análisis global del perfil de expresión transcripcional de la cepa parental con y sin fenilalanina ha revelado un nuevo conjunto de genes que pueden participar en el metabolismo de este aminoácido aromático. La nueva ruta de degradación de fenilalanina, descrita en esta tesis doctoral, sitúa al fenilpiruvato como un metabolito clave, lo que no había sido descrito previamente en *Pseudomonas* (Evans *et al*., 1987). Sin embargo, existen tanto bacterias gram-positivas como bacterias gram-negativas que producen fenilpiruvato durante la degradación de fenilalanina (Lee and Desmazeaud, 1986 y Spaepen *et al*., 2007). Esta ruta alternativa incluye la transaminación del grupo amino de la fenilalanina a un cetoácido, produciendo fenilpiruvato y el correspondiente aminoácido. Mediante ensayos de microarrays hemos observado la inducción de un conjunto de genes que participan en el

catabolismo del fenilacetil-CoA, en presencia de fenilalanina, tales como los genes *paaGHIJ* y el gen *paaY* (Tabla 2, capítulo 2). Esto nos sugirió que el fenilpiruvato podría ser metabolizado mediante esta vía alternativa. Además encontramos que la expresión del promotor *paaGHIJK,* se induce *in vivo* en respuesta a fenilalanina y fenilapiruvato, pero no en presencia de tirosina (Tabla 3, capítulo 2), lo que confirma la existencia de una ruta alternativa en el catabolismo de la fenilalanina en la que el fenilpiruvato es un intermediario (Figura 1, capítulo 2). Sin embargo, las enzimas involucradas en esta ruta alternativa no han sido identificadas. Los genes PP3122/PP3123 y PP3458 que codifican una acyl-CoA transferasa y ligasa respectivamente, son posibles candidatos a ser responsables de la síntesis del fenilacetil-CoA a partir del fenilpiruvato. Los resultados obtenidos a partir del estudio de mutantes sugieren por una parte que el operón PP3122/PP3123 codifica una enzima que transfiere un grupo acyl-CoA al acetoacetato, producto final de la ruta catabólica de la tirosina, para finalmente ser canalizado al ciclo de Krebs, y por otra parte que el mutante en el gen PP3458 no está afectado en el catabolismo de la fenilalanina y de la tirosina (Tabla 4, capítulo 2).

Para identificar el o los genes que participan en la primera reacción en la degradación del fenilpiruvato, llevamos a cabo una mutagénesis al azar en el genoma de *P. putida* KT2440 sobre un mutante *hmgA*, que tiene bloqueada la ruta de la tirosina a nivel del homogentisato, y una posterior selección en un medio en el que el fenilpiruvato era la única fuente de carbono. Ya que el único mutante obtenido fue en un transportador de aminoácidos (PP0946), consideramos la posibilidad de que el fenilpiruvato no se degrade mediante una única vía, dificultando la obtención de mutantes incapaces de degradar el fenilpiruvato mediante esta técnica (Herrera, datos no publicados). Spaepen y colaboradores (2007) han sugerido recientemente que el producto del gen *ipdC* de *Azospirillum brasilense* tiene además de un papel en la síntesis de la fitohormona IAA, actividad fenilpiruvato decarboxilasa, ya que un mutante en este gen muestra una menor síntesis del ácido fenilacético, producto obtenido a partir de la decarboxilación del fenilpiruvato. Además, este mutante presenta un menor crecimiento comparado con la cepa silvestre en un medio donde la

fenilalanina es la única fuente de nitrógeno. Por lo que el producto del gen *ipdC* puede estar involucrado en una transaminación seguida de una decarboxilación de este aminoácio. En *P. putida* KT2440 el gen *ipdC* no presenta esta actividad (Spaepen *et al*., 2007), pero no se puede descartar la existencia de otra enzima que lleve a cabo esta reacción.

La función establecida de la fenilalanina hidroxilasa en organismos eucariotas y procariotas es la de catabolizar la L-fenilalanina como fuente de carbono, sin embargo *P. putida* utiliza este aminoácido aromático únicamente como fuente de nitrogeno. Ésto resulta extraño ya que la primera reacción catabólica de la fenilalanina da lugar a la formación de tirosina, que puede ser utilizada tanto como fuente de carbono como de nitrógeno. Es possible que la fenilalanina proporcione nitrógeno mediante la segunda vía descrita en esta tesis, proporcionando a su vez algún intermediario metabólico que pueda interferir con el catabolismo de la fenilalnina como fuente de carbono. Este deficiente uso de la fenilalanina como fuente de carbono, ha sido previamente observado en *P. aeruginosa*, donde se ha propuesto que el fenilacetato interfiere con el 4-hidroxifenilacetato como un sustrato en el paso de la monooxigenasa del catabolismo de la tirosina (Figura 1, capítulo 2). En nuestro laboratorio el fenilacetato añadido no inhibió el uso de la tirosina como fuente de carbono (M.C. Herrera, datos no publicados).

El regulón PhhR de *P. putida* KT2440

Se han realizado análisis transcriptómicos, mediante microarrays, con objeto de estudiar la respuesta de la cepa silvestre y de la cepa mutante *phhR* en la presencia y ausencia de la fenilalanina. Ésto nos ha permitido definir el regulón PhhR y concluir que éste es un regulador global que controla la expresión de las dos rutas catabólicas de la fenilalanina. Además, PhhR controla la expresión de una bomba de eflujo (MexEF/OprN) que parece ser importante en la homeostasis de este aminoácido aromático y algunos genes involucrados en la biosíntesis de la fenilalanina y la tirosina.

PhhR en *P. putida* KT2440 es un regulador global capaz de activar y reprimir la expresión de determinados genes tanto de forma dependiente como

independiente de la fenilalanina. Estos genes han sido identificados y divididos en 4 grupos, basándonos en el modelo de expresión de los ensayos de arrays. Éstos se han validado usando fusiones '*lacZ* de promotores representantes de cada grupo y haciendo una comparación de la actividad β-galactosidasa en presencia y en ausencia del regulador PhhR. En el primer grupo están incluidos aquellos genes que requieren la presencia del regulador PhhR y de la fenilalanina como efector (PP2595/PP2596, PP3122/PP3123, PP3434/PP3433, PP3458, PP3468, *phhAB* y *hmgABC*), mientras que en el segundo grupo se incluyen genes dependientes de PhhR, pero independientes de la fenilalanina (PP0913, PP2078, PP2646, PP2827, PP3425/PP3526/PP3427 y PP4858). Englobados en otro grupo se han situado a los genes reprimidos por PhhR (PP2520, PP2607/PP2608, PP3067, *phhR*, *aroF* y *paaY*) y un último grupo consta de un conjunto de genes que están inducidos en la presencia de fenilalanina pero cuya expresión está bajo el control de PhhR de forma indirecta, es decir, mediante otro regulador, PaaY, que a su vez, y a través de un mecanismo no definido, controla la expresión del operón *paaGHIJK.* La hipótesis actual es que PhhR reprime el gen *paaY* en la ausencia de fenilalanina, pero probablemente, en la presencia del aminoácido, PhhR se libera del promotor de *paaY* permitiendo que se produzca la activación de la transcripción de este regulador, estimulando éste la expresión del promotor de P_{paaG}. De acuerdo a esta hipótesis se ha observado que los niveles basales del promotor P_{paaG} son más altos en la cepa mutante *phhR*⁻ que en la cepa silvestre y similares a los obtenidos en la cepa silvestre en presencia de fenilalanina (Tabla 4, capítulo 2). Genes reprimidos por PhhR, como *aroF* y PP2607/PP2608, codifican enzimas que participan en la biosíntesis de aminoácidos aromáticos. Ésto sugiere que PhhR podría ejercer un control de tipo *feed-back* a nivel transcripcional en la biosíntesis de aminoácidos aromáticos, como previamente se ha descrito en otros microorganismos (Pittard, 1996).

Nuestros resultados confirmaron un efecto positivo de PhhR sobre la transcripción del operón *phhAB* donde se observó que se requieren dos secuencias aguas arriba definidas por la secuencia consenso 5'-

NNNAAAANTNTNNTTNCG-3', a las que hemos denominado cajas PhhR. Nosotros hemos buscado esta caja PhhR en la secuencia promotora de los genes u operones que pertenecen al regulón, con objeto de identificar aquellos que se encuentran regulados directamente por PhhR. Así mediante un algoritmo basado en la secuencia consenso arriba mencionada (A.J. Molina-Henares y M.C. Herrera, datos no publicados), hemos encontrado 8 operones con al menos una caja PhhR. Existen dos cajas PhhR en los promotores *phhABR*, PP3434/PP3433, que codifican la *p*-hidroxifenilpiruvato dioxigenasa y en el operón PP3122/PP3123 que codifican una acil-CoA transferasa, mientras que en el resto encontramos una única caja (Figura 1). Dos de los genes que presentan una caja PhhR, los genes *paaY* y PP2078, codifican reguladores transcripcionales que pueden regular otros genes cuya expresión varía en presencia de fenilalanina. Otra única caja PhhR se localizó en el promotor del operón que codifica enzimas incluidas en el metabolismo de *p*-hidroxifenilpiruvato a maleilacetoacetato, denominado *hmgABC* (PP4621/4620/4619) y en otro gen que parece codificar una alcohol deshidrogenasa dependiente de zinc (PP2827) (Figura 1) (Tabla 5, capítulo 2).

Ensayos de *footprint*, de EMSA y estudios de microcalorimetría (ITC) han confirmado que PhhR se une específicamente a las cajas encontradas en los promotores de los genes que pertenecen al regulón. Además, ésto ocurre de forma independiente de la fenilalanina, por lo que, probablemente el efecto de este aminoácido aromático sobre el regulador PhhR sea posterior, es decir, una vez que éste se encuentra unido al ADN.

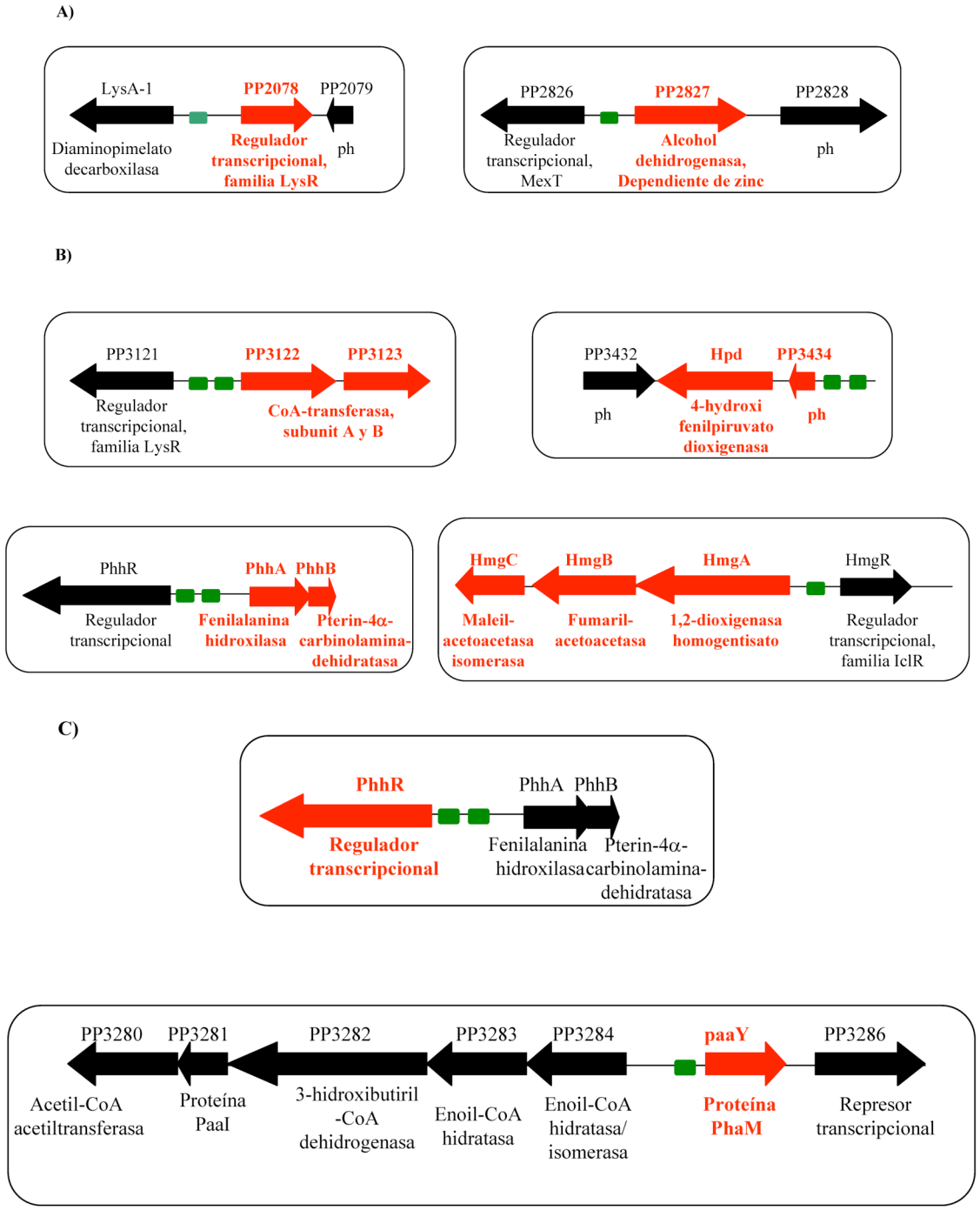

Figura 1. Organización génica de los genes del regulón PhhR regulados directamente por PhhR (marcados en rojo). Presentan al menos una caja PhhR (marcada en verde) tanto genes activados de forma independiente (A) y dependiente de fenilalanina (B) como reprimidos por PhhR (C).

La caracterización termodinámica del reconocimiento de PhhR con cada una de las secuencias diana encontradas en los promotores, reveló que todas las interacciones eran favorables entálpicamente y que la afinidad del regulador variaba entre 30 nM por un sitio de alta afinidad (el promotor *phhA*) y 6600 nM por un sitio de baja afinidad (el promotor PP3434), estando comprendida generalmente entre 500 y 2500 nM.

La técnicas utilizadas en esta búsqueda del regulón PhhR, como los ensayos de microarrays, no incluyen aquellos genes activados o reprimidos por PhhR en presencia de otro aminácido aromático como por ejemplo la tirosina. Ya que sabemos que la tirosina es también un efector del regulador cabría esperar la activación de otros genes inducidos sólo en presencia de este aminoácido. Mediante el estudio *in sílico* de las cajas PhhR en el genoma de la KT2440, se han determinado la presencia de posibles sitios diana de PhhR, por ejemplo en el gen que codifica para la tirosina descarboxilasa (PP2552). Esta enzima descarboxila la tirosina para rendir tiramina, la cual a su vez se desamina para ser finalmente metabolizada. El centro de la caja PhhR encontrada en este promotor se sitúa a 74 pb del ATG del gen. En la región promotora del gen *gltB*, que codifica para la subunidad mayor de la glutamato sintasa, se han encontrado dos cajas cuyos centros se disponen a 44 y a 113 pb del ATG. En el promotor del gen *aroC,* que codifica la corismato sintasa, responsable del último paso de la síntesis del corismato, también se ha encontrado una caja PhhR situada a 108 pb desde su centro hasta el ATG del gen. Todos estos genes podrían pertenecer al regulón PhhR, pero se requieren investigaciones más detalladas para su confirmación.

El regulador PhhR: un peculiar miembro de la familia NtrC

A diferencia de la gran mayoría de los miembros de la familia NtrC, pero al igual que ocurre en HupR y NtrC de *Rhodobacter capsulatus* (Davies *et al*., 2006) y TyrR de *E. coli* (Pittard *et al*., 2005), que también pertenecen a la familia de reguladores NtrC, la proteína PhhR en *P. putida* estimula la transcripción con RNA polimerasa dependiente de σ^{70}. Una serie de resultados, obtenidos en esta tesis doctoral, apoyan estas conclusiones. En primer lugar se

observó que la actividad β-galactosidasa del promotor del gen *phhA* en un mutante σ^{54} de *P. putida*, era similar a la determinada en la cepa silvestre, lo que indica que la expresión del operón *phhAB* en *P. putida* es independiente de σ^{54}. En segundo lugar, la secuencia consenso –24/–12 $GGN_{10}GC$, que es reconocida por la RNA polimerasa dependiente de σ^{54} no se ha encontrado aguas arriba del punto de inicio de la transcripción de ninguno de los genes definidos como miembros del regulón PhhR en *P. putida*; y en tercer lugar, PhhR en *P. putida* exhibe una glicina en lugar de un aspartato en el dominio Walker el cual participa en la hidrólisis del ATP, imprescindible en la oligomerización de estas proteínas e interacciones con σ^{54}. Cabe mencionar que la presencia de una variante del motivo GAFTGA, como puede ser la secuencia GAFEGA, presente en PhhR, puede provocar una interrupción en la interacción con σ^{54} como ocurre en PspF (Canon *et al*., 2000).

Excluyendo las *Pseudomonáceas*, el homólolgo más próximo de la proteína PhhR de *P. putida*, es TyrR de *E. coli* y *Haemophilus influenzae* (Pittard *et al*., 2005; Yang *et al*., 1993 y MacPherson *et al*., 1999). Tanto PhhR como TyrR, regulan el metabolismo de la fenilalanina y la tirosina, sin embargo mientras PhhR es un regulador global encargado de activar el catabolismo de estos aminoácidos, activando la degradación e inhibiendo la síntesis, TyrR, en *E. coli*, solamente activa la transcripción de enzimas que participan en la síntesis. PhhR es un homodímero en solución que puede funcionar como activador o como represor. La secuencia consenso reconocida por PhhR es similar a las denominadas cajas fuertes y débiles de TyrR (Pittard *et al*., 2005 y Andrews *et al*., 1991), pero a diferencia de lo que ocurre en TyrR de *E. coli*, que no reconoce las cajas débiles si no es en presencia de algún aminoácido aromático o de otra caja fuerte, PhhR se une a todas las cajas con mayor o menor afinidad pero en ausencia de los aminoácidos aromáticos (Tabla 2, capítulo 3). Por lo tanto no consideramos la necesidad de clasificar las cajas como fuertes o débiles, sólo en función de la afinidad que tenga por ellas, ya que ésto no determina el grado de expresión del gen.

PhhR como activador

PhhR activa la transcripción de casi el total de genes que participan en las rutas catabólicas de la fenilalanina y la tirosina, asegurándose la funcionalidad de estas rutas en presencia de los aminoácidos aromáticos. Además, activa la transcripción de un regulador de la familia LysR y una alcohol deshidrogenasa, independientemente de la fenilalanina. Por lo tanto, a diferencia de lo que ocurre en TyrR de *E. coli*, que es capaz de activar la transcripción solamente en respuesta a fenilalanina, tirosina o triptófano (Andrews *et al*., 1991; Argaet *et al*., 1994 y Lawley and Pittard, 1994), PhhR, permite la transcripción tanto en la presencia como en la ausencia de la fenilalanina. Como consecuencia, el mecanismo por el cual activa la transcripción en cada uno de los casos tiene que ser diferente. Nosotros proponemos además que esta activación viene dada por el número y la posición de las cajas PhhR con respecto al punto de inicio de la transcripción en cada promotor. Nuestros resultados sugieren que es un requisito imprescindible la presencia de dos cajas PhhR en aquellos promotores que son dependientes de fenilalanina, excepto en el promotor del operón *hmgABC*, cuya actividad β-galactosidasa es casi 5 veces superior en la presencia de fenilalanina con respecto a su ausencia (Tabla 4, capítulo 2). El promotor del gen *phhA* no es funcional cuando uno o dos de los sitios de unión identificados era eliminado o mutado por mutagénesis dirigida, lo cual indica que la unión a ambos sitios es *sine qua non* para la activación de la transcripción en presencia de la fenilalanina (Figura 2C, capítulo 1). Posiblemente esto mismo ocurra para el resto de los promotores que presentan dos cajas PhhR. La distancia que existe entre las dos cajas de un mismo promotor en estos sistemas oscila entre 51 y 58 pb. Cuando PhhR activa la transcripción de forma independiente a la fenilalanina, como en el caso de los genes PP2078 y PP2827, sólo se ha encontrado la presencia de una caja PhhR en cada promotor.

La inducción del operón *hmgABC* está también regulada por PhhR y es dependiente de fenilalanina. Sin embargo, Arias-Barrau y colaboradores (2004) observaron que los genes catabólicos del homogentisato se inducen cuando *P. putida* se crece en este intermediario de la ruta catabólica de la fenilalanina y

tirosina. En un principio se puede pensar que la inducción de los genes *hmgABC* se deba a la síntesis de homogentisato a partir de fenilalanina, sin embargo, el hecho de que se mantenga la expresión de este promotor en un mutante *hpd*⁻ sugirió que el efecto no podía ser atribuido a la síntesis interna del homogentisato a partir de la fenilalanina. Estudios previos revelaron que la expresión de este operón estaba mediado por el producto del gen *hmgR* (Arias-Barrau *et al*, 2004), el cual reconoce una secuencia en el promotor P_{hmg} que se extiende desde la posición -16 a la posición -29 con respecto al punto de inicio de la transcripción donde se encontró una perfecta secuencia palindrómica (5′-TCGTAATCTGATTACGA-3′). La caja identificada en nuestro estudio, solapa con este sitio y cubre desde la posición -18 hasta la -1. Dada la naturaleza solapante de los sitios, nuestra hipótesis es que PhhR puede inhibir la unión de HmgR a su sitio diana y facilitar el reclutamiento de la RNA polimerasa al promotor. Usando la fusión *hmgA*:*lacZ* encontramos que PhhR estimula la transcripción de *hmgA* en respuesta a fenilalanina, tirosina, y *p*-hidroxifenilpiruvato, pero no a homogentisato, indicando que el operón *hmg* responde a intermediarios por encima de la ruta, mediante el control de dos reguladores, HmgR y PhhR. En un fondo *phhR*⁻ la expresión del promotor de *hmgA* todavía incrementa en presencia de homogentisato, pero no en presencia de 4-hidroxifenilpiruvato, lo que confirma el control dual de esta ruta (M.C. Herrera, no publicado).

Además de la fenilalanina, la tirosina puede actuar como efector cuando PhhR regula las rutas catabólicas de estos dos aminoácidos, a diferencia de lo que ocurre con el triptófano. En casi todos los promotores estudiados donde la fenilalanina actúa como inductor, la presencia de tirosina también induce la expresión, excepto en el caso de los genes *phhR*, *paaY* del operón *paaGHIJK*. Ésto sugiere que PhhR es capaz de distinguir entre fenilalanina y tirosina, como ocurre en TyrR (Pittard, 2005) e inducir o reprimir genes de diferente manera en presencia de uno u otro. PhhR permite la activación de la segunda ruta catabólica, vía fenilpiruvato, en presencia de fenilalanina, pero no en presencia de tirosina, por lo que parece que esta vía es exclusiva de la fenilanina. Otra diferencia entre ambos aminácidos aromáticos es que en el promotor del

operón PP3122/PP3123, la activación de la fenilalanina es totalmente dependiente de PhhR, mientras que la tirosina es capaz de mantener la activación en ausencia de PhhR, aunque en menor grado, sugiriendo un segundo regulador en el sistema.

La interacción entre la RNA polimerasa y los activadores viene marcada por la disposición en la que se unen al promotor, por lo que la distancia que existe entre ellas una vez unidas al ADN es relevante. Se ha estudiado en TyrR de *E. coli*, que el α-CTD de la RNA polimerasa y el dominio N-terminal del regulador, poseen conectores de unión flexibles, de modo que facilitan un amplio rango de posibilidades de interacción entre ambas proteínas estando a diferentes distancia (Pittard *et al*., 2005). Probablemente PhhR sea capaz de interaccionar con la subunidad α de la ARN polimerasa desde diferentes posiciones, aunque exista una disposición óptima. En *E. coli* se ha observado que la activación transcripcional mediada por TyrR, es óptima cuando los centros de las cajas que reconoce el regulador y la caja -35 del promotor están separados por una distancia de 31 ó 42 pb. En *P. putida* hemos obtenido la mayor actividad mediada por PhhR en el promotor del gen PP2827 (próximo a 5000 UM), cuya distancia entre ambas cajas correponde a 31 pb. Para el resto de los promotores activados por PhhR, esta distancia se encuentra por encima incluso de 42 pb, lo que da lugar a una activación transcripcional inferior (Tabla 3, capítulo 2). Nuestros resultados sugieren que PhhR ocupa diferentes posiciones en cada promotor (desde -40 hasta -277), por lo que la interacción con la RNA polimerasa debe ser también diferente, de manera que contacta a través del α-CTD o a través de la región 4 de σ^{70} o de ambos (Pittard *et al*., 2005 y Busby *et al*., 1994). De esta forma PhhR actuaría tanto como un regulador de clase I como de clase II. En aquellos promotores en los que PhhR se une por encima de -75 pb, se ha observado la participaión de IHF, que permite que se produzca un plegamiento en el ADN facilitando que PhhR interaccione con la RNA polimerasa, de manera similar a lo que ocurre en los activadores dependientes de σ^{54}.

La función de PhhR como activador del catabolismo de la fenilalanina y de la tirosina puede consistir en asegurar la completa degradación de estos

aminoácidos aromáticos hasta intermediarios del metabolismo central. Una regulación postranscripcional sobre las enzimas catabólicas, puede desviar de la ruta a los sustratos intermediarios hacia la síntesis de otros compuestos. Es de esperar que la actividad enzimática de la fenilalanina hidroxilasa, por ejemplo, no sea igual que la Hpd o la Hmg, ya que la tirosina es un aminoácido utilizado también como precursor a partir del cual se sintetizan otros compuestos necesarios para la bacteria e incluso para la síntesis de proteínas. PhhR puede activar el catabolismo de la fenilalanina y la tirosina desde diferentes puntos a lo largo de las rutas, con objeto de que todas las enzimas necesarias para metabolizar completamente estos aminoácidos aromáticos se sintetizen en presencia de los aminoácidos, pero además reguladas de forma diferente ya que los operones a partir de los cuales se codifican están agrupados.

PhhR como represor

Hemos comprobado que PhhR de *P. putida* KT2440 actúa como un represor tanto de forma dependiente de fenilalanina, sobre el gen *paaY*, como independiente de la fenilalanina, sobre el gen *aroE-2*, *aroF*, el PP3067 y el propio gen *phhR*. En *E. coli*, TyrR actúa como un represor formando un hexámero en la presencia de tirosina y ATP, uniéndose tanto a las cajas fuertes como débiles y provocando un lazo entre ellas, lo cual impide que la RNA polimerasa contacte con el promotor (Bailey *et al*., 1996). Sin embargo, estudios de filtración en gel con PhhR en la presencia de fenilalanina y tirosina con y sin ATP han revelado que la estructura cuaternaria predominante de este regulador es un dímero, a pesar de la presencia de aminoácidos aromáticos y de ATP. Además, PhhR *in vitro* no se une al ATP (Herrera, datos no publicados). Por otro lado hemos observado que este estado oligomérico no se encuentra bajo la influencia de la naturaleza del promotor al que se une, ya que no se modifica en presencia de ADN (Figura 1C, capítulo 3), tanto con un promotor cuyo operón es activado (PP3122/PP3123), como con un promotor de un gen reprimido (PP3285). Por lo tanto parece que el mecanismo mediante el

cual PhhR reprime ciertos promotores del regulón, es diferente del mecanismo utilizado por TyrR.

En *P. putida* KT2440, PhhR reprime la transcripción uniéndose al promotor e impidiendo que la RNA polimerasa lleve a cabo la transcripción, ya que las cajas PhhR reconocidas por el regulador se localizan próximas al +1 de los genes *phhR* (+16) y *paaY* (+10) (Figura 4, capítulo 3). De esta forma PhhR unida al AND impide una correcta activación de la transcripción por parte de la RNA polimerasa. En ambos casos también se ha observado un papel represor de la proteína IHF, que compite con la RNA polimerasa por los sitios de unión en el promotor (Figura 4, capítulo 3).

En el caso del gen *phhR*, nuestros resultados actuales apoyan la posibilidad de que PhhR controle modestamente su propia expresión. Pensamos que esta represión se debe a la competición que existe entre la RNA polimerasa y la proteína PhhR por el mismo sitio de unión ya que la región -35 del promotor de *phhR* y la caja PhhR solapan. A partir de los ensayos *in vivo* sobre el promotor del gen *phhR* hemos obtenido un resultado inesperado ya que obsrevamos que la expresión se reprime 3 veces en presencia de la fenilalanina. PhhR reconoce la caja proximal y distal de la región intergénica *phhA* y *phhR* situándose de forma que permite la transcripción del operón *phhAB* y a su vez reprime su propia transcipción. La presencia de fenilalanina induce la activación del operón *phhAB* probablemente favoreciendo la estabilidad en el tiempo de la unión del regulador a las cajas PhhR, lo que provoca de forma simultánea una mayor represión del gen *phhR*. En ausencia de PhhR y de fenilalanina la RNA polimerasa se une preferentemente al promotor de *phhR* (Figura 6C, capítulo 1), como era de esperar ya que las cajas -10/-35 del este gen, están bastantes más conservadas que en el operón *phhAB*, permitiendo una expresión basal de este promotor. En presencia del regulador y de la fenilalanina, la RNA polimerasa se asienta sobre el promotor del operón *phhAB* para comenzar la transcripción de éste, secuestrando la RNA polimerasa ya que ésta se encuentra en cantidades limitantes y quedando en desventaja en la competición con PhhR por su propio promotor, lo que se traduce en una menor síntesis del regulador. Así se mantiene un equilibrio en el

resultado final de la síntesis de PhhR ya que una menor concentración del regulador en la célula disminuye la transcripción del operón *phhAB* permitiendo de nuevo una expresión basal de *phhR*.

VI. Conclusiones

Existen al menos dos rutas catabólicas de la fenilalanina en *P. putida* KT2440. Una en la que este aminoácido se hidroxila para dar lugar a la tirosina y otra en la que la fenilalania es deaminada para formar el fenilpiruvato.

PhhR es un regulador global que actúa tanto como activador como represor conformando el denominado regulón PhhR. Este regulador dual pertenece a la familia de proteínas NtrC pero a diferencia de éstas, activa la transcripción mediante la RNA polimerasa dependiente de σ^{70}.

PhhR activa y reprime los genes necesarios para la degradación de la fenilalanina y la tirosina mediante ambas rutas. También reprime genes involucrados en la biosíntesis de los aminoácidos aromáticos además de modular el nivel de expresión de las bombas de eflujo MexEF/Operón y otras proteínas que pueden participar en la homeostasis de la fenilalanina.

PhhR reconoce la denominada caja PhhR en los promotores de algunos genes del regulón. La secuencia consenso de esta caja es 5´- T**GTAAA**GA**T**A**G**T**TTTAC**A**-3´.**

Los promotores dependientes de PhhR y de fenilalanina requieren la presencia de dos cajas PhhR en la secuencia promotora para su

activación, mientras que los promotores que poseen una única caja son dependientes de PhhR, pero independientes de fenilalanina.

El estado oligomérico del regulador PhhR es predominantemente dimérico tanto en presencia como en ausencia de aminoácidos aromáticos, de ATP y ADP. Tampoco se encuentra bajo la influencia de la naturaleza del promotor al que regula.

PhhR activa la transcripción de genes tanto en ausencia como en presencia de aminoácidos aromáticos actuando tanto como un regulador de clase I como de clase II y actúa como represor compitiendo por el mismo sitio con la RNA polimerasa.

VII. Material suplementario

FÉ DE ERRATAS

Capítulo 1

Figura 3; a) N_{17} en lugar de N_{18}

Figura 4; a) La caja -35 del promotor del gen *phhR* es GTAAAA en lugar de TGTAAA

Figura 6; b) La caja -35 del promotor del gen *phhR* es GTAAAA en lugar de TGTAAA

Figura 6; b) Las cajas -10 y -35 del gen *phhA* están señaladas al revés, es decir la que indica -10 es la -35 y viceversa.

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