Control catabólico y factores genéticos implicados en la respuesta de *Pseudomonas putida* KT2440 a situaciones ambientales

Memoria presentada por la licenciada en
Ciencias Biológicas, Isabel Aranda Olmedo
para aspirar al Título de Doctora

Fdo. Isabel Aranda Olmedo

Vº Bº
Los directores

Fdo. Juan Luís Ramos Martín
Doctor en Biología
Profesor de investigación del CSIC

Fdo. Silvia Marqués Martín
Doctora en Biología
Científica titular del CSIC

Universidad de Granada
2006

Editor: Editorial de la Universidad de Granada
Autor: Isabel Aranda Olmedo
D.L.: Gr. 1408 - 2006
ISBN: 978-84-338-4041-7

Esta Tesis Doctoral ha sido realizada en la Unidad Estructural
de Bioquímica y Biología Molecular y Celular de Plantas
de la Estación Experimental del Zaidín. CSIC, Granada

A mi familia

A mi comunidad

... es preferible tener poca cultura e ingenio con humildad, que poseer grandes tesoros de doctrina con vana suficiencia.

TOMÁS H. DE KEMPIS

AGRADECIMIENTOS

El trabajo que aquí se presenta nunca hubiera existido sin la ayuda de todos aquellos que me han arropado durante estos años. Quiero agradecer a mis directores de tesis su esfuerzo y su disponibilidad. Al Profesor Juan Luís Ramos le agradeceré siempre el haberme incorporado a su tripulación (¡que sigue creciendo!). He ganado mucho gracias a aquella decisión. Gracias por abrirme puertas. A la Dra. Silvia Marqués le agradezco el haberme acompañado en esta "gymkhana" agotadora, de la que supongo que con el tiempo nos recuperaremos. Gracias por gastarte por mí. Te deseo éxito en tus nuevos proyectos. A mi tutor el Dr. Juan Pedro Martínez y a las Dras. Pepi Cabrero y Lola López León del Dpto. de Genética, gracias por iniciarme en la investigación. Fueron buenos tiempos que recuerdo con cariño. A Mai, Carmen e Inés, gracias por encargarse del papeleo que todo esto conlleva.

A mis "hermanitos" del 2.6. ("las Patris", M. y D., y "los niños", Javier, Alejandro y Pedro), gracias por aguantar tantas veces mi carácter "granaíno". Cuidad a los nuevos fichajes y conservad algún bote que ponga "Miao", para sentir que aun sigo por ahí. Gracias a Euge (mi "amiga") y a Mariam S. por las tertulias y por las cenas. Ya podéis ir organizando la próxima. Gracias a Patri B., por tener siempre "glucosa" en su mesa (las barritas de regaliz, las mejores). Gracias a Ana H. por quitarle las "enes" a mis secuencias y a Raquel T. por ayudarme a descifrarlas. Gracias a Vanina por contrarrestar mi estrés con ese toque argentino. Gracias a Jesús L., Antonio Jesús y Miguel por la cara de felicidad que llevan siempre puesta (me habéis alegrado los días). Gracias a Ana S. por tanto ir y venir, a Chechu y M. Trini por ayudarme con su experiencia, a Carmen H., Tesi y Amalia por el punto de fiesta que le dan al día a día, a Andreas por llegar a los "kits" de arriba (en este mundo de altos), a Patri G. y Nuria H. por acogerme en el "lab del TNT" cuando me daba mono de Mac. Gracias a Estrella por su apoyo y por tanto vale de fósforo que me ha firmado. Gracias a M. Angustias por autoclavar los biberones de 5 L para mis bichos y por los ratos de risas en la cocina (habrá que escribir ese libro). Gracias a Manolo y a Maribel, los dos me habéis tratado siempre con cariño y os lo agradezco. Y a María T., por ser tan sencilla. Gracias a Toñi, a Jesús de la Torre y a Marichu por compartir conmigo sus "soluciones". Gracias a los más "guiris" Dietmar, Pieter, Rolf y Tino, por los matices que le dais al andaluz. Y a Jesús M., por ser un amigo.

Gracias a los que me precedieron, Lázaro, Maxi, Susana, Mar, Raquel R., Miguel A., Gilberto, M. Carmen R., Antonia, Wilson, Olga, Nene, Manolo M., por hacerme ver que es posible, y en especial a Marian Ll., Antonio C., Abraham y Carlota. Hemos vivido buenos momentos. Gracias por el empujón de última hora.

Gracias a los "ñiños y ñiñas" de seguridad, limpieza, compras, administración, centralita, biblioteca, informática y mantenimiento, por cuidar de los "precarios".

Gracias a mi familia, en especial a mis padres, a mi hermana y a mi cuñaillo y, por supuesto, a la alegría de la casa, mi sobrina Rocío, por ayudarme a no reducir mi vida al trabajo. Gracias a mi comunidad, por la oración y por soportar mis "ya no puedo más". Esta tesis es tan vuestra como mía. ¡A ver si os la leéis!

Gracias a Dios por el misterio de todo lo creado. Gracias por el detalle con el que diseñaste la vida (¡en especial por el "tinglado" de la represión catabólica!) y gracias por regalarme la posibilidad de estudiarla.

(Gracias a todos los que no he nombrado, por no molestarse).

ÍNDICE GENERAL

I. APÉNDICES

I.1. ABREVIATURAS

Ap	ampicilina
Cm	cloranfenicol
αCTD	extremo carboxilo de la subunidad α de la RNAP
C-terminal	extremo carboxilo
D	Tasa de dilución
Eσ^{54}	RNAP asociada a σ^{54}
Gm	gentamicina
HTH	hélice-giro-hélice
Kb	Kilobase(s)
Km	kanamicina
LB	Luria-Bertani
MU	unidades Miller
N-terminal	extremo amino
ORF	marco abierto de lectura
PAS	dominio sensor de ciertas proteínas (*PER-ARNT-SIM*)
pb *o* **bp**	par(es) de bases
ppGpp	guanosina 3',5'-bipirofosfato
pppGpp	guanosina 3'-difosfato, 5'-trifosfato
(p)ppGpp	ppGpp + pppGpp
PTS	sistema de fosfotransferasas
REP	secuencias repetidas extragénicas palindrómicas
RNAPσ^{38}	RNAP asociada a σ^{38}
RNAPσ^{54}	RNAP asociada a σ^{54}
RNAPσ^{70}	RNAP asociada a σ^{70}
RT-AMV	transcriptasa reversa del virus de la mieloblastosis de ave
RT-PCR	transcripción reversa-reacción en cadena de la polimerasa
Sm	estreptomicina
Tc	tetraciclina
UAS	*upstream activator sequence*
μ_{max}	tasa máxima de crecimiento
X-gal	5-bromo-4-cloro-3-indolil-β-D-galactopiranósido
YE	extracto de levadura

I.2. ÍNDICE DE FIGURAS

En la Introducción General: Página

En Resultados:

Capítulo 1

Capítulo 2

Página

Capítulo 3

Capítulo 4

I.3. ÍNDICE DE TABLAS

II. INTRODUCCIÓN GENERAL

Los miembros del género *Pseudomonas* son bacterias Gram negativas pertenecientes al grupo de las γ-proteobacterias, conocidas por su amplia distribución en ambientes naturales y por su extraordinaria flexibilidad metabólica. En los nichos que ocupan, las especies de *Pseudomonas* están expuestas a estímulos muy diversos provocados por variaciones en los parámetros medioambientales y biológicos. La adaptabilidad a estos cambios exige un sistema sofisticado de transducción de señales, que permita detectar de forma continua cambios en el ambiente y una respuesta rápida por parte de la célula. La adaptación fisiológica se consigue en gran medida controlando la expresión de diferentes grupos de genes bajo diferentes circunstancias (44) .

El contacto de estos microorganismos, durante períodos largos de tiempo, con determinados compuestos aromáticos de su entorno ha favorecido el desarrollo de una serie de rutas catabólicas para la degradación de una gama amplia de sustratos (en ocasiones tóxicos) que convergen en un número limitado de intermediarios comunes. El procesamiento subsiguiente de estos compuestos intermediarios a través de rutas centrales del metabolismo celular permite su incorporación al ciclo de Krebs (85). Los elementos genéticos móviles han jugado un papel importante en la adquisición de estas rutas catabólicas. Su localización en plásmidos, transposones o elementos cromosómicos con movilidad intercelular ha favorecido la transferencia de genes catabólicos entre diferentes cepas y ha permitido el que se encuentren ampliamente distribuidas en procariotas (131, 206).

II.1. El plásmido pWW0. Ruta TOL de degradación de tolueno.

Uno de los plásmidos catabólicos bacterianos mejor caracterizados es el plásmido pWW0 de *Pseudomonas putida* mt-2, que dispone de los genes necesarios para el catabolismo aerobio de tolueno y xilenos (rutas TOL). Tiene un tamaño de aproximadamente 117 Kb, pertenece al grupo de incompatibilidad IncP-9, es autotransmisible y de bajo espectro de huésped (69). La cepa KT2440 es una bacteria derivada de *P. putida* mt-2 que no porta el plásmido pWW0 (126).

Los genes de la ruta TOL codificada en el plásmido pWW0 se organizan en dos bloques diferenciados que se transcriben en la misma dirección (Figura 1). Estos dos segmentos son conocidos como operón superior (*upper*), que incluye los genes *xylUWCMABN*, y operón inferior (*lower*) o *meta*, con los genes *xylXYZLTEGFJQKIH*. Los operones *upper* y *meta* están separados entre sí por un fragmento de unos 11 Kb de DNA que contiene 14 marcos de lectura abierta (ORFs). Además, próximos al extremo 3' de la ruta inferior se encuentran *xylR* y *xylS*, dos genes divergentes entre sí que codifican los reguladores de la ruta TOL (69).

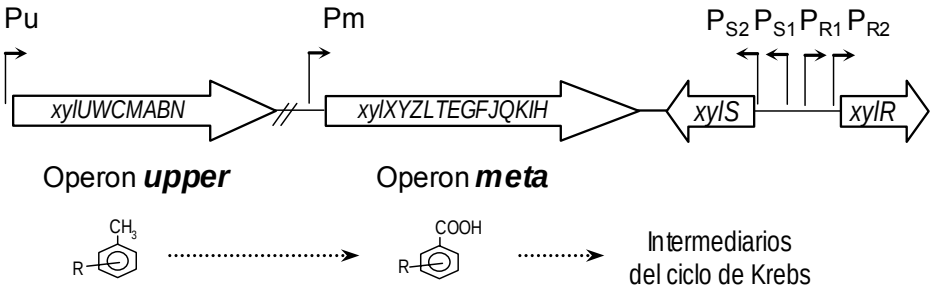

Figura 1. Esquema de la ruta de degradación de tolueno de pWW0. Están representados los dos conjuntos de genes que constituyen la ruta y sus reguladores. En la parte superior se marcan con flechas los promotores de las cuatro unidades transcripcionales.

El grupo metilo del tolueno se oxida de forma secuencial hasta benzoato por acción de las enzimas que codifica el operón *upper*. La primera enzima de este operón es una monooxigenasa que oxida el grupo metilo del tolueno para dar alcohol benzílico. La oxidación posterior de la cadena lateral se lleva a cabo por dos deshidrogenasas, que transforman el alcohol benzílico en benzaldehido, y éste en benzoato. El anillo del benzoato es entonces activado por incorporación de dos sustituyentes hidroxilo y abierto en posición *meta* (adyacente a los grupos hidroxilo) por acción de una dioxigenasa extradiol codificada en el operón *meta*. Este tipo de rotura, en contraposición a la rotura en *orto* (entre los carbonos que portan los grupos hidroxilo), caracteriza a este segmento de la ruta y le confiere el nombre (172).

La región catabólica de pWW0 se encuentra dentro del transposón Tn*4651* (de 56 Kb) que a su vez está incluido en un transposón de mayor tamaño llamado Tn*4653* (de 78 Kb) (Figura 2). Ambos transposones tienen su propia transposasa funcional (codificada por el gen *tnpA*) pero comparten los genes de resolución (*tnpT*, *tnpS* y *res*). A esto hay que sumar la identificación dentro de Tn*4651* de una secuencia de inserción, conocida como IS*Ppu*12, que también presenta una transposasa funcional. Es probable que el núcleo del plásmido original (con las funciones de replicación, herencia estable y conjugación) incorporara en primer lugar el precursor del Tn*4653*, seguido por la inserción dentro de éste del precursor del Tn*4651* y las posteriores incorporaciones de IS*Ppu*12 y la región catabólica con los genes *xyl* (202, 203, 216).

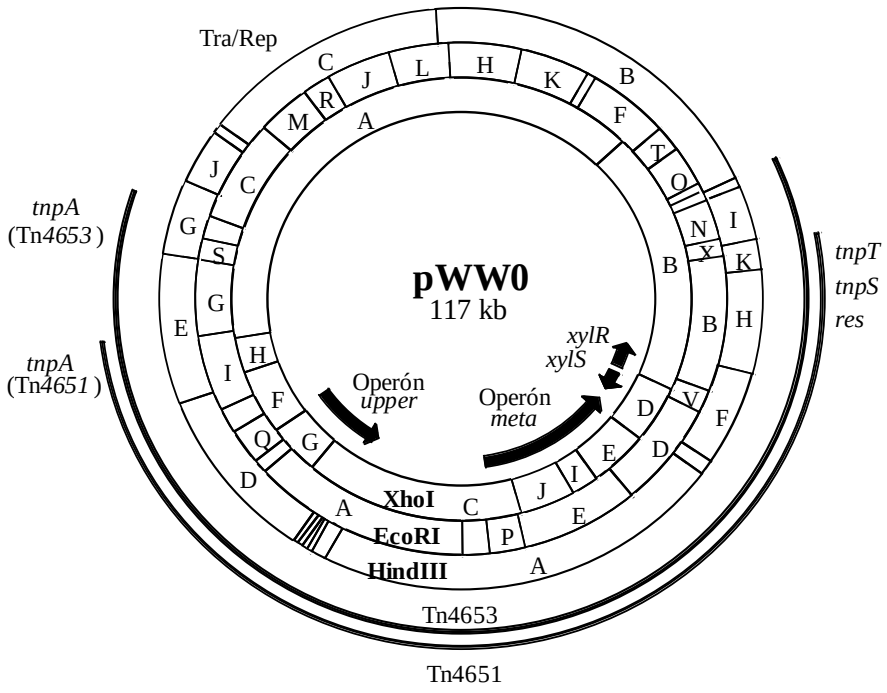

Figura 2: Mapa de restricción de pWW0. Se representan los sitios de corte para las enzimas *Xho*I, *Eco*RI y *Hin*dIII. Los fragmentos se nombran por orden alfabético en orden decreciente de acuerdo con su tamaño. Las flechas indican las regiones que contienen los operones *upper* y *meta*, así como los genes reguladores *xylS* y *xylR*, y su sentido indica el sentido de la transcripción. Se muestra la localización de los transposones Tn*4651* y Tn*4653*, así como los genes que codifican las transposasas (*tnpA*) específicas de cada transposón, y resolvasas (*tnpS* y *tnpT*) y el sitio res de Tn*4651*, común para ambos transposones. También se indica la posición de las regiones de transferencia (Tra) y replicación (Rep) del plásmido.

II.1.1. Elementos de la ruta TOL de degradación de tolueno de pWW0.

Los genes *xyl* están organizados en cuatro unidades transcripcionales (Figura 1). El promotor Pu dirige la transcripción de los genes de la ruta *upper*. Los genes de la ruta *meta* se transcriben desde el promotor *Pm*. Los genes reguladores *xylS* y *xylR* presentan cada uno dos sitios de inicio de la transcripción, los promotores P_{S1} y P_{S2} y los promotores P_{R1} y P_{R2}, respectivamente (157).

II.1.1.1. Estructura de los promotores que controlan la expresión de la ruta TOL

El promotor Pu del operón *upper* es un promotor dependiente del factor sigma alternativo σ^{54} y muestra la organización típica de esta clase de promotores (Figura 3A) (41): i) una secuencia del tipo GG-N_{10}-GC en posición -12/-24, para la unión de la RNA polimerasa asociada a σ^{54} (RNAPσ^{54}). ii) secuencias características UAS (de *upstream activator sequence*) que son dos palíndromos imperfectos para la unión de un activador a distancia, facilitando así un aumento de la concentración local del regulador. Las secuencias UAS pueden estimular la transcripción con relativa independencia de su distancia al sitio de unión de la RNAP y de la orientación que presenten (99, 190). En el caso de Pu el activador es XylR (el producto del gen *xylR*) y

las UAS (conocidas como proximal y distal, en función de la distancia al promotor) se localizan entre las posiciones -176 a -170 y -138 a -122 con respecto al sitio de inicio de la transcripción (76). Recientemente se ha observado que además de ser sitios de unión de XylR, las UAS evitan la transcripción de los genes *xyl* desde promotores situados por delante del operón *upper* (209). iii) un sitio característico de unión para la proteína IHF (de *integration host factor*) (1, 32). En los promotores dependientes de σ^{54} la secuencia diana de IHF se suele encontrar entre el sitio de unión de la RNAPσ^{54} (-12/-24) y el sitio de unión del activador (UAS). La unión de IHF produce una curvatura del DNA que es principalmente de naturaleza plana (192, 224) y que conduce a un acercamiento entre el activador y la RNAP unidos a sus sitios en el DNA. El sitio de unión de IHF en el promotor Pu se encuentra en la posición -52 a -79 con respecto al sitio de inicio de la transcripción y se dispone en la misma cara del DNA que el sitio proximal de unión de XylR. IHF puede dejar de ser un requerimiento para la activación de Pu si su sitio de unión en el DNA es reemplazado por una secuencia de curvatura intrínseca (147). En los últimos años se ha avanzado considerablemente en el conocimiento del funcionamiento de los promotores dependientes de σ^{54}, especialmente gracias a la obtención de cristales y a la resolución de la estructura de las proteínas implicadas. El modelo propuesto más reciente, basado en la estructura de PspF y en estudios con promotores quiméricos derivados de *glnA*p2 sugiere que la RNAPσ^{54} y el activador entrarían en contacto con la zona de inicio de la transcripción desde caras opuestas del DNA, de tal forma que éste quedaría cubierto por ambas proteínas (82, 161).

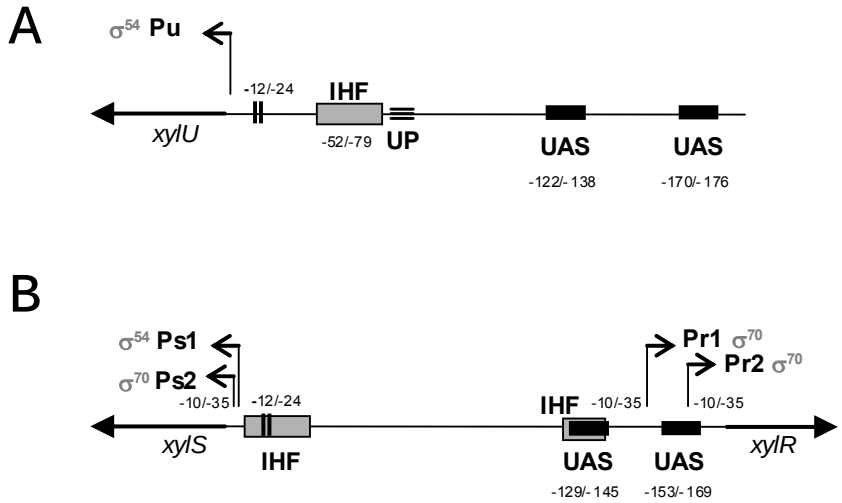

Figura 3. Esquema de las regiones promotoras de Pu (A) y P_S/P_R (B). Están representados los sitios de unión de las diferentes proteínas implicadas en la transcripción desde estos promotores (IHF, XylR, RNAP). Junto a cada promotor se indica el factor sigma del que depende la transcripción.

Además, en el promotor Pu, por encima del sitio de unión para IHF, existe una secuencia similar a la de los elementos UP de *Escherichia coli*, que interaccionan con el extremo carboxilo de la subunidad α de la RNAP para estimular la transcripción. Si IHF está presente, la curvatura del DNA permite el contacto con las dos subunidades α de la RNAP (102).

La transcripción *in vivo* del promotor Pm del operón *meta* está mediada por dos RNA polimerasas (RNAPs) diferentes, en función de la fase de crecimiento (Figura 4). En fase exponencial temprana, se requiere una RNAP asociada al factor σ^{32}, mientras que en fase de crecimiento exponencial tardía y en fase estacionaria se requiere σ^{38}. Es importante resaltar que el punto de inicio de la transcripción (+1) es el mismo en ambos casos (108). La secuencia de reconocimiento para σ^{32} en *Pm* ocupa las posiciones -18 a -7 con respecto al punto de inicio de la transcripción. Las posiciones fundamentales para la transcripción con la RNAP asociada a este factor sigma son la -7 (T), -9 (G), -11 (A), -12 (T), y -14 a -17 (C). La posición -10 es clave para el reconocimiento del promotor por la RNAP asociada a σ^{38}. Sin embargo, la transcripción asistida por σ^{38} es poco eficiente comparada con la llevada a cabo con la participación de σ^{32} (42). El regulador XylS se une a Pm en dos repeticiones directas situadas entre las posiciones -69 a -55 y -48 a -34 por encima del punto de inicio de la transcripción, solapando por tanto en uno de los casos con una base del sitio de unión de la RNAP (65, 88). Este solapamiento parece ser crítico para la transcripción. Por otra parte, las posiciones -48 a -45, -58, -59- -61 y -69 del promotor son necesarias para la interacción XylS/*Pm* (54, 65, 92).

La región intergénica de 300 pb existente entre los genes reguladores *xylS* y *xylR*, contiene cuatro promotores (Figura 3B): los dos promotores dependientes de σ^{70} de *xylR*, P_{R1} y P_{R2}, y divergentes a éstos los promotores de *xylS* P_{S1} y P_{S2}, dependientes de σ^{54} y de σ^{70} respectivamente . El promotor P_{S1} presenta las características de los promotores transcritos por la RNAPσ^{54} descritas previamente para el promotor del operón *upper*, Pu. El activador de P_{S1} es la proteína XylR. En P_{S1} las UAS, que se encuentran de la posición -145 a la -129 y de la -169 a la -153 respecto al punto de inicio de la transcripción solapan con los sitios de unión de la RNAP para *xylR* (76). Por otra parte, hay dos sitios a los que la proteína IHF se une con diferente afinidad, uno que solapa con el sitio de unión de la RNAPσ^{54} (-12/-24) y otro que solapa con una de las UAS (Figura 3B) (79).

II.1.1.2. La proteína reguladora XylR.

La proteína XylR, producto del gen *xylR*, actúa como activador de los promotores dependientes de σ^{54} de la ruta TOL. Pertenece a la familia NtrC de reguladores transcripcionales (185) y presenta cuatro dominios característicos: el dominio A, en el extremo N-terminal de la proteína, es el más variable entre los distintos miembros de la familia y es responsable de la interacción con el efector aromático para activación de la proteína (33, 35, 52, 140, 142); el dominio B o eslabón Q (*linker* Q), un dominio corto (20 residuos) con estructura de *coiled-coil* que une los dominios A y C, que contribuye al mantenimiento de una estructura adecuada para que el dominio A reconozca al efector y que parece estar implicado en la

oligomerización de XylR. Cambios en los residuos que componen este dominio provocan alteraciones en el bolsillo de reconocimiento del dominio A y modifican el perfil de moléculas efectoras (56, 132, 218); el dominio C, de unión e hidrólisis de ATP, es el más conservado entre los miembros de la familia y contacta con el factor sigma de la RNAP; y el dominio D, en el extremo C-terminal de la proteína, con un motivo HTH (hélice-giro-hélice) de unión al DNA en las UAS (145). En la actualidad no se dispone de cristales de la proteína XylR debido a su insolubilidad y a la dificultad para obtener la concentración necesaria de proteína soluble. Para tratar de explicar su modo de actuación, se ha predicho la estructura de XylR basándose en datos experimentales y en semejanzas con miembros de la familia. Según estas predicciones, el dominio N-terminal de XylR (y de su homólogo DmpR) constaría de 8 hélices α y 7 cadenas β y el extremo carboxilo de la hélice α7 podría ser la región principal de interacción entre los dominios A y C de XylR (35).

XylR es activado por compuestos aromáticos con una amplia variedad de sustituyentes, como grupos alquilo de diferentes longitudes o intermediarios oxidados del grupo metilo del tolueno, tales como alcohol benzílico, benzaldehido y derivados. El cambio estructural sufrido por la unión del efector libera a XylR de la represión intramolecular que el dominio A (N-terminal) ejerce en ausencia de efector sobre el dominio central C. Un derivado de XylR en el que se ha eliminado el dominio A (los 220 primeros aminoácidos) es activo de forma constitutiva y puede promover la transcripción desde Pu y P_{S1} en ausencia del inductor aromático tanto *in vivo* como *in vitro* (140, 143). Además, mutaciones puntuales en determinados residuos del dominio amino terminal de XylR hacen que éste sea capaz de activar la transcripción de Pu en ausencia de efectores (34).

Se ha propuesto que, al igual que NtrC (166), el regulador XylR forma dímeros en solución. En ausencia de ATP estos dímeros tienen poca afinidad por las UAS y la unión es principalmente al sitio proximal. La afinidad de XylR por las UAS aumenta tras la unión del ATP, sin que sea necesaria su hidrólisis. Cuando el ATP se hidroliza, XylR sufre un cambio conformacional que mejora su unión al DNA en las UAS y, además, esta unión pasa a ser cooperativa. El resultado es la formación de un complejo multimérico que podría estar formado por 6 u 8 subunidades. Aunque se ha sugerido que este complejo multimérico de XylR sería un octámero constituido por dos dímeros de dímeros en el que dos de los dímeros contactarían con sus sitios en el DNA y a su vez cada uno de ellos contactaría con otro dímero (56), la estructura resuelta de PspF, un miembro de la familia NtrC, indica que la forma activa de la proteína es un hexámero (161).

Pero la unión de XylR a sus UAS en el DNA no es permanente. En cada ronda de transcripción se ensambla y se disocia el complejo multimérico que este regulador forma sobre las UAS. En ausencia del inductor, la unión de XylR sólo se hace más estable cuando se sobreexpresa la proteína. La unión del inductor a XylR convierte la unión al DNA en un proceso transitorio asociado a la hidrólisis de ATP (57, 141, 205) en el que el multímero se desensambla cada vez que el ATP se hidroliza (57). En ausencia de ATP la unión de XylR a las UAS es débil y no cooperativa. No se sabe aún si la multimerización de XylR ocurre en el DNA o antes de la unión a las UAS. La

unión del complejo regulador al DNA podría ocurrir incluso después de la unión al factor σ^{54}.

El número de moléculas de XylR en la célula es muy bajo, unos 30 monómeros por célula en fase exponencial. Pero además, la concentración de XylR depende de la fase de crecimiento, aumentando en fase estacionaria hasta alcanzar unos 140 monómeros por célula (53). Se ha sugerido incluso que los niveles de XylR durante la fase exponencial de crecimiento podrían ser insuficientes para saturar al promotor Pu (204).

II.1.1.3. La proteína reguladora XylS.

La proteína XylS, de 321 aminoácidos, es el producto del gen *xylS* y actúa como activador del operón *meta*. XylS, que pertenece a la familia de reguladores transcripcionales AraC/XylS (113), es una proteína extremadamente insoluble (no cristalizada aún) que forma dímeros que se estabilizan en presencia de moléculas efectoras. Los reguladores de esta familia presentan tres dominios característicos: un dominio conservado de unión al DNA, que en la mayoría de las proteínas de la familia es el dominio C-terminal y que en XylS consta de siete hélices α plegadas formando dos motivos HTH que interaccionan con dos surcos mayores vecinos en una cara del DNA; un dominio de unión (*linker*) que conecta los dominios N-terminal y C-terminal y que incluye los residuos Leu193 y Leu194 implicados en dimerización de la proteína (173) y un dominio no conservado, que en XylS es el dominio N-terminal, implicado en unión al efector y activación (89, 120, 158). Alterando las propiedades de este dominio se han construido proteínas derivadas de XylS capaces de reconocer otras moléculas como efectores (159) o capaces de activar los genes de la ruta *meta* en ausencia de inductor (229).

Una transcripción eficiente desde Pm requiere la interacción de la subunidad α de la RNAP con XylS y con el DNA. Se ha observado que, aunque el residuo 291 de XylS es el que interacciona con el extremo carboxilo de la subunidad α de la RNAP, la sustitución de los residuos 137 y 153 de XylS hace que sea el residuo 289 de XylS el que contacte con α (175). Estos residuos 137 y 153 tienen además un papel clave en el reconocimiento del efector y en la activación de la proteína, ya que mutaciones puntuales en estos residuos son suficientes para que XylS permita que Pm se transcriba con RNAPσ^{70} en lugar de RNAPσ^{38} (174).

II.1.2. Regulación de la ruta TOL de degradación de tolueno de pWW0.

A grandes rasgos, se puede hablar de dos niveles de regulación que afectan al funcionamiento de la ruta de degradación de tolueno del plásmido pWW0. Un primer nivel de regulación se ejerce por la participación coordinada de todos los elementos propios de la ruta. En este tipo de regulación son importantes factores como la presencia o ausencia de moléculas efectoras, la localización de los promotores, el factor sigma que se combina con el núcleo de la RNAP en las regiones promotoras, los sitios de unión de las proteínas específicas de la ruta y la interacción de unos elementos con otros. Sin embargo, aunque estén presentes todos los elementos necesarios y se den las condiciones adecuadas para el funcionamiento de la ruta, no siempre se observa la respuesta correspondiente del operón *upper* sino que, en

determinadas condiciones de crecimiento la ruta de degradación de tolueno está apagada. Ésto se debe a la existencia de un nivel de regulación más amplio que al que están sujetos los diferentes niveles específicos de regulación de la célula. Se trata de un sistema de regulación global que responde a cambios en el entorno celular, especialmente en el estado nutricional y energético de la célula, y que permite la integración óptima de la ruta en el metabolismo general bacteriano.

A continuación se describe lo conocido hasta ahora sobre estos dos niveles de regulación en TOL.

II.1.2.1. Regulación específica de la ruta de degradación de tolueno de pWW0.

La posición que ocupa cada uno de los elementos que participa en la regulación de la expresión del sistema de degradación de tolueno del plásmido pWW0 (promotores y sitios de unión de proteínas) permite una modulación ajustada de la ruta en respuesta a la presencia de efectores aromáticos en el medio. El modo de actuación es diferente en función de la naturaleza del efector.

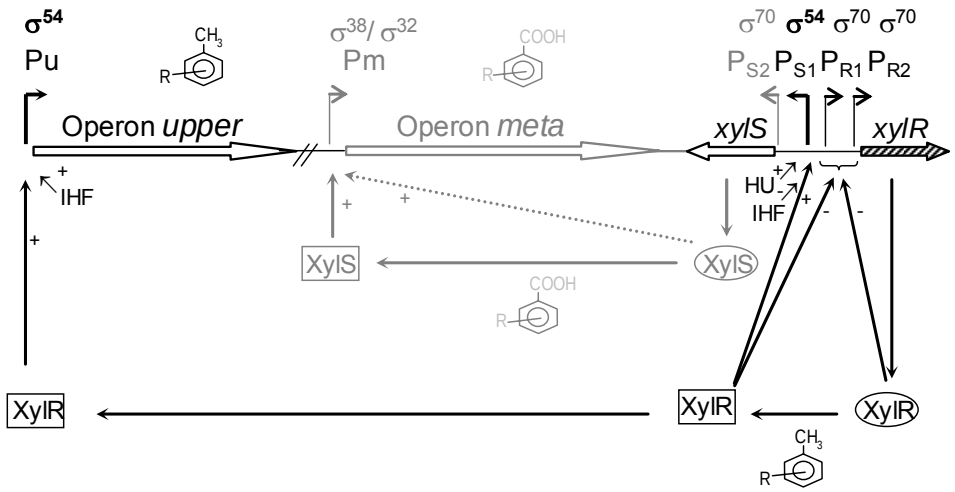

Figura 4. Esquema de regulación específica de la ruta de degradación de tolueno de pWW0. Se representan los dos operones de la ruta y sus genes reguladores. Sobre cada promotor se indica el factor sigma necesario para la transcripción. La forma inactiva de las proteínas reguladoras se representa con una elipse y la forma activa con un rectángulo. Las flechas indican las conexiones entre las proteínas y los promotores. El signo (+) indica activación de la transcripción y el signo (-) indica represión de la transcripción. La línea discontinua representa la sobreproducción de XylS en ausencia de efector de la ruta *meta*. Se indican también los sustratos aromáticos de la ruta.

II.1.2.1.a. <u>Expresión en presencia de un inductor de la ruta *upper*</u>.

Cuando el efector presente en el medio es tolueno u otro inductor de la ruta *upper*, *P. putida* asegura la expresión coordinada de los dos operones catabólicos para que el compuesto aromático sea degradado completamente hasta intermediarios del ciclo de Krebs (Figura 4). El regulador XylR interacciona directamente con la molécula efectora (33), cambia su conformación y adquiere una forma activa capaz de

promover la transcripción desde los dos promotores dependientes de σ^{54} del sistema, Pu y P_{S1}. En el caso de Pu la transcripción está asistida por la proteína IHF (62). Su unión distorsiona la topología del DNA, facilitando el contacto entre el activador y la RNAPσ^{54} unidos a distancia (182). Esta interacción RNAPσ^{54}/activador es requisito indispensable para la formación del complejo abierto antes del comienzo de la transcripción. En promotores dependientes de σ^{54} que carecen del sitio de unión para IHF, es la propia secuencia entre las UAS y el sitio de unión de la RNAPσ^{54} la que determina la capacidad del DNA para formar un ángulo adecuado (147). Sin embargo, en la región promotora de Pu no hay una secuencia intrínseca que permita que el DNA se curve por sí mismo y para que el DNA se doble es necesaria la unión de la proteína IHF (182). Como consecuencia de la interacción RNAPσ^{54}/XylR en Pu, los genes del operón *upper* se transcriben y sus enzimas inician la transformación del compuesto aromático que actuó de efector para la activación de XylR.

Se han propuesto dos modelos de activación de Pu por XylR. Según el primero de estos modelos, la unión de ATP al dominio central de XylR permitiría la multimerización del regulador en las UASs de Pu. La hidrólisis posterior de ATP dispararía el inicio de la transcripción dependiente de σ^{54} y el sistema volvería a su estado inicial no ensamblado (141). El modelo alternativo se basa en los resultados obtenidos con el regulador análogo DmpR. La proteína DmpR es el activador del promotor Po, dependiente de σ^{54}, de la ruta catabólica de (metil)fenol de *Pseudomonas* sp. CF600. De acuerdo con este modelo, el regulador en forma de dímero se activaría después de la unión del efector al dominio A, provocando un cambio estructural que haría posible la unión de ATP al dominio central y la formación de hexámeros requeridos probablemente para promover el inicio de la transcripción. Finalmente, la hidrólisis de ATP conduciría a disociación del hexámero y disociación del efector (215).

Por lo que respecta al otro promotor regulado por XylR, la interacción RNAPσ^{54}/XylR en P_{S1} provoca la sobreexpresión de la proteína XylS que activa, a su vez, la transcripción desde el promotor Pm, incluso en ausencia de un efector del operón *meta* (83, 114). La actividad de las enzimas del operón *meta* completan la transformación del compuesto aromático inicial hasta compuestos intermediarios del ciclo de Krebs.

Aunque en la transcripción desde P_{S1} también juega un papel importante IHF, existen diferencias claras con respecto a su papel en Pu (62). Se ha observado que, en un fondo *himA* (*ihfA*), mutante de *P. putida* incapaz de producir la proteína IHF, la transcripción de P_{S1} en presencia de tolueno alcanza niveles máximos muy superiores a los que se obtienen en la cepa silvestre . Esto podría ser reflejo de un mejor acceso de XylR y/o de la RNAPσ^{54} a sus sitios de unión en P_{S1}. Otra posibilidad es que la estructura tridimensional adquirida por la molécula de DNA al unirse IHF a sus dos sitios en P_{S1} restrinja el acceso de las proteínas reguladoras a la región promotora. En cualquier caso, IHF ejerce un efecto represor que parece mantener un nivel apropiado de transcripción (inferior al máximo) desde P_{S1} (77, 109). Por el contrario, la transcripción en presencia de efector desde el promotor Pu disminuye drásticamente en un mutante *himA*. Finalmente, al efecto negativo de IHF en la transcripción del

promotor P_{S1} hay que sumar el efecto positivo de la proteína HU (*heat-unstable nucleoid protein*), otra proteína de unión al DNA. Como la curvatura intrínseca del DNA en P_{S1} parece ser insuficiente para facilitar el contacto entre XylR unido a las UAS y la RNAPσ^{54} en su sitio, HU completaría esta función curvando el DNA para aumentar de este modo la flexibilidad en la región (146).

En P_{S1} los sitios de unión de XylR cubren parcialmente los promotores P_{R1} y P_{R2} del gen *xylR* (Figura 3B). Como en el caso de Pu, la unión del activador XylR a sus UASs es mucho más firme en presencia de efector y se observa tanto una huella más intensa del regulador en el DNA (en el caso de Pu (1)), como una mayor represión de los promotores P_{R1} y P_R en el caso de *xylS* (10, 63). De este modo el nivel de expresión de la proteína XylR se mantiene aproximadamente constante en la célula mediante la obstrucción y liberación controlada de los promotores de *xylR* (109). Otra consecuencia del solapamiento de promotores en la región intergénica que separa los genes reguladores es la represión de la transcripción desde P_{S2} cuando la RNAPσ^{54} está unida a su sitio (-12/-24) para transcribir desde P_{S1} (54).

II.1.2.1.b. Expresión en presencia de un inductor de la ruta *meta*.

Cuando el efector presente en el medio es 3-metil-benzoato u otro inductor de la ruta *meta*, el regulador XylS interacciona con la molécula efectora y adquiere una forma activa capaz de promover la transcripción desde *Pm* (Figura 4). La proteína XylS se sintetiza de forma constante a bajo nivel a partir del transcrito del promotor constitutivo P_{S2}. La concentración de XylS producida desde P_{S2} es suficiente para garantizar la inducción de la ruta *meta* en presencia de efector. Las enzimas codificadas en el operón *meta* transforman el compuesto aromático derivado de benzoato en intermediarios del ciclo de Krebs (157).

El incremento en la actividad del factor σ^{32}, necesario para la transcripción desde Pm durante el crecimiento en fase exponencial, está asegurado por la presencia del propio efector (3-metil-benzoato) que, además de ser una fuente de carbono y de energía para la bacteria, se detecta por la célula como un compuesto tóxico y desencadena una respuesta a estrés que conlleva un aumento de la concentración de σ^{32} (42).

Por otra parte, se ha observado que en ausencia de XylS el promotor Pm sigue siendo capaz de responder a benzoato, aunque no a 3-metil-benzoato. Se ha sugerido que esto es consecuencia de la regulación cruzada de la ruta *meta* por el regulador BenR de la ruta cromosómica *benABCD.* Tanto XylS como el regulador cromosómico BenR reconocen la misma secuencia en la región promotora de Pm (93).

II.1.2.2. Regulación global de la ruta de degradación de tolueno de pWW0.

Se considera que un regulador es global cuando modula operones y genes de diferentes rutas metabólicas. No se consideran reguladores globales a aquellas proteínas que forman parte de la maquinaria celular esencial. A su vez, los reguladores globales modulan frecuentemente a otros reguladores, controlando así a un amplio conjunto de genes de manera indirecta (67, 112). Entre las estrategias de coordinación global de la expresión génica se incluyen mecanismos de tipo diverso, como la metilación del DNA (en secuencias que pueden solapar con sitios de unión de

algún regulador global) (134) o el superenrrollamiento de la molécula de DNA asociado a nivel energético de la célula (71). Por último, algunos reguladores globales son a su vez controlados a nivel post-transcripcional. Las dianas de estos reguladores están, por tanto, sujetas a un control post-transcripcional indirecto (130).

Son muchos los frentes abiertos para tratar de entender cómo se ejerce este control global y cuales son los reguladores implicados. A continuación se describen algunos de los elementos que participan como reguladores globales en diferentes sistemas bacterianos.

II.1.2.2.a. Factores implicados en regulación global de sistemas bacterianos.

Complejo CRP-cAMP. La síntesis de AMP cíclico (cAMP) está catalizada por la enzima adenilato ciclasa, codificada por el gen *cya*. El gen *crp* codifica la proteína receptora de AMP cíclico, CRP. El complejo CRP-cAMP constituye uno de los sistemas de regulación global de la transcripción mejor caracterizados en bacterias Las bacterias entéricas responden a la disponibilidad de glucosa modulando la concentración intracelular de cAMP a nivel de su síntesis, excreción y degradación. En presencia de glucosa, condición en la cual la adenilato ciclasa está desfosforilada, un sistema de fosfotransferasas en cascada (PTS) fosforila al azúcar. La actividad de la enzima desfosforilada es baja y la tasa de síntesis de cAMP disminuye. Como consecuencia, la concentración del complejo CRP-cAMP es baja. En ausencia de glucosa, el PTS fosforila a la adenilato ciclasa que recupera una actividad alta. Los niveles de cAMP y, por tanto, los del complejo CRP-cAMP son elevados en esta condición (16, 178). En *Escherichia coli* CRP regula la expresión de más de 100 genes, muchos de ellos implicados en la utilización de fuentes de carbono y cuya expresión está sujeta a represión catabólica (228). CRP-cAMP actúa como regulador transcripcional activando la transcripción de determinados promotores (58, 177) y reprimiendo la de otros (153, 220). La unión del complejo CRP-cAMP en sitios específicos de la región promotora produce un cambio estructural del DNA cerca del sitio de unión de la RNAP y favorece su interacción con esta (55). La expresión de los genes *cya* y *crp* está a su vez controlada negativamente por el complejo CRP-cAMP (2, 3, 70).

Se han identificado homólogos de la proteína CRP de *E. coli* en muchas otras bacterias. El homólogo de CRP en *Pseudomonas aeruginosa* es Vfr (de *virulence factor regulator*, por su participación en la producción de varios factores de virulencia) (214). La proteína Vfr se une al cAMP con la misma afinidad que lo hace CRP y funciona también como un regulador global de la transcripción (afectando al menos a 60 proteínas de *P. aeruginosa*). Sin embargo, Vfr no parece intervenir en procesos de represión por glucosa como ocurre con CRP-cAMP en *E. coli*. Existen diferencias básicas en el modo de funcionamiento de Vfr con respecto a CRP de *E. coli*. En *P. aeruginosa* las fuentes de carbono preferidas y que dan lugar a represión catabólica son los ácidos orgánicos y no la glucosa (191). Por otra parte, los niveles de cAMP no fluctúan en esta bacteria en función de la fase de crecimiento o del tipo de fuente de carbono (151, 186). Además, aunque CRP y Vfr son muy similares (>90%) las principales diferencias se observan en el dominio de unión de cAMP. Es posible que Vfr requiera la unión de algún otro ligando (214).

Respuesta estricta. Efecto de (p)ppGpp. Otro de los sistemas de regulación global estudiados en profundidad es el de la "*stringent response*" (respuesta estricta), que se observó en cultivos de *E. coli* sometidos a escasez de aminoácidos. El fenómeno se caracterizó inicialmente por una disminución rápida de la transcripción de especies de RNA estables, como tRNA y rRNA, y un incremento en la transcripción de genes de enzimas implicadas en biosíntesis de aminoácidos, provocando la detención del crecimiento. La señal que dispara la respuesta estricta es la acumulación de la molécula efectora ppGpp (guanosina 3',5'-bipirofosfato), derivada de la hidrólisis de pppGpp (guanosina 3'-difosfato, 5'-trifosfato), conocidas conjuntamente como (p)ppGpp (25). Posteriormente se observó que el conjunto de (p)ppGpp no aumenta exclusivamente por privación de aminoácidos, sino también en respuesta a otros tipos de limitación de nutrientes (como carbono, fosfato o nitrógeno) y a diferentes factores que detienen el crecimiento (como por ejemplo los físico-químicos).

Las proteínas RelA y SpoT dirigen la síntesis de (p)ppGpp, que requiere GTP como sustrato (119). RelA es una sintetasa de (p)ppGpp asociada a ribosoma y responde principalmente a la presencia de tRNAs no cargados, acumulados en condiciones de escasez de aminoácidos (118). SpoT es una proteína bifuncional con actividades sintetasa e hidrolasa y parece regular los niveles de (p)ppGpp en respuesta a otras muchas condiciones además de la limitación de aminoácidos (179). Los mecanismos por los que (p)ppGpp reorienta el metabolismo celular se han estudiado principalmente en *E*. *coli*. La regulación se ejerce esencialmente a nivel de transcripción y los efectos pueden ser directos o indirectos. El efecto directo se debe a la interacción de la molécula de ppGpp con un sitio próximo al centro activo de la RNAP, lo que podría influir en la tasa de formación o estabilidad de los complejos abiertos o en la liberación del promotor. Entre los efectos indirectos destaca el referente a la competencia de factores sigma. Tanto en ensayos *in vitro* como en ensayos *in vivo*, se ha observado que los factores sigma alternativos compiten mejor con σ^{70} por su unión al núcleo de la RNAP en presencia de (p)ppGpp (86, 100). Además, el (p)ppGpp regula también la producción o actividad de algunos factores sigma, como se ha demostrado para RpoS en *E*. *coli* y en *P. aeruginosa* (17). Por último, se sabe que RelA y SpoT regulan también aspectos como percepción del quórum, formación de biopelículas, producción de antibióticos, esporulación, patogénesis e interacciones de simbiosis bacteria-hospedador (8, 84).

Topología del DNA. Determinados estímulos externos (estrés osmótico, tension de oxígeno, cambios nutricionales o de temperatura) afectan al superenrrollamiento del DNA. A su vez, el grado de superenrrollamiento del DNA puede influenciar la accesibilidad de la maquinaria transcripcional a determinadas regiones del cromosoma y afectar a la expresión de un conjunto de genes en un momento dado y bajo unas condiciones específicas (148, 200). Se puede por tanto hablar de un mecanismo de regulación global por el que ciertas señales ambientales son comunicadas al DNA para promover o inhibir la transcripción de genes.

Diferentes factores contribuyen a mantener la topología de la molécula de DNA, y entre ellos las proteínas asociadas al DNA desempeñan un papel fundamental. Dentro de este grupo de proteínas cabe destacar entre otras a H-NS (*histone-like nucleoid structuring protein*), HU, Fis (*factor for inversion stimulation*) e IHF, que

además de desempeñar un papel estructural, participan en una amplia variedad de procesos celulares. Se podría decir, por ejemplo, que la proteína IHF es por sí sola un regulador global (5). Entre sus funciones están la de reclutar y poner en contacto la RNAP con reguladores transcripcionales de toda una batería de promotores dependientes de σ^{54} (9, 19), evitar en estos promotores la activación cruzada de la transcripción por reguladores de otras rutas (144) y estimular o inhibir la formación y/o la estabilidad del complejo abierto (137). Se ha observado que la proteína IHF (71, 183) y también Fis (133) pueden unirse a puntos concretos del DNA donde la cadena se ha desestabilizado como consecuencia de algún tipo de estrés y desplazar esta desestabilización. Este traslado modifica el grado de superenrrollamiento local del DNA y favorece la formación de un complejo abierto en un promotor que se encuentre por debajo del sitio de unión de IHF. Por otra parte, tanto en *E. coli* como en *P. putida*, los niveles de IHF varían a lo largo de la curva de crecimiento, siendo la concentración de IHF mayor en fase estacionaria (40, 204). Esta característica lo convierte en un factor limitante de la transcripción desde determinados promotores durante la fase exponencial. A ésto hay que sumar que, al menos en *E. coli*, los niveles de IHF están bajo control parcial por (p)ppGpp (6).

La proteína Fis participa en la regulación de numerosos genes (15, 64, 111, 128, 170, 221, 222) y a su vez está regulada por el estado nutricional de la célula (7, 129). Los niveles de mRNA y proteína de Fis aumentan durante crecimiento exponencial en medio rico y disminuyen en fase estacionaria. La expresión de Fis se inhibe por el mecanismo de "respuesta estricta" en condiciones de escasez de aminoácidos, y su transcripción depende del grado de superenrrollamiento (180). Todo esto señala a Fis como candidato para la regulación global de genes en respuesta a la detección de cambios en la composición del ambiente.

La proteína H-NS no muestra especificidad por una secuencia particular sino que se une a moléculas de DNA y RNA en regiones de curvatura intrínseca (ricas en A) (223), desempeñando un papel importante en la arquitectura del cromosoma y en la expresión de genes implicados principalmente en adaptación de la célula a cambios medioambientales (80, 181). Se ha observado además que H-NS controla positivamente la expresión de genes regulados por el fenómeno de "respuesta estricta", entre los que se encuentran genes como *crp* y *fis*, que codifican otros reguladores globales (87). Las bacterias que contienen más de una copia del gen *hns* muestran gran diversidad ecológica y metabólica, como es el caso de las especies del género *Pseudomonas* (196). Recientemente se ha descrito el papel de TurA, una proteína de la familia MvaT semejante a H-NS, en la represión de la actividad del promotor Pu de la ruta de degradación de tolueno del plásmido pWW0 a temperaturas subóptimas de crecimiento (165).

Tampoco la proteína HU reconoce una secuencia específica en el DNA. Se une a DNA y a RNA tanto de cadena doble como de cadena sencilla pero preferentemente a regiones con alto grado de superenrrollamiento negativo. En la mayoría de los casos HU es un homodímero aunque en *E. coli* y otras enterobacterias está constituido por dos subunidades diferentes. Actúa como IHF produciendo una curvatura en el DNA, y los cambios de compactación que provoca la unión de HU al DNA afectan a la expresión de diferentes genes (90).

Las topoisomerasas son enzimas que se unen al DNA y alteran su grado de enrrollamiento. La girasa es una topoisomerasa que introduce superenrrollamiento negativo en el DNA, la topoisomerasa IV elimina superenrrollamientos positivos y la topoisomerasa I elimina superenrrollamientos negativos. De la actuación de estas enzimas en conjunto depende el estado general de enrollamiento del DNA y el patrón de expresión de cientos de genes bacterianos susceptibles a este tipo de cambios (171). La propia expresión de las topoisomerasas está regulada por este grado de enrrollamiento del DNA (148, 200).

En relación a las proteínas asociadas a DNA que participan en la regulación de la expresión global de genes, cabe mencionar el papel indirecto que se ha atribuido a las llamadas secuencias REP (*repetitive extragenic palindromic sequences*). Estas secuencias fueron halladas originariamente en el cromosoma de *E. coli* y actualmente se sabe que están presentes en muchos genomas bacterianos (51, 139, 198, 199) en un elevado número de copias. Entre otras funciones, se ha sugerido que las secuencias REP influyen en la topología global del DNA por ser sitios reconocidos por dos de las proteínas anteriormente señaladas: la proteína IHF (13) y la girasa (47, 225), que afectan al plegamiento y superenrrollamiento del DNA respectivamente y, por tanto, al patrón de expresión génico. A ésto hay que añadir el hecho de que las secuencias REP participan también en procesos de estabilización de mRNA, reforzando la idea de su implicación en la regulación génica global (26, 94, 127, 187).

Cadena de transporte de electrones. Es frecuente en bacterias que algunos de los componentes de la cadena respiratoria sean redundantes de tal forma que el flujo de electrones esté ramificado (154). La combinación de diferentes elementos de estas cadenas respiratorias bajo diferentes condiciones ambientales asegura una modulación adecuada para un funcionamiento óptimo. Pero, además de proporcionar la energía necesaria, la cadena respiratoria es fuente de información del estado energético general de la célula. Como resultado del bombeo de protones a través de la membrana citoplasmática concomitante al transporte de electrones, se genera una fuerza protón motriz. Las bacterias disponen de sistemas que detectan cambios en el transporte de electrones o en la fuerza protón motriz durante la reducción de oxígeno (154). Estas señales son transmitidas para el control de la expresión de un conjunto de genes. Una de las proteínas que actúa como sensor de esta señal es la proteína Aer, que parece detectar cambios en el estado redox del sistema de transporte electrónico (12, 162). Variaciones en el flujo de electrones a través del sistema de transporte electrónico provocan cambios del estado redox del cofactor FAD unido a la proteína Aer. Se ha sugerido que el dominio PAS de Aer, implicado en transducción de señales, interacciona con alguno de los componentes de la cadena de transporte de electrones, provocando un cambio de conformación en la proteína que es transmitido como señal para la autofosforilación de proteínas implicadas en taxis (164, 195). Otra de las proteínas transmisoras de señales de la cadena respiratoria es la proteína Tsr, que no tiene un grupo prostético redox y que responde posiblemente a cambios en la fuerza protón motriz (195). Finalmente, se ha demostrado que la oxidasa terminal *cyo*, uno de los componentes de la cadena de transporte electrónico, participa en el control global de algunas rutas catabólicas. La ruta de degradación de alcanos codificada en el plásmido OCT de *P. putida* GPo1 y la ruta de degradación de fenol codificada en el

plásmido pPGH1 de *P*. *putida* H están reprimidas cuando junto con los alcanos o el fenol, respectivamente, está presente una fuente de carbono preferida. En un mutante *cyoB*, incapaz de producir la subunidad CyoB de la oxidasa, la represión en ambas rutas de degradación se reduce (39, 150). La expresión de esta oxidasa terminal en *P. putida* depende de la fuente de carbono que la bacteria esté consumiendo. Es mayor cuando las células crecen en glutamato y decrece progresivamente en medio rico LB (Luria-Bertani), glucosa y glicerol. En *E. coli* existe una relación entre la expresión de la oxidasa terminal y la concentración de cAMP. Además, en *E. coli* se ha descrito que la expresión de esta oxidasa terminal es mayor a altas concentraciones de oxígeno. Resultados recientes sugieren que en *P. putida* no existe tal dependencia.

Crc. Crc (*catabolite repression control*) fue la primera proteína a la que se implicó en mecanismos de represión catabólica en *Pseudomonas* (217). Es una proteína soluble con un peso molecular de 30 kDa codificada por el gen *crc* (104). Aunque Crc es semejante (con un 25-32% de identidad) a enzimas reparadoras del DNA de procariotas y de eucariotas, no parece tener actividad endonucleasa ni unirse al DNA. Además, mutaciones en residuos muy conservados de los dominios que componen el bolsillo catalítico de esta superfamilia no afectan a la actividad de Crc, lo que sugiere que con gran probabilidad su función sea otra y que no se trate simplemente de un regulador negativo de unión al DNA (103, 176). El mecanismo molecular preciso de acción de Crc se desconoce, pero no todos los genes regulados por represión catabólica en *Pseudomonas* están influenciados por Crc. Se ha propuesto que en *P. putida* PpG2, el control de Crc sobre los niveles del regulador BkdR del operón *bkd* que codifica la deshidrogenasa de cetoácidos de cadena ramificada podría ejercerse a nivel post-transcripcional (73).

Crc reprime una serie de sistemas de transporte y de rutas catabólicas inducibles en *P. aeruginosa* y *P. putida* en presencia de ácidos orgánicos (217), regula la expresión de la ruta de degradación de alcanos, de la deshidrogenasa de ceto ácidos de cadena ramificada, de la glucosa-6-fosfato deshidrogenasa y de la amidasa de *P. aeruginosa* y *P. putida* (38, 72, 73). Está implicada en regular la expresión de genes que pertenecen a varias de las rutas cromosómicas centrales para la asimilación de compuestos aromáticos (rutas del homogentisato, del catecol y del protocatecuato) (123), inhibe la expresión de algunas funciones de la ruta de Entner-Doudoroff (208), es importante para la resistencia de *P. aeruginosa* a arsenito bajo condiciones aerobias (138) y es necesaria para la movilidad y formación de biopelículas en *P. aeruginosa* (135). Se ha sugerido que Crc podría ser parte de una ruta de transducción de señales capaz de detectar y responder a señales nutricionales.

IIA^{Ntr}. Es el producto del gen *ptsN*, que en bacterias Gram negativas aparece generalmente asociado a un conjunto de ORFs bien conservadas, entre las que se encuentra el gen *rpoN* que codifica la subunidad σ^{54} de la RNAP. Se denomina IIA^{Ntr} por su homología con las proteínas del PTS para el transporte de azúcares, y se ha sugerido que sirve de enlace entre el metabolismo del nitrógeno (a través de una conexión con σ^{54} aún no esclarecida) y el metabolismo del carbono (163). Facilita la utilización de fuentes de nitrógeno orgánico en ambientes con múltiples fuentes de

carbono (101, 121, 155), y en *Bradyrhizobium japonicum* se le atribuye un papel en el transporte de solutos no azúcares (95). También se ha sugerido que podría formar parte de un sistema sensor capaz de detectar el estado energético de la célula y modular la transcripción en consecuencia (21, 115, 116).

Se ha determinado la estructura de IIA^{Ntr} mediante cristalografía por rayos X (14). A pesar de ser muy similar a la proteína IIA para manitol del PTS y de proceder probablemente de un ancestro común, existen sutiles diferencias entre las estructuras tridimensionales de estas proteínas que dan lugar a considerables diferencias funcionales (207). IIA^{Ntr} tiene un residuo de histidina en su sitio activo que es fosforilado por la proteína NPr (155), pero se desconoce qué elemento sigue a IIA^{Ntr} en la cadena de fosforilación. Estudios recientes sugieren que la región N-terminal es importante para su interacción con otras proteínas y que no es necesaria para su interacción con NPr (213).

En *P. putida* los niveles de gran número de proteínas no relacionadas entre sí varían positiva o negativamente en un mutante *ptsN*. El efecto de IIA^{Ntr} es en muchos casos independiente del efecto represor provocado por la presencia de glucosa en el medio y no está limitado a genes que requieran el factor σ^{54} para su transcripción. Por este motivo, IIA^{Ntr} puede ser también considerado como un regulador global, al menos en *P. putida* (23).

RNAs reguladores. Un nuevo aspecto del control global es el descubrimiento tanto en bacterias como en otros grupos taxonómicos de un grupo particular de RNAs no codificantes (211). El número de estos RNAs, que son transcritos pero no se traducen, es mucho mayor que el anticipado (188, 212). Según las predicciones bioinformáticas puede haber cientos de ellos en los genomas bacterianos. Tienen la capacidad de unirse de forma selectiva a un grupo limitado de proteínas o de reconocer de manera específica un RNA diana por complementariedad de secuencia. En la mayoría de los casos descritos, el complejo formado por el RNA regulador y el mRNA del gen diana impide la traducción de este mRNA (66, 189). Sólo en algunos casos se ha observado un papel activador del RNA regulador (125). Investigaciones recientes apuntan a una actuación generalizada de estos RNAs reguladores en bacterias. Aunque queda por interpretar cual es la función concreta que desempeñan muchos de ellos, se les ha implicado, entre otras cosas, en el control del flujo de carbono en *E. coli* y se ha sugerido que podrían existir sistemas análogos en *Pseudomonas*.

II.1.2.2.b. Represión catabólica de la ruta *upper* de TOL en pWW0.

Como consecuencia del complejo sistema global de regulación que coordina todo el metabolismo bacteriano en *P. putida*, es posible observar represión de ciertas rutas catabólicas para la utilización de fuentes alternativas de carbono cuando está presente en el medio una fuente de carbono preferida. Es el fenómeno clásico conocido como “represión catabólica”.

En observaciones tempranas del funcionamiento de la ruta TOL, Hugouvieux-Cotte-Pattat y colaboradores propusieron la idea de una inducción sujeta a la fase de crecimiento (81). Posteriormente, se observó que existía un retraso en el tiempo de

respuesta al inductor de algunos de los promotores de la ruta (31). Se caracterizó finalmente en detalle la observación recurrente de una dependencia de la fase de crecimiento cuando la inducción se realizaba en medio rico. Para ello se analizó la expresión desde los promotores de la ruta, midiendo el tiempo requerido para la inducción de la síntesis de mRNA tanto en cultivos en medio rico (LB) como en cultivos en medio definido. Se concluyó que el funcionamiento de la ruta TOL dependía de la composición del medio de crecimiento de modo que, en células creciendo en fase exponencial en medio mínimo la activación del operón *upper* se producía inmediatamente después de la adición de un efector de XylR, mientras que la activación se retrasaba cuando las células crecían en fase exponencial en medio rico. Se atribuyó la represión a la presencia de uno o más compuestos inhibidores en el medio rico y se señaló a los aminoácidos como uno de estos factores de inhibición. La represión se ejercía a nivel transcripcional y las dianas primarias de esta represión eran los promotores dependientes de σ^{54}, Pu y P_{S1}. (107). Como la ruta TOL estaba reprimida en fase exponencial de crecimiento en medio rico pero la represión desaparecía cuando las células entraban en fase estacionaria, se denominó "silenciamiento exponencial" a este fenómeno (22). Una caracterización posterior permitió concluir que, además, algunas fuentes de carbono (glucosa, gluconato, α-cetogluconato, lactato y acetato) aunque no otras (succinato, citrato, piruvato, glicerol, fructosa y arabinosa) inhibían la activación de Pu en medios definidos. Sin embargo, este tipo de inhibición no parecía ajustarse al clásico fenómeno de represión catabólica descrito en *E. coli*, ya que fuentes de carbono como el succinato o el citrato, que son excelentes sustratos para *Pseudomonas*, no provocaban un retraso en la inducción (78).

Todas las observaciones anteriores corresponden a cultivos estanco en matraces. En este tipo de cultivo la composición del medio y el estado fisiológico de las bacterias no es constante. Por el contrario, el cultivo continuo de microorganismos permite mantener a la población bacteriana a una velocidad de crecimiento constante por un tiempo indefinido. De este modo es posible estudiar el efecto de un cambio en la composición o en las características del medio sin que éste se vea enmascarado por los efectos de un cambio en la fase de crecimiento. Células cultivadas en un sistema continuo a tasa de crecimiento no limitada (μ_{max}) no expresaban las enzimas de la ruta TOL en respuesta a la adición del inductor (45). En esta condición de crecimiento existe en el medio un exceso de fuente de carbono y de todos los demás nutrientes. Sin embargo, cuando se limitó la fuente de carbono en los cultivos continuos disminuyendo la tasa de dilución, la expresión de TOL se vio liberada de la represión. Esta disminución de la represión no era consecuencia del cambio en la velocidad de dilución del cultivo, ya que el efecto represor se observó también a la tasa inferior de dilución en condiciones de limitación de fosfato, sulfato o nitrógeno (situaciones en las que el carbono vuelve a estar en exceso). Los niveles más bajos de expresión de la ruta *upper* se observaron en condiciones de limitación de P o S. Bajo limitación de N la represión fue algo menor pero significativa. Por el contrario, como ocurre en el caso de cultivos en los que el factor limitante es el carbono, la limitación de la concentración de O_2 permitió niveles de expresión altos de la ruta *upper*. Estos resultados sugirieron que el estado energético de la célula podría estar jugando un

papel en la represión catabólica. De este modo, cuando el factor limitante del crecimiento era un sustrato anabólico (P, S, o N), situación que equivale a un elevado nivel energético en la célula, la ruta TOL de degradación estaba reprimida. Por el contrario, cuando el factor limitante del crecimiento era un sustrato catabólico (C o O_2), equivalente a un bajo nivel energético, la ruta TOL de degradación era funcional (46).

II.1.2.2.c. Estado actual del estudio de la regulación global de TOL en pWW0.

Al inicio de esta tesis doctoral, se disponía de un amplio conocimiento de los factores implicados en la regulación específica de la ruta TOL de pWW0. Por otra parte, se habían descrito detalladamente una serie de condiciones de crecimiento capaces de provocar la represión de la ruta, aún estando presentes todos los elementos necesarios para su funcionamiento. Sin embargo, nada se sabía de los factores genéticos que participaban en este fenómeno de represión. Los trabajos emprendidos en los últimos años han aportado nuevos datos y han puesto de manifiesto la implicación de algunas proteínas.

En general, los mecanismos responsables de represión catabólica en *P. putida* y en otras especies del género difieren significativamente de los descritos en enterobacterias. Para empezar, aunque *Pseudomonas* codifica en su cromosoma Vfr, una proteína análoga al principal regulador global de *E. coli*, CRP, esta proteína no parece tener un papel en la represión de rutas alternativas de carbono (entre las que se encuentra la ruta TOL codificada en el plásmido pWW0). En cuanto a la molécula efectora (p)ppGpp, se ha observado que juega un papel clave en represión de la expresión del promotor *Po*, dependiente de σ^{54}, de la ruta catabólica de (metil)fenol de *Pseudomonas* sp. CF600. Sin embargo, a pesar de la semejanza fisiológica y mecanística entre los sistemas formados por DmpR/*Po* y XylR/Pu, sólo se pudo observar un moderado efecto de ppGpp sobre Pu (193). Esto sugiere que las diferencias en la arquitectura de los dos promotores conducen a diferencias en su regulación, posiblemente por ser susceptibles a distintas señales metabólicas.

De la participación del regulador global Crc, ya implicado en una variedad de procesos relacionados con represión catabólica, o del papel de las señales transmitidas desde la cadena de transporte electrónico, no existían referencias al comienzo de este trabajo. Las proteínas IHF y HU, involucradas en la regulación específica de la ruta y, posteriormente, la proteína IIA^{Ntr}, fueron los únicos candidatos a los que se les pudo atribuir una función en la regulación global de la ruta TOL.

Este proyecto de tesis se planteó con idea de precisar el papel que desempeñaban en la regulación de la ruta TOL del plásmido pWW0 determinadas proteínas que participan en la represión de otros sistemas. Como el propio factor σ^{54}, codificado por el gen *rpoN*, había sido considerado uno de los posibles blancos de esta regulación, y por tratarse de una región muy conservada en un amplio grupo de especies bacterianas, se consideró de interés la caracterización del entorno cromosómico del gen *rpoN*.

III. OBJETIVOS

Las bases moleculares de la represión catabólica de la ruta TOL codificada en el plásmido pWW0 de *P. putida* mt-2 han sido objeto de estudio desde principio de los años 90. Sin embargo, aún no se han identificado todos los elementos implicados, ni se ha podido esclarecer cuál es el papel regulador específico de algunos de ellos. Este proyecto se diseñó con el fin de ampliar nuestro conocimiento sobre el mecanismo de regulación global que integra a la ruta TOL en el metabolismo bacteriano. Para la identificación de nuevos factores reguladores de la expresión de esta ruta, nos basamos en el estudio de proteínas involucradas en la represión de otros sistemas presentes en *Pseudomonas*. Además, se consideró de particular interés la caracterización del entorno cromosómico del gen *rpoN* por codificar el factor σ^{54}, del que depende la transcripción de los promotores de la ruta TOL susceptibles de represión. Por otra parte, se propuso la caracterización de unas secuencias repetidas encontradas en el cromosoma de *P. putida* KT2440.

Los objetivos específicos planteados fueron:

1.- Estudio de los promotores de las rutas de degradación del plásmido pWW0 susceptibles de regulación en función del estado metabólico de las células.

2.- Identificación de factores genéticos que participan en la regulación de las rutas de degradación del plásmido pWW0.

3.- Estudio del entorno cromosómico del gen *rpoN*.

4.- Estudio de secuencias repetidas en el cromosoma de *Pseudomonas putida* KT2440

Los dos primeros objetivos son abordados en los capítulos 1 y 2 de esta tesis doctoral, en los que se estudia la integración de señales en el fenómeno de la represión catabólica de los promotores Pu y P_{S1}. Para cubrir el objetivo tercero, que se corresponde con el capítulo 3 de esta tesis doctoral, se ha llevado a cabo el análisis *in silico* de la región del gen *rpoN* y una serie de experimentos que revelan un modelo de control complejo de esta región cromosómica. Si bien este trabajo no esclarece todos los pormenores de la regulación de *rpoN*, sienta las bases de futuros estudios en *P. putida* y otros microorganismos. Finalmente, el capítulo 4 de esta tesis doctoral abarca el cuarto objetivo, con resultados que han sido de interés taxonómico para esta especie.

IV. RESULTADOS

IV.1. CAPÍTULO 1.

Integración de señales a través de Crc y PtsN en la represión catabólica de la ruta TOL del plásmido pWW0 de *Pseudomonas putida*.

Autores: Isabel Aranda-Olmedo, Juan Luís Ramos y Silvia Marqués.

Publicado en: Appl Environ Microbiol. 2005 Aug;71(8):4191-8.

Resumen:

La degradación de tolueno por el plásmido pWW0 de *Pseudomonas putida* KT2440 está sujeta a represión catabólica. Los promotores Pu y P_{S1} del plásmido, que dependen del factor σ^{54} para su transcripción, están reprimidos *in vivo* en células creciendo exponencialmente en medio rico. En células creciendo en medio mínimo, la adición de extracto de levadura (YE) simula la represión de estos promotores en fase exponencial en medio rico. Hemos construido mutantes en una serie de reguladores globales descritos en *Pseudomonas* y hemos analizado en ellos la expresión de los promotores Pu y P_{S1}. La interrupción del gen *crc* (*catabolite repression control*) alivia parcialmente la represión del nivel inducido de expresión de los dos promotores por YE. La hibridación del RNA total del mutante *crc* con sondas para distintos genes de *P. putida* KT2440 mostró un aumento considerable del transcrito de *crc* en presencia de YE, lo que se correlaciona con la represión de las rutas de degradación del plásmido en esa condición. Por otra parte, la interrupción del gen *ptsN* también liberó parcialmente la represión por YE de los niveles inducidos de Pu y P_{S1}. PtsN (IIA^{Ntr}), pero no Crc, parece interferir de forma directa con el mecanismo de activación del regulador XylR en sus promotores diana (Pu y P_{S1}). Un mutante doble, con los genes *ptsN* y *crc* interrumpidos no presenta un fenotipo que refleje la suma de los efectos de cada mutación independiente, lo que sugiere que ambos reguladores globales deben formar parte de una ruta común de regulación. El nivel basal de expresión desde Pu y P_{S1} en ausencia de inductor sigue siendo dependiente de XylR y está también reprimido en presencia de extracto de levadura. Sin embargo, ni el mutante *crc* ni el mutante *ptsN* se ven libres de esta represión.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2005, p. 4191–4198 Vol. 71, No. 8
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.8.4191–4198.2005

Integration of Signals through Crc and PtsN in Catabolite Repression of *Pseudomonas putida* TOL Plasmid pWW0

Isabel Aranda-Olmedo, Juan L. Ramos, and Silvia Marqués*

Department of Biochemistry and Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Apdo. Correos 419, E-18008 Granada, Spain

Received 17 November 2004/Accepted 21 February 2005

Toluene degradation in *Pseudomonas putida* KT2440 pWW0 plasmid is subjected to catabolite repression. Pu and P_{S1} promoters of the pWW0 TOL plasmid are down-regulated in vivo during exponential growth in rich medium. In cells growing on minimal medium, yeast extract (YE) addition mimics exponential-phase rich medium repression of these promoters. We have constructed and tested mutants in a series of global regulators described in *Pseudomonas*. We describe that a mutant in *crc* (*c*atabolite *r*epression *c*ontrol) partially relieves YE repression. Macroarray experiments show that *crc* transcription is strongly increased in the presence of YE, inversely correlated with TOL pathway expression. On the other hand, we have found that induced levels of expression from Pu and P_S in the presence of YE are partially derepressed in a *ptsN* mutant of *P. putida*. PtsN but not Crc seems to directly interfere with XylR activation at target promoters. The effect of the double mutation in *ptsN* and *crc* is not the sum of the effects of each independent mutation and suggests that both regulators are elements of a common regulatory pathway. Basal expression levels from these promoters in the absence of inducer are still XylR dependent and are also repressed in the presence of yeast extract. Neither *crc* nor *ptsN* could relieve this repression.

The TOL plasmid catabolic pathway for the degradation of toluene and xylenes is a paradigm of specific and global regulation (5, 40, 41, 42). Expression of the catabolic operons involves the TOL plasmid-encoded XylR and XylS regulators, a set of sigma factors (σ^{70}, σ^{54}, σ^{32}, and σ^{38}), and DNA-bending proteins such as integration host factor and HU. Above this interplay of specific plasmid regulators and host transcriptional factors, catabolite repression plays a key role in the control of the expression of these pathways (5, 7, 9, 16, 17, 24, 30, 31, 41, 42).

The current model of specific regulation of TOL plasmid expression is shown in Fig. 1 and can be summarized as follows: the so-called *meta*-regulatory loop operates when cells grow on toluates, whereas a more complex system, the cascade loop, operates when cells grow on xylenes and ensures that both the upper and the *meta* pathways are coordinately expressed. The master regulator involved in transcriptional control of the catabolic pathways in cells growing on xylenes is XylR. The *xylR* gene is transcribed from two σ^{70}-dependent tandem promoters, P_{R1} and P_{R2}. The cascade model is operational when cells grow in the presence of toluene or xylenes. In these conditions, XylR binds the aromatics and the inactive protein ($XylR_i$) becomes activated ($XylR_a$) to stimulate transcription from the upper pathway operon Pu promoter (1, 19, 35). This process requires σ^{54}-containing RNA polymerase and the DNA-bending protein integration host factor (1, 37). In a similar pattern, $XylR_a$ also stimulates expression of the divergent *xylS* gene, normally transcribed at low levels by the constitutive P_{S2} promoter, by inducing transcription from a second σ^{54}-dependent promoter, P_{S1} (18), a process that may be assisted by the chromatin-associated DNA-bending protein HU (36). In addition, translation of P_{S1} mRNA is more efficient than that of P_{S2} (20). As a consequence, in the presence of *o*-xylene, a nonmetabolizable XylR effector, the XylS protein is overproduced and transcription from the lower pathway promoter Pm is achieved even in the absence of *meta* pathway effectors.

The P_R and P_S promoters are clustered in the 300-bp DNA region between the divergent *xylR* and *xylS* genes. The P_{S1} promoter shows the typical organization of σ^{54}-dependent promoters. XylR upstream activator sequences (UASs) in P_{S1} overlap the two *xylR* tandem promoters so that XylR binding to its UASs to activate P_{S1} results in the repression of the two σ^{70}-dependent P_R promoters and consequently in its own synthesis (3, 29). The *meta* loop is operational when cells grow on benzoates. In these conditions, *xylS* is transcribed only from the constitutive, XylR-independent P_{S2} promoter. The basal levels of XylS protein are activated by a benzoate effector to promote transcription from Pm.

The expression of TOL plasmid operons is integrated into the overall metabolic control in *Pseudomonas putida*: both the upper pathway operon promoter Pu and the *xylS* P_{S1} promoter are subject to catabolite repression. As a consequence of the latter, expression of the *meta*-cleavage operon is also subject to moderate catabolite repression (16, 40). Early studies showed that *P. putida* (pWW0) cells did not express the TOL pathways during exponential growth in rich medium (24, 30), a phenomenon also referred to by Cases et al. as exponential silencing (7). These authors distinguished a second regulatory circuit based on their observation that specific carbon sources could reduce Pu activity to one-third (6). The rich medium exponential switch-off was overcome when spent Luria-Bertani (LB) medium was used instead (30). The TOL operons could be silenced in a similar way in cells growing on minimal medium with toluene if yeast extract (YE) was added (30).

* Corresponding author. Mailing address: EEZ-CSIC, Apdo. de Correos 419, E-18080 Granada, Spain. Phone: 34 958 181600. Fax: 34 958 135740. E-mail: silvia@eez.csic.es.

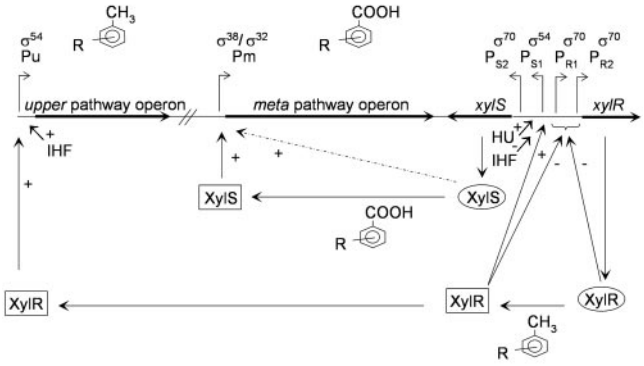

FIG. 1. TOL pathway regulatory network. The thick horizontal line depicts the TOL region including the upper and *meta*-cleavage pathways and the two regulatory genes XylR and XylS. Elliptical boxes indicate the inactive form of the regulatory proteins. Rectangular boxes indicate the active form of the regulatory proteins. Thin lines represent the connections between regulatory proteins and promoters, where a plus sign indicates transcription activation and a minus sign indicates inhibition of transcription. The dotted line indicates transcription activation of overproduced XylS in the absence of effector. The sigma factor(s) involved in transcription initiation is indicated above each promoter. Aromatic substrates of the pathways acting as effectors of the regulatory proteins are indicated. The regulatory circuits are explained in the text. R indicates possible substituent groups of the ring.

Definitive proof of catabolite repression was provided by Duetz et al. (17), who showed that *o*-xylene-induced expression of the TOL catabolic pathways did not occur in continuous cultures growing either at a high rate under nonlimiting conditions (i.e., excess of all nutrients) or at a low rate in cultures limited in N, P, or S, conditions which all result in an excess of carbon in the medium. However, when the culture was limited in C, the operon was expressed at a high level.

Catabolite repression in *Pseudomonadaceae* does not involve cyclic AMP (cAMP) as in *Enterobacteriaceae* (41). In fact, in *P. putida* and *P. aeruginosa*, cAMP levels are relatively constant regardless of the growth conditions (39, 44). Instead, catabolite repression seems to integrate different signals, a feature which increases the complexity of the system. Up to five different potential regulators have been related to catabolite repression in *P. putida*, namely, Crc (34, 41), Crp, called Vfr in *P. aeruginosa* (45, 49), CyoB (14, 38), RelA (25, 47), and the PTS system (8–10).

Crc is responsible for the catabolic repression of a number of functions in *P. aeruginosa* and *P. putida*, such as the expression of glucose-6-phosphate dehydrogenase and amidase activities and the branched-chain keto acid dehydrogenase (23). Recently, the alkane degradation pathway encoded by the OCT plasmid from *P. putida* GPo1 has also been shown to be under the control of Crc (51). Although the molecular mechanism underlying Crc activity is unknown, available data suggest that Crc is a component of a signal transduction pathway that modulates carbon metabolism in *Pseudomonas*.

P. aeruginosa vfr gene encodes a regulator homologous to Crp able to bind cAMP. Proteome analysis showed that the synthesis of at least 60 proteins is affected in a *P. aeruginosa vfr* mutant, confirming the role of this protein as a global regulator. However, *vfr* was not required for catabolite repression control in this strain (45). The *cyoB* gene codes for a subunit of one of the terminal oxidases in the branched respiratory system of *Pseudomonas*. It has been involved in catabolite repression of the phenol pathway of *P. putida* (38) and the alkane degradation pathway of *P. putida* GPo1 (previously *P. oleovorans*) (14). CyoB-deficient mutants partially escape from catabolite repression.

RelA and its counterpart SpoT are involved in the biosynthesis of (p)ppGpp. The level of these alarmones influences RNA polymerase activity at certain promoters and σ^{54} competitiveness for the RNA polymerase core. σ^{54} occupancy of RNA polymerase modulates the expression of the DmpR-dependent Po promoter that controls the phenol degradation operon in *Pseudomonas* sp. CF600 (25).

The *ptsN* gene product IIA^{Ntr} shares characteristics with phosphotransferases of the PTS family. A knockout mutant in the *ptsN* gene relieves C source inhibition of the TOL plasmid upper pathway operon in cells growing exponentially in minimal medium with glucose or gluconate as a C source (9). However, expression from Pu in cells growing on rich medium in the exponential phase of growth was not affected (9).

In this study we used *P. putida* KT2440 and a series of knockout mutants in *relA*, *cyoB*, *crp*, *crc*, and *ptsN* to analyze expression from the TOL promoters. The approach we used reproduces rich medium repression under batch growth conditions by adding YE to cells growing exponentially on minimal medium. We also used macroarrays to simultaneously monitor variations in the level of expression of the potential global regulators and the expression of the TOL genes under repressive conditions. Our results show that repression of the TOL genes was concomitant with increase in the expression of the *crc* gene.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were routinely grown at 30°C in liquid LB medium (43). *P. putida* strains were grown at 30°C in modified M9 minimal medium with glucose (25 mM) as the sole carbon source (30). When indicated, YE, which consists basically of a mixture of amino acids plus micrograms of vitamins, was added to a final concentration of 1%. This gave a final concentration of each amino acid in the culture medium ranging between 0.5 and 3 mM. When required, antibiotics were used at the following final concentrations (in micrograms per milliliter): kanamycin (Km), 50; tetracycline (Tc), 8; gentamicin (Gm), 100; chloramphenicol (Cm), 30; and streptomycin (Sm), 100.

Construction of mutants. The *P. putida* KT2440 genome (http://tigrblast.tigr.org) was screened for the target genes, and the flanking region was identified. The different genes responsible for global regulation were cloned using PCR amplification of each region with specific oligonucleotides located approximately 1 kb upstream and downstream from the gene of interest. Every cloned gene was interrupted with an antibiotic resistance cassette and transferred to *P. putida* KT2440 by reverse genetics. To generate a *crc* mutant, we screened the genome for a sequence homologous to the *crc* gene of *P. aeruginosa*. Oligonucleotides 5′-GTAGCGTAGTGTGACTTGAAGGG-3′ and 5′-TGTACCGCGCTTCCTCAAAGGC-3′ located 1 kb upstream and downstream from the *crc* gene, respectively, were used to amplify a 2.8-kb fragment from the *P. putida* KT2440 chromosome. The fragment was first cloned in pGEM-T and sequenced to detect any unwanted mutation. A gentamicin cassette was introduced at the unique SmaI site, thus interrupting the *crc* open reading frame (ORF) in the 225th codon. The knockout gene was then cloned in the suicide delivery plasmid pKNG101 between the SmaI and SpeI sites. The resulting plasmid was transferred to *P. putida* KT2440 in a triparental mating where transfer functions were provided in *trans* by *E. coli* HB101 (pRK600). *P. putida* mutant strains bearing the knockout *crc* gene were directly selected as streptomycin-sensitive, gentamicin-resistant, sucrose-resistant colonies. Accuracy of the double recombination event was confirmed by PCR and Southern blot analysis.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Characteristics	Reference
Strains		
P. putida KT2440	mt-2 pWW0 cured	2
P. putida KT2440 (pWW0)	mt-2; ATCC 33015	50
P. putida KT2440/102	Km^r, *P. putida* KT2440 with a Km resistance cassette interrupting ORF102	This study
P. putida KT2440/*ptsN*	Km^r, *P. putida* KT2440 with a Km resistance cassette interrupting the *ptsN* gene	This study
P. putida KT2440/284	Km^r, *P. putida* KT2440 with a Km resistance cassette interrupting ORF284	This study
P. putida KT2440/*ptsO*	Gm^r, *P. putida* KT2440 with a Gm resistance cassette interrupting the *ptsO* gene	This study
P. putida KT2440/*crc*	Gm^r, *P. putida* KT2440 with a Gm resistance cassette interrupting the *crc* gene	This study
P. putida KT2440/*crp*	Km^r, *P. putida* KT2440 with a Km resistance cassette interrupting the *crp* gene	This study
P. putida KT2440/*cyoB*	Tc^r, *P. putida* KT2440 with a Tc resistance cassette interrupting the *cyoB* gene	F. Rojo
P. putida KT2440/*aer*	Km^r, *P. putida* KT2440 with a Km resistance cassette interrupting the *aer* gene	V. Shingler
P. putida KT2440/*relA*	Km^r, *P. putida* KT2440 with a Km resistance cassette interrupting the *relA* gene	V. Shingler
P. putida KT2440/*spoT-relA*	Km^r, Gm^r, *P. putida* KT2440/*relA* with a Gm resistance cassette interrupting the *spoT* gene	V. Shingler
P. putida KT2440/*crc-ptsN*	Gm^r, Km^r, *P. putida* KT2440/*ptsN* with a Gm resistance cassette interrupting the *crc* gene	This study
E. coli DH5α	F$^-$ Φ80d *lacZ*ΔM15Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* R$^-$ M$^+$ *supE44 thi1 gyrA relA1*	21
E. coli HB101	Sm^r, *hsd* R$^-$ M$^+$ pro leu thi *recA*	43
Plasmids		
pGEM-T	PCR product cloning vector	Promega
pKNG101	Sm^r, sucrose sensitive, ori RK2	26
pKNG102	Sm^r, Km^r (with a Km resistance cassette inserted in ORF102)	9
pKNG154	Sm^r, Km^r (with a Km resistance cassette inserted in the *ptsN* gene)	9
pKNG284	Sm^r, Km^r (with a Km resistance cassette inserted in ORF284)	9
pKNGptsO	Sm^r, Gm^r (with a Gm resistance cassette inserted in the *ptsO* gene)	This study
pKNGcrc	Sm^r, Gm^r (with a Gm resistance cassette inserted in the *crc* gene)	This study
pKNGcrp	Sm^r, Gm^r (with a Km resistance cassette inserted in the *crp* gene)	This study
pRK600	Cm^r, oriColE1, mobRK2, traRK2	27
pS10	IncP1, Sm^r, *xylR*, transcriptional Pu::*lacZ*::*tet* fusion	This study
pWW0	IncP9, mob$^+$, tra$^+$, 3MB$^+$	50

The knockout *cyoB* mutant where the *cyoB* gene was interrupted by a tetracycline resistance cassette was obtained from F. Rojo. Delivery plasmids containing a knockout mutant of ORF284, *ptsN*, and ORF102 were obtained from V. de Lorenzo and were transferred to *P. putida* as described above. Mutants were selected as kanamycin-resistant, sucrose-resistant, streptomycin-sensitive strains and checked by PCR and Southern blot analysis. To obtain a *ptsO* mutant, oligonucleotides located ca. 1 kb upstream (5′-GCCCACCTTGAACTTCTGC G-3′) and downstream (5′-GTCCGGAATACATCGGTGCC-3′) of the *ptsO* gene were designed and a 2.3-kb fragment was amplified from *P. putida* KT2440 chromosome. The fragment was first cloned in pGEM-T and sequenced to detect any unwanted mutation. A Gm cassette was introduced in the unique SmaI site, thus interrupting the *ptsO* ORF. The knockout gene was then cloned in the suicide delivery plasmid pKNG101 between the SspI and SpeI sites. The resulting plasmid was transferred to *P. putida* KT2440 in a triparental mating as described above.

To obtain a *crp* mutant, oligonucleotides located 1 kb upstream (5′-GGCTC ACCGTTCAGTTGGG-3′) and downstream (5′-GGATACGCCGCTGGTGG G-3′) from the *crp* gene were used to amplify a 2.75-kb fragment from the *P. putida* KT2440 chromosome. The fragment was first cloned in pGEM-T and sequenced. A kanamycin cassette was introduced in the unique NruI site, thus interrupting the *crp* ORF in the 116th codon. The knockout gene was then cloned in the suicide delivery plasmid pKNG101 and transferred to *P. putida* KT2440 as described above, and mutants were selected as kanamycin-resistant, sucrose-resistant, streptomycin-sensitive strains and checked by PCR and Southern blot analysis.

In addition to the previously described mutants, *aer*, *relA*, and *relA*/*spoT P. putida* KT2440 mutant strains were obtained from V. Shingler. Finally, the TOL plasmid pWW0 was transferred to each mutant strain by conjugation. To obtain the *crc-ptsN* double mutant, the above approach was used, except that the recipient strain was *P. putida* KT2440 containing the *ptsN* mutation.

Sampling and isolation of RNA. Cells grown overnight in M9 minimal medium with glucose as the carbon source were diluted to a turbidity of 0.2 at 660 nm in the same medium. When the cultures reached a turbidity of 0.7 (in the exponential growth phase), they were divided into four fractions, which were supplemented with *o*-xylene (a nonmetabolizable inducer of the Pu and P_{S1} promoters in the TOL plasmid pWW0), 1% (wt/vol) yeast extract as repressor agent, or both. The fourth fraction was left unsupplemented as a control. Cultures were incubated at 30°C for 30 min, and 10-ml samples were harvested by centrifugation at 4°C in disposable plastic tubes precooled in liquid N_2 and were kept at −80°C until use. Total RNA was extracted with the phenol-guanidine thiocyanate mixture Tri Reagent LS (Molecular Research Center, Inc.) according to the manufacturer's instructions, except that the initial lysis step was carried out at 60°C. The relative levels of each specific messenger were estimated by reverse primer extension analysis of equal amounts of total RNA, using the following oligonucleotides: 5′-GGCCAGCGTCACAGACTCCAGGCG-3′ for Pu-dependent transcripts, 5′-GAGACTGCATAGGGCTCGGCGTGG-3′ for P_S, and 5′-ACGGATCTGGCTGCTAAGGTCTTGC-3′ for P_R transcripts. Primer extension analysis of 10 to 20 μg total RNA samples was carried out as described previously (31) using the ^{32}P-end-labeled oligonucleotides described above. Samples were run in urea sequencing gels, and gels were exposed to a phosphorimaging screen (Fuji Photo Film Co. Ltd.) for 5 to 12 h. Phosphorimaging screens were scanned with a phosphorimaging instrument (Molecular imager FX; Bio-Rad). Data were quantified with Quantity One software (Bio-Rad).

Construction of the macroarray and cDNA labeling. The DNA arrays used in the hybridization experiments were produced by Newbiotechnic (Seville, Spain). Each DNA array consisted of a positively charged nylon membrane on which each PCR-amplified, ORF-specific DNA fragment was printed in duplicate with a robotic technique. For radioactive cDNA labeling, we used 20 μg total RNA in 12 μl diethyl pyrocarbonate-treated H_2O containing 250 ng random hexamer oligonucleotides (Amersham). Samples were heated to 70°C for 10 min and chilled on ice. Probe synthesis was carried out at 42°C for 2 h in a 50-μl reaction volume containing 50 mM Tris-HCl (pH 8.5), 30 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dGTP, 0.05 mM dCTP, 100 μCi [α-^{32}P]dCTP at 3000 Ci/mmol (Amersham), 40 U RNAsin (Promega), and 200 U Superscript II RNase H$^-$ reverse transcriptase (Invitrogen LifeTechnologies). Unincorporated nucleotides were removed in Micro Bio-Spin chromatography columns (Bio-Rad Laboratories), and samples were treated with RNase H (U.S. Biochemicals) for 20 min at 37°C. Prior to hybridization, high density arrays were prewetted in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) and prehybridized for 2 h at 64°C in roller bottles containing 20 ml 1× hybridization buffer (0.5% blocking reagent [Roche], 5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 0.1 mg/ml sheared salmon sperm DNA). Prehybridization buffer was removed and replaced with 1× hybridization buffer containing 4×10^7 cpm/ml cDNA probe, and hybridization

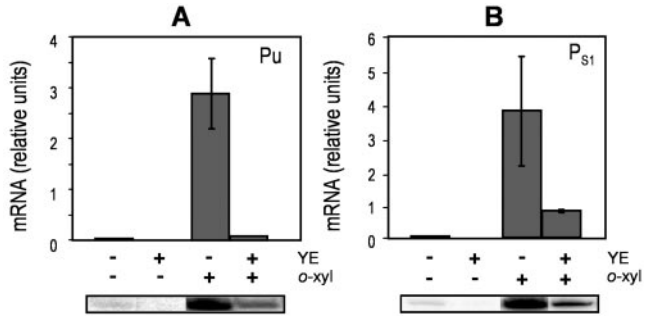

FIG. 2. Yeast-extract-mediated repression of mRNA expression from Pu (A) and P_{S1} (B) promoters in *P. putida* KT2440 (pWW0). Cells grown overnight in M9 minimal medium with glucose as the carbon source were diluted to an optical density at 660 nm of 0.2. When the cultures reached exponential growth, they were divided into four fractions, three of which were supplemented with *o*-xylene (*o*-xyl), 1% (wt/vol) yeast extract, or both. The fourth fraction was left unsupplemented as a control. Cultures were incubated at 30°C for 30 min, and samples were collected for primer extension mRNA analysis. cDNA bands corresponding to each promoter (134 nucleotides for Pu and 206 for P_{S1} [bottom]) were quantified and compared.

continued at 64°C for 40 h. After hybridization, arrays were washed twice at room temperature and once at 65°C in 2× SSC with 0.5% SDS, followed by one wash at 65°C in 0.1× SSC with 0.5% SDS. Arrays were then sealed in thin polypropylene bags to avoid drying and exposed to a phosphorimaging screen (Fuji Photo Film Co. Ltd.) for 12 h. Phosphorimaging screens were scanned with a phosphorimaging instrument (Molecular imager FX; Bio-Rad).

β-Galactosidase activity assay. The wild-type *P. putida* cells and a series of isogenic mutants deficient in the synthesis of Aer, RelA, and SpoT proteins were transformed with pS10, a Km^s derivative of the low-copy-number plasmid pJB3 (4) bearing the *xylR* gene and a transcriptional Pu::*'lacZ*::*tet* fusion preceded by a Sm^r cassette to prevent read-through transcription from vector promoters. Cells grown overnight on M9 minimal medium with glucose as the carbon source were diluted to an optical density at 660 nm of 0.5 in the same medium. After 30 min at 30°C with shaking, cultures were divided into four fractions: one was kept as a control, and to the other three, we added *o*-xylene, 1% (wt/vol) yeast extract, or both. After 90 min, culture samples were analyzed for β-galactosidase activity with the standard colorimetric assay described by Miller (32). At least two independent assays with duplicate samples were done in each case.

RESULTS AND DISCUSSION

Expression of the TOL plasmid Pu and P_S promoters. As a first step in identifying the global regulators involved in catabolite repression of the TOL plasmid promoters, we analyzed the *o*-xylene induction of TOL catabolic promoters in batch cultures in the presence and in the absence of 1% (wt/vol) YE. *o*-Xylene is a nonmetabolizable inducer able to activate XylR, which promotes transcription from both σ^{54}-dependent promoters Pu and P_{S1}, while the addition of YE reproduces rich medium repression (30). *P. putida* (pWW0) cells were grown on M9 minimal medium with glucose as the carbon source. When the culture reached a turbidity of 0.7, it was split into four fractions: one was kept as a control, one was supplemented with 1% YE, and *o*-xylene in the gas phase with or without 1% YE was added to the other two fractions. After 30 min of incubation at 30°C, mRNA was extracted and expression levels from the TOL promoters were determined by primer extension analysis. As expected, Pu and P_{S1} were strongly induced when *o*-xylene was added (Fig. 2). However, *o*-xylene-induced Pu and P_{S1} expression in the presence of YE was about 80 to 90% lower than that in its absence (Fig. 2A and B). High-level expression from Pu and P_{S1} was strictly dependent on the presence of the inducer *o*-xylene. Overall, these results confirm previous findings that the two σ^{54}-dependent TOL plasmid promoters are the targets of catabolite repression control.

The same assays were repeated with the whole series of *P. putida* mutants in genes that could potentially be involved in catabolite repression, as well as in mutants in the open reading frames adjacent to *ptsN*. Our results showed that the pattern described above for the wild type was almost identical in the mutants ORF102 and ORF284 of the *rpoN* gene cluster and for *crp*. In the cyoB mutant, only a moderate relief of YE-dependent Pu and P_{S1} repression was observed (not shown). These results ruled out the involvement of these proteins in TOL pathway catabolite repression. By using pS10, we measured expression from Pu in the presence of *o*-xylene and 1% (wt/vol) YE in isogenic *P. putida* backgrounds lacking the Aer protein, an aerotaxis and energy sensor thought to monitor the redox state of the electron transport chain in *E. coli* (22), and RelA- and SpoT-deficient backgrounds. The RelA and SpoT proteins are involved in the synthesis of (p)ppGpp, which influences RNA polymerase activity (11), and they are known to respond to amino acid and nutrient limitations. Although the inhibition caused by YE points toward (p)ppGpp playing a role in this repression, this does not seem the case. In all these mutants, *o*-xylene-induced β-galactosidase levels were similar to levels in the wild type, therefore indicating that these genes were not involved in catabolite repression of the Pu promoter either. This is in accordance with previous results comparing the (p)ppGpp effect on the activity of the two analogous systems DmpR/Po and XylR/Pu (46). Despite the clear contribution of this alarmone to upregulate the DmpR-dependent Po transcription, it appeared to play a minor role in XylR-dependent Pu transcription (46), which is consistent with our findings.

In contrast, pronounced derepression was observed in the *ptsN* and *crc* mutants (Fig. 3). The level of repression from Pu and P_{S1} in the *ptsN*- and *crc*- deficient mutants was only about 50%. PtsN repression of Pu (9) and P_{S1} (15) had been described before, but this is the first report of Crc-mediated repression of Pu and P_{S1}. To test the potential synergistic effects of these two factors on catabolite repression, we constructed a *ptsN-crc* double mutant and tested the expression from Pu and P_{S1}. The results revealed that the degree of derepression was not increased with respect to that observed in the *crc* or *ptsN* single mutant, which yielded the highest level of derepression.

In the wild-type strain, basal levels of Pu and P_{S1} promoters in the absence of inducer were low but measurable and were XylR dependent (results not shown). In the presence of YE, these levels were repressed to 10% of their initial value. We measured YE effect on basal expression levels from both promoters in the *ptsN* and *crc* mutants. Neither *ptsN* nor *crc* mutants were able to relieve the strong repression of these uninduced levels (not shown). This suggests that the effect of YE is partially mediated by PtsN or Crc only in the presence of effector.

PtsN but not Crc directly interferes with XylR activation at target promoters. Expression from both promoters of the *xylR* gene is known to be repressed in the presence of an effector for

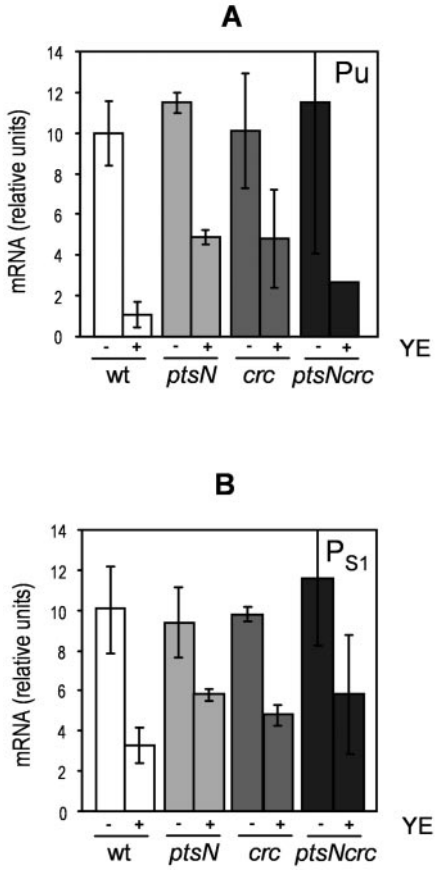

FIG. 3. Yeast-extract-mediated repression of mRNA expression from Pu (A) and P_{S1} (B) promoters in mutants *P. putida* KT2440/*ptsN* (pWW0), *P. putida* KT2440/*crc* (pWW0), and *P. putida* KT2440/*ptsNcrc* (pWW0) compared to the control strain *P. putida* KT2440 (pWW0). Cell growth, sampling, and analysis done were as described in the legend to Fig. 2.

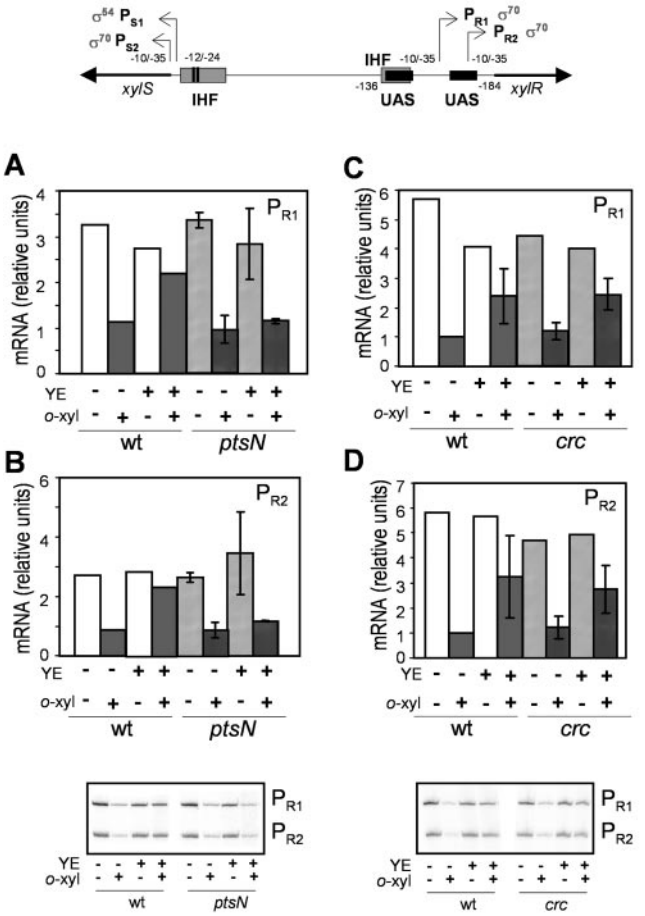

FIG. 4. Expression from P_{R1} (A and C) and P_{R2} (B and D) promoters in mutants *P. putida* KT2440/*ptsN* (pWW0) and *P. putida* KT2440/*crc* (pWW0) compared to the control strain *P. putida* KT2440 (pWW0) in the presence (+) or absence (−) of effector (*o*-xylene [*o*-xyl]) and repressor agent (yeast extract). Cell growth, sampling, and analysis were done as described in the legend to Fig. 2, except that oligonucleotides complementary to *xylR* were used. cDNA bands corresponding to each P_R promoter (208 n for P_{R1} and 180 n for P_{R2} [bottom]) were quantified and compared. IHF, integration host factor; wt, wild type.

XylR, which was attributed to the stronger binding of activated XylR to its target UASs in the −140 region with respect to the +1 region of P_{S1}. In fact, the UASs overlap $E\sigma^{70}$ binding sites at P_{R1} and P_{R2} (3, 29) (Fig. 4). In vitro analysis of P_R promoter expression has previously shown that $E\sigma^{70}$-dependent transcription from P_{R1} and P_{R2} decreases markedly in the presence of a truncated version of XylR which mimics the effector activated conformation. This was apparently a consequence of the binding and multimerization of the regulator at the UASs (3). Similar conclusions were obtained in vivo by comparing P_{R1} and P_{R2} promoter activity in a wild type and a *xylR*-deleted mutant of TOL plasmid. A strong derepression (more than fivefold) of both promoters was observed in the absence of XylR, and consistently, this expression, which was the highest observed for *xylR* in vivo in any condition, was independent of the presence of an aromatic effector (29). Based on these previous observations, we can assume that XylR occupancy of the UASs can be inferred from the activity of these two P_R promoters. XylR binding to its UASs is influenced by a number of factors. Besides the presence of effector, which increases XylR affinity for its targets (1, 3, 29), the presence and activity of $E\sigma^{54}$ seems to influence UAS occupancy by allowing XylR release after each transcription cycle (29). The results in Fig. 4 show that the presence of 1% (wt/vol) YE partially relieved *o*-xylene-dependent P_R repression in the wild-type strain. Therefore, the presence of YE could be influencing any of the above-mentioned processes to produce a lower occupancy of UASs by XylR, as reflected by the higher activity of P_{R1} and P_{R2}. This lower binding of XylR correlates with the low level of P_{S1} (and hence of Pu) expression in the presence of YE (Fig. 2). However, in the *ptsN* mutant, P_R promoter expression with *o*-xylene was not affected by the presence of YE (Fig. 4A and B). This indicates that PtsN was in part responsible for YE effect on XylR binding to its UASs and suggests that PtsN exerts its influence directly at the level of the transcriptional machinery at the TOL σ^{54}-dependent promoters, somehow interfering with *o*-xylene-XylR interaction to bind its UASs or with the $E\sigma^{54}$-binding and activation mechanism.

The behavior of the *crc* mutant was different from that of *ptsN*, namely, XylR expression in this mutant in the presence of

YE was similar to its expression in the wild type, i.e., YE partially relieved the *o*-xylene-dependent repression of P_{R1} and P_{R2} (Fig. 4C and D). Therefore, Crc does not seem to influence XylR UAS occupancy in the presence of YE. Then Crc effect on P_{S1} expression (and hence Pu expression) is not likely to be exerted directly at the level of the transcription machinery, which would probably influence UAS occupancy. The molecular mechanism behind Crc activity is unknown. Sequence similarities suggest a relation with endo- and exonucleases, which would point out to processes such as messenger stability. However, such a function would produce an additive phenotype in a double mutant, *ptsN-crc*, which was not observed. A general role of Crc generating a metabolic signal is more plausible. Recently, Velázquez et al. (48) suggested that catabolites of the Entner-Doudoroff pathway were responsible for C-source repression of Pu. The authors observed a degree of relief of glucose repression of the Pu promoter in a *crc* knockout mutant, connecting this result with the observation in a *crc* mutant of *P. aeruginosa* of an increase in Entner-Doudoroff pathway activity, detected as a higher level of glucose-6-phosphate dehydrogenase (12). Although this effect on the Entner-Doudoroff pathway has not been confirmed in *P. putida*, we cannot rule out this possibility that it is responsible for the *crc* effect on YE repression. However, this scenario is hardly conceived when YE, containing essentially a mixture of amino acids, is added as a repressing agent.

Expression of the *crc* gene inversely correlates with levels of catabolite repression from Pu and P_{S1}. We set up a macroarray to monitor the mRNA level of *rpoN* and *ihf*, whose gene products are directly involved in the transcription of Pu and P_{S1}, together with genes of the TOL upper pathway and *xylS*. We also monitored expression of the genes in the *rpoN* gene cluster (*orf102*, *ptsN*, *orf284*, and *ptsO*) and genes potentially involved in catabolite repression, such as *crp*, *crc*, and *cyoB*. Total RNA was isolated from *o*-xylene-induced *P. putida* KT2440 (pWW0) cells growing in the presence and in the absence of YE, and its derived cDNA was radioactively labeled and hybridized to the macroarray membrane as described in the Materials and Methods section. Figure 5 shows that expression of *xylS* and of the Pu-dependent catabolic genes was repressed in the presence of YE. Interestingly, these results also revealed that, in general, YE did not affect the level of expression of the genes directly involved in the expression of Pu. This was also the case for the genes in the *rpoN* cluster, although *ptsO* expression levels were below the detection limits and no conclusion could be drawn out for this gene. This was also the case for *crp*, *ihfB*, and *cheA*. However, we observed a marked increase in the expression of *crc*, *cyoA*, and *cyoB* transcripts. Interestingly, *crc* expression increase correlated with a decrease in the expression from Pu and P_{S1}. The increase in the mRNA of the two subunits of the terminal oxidase *cyoA* and *cyoB*, which are encoded by the same operon, can be related to an increase in respiratory rates, in agreement with the observation made in *E. coli* in response to the addition of Casamino Acids (33). The terminal oxidase levels have been shown to play a minor role in the YE repression of TOL catabolite promoters.

Concluding remarks. Several functions have been described in *Pseudomonadaceae* as responsible for the catabolite repression of different genes and pathways. Among these genes,

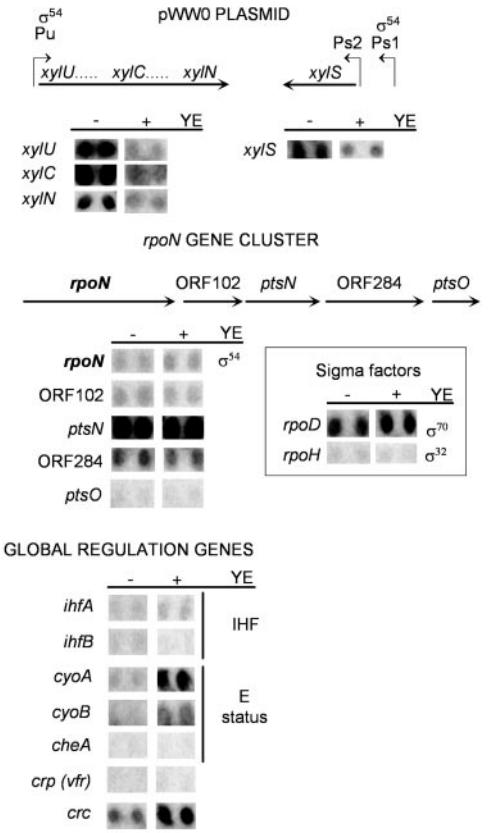

FIG. 5. Effect of the presence of yeast extract on the mRNA levels of genes involved in the control of the expression in TOL plasmid catabolic operons. Genes in the DNA macroarray used to determine the mRNA levels are displayed in the panels. *P. putida* KT2440 (pWW0) cells were grown on M9 minimal medium with glucose as the carbon source. When the cultures reached exponential growth, they were divided into two fractions, both of them supplemented with *o*-xylene in the gas phase, and to one of them, 1% (wt/vol) yeast extract was added. Cultures were incubated at 30°C for 30 min, and samples were collected for mRNA isolation. Radioactively labeled cDNA was synthesized and hybridized to the DNA macroarray as described in Materials and Methods. IHF, integration host factor.

cyoB, *crc*, and *ptsN* have been directly related to the global regulation of different operons encoding enzymes for the metabolism of hydrocarbon (6, 14, 46, 51). However, except for (p)ppGpp in the DmpR-activated Po promoter of the phenol degradation pathway (28), the molecular mechanism underlying each process remains to be elucidated.

PtsN, the IIA^{Ntr} phosphotransferase present in the *rpoN* gene cluster, has been shown to mediate the glucose-dependent repression of Pu transcription, although the phenomenon known as exponential silencing, i.e., the repression of transcription in the early-exponential phase during growth in LB medium, was unaltered in this mutant. Using primer extension analysis to directly track the transcription process, we found that Pu and P_{S1} expression in the presence of YE is derepressed in a *ptsN* mutant. The addition of YE seems to interfere with XylR binding at its UASs, and the absence of PtsN prevents this interference. We envisage that PtsN may function by affecting XylR activity (46) or at least by modulating the

overall mechanism of XylR activation of both σ^{54}-dependent promoters.

Crc has been implicated in carbon source regulation of the *alk* operon for alkane degradation in *P. putida* GPo1 (51), although in this pathway, *cyoB*, which appears to "sense" the energy status of the cell, is the main player in catabolite repression (13). We have shown here that *crc* transcription is markedly increased in the presence of YE, which inversely correlates with TOL pathway expression. A *crc* mutant was partially derepressed in the presence of YE. However, this derepression does not seem to interfere with XylR binding at its UASs.

Our data show that the effect of the double mutation in *ptsN* and *crc* is not the sum of the effects of each independent mutation. A genetic interpretation of this result suggests that both regulators are elements of a common regulatory pathway. However, on the basis of current knowledge on the phosphotranferase mechanism in the PTS systems and the observations of XylR binding in both mutants, it is difficult to envisage such a scenario. The precise function and mechanism of Crc in the cell are still unknown, but current evidence points to a very different mechanism. The only plausible explanation for our findings at this time appears to be that Crc acts by sensing the presence of YE in an early step of the regulatory process.

ACKNOWLEDGMENTS

We thank Patricia Marín for skillful technical assistance, V. Shingler, F. Rojo, and V. de Lorenzo for generous gifts of strains and plasmids, and Manuel Rey from Newbiotechnic, Seville, Spain, for imprinting the macroarrays and technical advice. We thank M. M. Fandila for secretarial assistance.

This work was supported by EC grant Biocarte (CLK-2002-01923) and MCYT grant BMC2001-0515.

REFERENCES

1. **Abril, M. A., M. Buck, and J. L. Ramos.** 1991. Activation of the *Pseudomonas* TOL plasmid upper pathway operon. Identification of binding sites for the positive regulator XylR and for integration host factor protein. J. Biol. Chem. **266:**15832–15838.
2. **Bagdasarian, M., R. Lurz, B. Ruckert, F. C. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. Gene **16:**237–247.
3. **Bertoni, G., S. Marqués, and V. de Lorenzo.** 1998. Activation of the toluene-responsive regulator XylR causes a transcriptional switch between sigma54 and sigma70 promoters at the divergent P_R/P_S region of the TOL plasmid. Mol. Microbiol. **27:**651–659.
4. **Blatny, J. M., T. Brautaset, H. C. Winther-Larsen, K. Haugan, and S. Valla.** 1997. Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. Appl. Environ. Microbiol. **63:**370–379.
5. **Cases, I., and V. de Lorenzo.** 2001. The black cat/white cat principle of signal integration in bacterial promoters. EMBO J. **20:**1–11.
6. **Cases, I., and V. de Lorenzo.** 1998. Expression systems and physiological control of promoter activity in bacteria. Curr. Opin. Microbiol. **1:**303–310.
7. **Cases, I., V. de Lorenzo, and J. Pérez-Martín.** 1996. Involvement of sigma factor σ^{54} in exponential silencing of the *Pseudomonas putida* TOL plasmid Pu promoter. Mol. Microbiol. **19:**7–17.
8. **Cases, I., J.-A. López, J.-P. Albar, and V. de Lorenzo.** 2001. Evidence of multiple regulatory functions for the PtsN (IIA^{Ntr}) protein of *Pseudomonas putida*. J. Bacteriol. **183:**1032–1037.
9. **Cases, I., J. Pérez-Martín, and V. de Lorenzo.** 1999. The IIANtr (PtsN) protein of *Pseudomonas putida* mediates the C source inhibition of the sigma54-dependent Pu promoter of the TOL plasmid. J. Biol. Chem. **274:**15562–15568.
10. **Cases, I., F. Velázquez, and V. de Lorenzo.** 2001. Role of *ptsO* in carbon-mediated inhibition of the *Pu* promoter belonging to the pWW0 *Pseudomonas putida* plasmid. J. Bacteriol. **183:**5128–5133.
11. **Cashel, M., D. Gentry, V. J. Hernandez, and D. Vinella.** 1996. The stringent response, p. 1458–1496. *In* F. C. Neidhardt, R. Curtis III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
12. **Collier, D. N., C. Spence, M. J. Cox, and P. V. Phibbs.** 2001. Isolation and phenotypic characterization of Pseudomonas aeruginosa pseudorevertants containing suppressors of the catabolite repression control-defective crc-10 allele. FEMS Microbiol. Lett. **196:**87–92.
13. **Dinamarca, M. A., I. Aranda-Olmedo, A. Puyet, and F. Rojo.** 2003. Expression of the *Pseudomonas putida* OCT plasmid alkane degradation pathway is modulated by two different global control signals: evidence from continuous cultures. J. Bacteriol. **185:**4772–4778.
14. **Dinamarca, M. A., A. Ruíz-Manzano, and F. Rojo.** 2002. Inactivation of cytochrome *o* ubiquinol oxidase relieves catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. J. Bacteriol. **184:**3785–3793.
15. **Du, Y., A. Holtel, J. Reizer, and M. H. Saier, Jr.** 1996. Sigma54-dependent transcription of the *Pseudomonas putida xylS* operon is influenced by the IIANtr protein of the phosphotransferase system in *Escherichia coli*. Res. Microbiol. **147:**129–132.
16. **Duetz, W. A., S. Marqués, C. de Jong, J. L. Ramos, and J. G. van Andel.** 1994. Inducibility of the TOL catabolic pathway in *Pseudomonas putida* (pWW0) growing on succinate in continuous culture: evidence of carbon catabolite repression control. J. Bacteriol. **176:**2354–2361.
17. **Duetz, W. A., S. Marqués, B. Wind, J. L. Ramos, and J. G. van Andel.** 1996. Catabolite repression of the toluene degradation pathway in *Pseudomonas putida* harboring pWWO under various conditions of nutrient limitation in chemostat culture. Appl. Environ. Microbiol. **62:**601–606.
18. **Gallegos, M.-T., S. Marqués, and J. L. Ramos.** 1996. Expression of the TOL plasmid *xylS* gene in *Pseudomonas putida* occurs from a σ^{70}-dependent promoter or from σ^{70}- and σ^{54}-dependent tandem promoters according to the compound used for growth. J. Bacteriol. **178:**2356–2361.
19. **Garmendia, J., and V. de Lorenzo.** 2000. The role of the interdomain B linker in the activation of the XylR protein of *Pseudomonas putida*. Mol. Microbiol. **38:**401–410.
20. **González-Pérez, M. M., J. L. Ramos, and S. Marqués.** 2004. Cellular XylS levels are a function of transcription of *xylS* from two independent promoters and the differential efficiency of translation of the two mRNAs. J. Bacteriol. **186:**1898–1901.
21. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
22. **Herrmann, S., Q. Ma, M. S. Johnson, A. V. Repik, and B. L. Taylor.** 2004. PAS domain of the Aer redox sensor requires C-terminal residues for native-fold formation and flavin adenine dinucleotide binding. J. Bacteriol. **186:**6782–6791.
23. **Hester, K. L., J. Lehman, F. Najar, L. Song, B. A. Roe, C. H. MacGregor, P. W. Hager, P. V. Phibbs, Jr., and J. R. Sokatch.** 2000. Crc is involved in catabolite repression control of the *bkd* operons of *Pseudomonas putida* and *Pseudomonas aeruginosa*. J. Bacteriol. **182:**1144–1149.
24. **Hugouvieux-Cotte-Pattat, N., T. Köhler, M. Rekik, and S. Harayama.** 1990. Growth phase dependent expression of the *Pseudomonas putida* TOL plasmid pWWO catabolic genes. J. Bacteriol. **172:**6651–6660.
25. **Jishage, M., K. Kvint, V. Shingler, and T. Nystrom.** 2002. Regulation of sigma factor competition by the alarmone ppGpp. Genes Dev. **16:**1260–1270.
26. **Kaniga, K., I. Delor, and G. R. Cornelis.** 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137–141.
27. **Kessler, B., V. de Lorenzo, and K. N. Timmis.** 1992. A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. **233:**293–301.
28. **Laurie, A. D., L. M. Bernardo, C. C. Sze, E. Skarfstad, A. Szalewska-Palasz, T. Nystrom, and V. Shingler.** 2003. The role of the alarmone (p)ppGpp in sigma N competition for core RNA polymerase. J. Biol. Chem. **278:**1494–1503.
29. **Marqués, S., M. T. Gallegos, M. Manzanera, A. Holtel, K. N. Timmis, and J. L. Ramos.** 1998. Activation and repression of transcription at the double tandem divergent promoters for the *xylR* and *xylS* genes of the TOL plasmid of *Pseudomonas putida*. J. Bacteriol. **180:**2889–2894.
30. **Marqués, S., A. Holtel, K. N. Timmis, and J. L. Ramos.** 1994. Transcriptional induction kinetics from the promoters of the catabolic pathways of TOL plasmid pWW0 of *Pseudomonas putida* for metabolism of aromatics. J. Bacteriol. **176:**2517–2524.
31. **Marqués, S., and J. L. Ramos.** 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Mol. Microbiol. **9:**923–929.
32. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Minagawa, J., H. Nakamura, I. Yamato, T. Mogi, and Y. Anraku.** 1990. Transcriptional regulation of the cytochrome b562-o complex in *Escherichia coli*. Gene expression and molecular characterization of the promoter. J. Biol. Chem. **265:**11198–11203.
34. **Morales, G., J. F. Linares, A. Beloso, J. P. Albar, J. L. Martínez, and F. Rojo.** 2004. The *Pseudomonas putida* Crc global regulator controls the expression

of genes from several chromosomal catabolic pathways for aromatic compounds. J. Bacteriol. **186:**1337–1344.
35. **Pérez-Martín, J., and V. de Lorenzo.** 1995. The amino-terminal domain of the prokaryotic enhancer-binding protein XylR is a specific intramolecular repressor. Proc. Natl. Acad. Sci. USA **92:**9392–9396.
36. **Pérez-Martín, J., and V. de Lorenzo.** 1995. The σ^{54}-dependent promoter *Ps* of the TOL plasmid of *Pseudomonas putida* requires HU for transcriptional activation in vivo by XylR. J. Bacteriol. **177:**3758–3763.
37. **Pérez-Martín, J., K. N. Timmis, and V. de Lorenzo.** 1994. Co-regulation by bent DNA. Functional substitutions of the integration host factor site at sigma 54-dependent promoter Pu of the upper-TOL operon by intrinsically curved sequences. J. Biol. Chem. **269:**22657–22662.
38. **Petruschka, L., G. Burchhardt, C. Muller, C. Weihe, and H. Herrmann.** 2001. The *cyo* operon of *Pseudomonas putida* is involved in carbon catabolite repression of phenol degradation. Mol. Genet. Genomics **266:**199–206.
39. **Phillips, A. T., and L. M. Mulfinger.** 1981. Cyclic adenosine 3′,5′-monophosphate levels in *Pseudomonas putida* and *Pseudomonas aeruginosa* during induction and carbon catabolite repression of histidase synthesis. J. Bacteriol. **145:**1286–1292.
40. **Ramos, J. L., S. Marqués, and K. N. Timmis.** 1997. Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid- encoded regulators. Annu. Rev. Microbiol. **51:**341–373.
41. **Rojo, F., and A. Dinamarca.** 2004. Catabolite repression and physiological control, p. 365–387. *In* J. L. Ramos (ed.), Pseudomonas. Kluwer Academic/Plenum Publishers, London, United Kingdom.
42. **Ruíz, R., M. I. Aranda-Olmedo, P. Domínguez-Cuevas, M. I. Ramos-González, and S. Marqués.** 2004. Transcriptional regulation of the toluene catabolic pathways, p. 509–537. *In* J. L. Ramos (ed.), Pseudomonas. Kluwer Academic/Plenum Publishers, London, United Kingdom.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Siegel, L. S., P. B. Hylemon, and P. V. Phibbs, Jr.** 1977. Cyclic adenosine 3′,5′-monophosphate levels and activities of adenylate cyclase and cyclic adenosine 3′,5′-monophosphate phosphodiesterase in *Pseudomonas* and *Bacteroides*. J. Bacteriol. **129:**87–96.
45. **Suh, S. J., L. J. Runyen-Janecky, T. C. Maleniak, P. Hager, C. H. MacGregor, N. A. Zielinski-Mozny, P. V. Phibbs, Jr., and S. E. West.** 2002. Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*. Microbiology **148:**1561–1569.
46. **Sze, C. C., L. M. D. Bernardo, and V. Shingler.** 2002. Integration of global regulation of two aromatic-responsive σ^{54}-dependent systems: a common phenotype by different mechanisms. J. Bacteriol. **184:**760–770.
47. **Sze, C. C., and V. Shingler.** 1999. The alarmone (p)ppGpp mediates physiological-responsive control at the sigma 54-dependent Po promoter. Mol. Microbiol. **31:**1217–1228.
48. **Velázquez, F., I. di Bartolo, and V. de Lorenzo.** 2004. Genetic evidence that catabolites of the Entner-Doudoroff pathway signal C source repression of the σ^{54} *Pu* promoter of *Pseudomonas putida*. J. Bacteriol. **186:**8267–8275.
49. **West, S. E., A. K. Sample, and L. J. Runyen-Janecky.** 1994. The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. J. Bacteriol. **176:**7532–7542.
50. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124:**7–13.
51. **Yuste, L., and F. Rojo.** 2001. Role of the *crc* gene in catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. J. Bacteriol. **183:**6197–6206.

IV.2. CAPÍTULO 2.

Papel del producto del gen *ptsN* en la represión catabólica de la ruta TOL de degradación de tolueno de *Pseudomonas putida* en cultivos en quimiostato.

Autores: Isabel Aranda-Olmedo, Patricia Marín, Juan Luís Ramos y Silvia Marqués.

Enviado a Appl Environ Microbiol. para su publicación.

Resumen:

El cultivo en sistemas de alimentación continua permite mantener constantes una serie de variables que afectan al crecimiento de microorganismos, facilitando así el estudio de otros parámetros de interés. El fenómeno de represión de la ruta TOL de degradación de tolueno codificada en el plásmido pWW0 de *Pseudomonas putida* mt-2, ha sido observado en poblaciones que crecen en cultivo continuo a tasa de dilución alta (μ_{max}). En esta condición todos los nutrientes que se proporcionan para el crecimiento están en exceso en el medio. Este trabajo centra su atención en el estudio del papel de los reguladores globales IIA^{Ntr} (o PtsN) y Crc en este tipo de represión. Aunque ambos reguladores participan en la represión catabólica de la ruta bajo condiciones de crecimiento en cultivos estanco, sólo la proteína IIA^{Ntr} está implicada en la represión de la ruta en cultivo continuo a tasa de dilución alta. Por otra parte, se ha observado que Crc tampoco es responsable de la represión que provoca el exceso de fuente de carbono en poblaciones de *P. putida* creciendo en cultivos estanco. Estos resultados sugieren que la bacteria cuenta con más de una estrategia para responder a diferentes cambios ambientales que requieran que la ruta de degradación de tolueno esté apagada. Los elementos genéticos implicados variarán en función del factor que desencadene el mecanismo de represión.

SHORT-FORM PAPER

Role of the *pstN* gene product in catabolite repression of *Pseudomonas putida* TOL toluene degradation pathway in chemostat cultures.

Isabel Aranda-Olmedo, Patricia Marín, Juan L. Ramos, and Silvia Marqués

Consejo Superior de Investigaciones Científicas, Estación Experimental del Zaidín, Dept. of Environmental Protection, C/. Profesor Albareda nº 1, E-18008-Granada, Spain

The *Pseudomonas putida* KT2440 TOL plasmid upper pathway is strongly repressed under non-limiting conditions in cells growing in continuous culture. We show that IIA^{Ntr} protein is involved in this repression. The Crc protein, which was previously shown to control this repression in batch cultures, did not influence expression when cells are growing in a chemostat.

Bacteria have developed different mechanisms to degrade aromatic compounds widely distributed in natural environments. *Pseudomonas putida* KT2440 *mt*-2 is able to degrade and grow on aromatic substrates such as toluene and xylenes. The upper and *meta*-cleavage pathways required for complete degradation are coded by the TOL plasmid pWW0 present in this strain, where XylR is the master regulator of the concerted expression of the two pathways (20). The *xylR* gene is transcribed from two σ^{70}-dependent tandem promoters, P_{R1} and P_{R2} (Figure 1). In the presence of substrates of the pathway or of certain analogues, XylR protein becomes active (1) and induces transcription from the two σ^{54}-dependent promoters of this system, the upper pathway promoter Pu and the *xylS* promoter P_{S1}. XylR binding sites to activate P_{S1} overlap the two *xylR* tandem promoters, thus repressing their expression and consequently its own synthesis (3, 20, 21).

Induction of the TOL degradation pathway by XylR is silenced under several growth conditions. Repression is exerted at the transcriptional level, the catabolic repression targets being the TOL σ^{54}-dependent promoters Pu and P_{S1} (7, 16). Initially, this phenomenon was described as a result of growth phase dependence because TOL induction was repressed in the exponential growth phase in rich medium, and repression disappeared when cells entered the late-exponential growth phase (6, 15). This phenomenon was thus referred as "exponential silencing" (4). It was subsequently observed that TOL pathway operation was dependent on growth medium composition. In cells growing in the exponential phase on minimal medium, TOL upper pathway activation occurred immediately after the addition of a XylR effector, whereas activation was delayed when cells were growing in exponential phase on complex rich medium. This was attributed to the presence of one or more inhibitory compounds in this medium. Amino acids were implicated as the factor responsible for the repression (16). Additional evidence came from the observation that some carbon sources (glucose, gluconate, α-ketogluconate, lactate and acetate) but not others (succinate, citrate, pyruvate, glycerol, fructose, and arabinose) inhibited activation of Pu in batch cultures (4, 13, 22). Moreover, total repression of the TOL pathway was observed in cells growing in continuous culture at a non-limiting rate (μ_{max}) (7). When cultures where limited in carbon (regardless of the carbon source used) TOL expression was completely derepressed. However, when a similar low growth rate was maintained through conditions of phosphate, sulphate or nitrogen limitation (i.e., with excess carbon in all cases) TOL induction was repressed. This suggests that catabolite repression is mediated by excess of carbon rather than growth rate. In accordance with this statement, we observed that when an anabolic substrate (P, S, or N) was the growth-limiting factor, which is equivalent to a high energy state in the cell (11), the TOL degradation pathway was repressed, but when a catabolic substrate (C or O_2) was the growth-limiting factor, which is equivalent to a low energy state (11), the TOL degradation pathway was functional (8).

Although the molecular basis of the physiological control of the TOL degradation pathway is unknown, some proteins have been suggested to be involved in this process. IIA^{Ntr}, the *ptsN* gene product, contributes to Pu repression by glucose or gluconate, but does not seem to promote Pu down-regulation in cells growing in the exponential phase on LB (5). These results point to differences in the genetic factors used by the cell to adapt to environmental conditions. However, it was recently shown that IIA^{Ntr} is also involved in the rich medium-dependent repression exerted on Pu and P_{S1} promoters (2). In addition, the Crc protein, a protein found involved in several catabolic repression mechanisms in *Pseudomonas* (12, 19, 22, 23), has been shown to contribute to TOL σ^{54}-dependent promoter repression in rich medium. The role of the Crc protein may be to "sense" the energy state of the cell and thus help the cell to launch an appropriate response (2)

Nevertheless, our knowledge of the genetic factors involved in TOL catabolic repression observed in continuous culture has thus far been limited. To clarify whether cells respond differently in terms of catabolic repression of the TOL pathways under optimal growth conditions, we investigated the extent to which TOL inhibition in cells growing at maximum growth rate

* Corresponding autor. Mailing address: CISC (Consejo Superior de Investigaciones Científicas), Estación Experimental del Zaidín, Dept. of Environmental Protection, C/. Profesor Albareda nº 1, E-18008-Granada, Spain. Phone: (+34) 958181600, ext. 285. Fax: (+34) 958129600. E-mail: silvia@eez.csic.es.

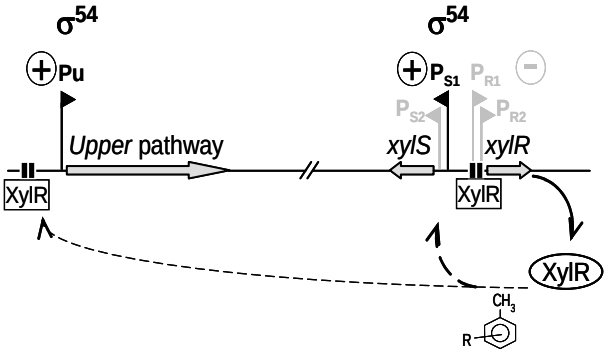

Figure 1. pWW0 TOL plasmid upper-pathway regulation. The diagram shows stimulation by the XylR protein of the two TOL pathway σ^{54}-dependent promoters, Pu and P_{S1}. The TOL upper pathway and regulatory genes *xylS* and *xylR* are shown as grey arrows. R is any substituent group in the aromatic ring. Elliptical and square boxes indicate the inactive and active forms of XylR, respectively. Plus and minus signs indicate transcription activation or repression by XylR, respectively. Black small boxes represent XylR binding sites to activate Pu and P_{S1} (UASs). Promoters are outlined as flags.

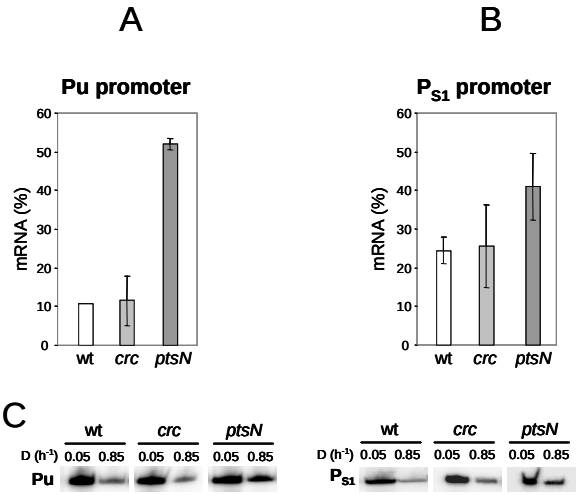

Figure 2. Level of mRNA derived from Pu and P_{S1} promoters from *P. putida* (pWW0) and its *ptsN* and *crc* mutants grown in continuous culture, in response to *o*-xylene at D = 0.05 h^{-1} (carbon limitation) and D = 0.85 h^{-1} (carbon excess). The presence of messengers was analysed by reverse primer extension of equal amounts (10 or 20 µg) of total RNA with the corresponding labelled specific oligonucleotide (2). Samples were run in urea sequencing gels, which were exposed to a phosphor screen (Fuji Photo Film Co; LTD) for 5-12 h. Phosphor screens were scanned with a phosphor-imaging instrument (Molecular Imager FX, Bio-Rad). Data were quantified with Quantity One software (Bio-Rad). The graph shows the mRNA level of Pu (A) and P_{S1} (B) promoters in the three strains at D = 0.85 h^{-1} as a percentage of the levels observed in each strain at low growth rate (D = 0.05 h^{-1}). (C) Representative urea-PAGE results corresponding to mRNA-derived cDNA from the two promoters at each growth rate are shown.

(excess of all nutrients including carbon) was affected by the inactivation of genes previously shown to play a role in repression under batch culture conditions. We set up a number of continuous cultures to grow the cells for many generations under selected constant conditions.

The strains tested here were *P. putida* KT2440 (pWW0) and its *ptsN* and *crc* mutants (2). Custom-made reactors with working volumes of 100 ml of mineral salt medium (9) supplemented with 10 mM succinate, a primary carbon and energy source for pseudomonads (10), were used. Flasks were inoculated with 15 ml of an overnight culture and were maintained at 30 ºC and stirred at 150 rpm with a constant airflow. Cells were grown at a low dilution rate mimicking environmental conditions (D = 0.05 h^{-1}, with carbon source as the growth-limiting factor) to determine full induction level, and at a high dilution rate (D = 0.85 h^{-1}, with excess carbon) to set up catabolite repression conditions (7). Succinate concentration remaining in the vessel was checked by high-performance liquid chromatography with a Water Nova-Pak C18 column and a UV detector. Chromatography was performed at 0.8-ml min^{-1} in 0.2 M H_3PO_4, and succinate was detected at 210 nm. Continuous cultures subjected to a high dilution rate (D = 0.85 h^{-1}) showed a residual concentration of succinate in the supernatant of 1 to 6 mM (not shown), similar to previously reported values (9). As expected, in cultures growing at D = 0.05 h^{-1}, no succinate could be detected in the effluent. After induction for 5 min of the cultures with *o*-xylene (a non-metabolizable effector of XylR) through the gas phase via an additional airflow, 10-ml samples were rapidly taken from the reactors for RNA isolation and colony counts in selective and non-selective media, and to measure pH and the residual concentration of succinate. Total RNA extraction and primer extension analysis of 10-20 µg of total RNA were carried out as described previously (2, 17).

Figure 2 compares induction values of Pu and P_{S1} at the two dilution rates. As shown before, transcription from the Pu and P_{S1} promoters in the wild-type strain was repressed in cells growing under conditions of excess carbon (D = 0.85 h^{-1}) compared to cells growing with the carbon source as the growth-limiting factor (D = 0.05 h^{-1}) (7). When an excess of succinate was present in the growth medium, Pu transcription was reduced to about 10% of the Pu transcription level in a carbon-limited medium. P_{S1} transcription was also reduced at the highest dilution rate to about 25% of the values observed under succinate limitation. Differences between the promoter regions in Pu and P_{S1} promoters (20) may account for the different repression levels in the two promoters under the same growth conditions.

Analysis of σ^{70}-dependent *xylR* tandem promoter transcription is a useful tool to indirectly tract the efficiency of P_{S1} transcription. As mentioned above, XylR activation of P_{S1} blocks P_{R1} and P_{R2} transcription through binding to its upstream activation sites (UAS) overlapping P_{R1} and P_{R2} promoters (20). Therefore, when P_{S1} transcription is favoured, P_{R1} and P_{R2} transcription is significantly reduced (3, 14). Figure 3 shows that in the wild-type strain, *xylR* promoter transcription was higher in cells growing under conditions of excess carbon (D = 0.85 h^{-1}) than in cells under carbon limitation (D = 0.05 h^{-1}). With excess succinate in the growth medium, P_{R1} and P_{R2} transcription was practically double (Figure 3) in cultures growing at maximum rate. These results confirm a lower occupancy of XylR UAS in P_{S1} at high growth rates, in good correlation with the observed decrease in P_{S1} activity.

Repression of Pu promoter transcription in the *ptsN* mutant when the culture was subjected to excess carbon was lower than in the wild-type strain. In this mutant, Pu transcription was reduced only by about 50% compared to Pu transcription in the carbon-limited medium. A similar behaviour was observed for P_{S1} promoter transcription, although P_{S1} derepression was less apparent because of the weaker repressive effect in this promoter in the wild type (Figure 2). As expected, the increase in P_{R1} and P_{R2}

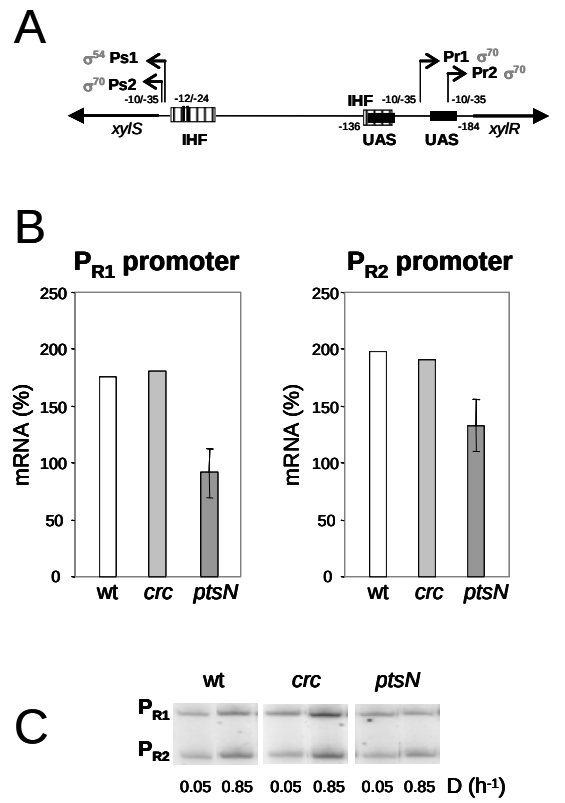

Figure 3. Level of mRNA derived from the P_{R1} and P_{R2} promoters from *P. putida* (pWW0) and its *ptsN* and *crc* mutants grown in continuous culture, in response to *o*-xylene at D = 0.05 h^{-1} (carbon limitation) and D = 0.85 h^{-1} (carbon excess). The presence of messengers was analysed by reverse primer extension of equal amounts (10 or 20 µg) of total RNA with the corresponding labelled specific oligonucleotide (2). Samples were run in urea sequencing gels, which were exposed to a phosphor screen (Fuji Photo Film Co; LTD) for 5-12 h. Phosphor screens were scanned with a phosphor-imaging instrument (Molecular Imager FX, Bio-Rad). Data were quantified with Quantity One software (Bio-Rad). A) The diagram at the top of the figure represents the *xylR*/*xylS* intergenic region (14). B) The graph shows the mRNA level of P_{R1} and P_{R2} promoters in the three strains at D = 0.85 h^{-1} as a percentage of the levels observed in each strain at low growth rate (D = 0.05 h^{-1}). C) Representative urea-PAGE results corresponding to mRNA-derived cDNA from the two promoters at each growth rate are shown.

transcription was smaller when P_{S1} transcription was more markedly enhanced under non-limiting growth conditions (Figure 3).

When we analysed transcription of Pu and P_{S1} in the *crc* mutant as above, we found no significant difference from the wild-type strain (Figure 2). Accordingly, P_{R1} and P_{R2} transcription remained unchanged (Figure 3). In the light of these results, a question mark hangs over the role of Crc in TOL catabolic repression in batch cultures when glucose is the repressor agent. Crc has been shown to contribute to TOL regulation in rich medium-mediated repression (2), but no data are available on its putative role in glucose-mediated repression in batch cultures. We therefore investigated the role of Crc in TOL repression in cells growing on minimal medium in the presence of glucose. Our approach was to compare the β-galactosidase activity of wild-type and *crc* mutant strains carrying the pS10 plasmid, which bears a Pu::*lacZ* fusion and the *xylR* regulator gene (2), in the presence of 75 mM (1.5% [wt/vol]) glucose in the medium. The *P. putida ptsN* mutant was used as a positive control of derepression of the Pu promoter (5). Overnight cultures were grown on mineral salt medium supplemented with 0.2% (wt/vol) casamino acids as the carbon source (5). The cultures were diluted at OD_{660} = 0.2 in the same medium and were divided in two fractions. One of them was supplemented with both the Pu inducer *o*-xylene in the gas phase and with 1.5% glucose, and to the second fraction only the inducer was added in the absence of glucose. When cultures reached the exponential phase, we measured β-galactosidase activity as described (18) (Figure 4). Pu promoter expression was repressed in the presence of 1.5% (wt/vol) glucose in the wild-type strain *P. putida* KT2440 (4), but as shown previously, this repression was weaker in the *ptsN* mutant, which seemed to show no evidence of the repressive effect of glucose on Pu (5). In the *crc* mutant, glucose repression of Pu was similar to the repression seen in the wild type. This finding shows that despite its role in Pu repression in rich medium (2), Crc does not participate in the Pu repression caused by an excess of carbon either in continuous or in batch cultures (Figure 4). We can therefore conclude that the role of Crc in catabolic repression of the TOL plasmid is not directly related with the presence of a carbon source per se.

Taken together, these results help clarify some aspects of the processes controlling the toluene degradation pathway of the TOL plasmid pWW0 in *P. putida* KT2440. Bacterial growth in continuous culture avoids the drawbacks of the initial growth lag phase, changes in growth medium composition during culture, and the onset of the stationary phase. In short, continuous culture helps maintain a physiological steady state at a selected growth rate in a closed culture system. In this study we analyzed how knock-out of the genes known to play a role in TOL repression under batch culture conditions affected the intensity of repression of the TOL upper pathway in continuous culture with excess carbon. Previous work showed that the products of the *ptsN* and *crc* genes (IIA^{Ntr} and Crc proteins, respectively) played an important role in repression of the TOL plasmid catabolic pathways (2, 5). To establish whether these genetic factors were also involved in the catabolic repression observed in continuous culture, we analysed TOL promoter transcription in *ptsN* and *crc* mutants in continuous cultures. Primer extension analyses showed that, in

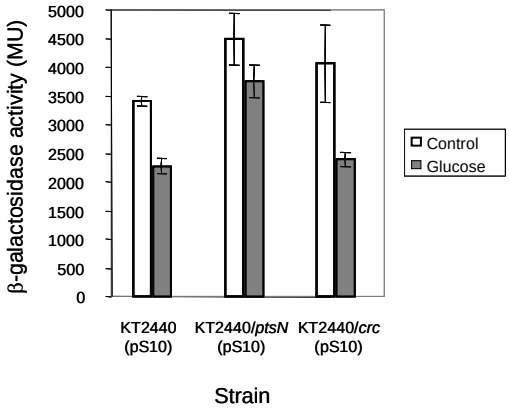

Figure 4. Effect of glucose on Pu expression. β-galactosidase activity (as Miller units, MU) was measured in *o*-xylene-induced batch cultures of *P. putida* strain KT2440 and its *ptsN* and *crc* mutants carrying the pS10 plasmid with Pu fused to the *lacZ* gene and the regulator gene *xylR* (2), in the presence or absence of glucose.

contrast with findings in batch cultures, *ptsN* but not *crc* was involved in the phenomenon of TOL catabolic repression under these conditions. Subsequent analysis of Pu expression in the *crc* mutant grown in batch cultures confirmed that Crc did not play a role in TOL repression by excess carbon. These results point to a difference in the genetic factors used by the cell to adapt to different environmental conditions that cause TOL catabolic repression. We conclude that no single response mechanism is suitable for all environmental conditions; instead, cells may resort to more than one strategy to react appropriately to a changing environment. Cells seem to be able to distinguish catabolic repression triggered by excess carbon from repression produced by some components of rich medium.

ACKNOWLEDGEMENTS

This work was supported by EC grant QLK3-CT-2002-01923, grant BCM 2001-0515 from the Spanish Ministry of Science and Education and grant VEM2004-08560 from Spanish Ministry of the Environment. We thank K. Shashok for checking the English in the manuscript.

REFERENCES

1. **Abril, M. A., C. Michán, K. N. Timmis, and J. L. Ramos.** 1989. Regulator and enzyme specificities of the TOL plasmid encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol **171:**6782-6790.
2. **Aranda-Olmedo, I., J. L. Ramos, and S. Marqués.** 2005. Integration of signals through Crc and PtsN in catabolite repression of *Pseudomonas putida* TOL plasmid pWW0. Appl Environ Microbiol **71:**4191-8.
3. **Bertoni, G., S. Marqués, and V. de Lorenzo.** 1998. Activation of the toluene-responsive regulator XylR causes a transcriptional switch between sigma54 and sigma70 promoters at the divergent Pr/Ps region of the TOL plasmid. Mol. Microbiol. **27:**651-659.
4. **Cases, I., V. de Lorenzo, and J. Pérez-Martín.** 1996. Involvement of sigma factor s^{54} in exponential silencing of the *Pseudomonas putida* TOL plasmid Pu promoter. Mol. Microbiol.
5. **Cases, I., J. Pérez-Martín, and V. de Lorenzo.** 1999. The IIANtr (PtsN) protein of *Pseudomonas putida* mediates the C source inhibition of the sigma54-dependent Pu promoter of the TOL plasmid. J Biol Chem **274:**15562-8.
6. **de Lorenzo, V., I. Cases, M. Herrero, and K. Timmis.** 1993. Early and late responses of TOL promoters to pathway inducers: identification of growth-phase-dependent promoters in *Pseudomonas putida* with *lacZ-tet* bicistronic reporters. J. Bacteriol. **175:**6902.
7. **Duetz, W. A., S. Marqués, C. de Jong, J. L. Ramos, and J. G. van Andel.** 1994. Inducibility of the TOL catabolic pathway in *Pseudomonas putida* (pWW0) growing on succinate in continuous culture: evidence of carbon catabolite repression control. J Bacteriol **176:**2354-61.
8. **Duetz, W. A., S. Marqués, B. Wind, J. L. Ramos, and J. G. van Andel.** 1996. Catabolite repression of the toluene degradation pathway in *Pseudomonas putida* harboring pWW0 under various conditions of nutrient limitation in chemostat culture. Appl Environ Microbiol **62:**601-6.
9. **Duetz, W. A., and J. G. van Andel.** 1991. Stability of TOL plasmid pWW0 in *Pseudomonas putida* mt-2 under non-selective conditions in continuous culture. J Gen Microbiol **137:**1369-74.
10. **Harder, W., and L. Dijkhuizen.** 1982. Strategies of mixed substrate utilization in microorganisms. Philos Trans R Soc Lond B Biol Sci **297:**459-80.
11. **Hellingwerf, K. J., I. Friedberg, J. S. Lolkema, P. A. Michels, and W. N. Konings.** 1982. Energy coupling of facilitated transport of inorganic ions in *Rhodopseudomonas sphaeroides.* J Bacteriol **150:**1183-91.
12. **Hester, K. L., J. Lehman, F. Najar, L. Song, B. A. Roe, C. H. MacGregor, P. W. Hager, P. V. Phibbs, Jr., and J. R. Sokatch.** 2000. Crc is involved in catabolite repression control of the *bkd* operons of *Pseudomonas putida* and *Pseudomonas aeruginosa*. J Bacteriol **182:**1144-9.
13. **Holtel, A., S. Marqués, I. Möhler, U. Jakubzik, and K. N. Timmis.** 1994. Carbon source-dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. J. Bacteriol. **176:**1773-1776.
14. **Holtel, A., K. Timmis, and J. Ramos.** 1992. Upstream binding sequences of the XylR activator protein and integration host factor in the *xylS* gene promoter region of the *Pseudomonas* TOL plasmid. Nucleic Acids Res. **20:**1755-1762.
15. **Hugouvieux-Cotte-Pattat, N., T. Köhler, M. Rekik, and S. Harayama.** 1990. Growth phase dependent expression of the *Pseudomonas putida* TOL plasmid pWW0 catabolic genes. J. Bacteriol **172:**6651-6660.
16. **Marqués, S., A. Holtel, K. N. Timmis, and J. L. Ramos.** 1994. Transcriptional induction kinetics from the promoters of the catabolic pathways of TOL plasmid pWW0 of *Pseudomonas putida* for metabolism of aromatics. J Bacteriol **176:**2517-24.
17. **Marqués, S., and J. L. Ramos.** 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Mol Microbiol **9:**923-9.
18. **Miller, J.** 1972. p. 352-355, Experiments in molecular genetics. Cold Spring Harbor, N.Y., USA.
19. **Morales, G., J. F. Linares, A. Beloso, J. P. Albar, J. L. Martínez, and F. Rojo.** 2004. The *Pseudomonas* putida Crc global regulator controls the expression of genes from several chromosomal catabolic pathways for aromatic compounds. J Bacteriol **186:**1337-44.
20. **Ramos, J. L., S. Marqués, and K. N. Timmis.** 1997. Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid- encoded regulators. Annu Rev Microbiol **51:**341-73.
21. **Ruíz, R., M. I. Aranda-Olmedo, P. Domínguez-Cuevas, M. I. Ramos-González, and S. Marqués.** 2004. Transcriptional regulation of the toluene catabolic pathways, p. 509-537. *In* Ramos JL (ed.), Pseudomonas, vol. 2. Kluwer Academic/Plenum Publishers, London.
22. **Velázquez, F., I. di Bartolo, and V. de Lorenzo.** 2004. Genetic evidence that catabolites of the Entner-Doudoroff pathway signal C source repression of the sigma54 Pu promoter of *Pseudomonas putida*. J Bacteriol **186:**8267-75.
23. **Yuste, L., and F. Rojo.** 2001. Role of the *crc* gene in catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. J Bacteriol **183:**6197-206.

IV.3. CAPÍTULO 3.

Análisis transcripcional de la agrupación génica *rpoN* de *Pseudomonas putida*.

Autores: Isabel Aranda-Olmedo, Patricia Marín, Juan Luís Ramos y Silvia Marqués.

Pendiente de envío para su publicación.

Resumen:

El gen *rpoN* codifica el factor sigma alternativo de la RNA polimerasa σ^{54}. El estudio del entorno cromosómico de *rpoN* reveló la existencia de un complejo sistema de regulación que engloba a *rpoN* y a los cuatro genes posteriores: *orf102*, *ptsN*, *orf284* y *ptsO*. Tiene especial interés la presencia en este grupo del gen *ptsN*, que codifica el regulador global IIA^{Ntr} implicado en represión catabólica de algunos promotores dependientes de σ^{54}. Dos promotores dirigen la transcripción del conjunto de genes estudiado. Uno de ellos se sitúa en la región anterior a *rpoN* y está autorregulado por el producto de *rpoN*, y otro solapa con la región que codifica a *rpoN* y dirige la transcripción del resto de genes. En este segundo promotor se ha demostrado la existencia de un elemento UP que estimula la transcripción mediante interacción con la subunidad α de la RNA polimerasa. La transcripción desde los dos promotores descritos produce transcritos con una región lider de tamaño considerable, sugiriendo que podrían existir además mecanismos de regulación post-transcripcional de estos mensajeros. Por otra parte, se ha detectado en esta región la presencia de dos transcritos antisentido de los que se desconoce aún el mecanismo de actuación y que parecen no codificar proteínas. Deducir el papel de estos RNAs no codificantes en la regulación de *rpoN* y de los genes posteriores permitirá un mejor entendimiento de la transcripción de genes por la RNA polimerasa asociada al factor alternativo σ^{54}.

Transcriptional analysis of the *Pseudomonas putida rpoN* gene cluster

Isabel Aranda-Olmedo, Patricia Marín, Juan L. Ramos and Silvia Marqués*

Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Apartado de Correos 419, E-18080 Granada, Spain

*Corresponding autor: Mailing ardes:, Estación Experimental del Zaidín, CSIC, C/. Profesor Albareda 1, E-18008 Granada, Spain, Phone: +34-958-181600, Fax: +34-958-129600, E-mail: silvia@eez.csic.es

The alternative sigma factor σ^{54} is involved in transcription of several catabolic genes in *P. putida* mt-2. As for most Gram negative bacteria, its coding gene *rpoN* is clustered with four additional ORFs in *P. putida* chromosome. These ORFs code for proteins of unknown function, which are thought to modulate σ^{54} activity. We have analyzed gene organization and expression regulation of all genes present in the *rpoN* cluster. Results show that *rpoN* promoter is auto-regulated and gives rise to two putative transcripts, a short one coding for σ^{54} and a long one encompassing the five genes in the cluster. A second promoter located at *rpoN* 3'-end controls expression of the four modulating genes present in the cluster. In addition, two anti-sense mRNA are produced complementary to *rpoN* and *ptsN*. *In silico* sequence analysis points to the *rpoN-orf102* intergenic region as the main target for gene expression regulation. Two potential transcriptional terminators, five *rpoN* binding sites and several inverted repeat sequences can be found in an 80 bp sequence stretch. The fact that *orf102*, the tightest regulated gene in the cluster, codes for a ribosome related protein suggests an additional regulatory step at the translational level.

The use of alternative sigma factors in RNA polymerase (RNAP) allows a fine modulation of global expression patterns in response to different cellular signals (growth phase, metabolic state, etc.) and especially to environmental signals, which plays a central role in cell adaptation to culture media. Eubacterial RNAP core is composed of five subunits: β,β', α_2 and ω. A sixth subunit, the sigma factor, transiently binds RNAP core and confers on it affinity for specific promoter regions in the DNA. The major σ factor in Gram negative bacteria is σ^{70} and the majority of alternative sigma factors present in prokaryotes show homology to this σ factor, suggesting a structural and functional similarity (43). σ^{54} is the only alternative sigma factor found in Gram negative bacteria not belonging to the σ^{70} family (38). Furthermore, its structure and activation mechanism are distinct from those of σ factors belonging to that group (38). Unlike in RNAP with σ^{70}-family sigma factors, transition from close to open complex in RNAP with σ^{54} ($E\sigma^{54}$) is strictly dependent on a special class of transcriptional activators (the NtrC family) recently classified within the broader AAA+ family of ATPases (42). σ^{54} has been involved in a variety of functions, including carbon source utilization, nitrogen metabolism, fermentation pathways, flagellar synthesis or bacterial virulence (11, 50). Because of the relevance of these σ^{54}-regulated processes, extensive studies have been carried out in the past 20 years to elucidate the activation mechanism of σ^{54}-dependent promoters. However, very little is known about the regulation of its own expression.

σ^{54} is widespread among bacteria, and its coding gene *rpoN* has been detected in all Gram negative strains sequenced so far. In all cases, *rpoN* was found clustered with several downstream open reading frames (ORFs) (2, 27, 28, 30, 56) which are highly conserved in most bacterial groups: *orf102*, *ptsN*, *orf284* and *ptsO*, named following *E. coli* nomenclature. Some of these proteins have been implicated in modulating σ^{54} activity (39) although their precise function has not yet been elucidated. Among them *ptsN*, the third gene in the cluster, shows interesting features (10). Sequence homology indicates that its gene product is a paralogue of the classical phosphotransferase transport system (PTS) IIA protein, and was thus designated IIA^{Ntr}. The nitrogen PTS would also include the protein NPr (*ptsO*), the last ORF in the cluster, and *ptsP*, an Enzyme I (EI) paralogue found in an unrelated location in the chromosome (12, 46). Attempts have been carried out to elucidate the physiological function of these proteins in different strains. In *Klebsiella pneumoniae*, mutants in either *ptsN* or the corresponding ORF102 gene (*orf102*) (ORF95 in *K. pseumoniae*) showed that these proteins exerted a negative effect on σ^{54}-dependent P_{nifH} transcription, while *ptsO* would have a positive effect (39). A similar result was observed in *Caulobacter crescentus*, although not for all σ^{54}-dependent promoters tested (27). Surprisingly, *ptsN* repressive effect was not observed in σ^{54}-promoters for pilin and flagellin expression in *P. aeruginosa*, although a *ptsN* mutation severely impaired growth in defined minimal medium (28). It seems therefore that the effects of mutations in the *rpoN* cluster genes are not common to all σ^{54}-dependent promoters, but probably specific sequences and/or regulators modulate the response in each case. The similarity of the *rpoN* cluster proteins to the carbon PTS and their effect on transcription of certain σ^{54}-promoter has led to the speculation that protein phosphorylation involving the three components of the Ntr phosphoryl relay could represent a link between carbon and nitrogen assimilation (45, 51).

P. putida mt-2 is a soil bacterium with versatile metabolic and environmental response capacities, which has been extensively used in bioremediation,

Table 1.- Strains and plasmids used in this work.

Strains	Characteristics	Reference
P. putida KT2440	mt-2 pWW0 cured	(6)
P. putida KT2440 *rpoN*::Km	Km^r, *rpoN* derivative of *P. putida* KT2440	(34)
P. putida KT2440 *orf284*::Km	Km^r, *orf284* derivative of *P. putida* KT2440	(10)
E. coli DH5αF	F′/*endA1 hsdR17*($r_k^-m_k^-$) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169* [ϕ*80dlac*Δ(*lacZ*)M15]	(19)
E. coli CAG1574 (pLAX185)	MC1061 *recA* with wild type *rpoA* in a plasmid.	(20)
E. coli CAG1574 (pLAD256)	MC1061 *recA* with mutant Δ256 *rpoA* in a plasmid.	(20)
E. coli JM109 (pHTf1α)	JM109 with wild type *rpoA* in a plasmid	(17)
E. coli JM109 (pHTf1αR265A)	JM109 with mutant R265A *rpoA* in a plasmid.	(17)
Plasmids		
pBluescriptII (SK+)	Ap^r	Stratagene, Co.
pMP220	IncP, Tc^r, *cat*::′*lacZ*. Broad-host range promoter-probe vector.	(54)
pM102-4	Transcriptional fusion of the intergenic *rpoN-orf102*, in pMP220.	This work
pM102-5	Transcriptional fusion of P_{102} promoter including the -10/-35 RNAP binding site, in pMP220.	This work
pM102-UP	Transcriptional fusion of P_{102} promoter including the -10/-35 RNAP binding site and the UP element, in pMP220.	This work
pM102-6	Transcriptional fusion of P_{102} promoter including the -10/-35 RNAP binding site, the UP element, and an inverted repeat, in pMP220.	This work
pM102-7	Transcriptional fusion of P_{102} promoter including the -10/-35 RNAP binding site, the UP element, and two inverted repeats, in pMP220.	This work
pM*arpoN*	Transcriptional fusion of $P_{anti\text{-}rpoN}$ promoter in pMP220 EcoRI/PstI.	This work
pM*aptsN*	Transcriptional fusion of $P_{anti\text{-}ptsN}$ promoter in pMP220 EcoRI/PstI.	This work
pBSrpoN	ScaI/HinDIII 1′86 Kb fragment including *P. putida* KT2440 *rpoN* gene, in pBluescript SmaI/HinDIII sites.	This work
pLAFR3*rpoN*	Tc^r, *P. putida* KT2440 library cosmid including *rpoN* gene cluster.	This work, (49)
pRK600	Cm^r, ori ColE1, mob RK2, tra RK2. Helper plasmid	(32)
pGEM-T	PCR product cloning vector	Promega, Co

rhizoremediation and environmental monitoring processes (37). The presence of the TOL plasmid enables this strain to degrade toluene, xylenes and a number of toxic derivatives. The TOL biochemical pathways responsible for aromatic degradation are tightly regulated at the transcriptional level. In the presence of a substrate of the pathway XylR, the master regulator of the pathway, activates both the upper pathway for oxidation of toluene to benzoate, and transcription of the XylS regulator which specifically controls the lower segment for benzoate degradation to Krebs cycle intermediates (47). XylR is homologous to NtrC family regulators and the two XylR-responsive promoters Pu and P_{S1} show the typical σ^{54}-dependent promoter architecture. In fact, expression of these promoters is abolished in a *P. putida rpoN* mutant despite the presence of toluene activated XylR, so degradation of the toxic aromatic compounds is fully dependent on this alternative sigma factor (14, 24, 34, 48). Recently, it has been shown that the toluene degradation pathway is subject to global regulation in the presence of alternative carbon sources through an unknown mechanism which involves the *ptsN* encoded protein IIA^{Ntr}. A knocked-out mutant in the *ptsN* gene was impaired in both carbon source and rich medium repression in *P. putida*. No other gene in the cluster produced any change in *P. putida* repression phenotype (4, 10). Indirect evidences suggested that IIA^{Ntr} could exert its repressive role directly at the level of the transcriptional machinery (4). Except for *rpoN*, no information is available on the transcriptional regulation of the different genes present in this cluster in *P. putida* (33). As a first attempt to unravel the physiological role of the *rpoN* gene cluster, we thoroughly analyzed the transcriptional organization and regulation of its different genes. RT-PCR revealed the presence transcripts in both directions. The location of promoters was determined by primer extension and confirmed with promoter-*lacZ* fusions. Transcriptional regulation of the operons was further investigated. Our results show that two promoters control expression of cluster genes, and two reverse transcripts are also synthesized in this region. Evidences suggest that many regulatory mechanisms control protein levels at both the transcriptional and post-transcriptional stage. The functional consequences of such an intricate organization are discussed.

MATERIAL AND METHODS

Strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* and *P. putida* strains were routinely grown at 30ºC in liquid Luria-Bertani (LB) medium (53). When required, antibiotics were used at the following final concentration (in μg/ml); kanamycin, 25; tetracycline, 20; gentamicin, 100; cloramphenicol, 30; and streptomycin, 100. The following plasmids were constructed in this work: plasmid pM102-4 was obtained by inserting a 154 bp fragment obtained through amplification of the *rpoN* region with oligonuleotides 1pstRP102 and 1ecoRP102 (Table 2) between the PstI/EcoRI sites in pMP220 (54). The cloned fragment included 83 bp of *rpoN* 3'-end and 71 bp of the flanking intergenic region. To obtain the transcriptional fusions plasmids pM102-5, pM102-UP, pM102-6 and pM102-7 (Table 1), a similar strategy was followed except that oligonucleotides 2AecoRP102, UP102eco, 2BecoRP102 or 2CecoRP102, respectively, were used with 2pstRP102 (Table 2). pMarpoN was obtained by inserting between the EcoRI and PstI sites of pMP220 a 319 bp fragment including the anti-*rpoN* putative promoter region amplified by PCR with 5RP-arpoN and 3RP-arpoN oligonulceotides. pMaptsN was obtained in a similar way by inserting in pMP220 a 237 bp fragment including the anti-*ptsN* putative promoter region amplified by PCR with 5RP-aptsN and 3RP-aptsN oligonucleotides. All PCR products were sequenced to rule out the presence of unwanted mutations. Plasmids were constructed in *E. coli* DH5α and transferred to

P. putida KT2440 by conjugation using pRK600 as helper plasmid.

Primer extension analysis. *P. putida* cells grown overnight in LB medium were diluted in the same medium to a turbidity at 660 nm of 0.2. When the cultures reached exponential growth 10 ml samples were harvested by centrifugation at 4ºC in disposable plastic tubes precooled in liquid N_2 and were kept at −80 ºC until use. Total RNA was extracted with the phenol-guanidine thiocyanate mixture Tri Reagent LS (Molecular Research Center, Inc.) according to the manufacturer's instructions, except that the initial lysis step was carried out at 60 ºC. The relative level of each specific messenger was estimated by reverse primer extension analysis of equal amounts of total RNA, using the following oligonucleotides (Table 2): PErpoN for *rpoN* transcripts, PE102, P102, and 220PE for *orf102*, P-anti1 for anti-*ptsN*

Table 2.- Oligonucleotides used in this work

Name	Sequence	Application[1]
1ecoRP102	5'-agaattccaggtagcccgtc-3'	RT-PCR
1pstRP102	5'-ttctgcagtcctttgttgccc-3'	RT-PCR
1RP-anti	5'- agaattcgttcagtgctcgt -3'	RT-PCR
220PE	5'-cattttagcttccttagctcctg -3'	PE
2AecoRP102	5'-tgaattcattgagtgacagcaag-3'	RT-PCR/PC
2BecoRP102	5'--tgaattctgaaatacttttttctcc-3'	RT-PCR/PC
2CecoRP102	5'-tgaattcgaccatttcgcggg-3'	RT-PCR/PC
2pstRP102	5'- tactgcagacggtgcgacg -3'	RT-PCR/ PC
2RP-anti	5'-tgaattcggtgctcttgcc-3'	RT-PCR
3RP-anti	5'-agaattcaatagaaaccgctg-3'	RT-PCR
3RPaptsN	5'-tgaattcgcagcaattcggt -3'	PC
3RParpoN	5'-agaattcggcgttggctaccac -3'	PC
3RT284	5'-gggaaaacgcatcaggtggct-3'	PE
3RTptsN	5'-tggcgacctttttccagggcg -3'	PE/ RT-PCR
3RTptsO	5'-ctgcggccagcatcatcacc-3'	PE/ RT-PCR
3RTrpoN	5'-gcatcgggttcgactccagc-3'	RT-PCR
5'102*rpoN*	5'-tagcccctgagatgtgcc-3'	RT-PCR
5RPaptsN	5'-aactgcaggaggtccgtg -3'	PC
5RParpoN	5'-atctgcagtccctcggcatcg -3'	PC
5RT102	5'-gagaatgctcgtccacggcg -3'	PE/ RT-PCR
5RT*ptsN*	5'-gagtcacttcgacaagatcacc-3'	RT-PCR
5RTptsO	5'-agccacctgatgcgttttccc-3'	PE/ RT-PCR
5RT*rpoN*	5'-ctgatcgccgaaggtgacgc-3'	RT-PCR
P102	5'-tacatcagtcgcttgcgttcg-3'	PE/RT-PCR
P-anti1	5'-tgcccaccttgaacttctgcg-3'	PE/ RT-PCR
P-anti2	5'-agccacctgatgcgttttccc-3'	PE
P-anti3	5'-cgtggaaagcgacttgttggg-3'	PE/ RT-PCR
PE102	5'-ctgtccactgatattgacttgc-3'	PE/RT-PCR
PErpoN	5'-gcccatttttaggacgagcgatg-3'	PE/RT-PCR
RT1	5'-caggggcttaatacctttgttcg-3'	RT-PCR
UP102eco	5'-cgaattcagaaaaagccattgag-3'	PC

[1] PE: primer extensión; RT-PCR: Reverse Transcriptase-Polymerase Chain reaction, PC: plasmid construction

transcripts and 5RT102 for anti-*rpoN* transcripts. The following oligonucleotides were used to search for putative promoters: 3RTptsN for *ptsN*, 3RT284 for *orf284* and 3RTptsO for *ptsO*. Oligonucleotides P-anti2, P-anti3 and 5RTptsO were used to check the presence of anti-messengers downstream *ptsN*, and oligonucleotide RT1 upstream *rpoN*. All gave negative results. Primer extension analysis of 50 to 100 µg total RNA samples was carried out as described previously (36) using the ^{32}P end-labeled oligonucleotides described above. To improve electrophoresis quality, a final RNAse digestion step with DNAse-free RNAse (Roche) was carried out after extension was completed. Samples were run in urea sequencing gels and gels were exposed to a phosphor screen (Fuji Photo Film Co; LTD) for 5-12 h. Phosphor screens were scanned with a phosphorimaging instrument (Molecular imager FX, Bio-Rad). Data were quantified with Quantity One software (Bio-Rad).

Reverse transcription-PCR (RT-PCR). For RT-PCR, 10 µg of total RNA isolated as above were treated with TURBO DNA-*free*™ (Ambion) according to the manufacturer's instructions, to exclude the possibility of contamination with traces of chromosomal DNA. RT-PCR was carried out in a final volume of 30 µl Avian Myeloblastoma Virus reverse transcriptase (AMV-RT) buffer (Roche) supplemented with 70 ng of total RNA samples, 0.5 µM of the reverse oligonucleotide, 1.25 µM of each dNTP, 15 U of RNase inhibitor (Amersham) and 5 U of AMV-RT. Reverse transcription was carried out as follows: incubation at 85ºC for 5 min, followed by 2 min annealing at 65ºC and 30 min extension at 50ºC. A final 2 min step at 94 ºC was added to inactivate the AMV-RT. The reaction volume was then divided in two 15 µl fractions. A PCR reaction was carried out with one of the fractions by adding 0.25 µM of the reverse oligonucleotide, 0.5 µM of the forward oligonucleotide and 0.75 U of Taq polymerase (Eppendorf) in a final volume of 30 µl Taq polymerase buffer. The second fraction was used as control by adding all mentioned components except the forward oligonucleotide. The cycling conditions were 94ºC for 5 min, followed by 25 cycles consisting of: 94ºC for 30 s, 30 s at a temperature 3 to 5ºC below oligonucleotide Tm, 30 s to 1 min extension at 72ºC, depending on the expected fragment length, and a final 10 min step at 72 ºC. In addition, the presence of DNA contamination in RNA samples was ruled out in a parallel PCR reaction where the initial reverse transcriptase step was omitted. Amplification products were run on 2% agarose gels.

Additional molecular biology methods. DNA preparation, digestion with restriction enzymes, analysis by agarose gel electrophoresis, isolation of fragments, ligation, transformation, and sequencing were done according to standard procedures (5) or to the manufacturer's recommendations.

β-Galactosidase activity. The wild-type *P. putida* or *E. coli* strains (see Table 1) and a series of isogenic mutants were transformed with the appropriate promoter::*lacZ* fusions in the broad-host range plasmid pMP220. Cells grown overnight on LB were diluted to an OD_{660} of 0.2 in the same medium. Cultures were grown until mid-exponential phase and culture samples were taken and analyzed for ß-galactosidase activity using the standard colorimetric assay described by Miller (40). At least two independent assays with duplicate samples were done in each case.

RESULTS

Operon organization. Early interest in *P. putida rpoN* gene came from its involvement in aromatic degradation pathway expression (34). As in most Gram-negative bacteria, *rpoN* gene is clustered with four highly conserved ORFs located at its 3'-end. The entire gene cluster was first cloned and sequenced (10, 33) before *P. putida* KT2440 complete genome sequence was available (41). The ORFs present in the gene cluster (Figure 1) are flanked by two apparently unrelated genes: an ABC transporter in the same strand and upstream from *rpoN* and a conserved ORF with homology to *Mixococcus gufA* downstream from the cluster and coded in the complementary strand. Within the cluster, ORFs of unknown function are named following nomenclature assigned in *E. coli*, which refers to ORF size. The short distance between them (79, 12, 2 and 18 bp, respectively) suggests the cluster is organized as a single operon (Figure 1). To assess the existence of a polycistronic messenger encompassing the *rpoN* gene cluster, we designed a series of RT-PCR experiments using total RNA isolated from

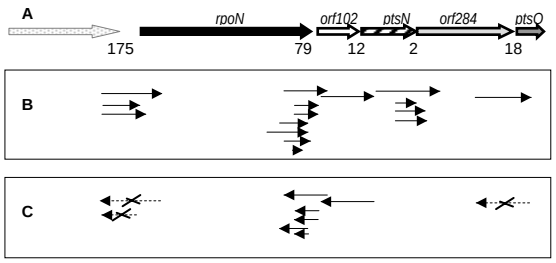

Figure 1: A) Schematic drawing of the *P. putida rpoN* cluster genes. Filled arrows represent the different ORFs. Numbers below the scheme indicate the distance between the stop and start codons of contiguous genes. B) The positions of the amplified fragments obtained in RT-PCR for the determination of transcription organization are shown with arrows. Only the reverse primer was used in the RT step, thus detecting forward transcripts, or C) only the forward primer was used in the RT step, thus detecting anti-mRNAs. A crossed dot-arrow indicates the absence of PCR product.

exponentially growing *P. putida* KT2440 cells as template. Discrepancies between macro- and microarray results in early experiments performed in our lab suggested the presence of reverse RNAs produced from the *rpoN* cluster (Aranda-Olmedo, unpublished). We thus decided to analyze transcription of both DNA strands. To only detect sense mRNAs, the oligonucleotide complementary to the mRNA of the downstream gene (reverse primer) was used in the RT reaction to synthesize cDNA and the oligonucleotide matching the upstream gene (forward primer) was added to the amplification reaction after RT had been heat-inactivated. Using these conditions, oligonucleotide pairs used to detect mRNA spanning from *rpoN* to *orf102* gave positive results yielding PCR products of the expected sizes (Figure 1B), thus indicating that *rpoN* and *orf102* were co-transcribed. The same was true when oligonucleotide pairs producing DNA spanning from *orf102* to *ptsN*, *ptsN* to *orf284* and *orf284* to *ptsO* were used, suggesting that either a single mRNA spanning the whole cluster was synthesized from a single promoter located upstream *rpoN*, or several mRNAs were synthesised from internal promoters, giving smaller RNA transcripts each spanning at least two genes. It is worth noting that control amplifications with oligonucleotides internal to the genes always gave positive results (not shown). When co-transcription of the ABC transporter and *rpoN* genes was tested, PCR products were obtained, pointing to a basal co-transcription of *rpoN* cluster upstream gene with *rpoN* (see below).

To detect anti-sense transcripts, a second set of RT-PCR reactions was carried out as above but using the oligonucleotide matching the upstream gene to synthesize cDNA, and the oligonucleotide complementary to the downstream gene for the amplification reaction. Figure 1C shows that the presence of anti-messenger molecules could be detected, spanning the intergenic *rpoN-orf102* region and the intergenic *orf102-ptsN* region. Additional RT-PCR reactions designed to delimit the ends of the detected anti-messenger(s) gave negative results upstream from *rpoN* and downstream *orf284*, but doubtful results within *rpoN* or *orf284* coding region. We therefore decided to analyze the gene cluster for the presence of internal promoters in both directions.

Promoter identification and mapping. To further analyze operon organization within the gene cluster, primer extension assays were carried out using labelled primers located in the coding region of each gene. Early works detected promoter activity in an 86 bp DNA fragment situated 69 bp upstream from *rpoN* start codon and proposed a potential σ^{70}-RNA polymerase binding region partially overlapping a σ^{54}-recognition consensus sequence (2, 25, 33). To identify *rpoN* start site, primer extension mapping was performed on RNA isolated from the wild-type strain *P. putida* KT2440. As shown in Figure 2, a 104 nt primer extension product was synthesized, which corresponded to an mRNA 5'-end mapping at the T residue located 74 n upstream from the *rpoN* translation start site. The T residue was designated +1 (Figure 2B). A potential promoter sequence that we called P_{rpoN} could be detected in the sequence upstream

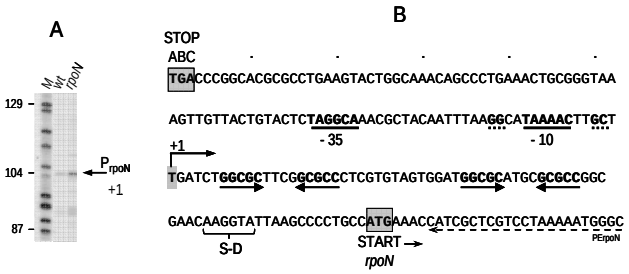

Figure 2: *rpoN* gene promoter mapping. (A) Primer extension analysis of the *rpoN* promoter region. RNA was isolated from *P. putida* cultures as described in the Methods section and 100 μg of total RNA were used as template for reverse transcriptase using PErpoN oligonucleotide complementary to the *rpoN* coding region (Table 2). First lane, molecular weight marker; second lane, wild-type *P. putida* KT2440; third lane, *P. putida* KT2440 *rpoN* mutant. (C) Sequence organization of the *rpoN* promoter region. Potential -10 and -35 boxes and Shine-Dalgarno sequences are underlined. The transcriptional start point is indicated with a right-angled arrow. The *rpoN* start codon and the upstream gene stop codon are boxed. A putative σ^{54} recognition consensus sequence is dot-underlined. Two identical palindromic sequences are marked with inverted arrows. The oligonucleotide used in primer extension analysis is underlined with a doted arrow.

from that start site showing -10 to -35 hexamers with correspondence to the standard *P. putida* σ^{70} consensus (15). Overlapping with this promoter, a well conserved -12/-24 σ^{54} binding sequence was found (Figure 2B). RpoN was previously suggested to mediate repression of its own synthesis, since a P_{rpoN}::*lacZ* fusion was 4 to 5 times more active in an *rpoN* mutant than in wild type (33). To confirm this proposal, primer extension analysis was carried out with total RNA from a *P. putida rpoN* mutant isolated as above. Figure 2A shows a three-time increase in P_{rpoN} expression in the mutant lacking *rpoN*, thus confirming negative autorregulation of σ^{54} expression. In primer extension analysis carried out to map P_{rpoN}, faint larger cDNA bands were sometimes visible in the electrophoretic lanes; this observation together with positive results in RT-PCR suggests that a certain level of *rpoN* and upstream genes co-transcription occurred. Two identical perfect inverted repeats were found in the mRNA leader region whose significance is unknown (Figure 2B). This sequence was not found at any other site in the *rpoN* cluster. A sequence complementary to *P. putida* 16S rRNA 3'-sequence located 15 nt upstream from the translation start site could be identified as *rpoN* Shine-Dalgarno motif.

orf102, located 79 bp downstream the *rpoN* gene, shows homology to a number of unrelated, ribosome associated proteins. It has been suggested to modulate σ^{54} activity although its specific function is unknown (39). We analyzed the presence of putative transcription start sites controlling independent transcription of *orf102*. The result of a primer extension using a labelled oligonucleotide complementary to *orf102* coding region is shown in figure 3A. A cDNA product of approximately 190 nt was clearly visible, which was further precisely mapped using a second oligonucleotide complementary to the expected leader region close to *rpoN* stop codon and rendering a shorter extension product (Figure 3B and D). A 90 nt cDNA product was obtained, corresponding to this new promoter that we called P_{102}. The mRNA 5'-end mapped at an A located within the *rpoN* coding region at 91 nt upstream from the *rpoN* stop codon and 170 n upstream from *orf102* start codon (Figure 3D). A potential promoter sequence could be detected 7 bp upstream from the mapped start site showing 9/12 matches with the -10/-35 hexamers of the standard *P. putida* σ^{70} consensus (15). A perfect inverted repeat of unknown function was found in *orf102* mRNA leader region. Extension with the farthest oligonucleotide (PE102) gave a defined group of shorter bands around 82 n in size consistent with endonucleolytic cleavage (see below). In addition faint cDNA bands of a larger size were visible corresponding to degradation fragments of the P_{rpoN}-derived transcript, thus suggesting a certain level of co-transcription of *rpoN* and *orf102*. To further confirm the presence of a promoter driving *orf102* transcription and to map its start site, pMP102-6 plasmid bearing a P_{102}::*lacZ* fusion was transferred to *P. putida* and primer extension analysis of total RNA isolated from the resulting strain was carried out. Results revealed the presence of a defined extension band of the expected size which confirmed previous results (Figure 3C).

Similar primer extension analyses were carried out with oligonucleotides complementary to the remaining ORFs, but no clear band beside the expected mRNA degradation bands could be detected. We can therefore conclude that two promoters control *rpoN* cluster expression. The first one probably controls transcription of the complete cluster starting at *rpoN*, while the second one produces an RNA transcript encompassing *orf102*, *ptsN* and most probably *orf284* and *ptsO*. In addition, RNAP read-through from the upstream operon would produce a basal transcription of the gene(s). Further analyses are required to determine the relative amount of each transcript.

According to our RT-PCR results, the presence of anti-mRNA was restricted to the region comprising from *ptsN* to *rpoN*. The start sites of newly detected anti-mRNA were determined using primer extension analysis with oligonucleotides matching the coding sequence of every gene present in this region. Only two of them gave defined extension products consistent with the presence of a transcription start site. The first one defined a promoter located 39 nucleotides upstream from *ptsN* stop codon (Figure 4) that we called $P_{anti\text{-}ptsN}$. Nine nucleotides upstream from the mapped transcription start, a putative σ^{70}-consensus was found (Figure 4B). Thus, the mRNA produced from this promoter would be complementary to *ptsN* coding region (see also Figure 7). A blast search against available data bases did not hit any protein homologue

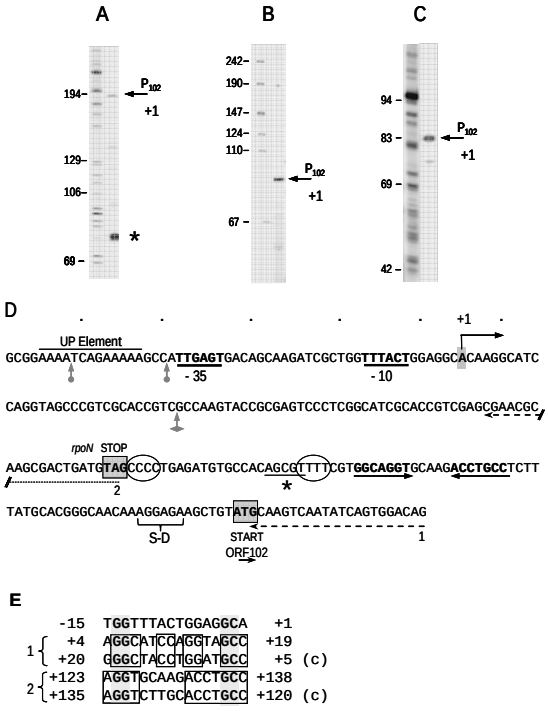

Figure 3: *orf102-ptsN-orf284-ptsO* operon promoter mapping. Primer extension analysis of *orf102* promoter region. RNA was isolated from *P. putida* cultures as described in the Methods section and 100 μg of total RNA were used as template for reverse transcriptase using (A) PE102 oligonucleotide complementary to the *orf102* coding region (Table II), (B) P102 oligonucleotide complementary to the 3'-end of *rpoN* gene (Table II) or (C) 220PE oligonucleotide complementary to the pMP220 plasmid sequence adjacent to the *orf102* promoter region inserted in pM102-6; in this experiment, RNA was isolated from *P. putida* KT2440 (pMP102-6). (D) Promoter structure of the *P. putida* P_{102} promoter. The transcriptional start point (+1) is shadowed and indicated with a +1 above a rigt-angled arrow. Potential -10 and -35 motifs are underlined. The Shine-Dalgarno sequence is underlined with a bracket. A line above the sequence denotes the identified UP element. Start and stop codons are boxed. Doted arrows show the position of oligonucleotides used in primer extension. Horizontal arrows underlining sequence stretches designate the presence of perfect palindromic sequences. A diamond-bound vertical arrow indicates the 3'-end of DNA fragments used in figure 6 to measure β-galactosidase. Dot-bound vertical arrows shows the 5'-end of the different fragments used in the deletion analysis in figure 6. (E) Alignment of σ54-consensus sequences present in the intergenic region. Numbers at both ends of each sequence indicates the position with respect to P_{102} transcription start site. A (c) indicates that the sequence is present in the complementary strand.

to the translated complementary DNA sequence in the *ptsN* region, thus pointing to a regulatory role for this mRNA. To confirm the existence of this promoter, a mutant in the *ptsN* downstream gene *orf284*, in which the complete ORF284 except its four first codons had been replaced with a kanamycin resistance cassette (10), was used in a similar primer extension analysis. The extension band obtained was the same as above (not shown), thus confirming that the 5'-end of this mRNA was determined by $P_{anti\text{-}ptsN}$ independently of the upstream sequence coding for *orf284*.

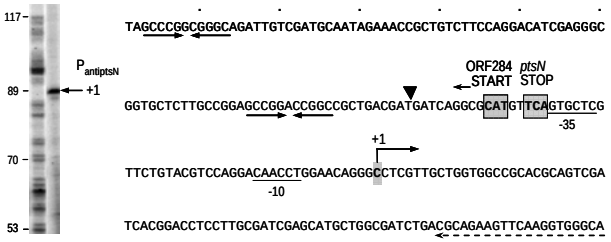

Figure 4: Anti-*ptsN* promoter search. (A) Primer extension analysis of the *ptsN-orf284* reverse intergenic region. RNA was isolated from *P. putida* cultures as described in the Methods section and 100 µg of total RNA were used as template for reverse transcriptase using P-anti1, an oligonucleotide located within the *ptsN* coding region (Table 2). (B) DNA sequence of the *ptsN-orf284* intergenic region. The transcriptional start point (+1) is indicated with a right-angled arrow. Potential -10 and -35 boxes are underlined. Two identical palindromic sequences are marked with inverted horizontal arrows. A triangle denotes the insertion point where the Km^R cassette replaced the internal gene region in mutant *P. putida ORF284*::Km.

The second positive primer extension experiment showed two close extension products which mapped 5 and 9 nucleotides upstream from the *rpoN* stop codon (Figure 5). This promoter was called $P_{anti\text{-}rpoN}$. Thus, the RNAP transcribing from this promoter will have its binding site in the *rpoN-orf102* intergenic region. These extension bands were also present in an *orf102* mutant interrupted with a kanamycin resistance cassette (not shown). No clear σ^{70} or σ^{54} consensus sequence could be unequivocally defined in this region although in figure 5 we suggest a putative RNAP binding site with 7/12 matches with σ^{70}-consensus and 18 nt distance between -10 and -35 boxes. The presence of a number of overlapping regulatory sequences within this intergenic region (see below) is probably the reason for this poorly conserved RNAP binding site. The transcript derived from this newly identified promoter would be complementary to *rpoN* transcript (see Figure 7). A blast search performed as above produced no hit with known protein sequences present in the databases.

Figure 5: Anti-*rpoN* promoter search. (A) Primer extension analysis of the *rpoN-orf102* reverse intergenic region. RNA was isolated from *P. putida* cultures as described in the Methods section and 100 µg of total RNA were used as template for reverse transcriptase using 5RT102 oligonucleotide within the *rpoN* coding region. (B) DNA sequence of the *rpoN-orf102* intergenic region. The transcriptional start points identified in primer extension (+1) are indicated with a right-angled arrow. Elements depicted in grey show the regulatory regions identified for P_{102} (see Figure 3 legend). Start and stop codons of *orf102* and *rpoN* respectively, are boxed. The putative *rpoN* rho-dependent terminator complementary sequence is dash-underlined. The putative *rpoN* rho-independent terminator complementary sequence is over lined with inverted arrows. The *orf102* Shine-Dalgarno sequence is over lined with a bracket.

P_{102} promoter is tightly controlled. To confirm the presence of the newly identified P_{102} promoter, pM102-7 plasmid bearing P_{102}::*lacZ* transcriptional fusion in the broad-host range reporter plasmid pMP220 (Table 1) was transferred to *P. putida* wild-type strain and β-galactosidase activity in exponentially growing cells was determined. The 239 bp fusion gave 200 Miller Units (MU) in rich medium (Figure 6, construct A). Two putative inverted repeats could be recognized in the cloned sequence, as well as an A/T rich sequence showing homology to *E. coli* UP elements (52). To analyze a possible role of these elements in P_{102} expression, sequential deletions of the initial fragment were cloned in the same site of pMP220 and β-galactosidase activity was determined as above. High activity levels were maintained with all clones deleted down to contain a fragment reaching at least position -48 with respect to the transcription start site (Figure 6, construct C). A deletion down to position -43, which completely eliminated the putative UP element, reduced activity to 25% (Figure 6, construct D). This confirmed

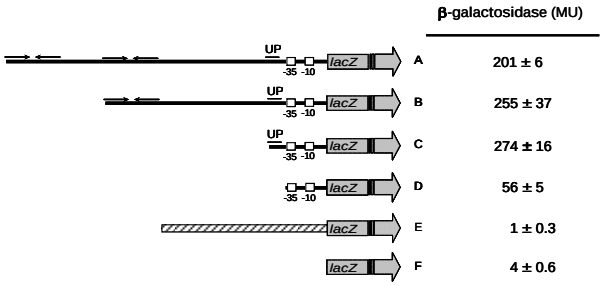

Figura 6: Defining P_{102} promoter structure. β-galactosidase activity of a series of P_{102} transcriptional fusions carrying different sizes of DNA inserts cloned in pMP220. P_{102} promoter regulatory regions present in each fragment are outlined as follows: the location of -10/-35 RNAP binding region are denoted with small white boxes, the UP element consensus is underlined and palindromic sequences present in the segments are marked with inverted arrows. A stripped box indicates the insertion of the 154 bp PCR fragment including the putative endonucleolytic cleavage site found in P_{102} mRNA leader region. Sequence details of the different cloned fragments can be found in figure 3D.

the stimulatory effect of this recognition sequence on P_{102} transcription. To further demonstrate the role of the UP element in P_{102} expression, we investigated αCTD binding to this region using the reporter plasmid pMP102-6 (Table 1) in *E. coli* strains over-expressing mutant α-subunits of RNA polymerase known to be impaired in UP-element interaction (1, 52). Two mutants were selected: mutant Δ256, which lacks the αCTD protein moiety, and mutant R265A known to abolish the *rrnB* P1 UP element function in *E. coli*. Isogenic strains expressing wild-type α subunits were used as controls. Table 3 shows that over-expression of the truncated αCTD mutant reduced β-galactosidase activity to 59%, confirming the stimulating role of the UP element in P_{102}. However, a mutant with a single amino acid substitution in Arg^{265} residue produced β-galactosidase activity levels similar to wild type. This was not unexpected, since α-CTD residues which are relevant to certain promoter activity do not necessarily participate in contacting the UP-element of others (57).

P_{102} sequence analysis. The short intergenic region between *rpoN* and *orf102*, which covers 79 bp, must accommodate several regulatory sequences: *orf102* ribosome binding site, $P_{anti\text{-}rpoN}$ RNAP binding site and all control elements necessary for a regulated expression of ORF102. A first eye search revealed the presence of a putative Rho-independent (intrinsic) terminator located 29 nt downstream *rpoN* stop codon (Figure 3D). We could identify a stem-loop structure composed of a GC-rich stem and a 5 nt loop, followed by a U-rich stretch in the correct orientation. If this terminator was operative and according to the proposed intrinsic terminator mechanisms (13), *rpoN* transcription would terminate approximately 50 nt downstream *rpoN* stop codon.

Table 3: Promoter P_{102} activity in different α-mutant backgrounds.

Plasmid	α-CTD genotype	β-galactosidase (MU)	%
pLAX185	WT	42 ± 0.7	100
pLAD256	Δ256	25 ± 6.8	59
pHTf1α	WT	22 ± 3	100
pHTf1αR265A	R265A	23 ± 2	105

Interestingly, when we performed primer extension analysis with PE102 oligonucleotide, a group of strong extension bands of 82±2 nt was clearly visible (Figure 3A, labeled with a star). To rule out the possibility of these bands corresponding to an independent promoter, we assayed a 154 bp fragment including 105 bp upstream from the mapped 5'-end of the central band, in the promoter probe vector pMP220 (pMP102-4 plasmid). As shown in Figure 6 (construct E), this sequence had no detectable promoter activity.

Finally, a thorough analysis of the intergenic sequence revealed the presence of five σ^{54} consensus sequences (Figure 3E) organized in a peculiar manner. The first one overlapped P_{102} -10 sequence, while the remaining four where organized as pairs symmetrically arranged in the two strands. One pair overlapped *orf102* transcription start site while the other one was located overlapping the intrinsic terminator. The strong base conservation among the four sequences (Figure 3E) rules out the possibility of these sequences being accidental. Their putative role in *rpoN* expression regulation is under analysis in our laboratory.

DISCUSSION

Several features of σ^{54} make it a unique element of RNA polymerase. Unlike σ^{70}-family sigma factors, σ^{54} is able to bind DNA in the absence of core polymerase, its activation mechanism is distinct and unique among sigma factors, it always requires activation by a specific activator protein which bind DNA distantly from the RNAP, and it is strictly required in promoters transcribed by $E\sigma^{54}$, which are silent with other RNAPs (55). As a consequence, promoters transcribed by $E\sigma^{54}$ are subject to a tight regulation which allows a fast response to changes in the cellular medium or in the environment. Promoters transcribed by $E\sigma^{54}$ belong to all type of metabolic routes or structural functions, although interestingly the initial idea of a nitrogen-related σ factor has been brought up again recently with the argument that all σ^{54}-regulated functions are either directly related to nitrogen or they indirectly alleviate conditions that are detrimental to nitrogen assimilation (50). Besides the intrinsic regulatory features, the extremely low intracellular levels of σ^{54} (close to one order of magnitude less than σ^{70}, depending on the strain) impose an additional control over σ^{54}-dependent promoters, which is exerted through core competition, mainly against the house-keeping major factor σ^{70} (29, 35). In this situation, it is also predictable that different σ^{54}-dependent promoters compete for limiting amounts of $E\sigma^{54}$. It is thus important to the cell to carefully control that the levels of σ^{54} meet the transcriptional needs at any moment.

Sigma factor concentration regulation mechanisms are specific to each factor, although some general patterns are found in almost all of them. Such mechanisms can affect *de novo* synthesis by preventing gene transcription or protein translation, or they are associated to mRNA or protein stability and post-translational processing (18, 26). In some cases the levels of active σ factor are regulated through an anti-σ factor which contacts directly with the sigma subunit to hinder its interaction with the RNAP (21, 23). Each of these processes doesn't exclude the others, so that sigma factor regulation is normally the result of several control levels (22). The contribution of these processes to σ activity has been extensively studied for the most relevant σ factors of the σ^{70}-family. Strikingly, little is known about σ^{54} transcription, translation or stability role in σ^{54} activity, except for the role of (p)ppGpp on σ^{54} competition for RNA polymerase core which has been deeply investigated with relevant results (8, 29). In that sense, it has been shown that in the presence of (p)ppGpp, σ^{54} is able to out-compete σ^{70} for RNAP core binding. The levels of σ^{54} have been analyzed in different species and strains, not always with similar results (9, 29). In *P. putida* KT2440, the published data indicate σ^{54} level is kept constant along the growth curve (31). This is in contrast with our observation, which points to a significant increase of σ^{54} levels with the growth phase (Aranda-Olmedo, unpublished). On the other hand, several σ^{54}-dependent promoters in *P. putida* are subject to catabolite repression, a process which involves at least the cluster protein IIA^{Ntr}, previously suggested to modulate σ^{54} and which target has been suggested to be the transcriptional machinery (4). Taken together, these findings can be considered an indication that σ^{54} expression must be controlled. Results obtained in this work confirm this prediction.

We have centred our analysis on the transcriptional regulation elements controlling σ^{54} expression and expression of the genes present in the flanking gene cluster, thought to be involved in σ^{54} modulation. The general conclusion of this work is that many processes seem to be involved in regulating this expression, where controlling the synthesis of some of the flanking gene proteins plays a central role. We have shown that two operons are responsible for the synthesis of the different genes of the cluster. One of them is controlled from the P_{rpoN} promoter and is subject to auto-regulation, which probably ensures that σ^{54} levels are maintained at the appropriate level in the cell. According to our RT-PCR results, this promoter probably renders two transcripts

of different length. A short one would be produced after transcription termination at the end of *rpoN*. Although two putative terminators are present in this region, our analysis does not define which one of them is functional. The longer transcript would be generated when RNAP escapes this terminator(s). The total length of this transcript is unknown, but it could include all genes in the cluster. Previous studies had detected promoter activity upstream *rpoN* gene in *P. putida* and had also suggested the possibility of an undetermined additional promoter in its downstream region (33). Results in figure 2 define the exact transcriptional start point of *rpoN* gene, discarding the presence of alternative promoters for *rpoN* and some *in silico* promoter predictions. The RNA polymerase binding site identified in this work differed from any of the previously proposed hypothetical sequences (25), though it was coincident with σ^{70}-binding site suggested for *P. putida* from the alignment of several *Pseudomonas rpoN* promoters (2). The second operon is controlled from the P_{102} promoter, which overlaps the *rpoN* gene. The presence of this promoter was anticipated in minicell experiments which detected a protein product corresponding to ORF102 when an interposon was inserted in *rpoN* gene (33). An independent promoter driving *orf102* expression was also found in *Acinetobacter calcoaceticus* (16). Interestingly, primer extension of P_{102} detected a group of smaller bands incompatible with promoter activity (Figures 3 and 6). This group of bands which mapped at a position centered 20-24 nt downstream *rpoN* stop codon (Figure 3A and D) was consistent with mRNA processing products. We envision two possibilities to explain their presence: i) these bands are formed *in vitro* during primer extension analysis as a consequence of reverse transcriptase posing or ii) the primer extension analysis reflects bands which originated after *in vivo* mRNA processing. In the first case, AMV-RT extension progress from PE102 binding site could be hindered because of the presence of a stable RNA secondary structure. Primer extension assay optimal temperature is lower than the predicted intrinsic terminator hairpin Tm (80ºC), thus the stem-loop could remain stably folded during the extension reaction. This hairpin-mediated hindrance would generate a truncated extension cDNA product with a 3'-end close to the hairpin structure. With a certain frequency, AMV-RT would overcome this obstacle and proceed with extension through the complete transcript, giving the larger band corresponding with the +1 position of P_{102} promoter. Transcripts derived from both P_{102} and P_{rpoN} promoters could render these short products.

On the other hand, a putative Rho-dependent terminator (44) could also be identified in the *rpoN-orf102* intergenic region, located directly downstream *rpoN* stop codon (Figure 3D). Some 60-70 nt upstream from this putative Rho-dependent termination element, a C-rich, G-poor sequence stretch could be found, consistent with a *rut* (Rho utilization) site (3). If effective, this terminator would generate an mRNA 3'-end mapping around 22 nt downstream *rpoN* stop codon, thus coinciding with mapped 5'-end of the set of shorter bands observed in figure 3. A premature-termination transcript generated by this terminator would not be detected in primer extension analysis with P_{102} oligonucleotide. The proportion of mRNA overcoming Rho termination would depend on a number of factors which, as a final consequence, would allow or not RNAP dissociation from DNA, and RNA release (7). The set of defined degradation bands found in primer extension with PE102 could suggest that a cleavage site could be present at this position, coincident with the putative Rho termination site. The mechanism leading to this cleavage could involve RNA secondary structure recognition or mRNA/anti-mRNA duplexes formation, which could also influence mRNA translation and or cleavage. It is worth noting that a *rho* homologue is present in *P. putida* KT2440 chromosome, and sequence homology of its putative promoter region predicts that its expression is dependent on $E\sigma^{54}$. The relative intensity of the 194 and 82 nt bands in figure 3A would point to a high proportion of RNAP overcoming the termination site, though leaving available an efficient cleavage target. Regardless of the mechanism underlying this observation, an mRNA bearing the necessary signals for *orf102* to be translated would be accumulated, facilitating ORF102 protein synthesis. Since no other promoter seems to be present downstream P_{102}, the longer P_{102}-derived transcript probably includes the *orf102*, *ptsN*, *orf284* and *ptsO* products.

Our results also suggested the presence of antisense transcripts in the *rpoN* cluster. In order to confirm this unexpected finding, a series of RT-PCR reactions were performed in which the oligonucleotide used to synthesize cDNA was complementary to the putative antisense mRNA. We paid special attention to control experimental conditions to avoid DNA contamination of the samples and false positive signals because of unspecific amplifications. The RT-PCR results confirmed the initial suggestion that there was antisense transcription. To determine the anti-mRNA 5'-end location, primer extension analyses were carried out with appropriate oligonucleotides. Two anti-mRNAs were detected and mapped. One of them would produce a transcript complementary to *ptsN* mRNA while the second one would be complementary to *rpoN*. Thus, the genes coding for these two main players are subject to an additional control, which probably involves RNA duplex formation to modulate mRNA half-life and/or translation efficiency. The physiological consequences of this regulation mechanism are difficult to address, since mutations in *rpoN* or *ptsN* genes would in parallel generate an anti-mRNA mutant. A scheme summarizing all mRNA molecules encompassing this region is shown in figure 7.

Although σ^{54} synthesis is auto-regulated ((33) and figure 2), the *rpoN-orf102* region seems to be the hot-spot in cluster gene regulation. In this short DNA sequence we found two divergent promoters able to produce complementary messengers, two putative transcription terminators, and a number of inverted repeats. In addition, five non-promoter σ^{54}-binding sites are found in this region, the function of which is being investigated. Although σ^{54} has been said to bind DNA as free subunit these sites could also be recognized by $E\sigma^{54}$. Binding of σ^{54} alone or of the RNAP holoenzyme could have different consequences on the expression of either divergent promoter. *In vitro* binding analyses are necessary to clarify this point.

Finally, P_{102} transcription is directly dependent on α-CTD binding to an UP element. It is interesting to note that ORF102 protein shows significant homology to several ribosome-associated proteins. This implies an

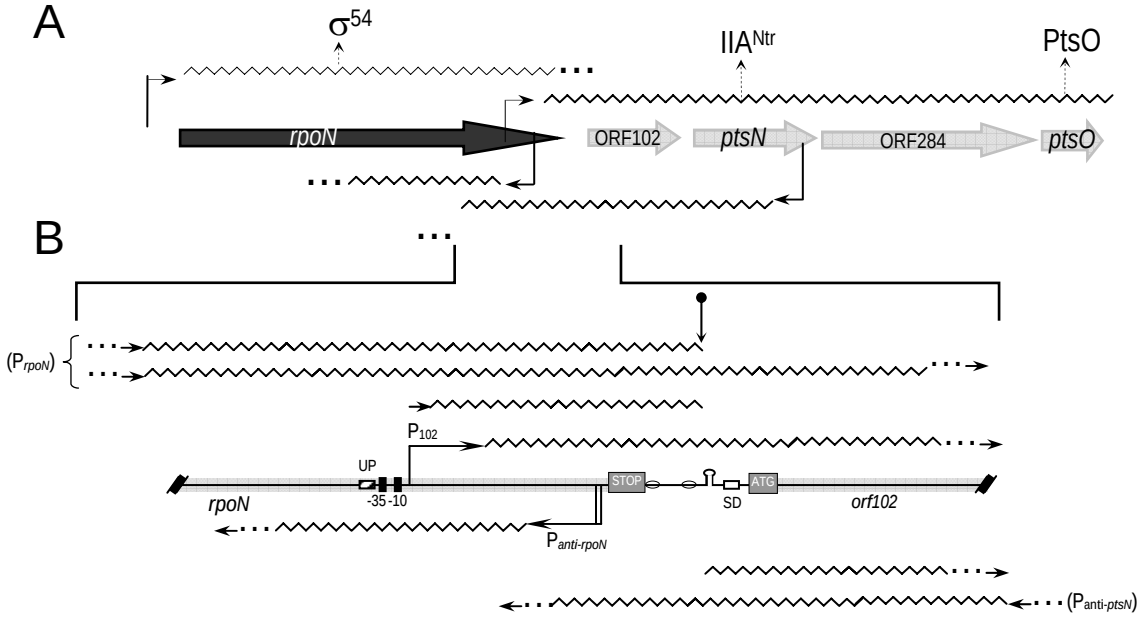

Figure 7: Schematic representation of *rpoN* cluster transcripts.

additional regulation level probably affecting RNA translation. However, the mRNA target of this post-transcriptional regulation is to be identified. Both *rpoN* and *ptsN* transcripts would be suitable candidates. The results presented in this work show that *rpoN* gene cluster offers a considerable number of possibilities for post-transcriptional regulation, with the final goal of obtaining a coordinated and fine-tuned regulation of all proteins in the cluster.

ACKNOWLEDGEMENTS

This work was supported by EC grant QLK3-CT-2002-01923 and grant BCM 2001-0515 from the Spanish Ministry of Science and Education. We thank A.J. Molina-Henares for helpful automated sequence analysis.

REFERENCES

1. **Aiyar, S. E., R. L. Gourse, and W. Ross.** 1998. Upstream A-tracts increase bacterial promoter activity through interactions with the RNA polymerase alpha subunit. Proc Natl Acad Sci U S A **95:**14652-7.
2. **Alarcón-Chaidez, F. J., and C. L. Bender.** 2001. Analysis of the *rpoN* locus in the plant pathogenic bacterium, *Pseudomonas syringae* pv. *glycinea*. DNA Seq **12:**77-84.
3. **Alifano, P., F. Rivellini, D. Limauro, C. B. Bruni, and M. S. Carlomagno.** 1991. A consensus motif common to all Rho-dependent prokaryotic transcription terminators. Cell **64:**553-63.
4. **Aranda-Olmedo, I., J. L. Ramos, and S. Marqués.** 2005. Integration of signals through Crc and PtsN in catabolite repression of *Pseudomonas putida* TOL plasmid pWW0. Appl Environ Microbiol **71:**4191-8.
5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1991. Current Protocols in Molecular Biology, Wiley, New York.
6. **Bagdasarian, M., R. Lurz, B. Ruckert, F. C. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene **16:**237-47.
7. **Banerjee, S., J. Chalissery, I. Bandey, and R. Sen.** 2006. Rho-dependent transcription termination: more questions than answers. J Microbiol **44:**11-22.
8. **Bernardo, L. M., L. U. Johansson, D. Solera, E. Skarfstad, and V. Shingler.** 2006. The guanosine tetraphosphate (ppGpp) alarmone, DksA and promoter affinity for RNA polymerase in regulation of sigma-dependent transcription. Mol Microbiol **60:**749-64.
9. **Brun, Y. V., and L. Shapiro.** 1992. A temporally controlled sigma-factor is required for polar morphogenesis and normal cell division in *Caulobacter*. Genes Dev **6:**2395-408.
10. **Cases, I., J. Pérez-Martín, and V. de Lorenzo.** 1999. The IIANtr (PtsN) protein of *Pseudomonas putida* mediates the C source inhibition of the sigma54-dependent Pu promoter of the TOL plasmid. J. Biol. Chem. **274:**15562-15568.
11. **Cases, I., D. W. Ussery, and V. de Lorenzo.** 2003. The sigma54 regulon (sigmulon) of *Pseudomonas putida*. Environ Microbiol **5:**1281-93.
12. **Cases, I., F. Velázquez, and V. de Lorenzo.** 2001. Role of *ptsO* in carbon-mediated inhibition of the Pu promoter belonging to the pWW0 *Pseudomonas putida* plasmid. J Bacteriol **183:**5128-33.
13. **d'Aubenton Carafa, Y., E. Brody, and C. Thermes.** 1990. Prediction of rho-independent *Escherichia coli* transcription terminators. A statistical analysis of their RNA stem-loop structures. J Mol Biol **216:**835-58.
14. **Dixon, R.** 1986. The *xylABC* promoter from the *Pseudomonas putida* TOL plasmid is activated by nitrogen regulatory genes in *Escherichia coli*. Mol. Gen. Genet**:**129-136.
15. **Domínguez-Cuevas, P., and S. Marqués.** 2004. Compiling sigma-70-dependent promoters, p. 319-343. *In* R. JL (ed.), Pseudomonas, vol. 2. Kluwer Academic/Plenum Publishers, London.
16. **Ehrt, S., L. N. Ornston, and W. Hillen.** 1994. RpoN (sigma 54) is required for conversion of phenol to catechol in *Acinetobacter calcoaceticus*. J Bacteriol **176:**3493-9.
17. **Gaal, T., W. Ross, E. E. Blatter, H. Tang, X. Jia, V. V. Krishnan, N. Assa-Munt, R. H. Ebright, and R. L. Gourse.** 1996. DNA-binding determinants of the alpha subunit of RNA polymerase: novel DNA-binding domain architecture. Genes Dev **10:**16-26.
18. **Gruber, T. M., and C. A. Gross.** 2003. Multuiple sigma subunits and the partitioning of bacterial transcription space. Annu Rev Microbiol **57:**441-466.
19. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166:**557-80.
20. **Hayward, R. S., K. Igarashi, and A. Ishihama.** 1991. Functional specialization within the alpha-subunit of *Escherichia coli* RNA polymerase. J Mol Biol **221:**23-9.

21. **Helmann, J. D.** 1999. Anti-sigma factors. Curr Opin Microbiol **2:**135-41.
22. **Hengge-Aronis, R.** 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev **66:**373-95, table of contents.
23. **Hughes, K. T., and K. Mathee.** 1998. The anti-sigma factors. Annu Rev Microbiol **52:**231-86.
24. **Inouye, S., Y. Ebina, A. Nakazawa, and T. Nakazawa.** 1984. Nucleotide sequence surrounding transcription initiation site of *xylABC* operon on TOL plasmid of *Pseudomonas putida*. Proc. Natl. Acad. Sci. USA**:**1688-1691.
25. **Inouye, S., M. Yamada, A. Nakazawa, and T. Nakazawa.** 1989. Cloning and sequence analysis of the *ntrA* (*rpoN*) gene of *Pseudomonas putida*. Gene **85:**145-152.
26. **Ishihama, A.** 2000. Functional modulation of *Escherichia coli* RNA polymerase. Annu Rev Microbiol **54:**499-518.
27. **Janakiraman, R. S., and Y. V. Brun.** 1997. Transcriptional and mutational analyses of the *rpoN* operon in *Caulobacter crescentus*. J Bacteriol **179:**5138-47.
28. **Jin, S., K. Ishimoto, and S. Lory.** 1994. Nucleotide sequence of the *rpoN* gene and characterization of two downstream open reading frames in *Pseudomonas aeruginosa*. J Bacteriol **176:**1316-22.
29. **Jishage, M., K. Kvint, V. Shingler, and T. Nystrom.** 2002. Regulation of sigma factor competition by the alarmone ppGpp. Genes Dev **16:**1260-70.
30. **Jones, D. H., F. C. Franklin, and C. M. Thomas.** 1994. Molecular analysis of the operon which encodes the RNA polymerase sigma factor sigma 54 of *Escherichia coli*. Microbiology **140 (Pt 5):**1035-43.
31. **Jurado, P., L. A. Fernandez, and V. de Lorenzo.** 2003. Sigma 54 levels and physiological control of the *Pseudomonas putida* Pu promoter. J Bacteriol **185:**3379-83.
32. **Kessler, B., V. de Lorenzo, and K. Timmis.** 1992. A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. **233:**293-301.
33. **Köhler, T., J. F. Alvarez, and S. Harayama.** 1994. Regulation of the *rpoN*, ORF102 and ORF154 genes in *Pseudomonas putida*. FEMS Microbiol Lett **115:**177-84.
34. **Köhler, T., S. Harayama, J. L. Ramos, and K. N. Timmis.** 1989. Involvement of *Pseudomonas putida* RpoN s Factor in regulation of various metabolic functions. J. Bacteriol**:**4326-4333.
35. **Maeda, H., N. Fujita, and A. Ishihama.** 2000. Competition among seven *Escherichia coli* sigma subunits: relative binding affinities to the core RNA polymerase. Nucleic Acids Res **28:**3497-503.
36. **Marqués, S., A. Holtel, K. N. Timmis, and J. L. Ramos.** 1994. Transcriptional induction kinetics from the promoters of the catabolic pathways of TOL plasmid pWW0 of *Pseudomonas putida* for metabolism of aromatics. J Bacteriol **176:**2517-24.
37. **Marqués, S., M. Manzanera, M. M. González-Pérez, R. Ruíz, and J. L. Ramos.** 1999. Biodegradation, plasmid-encoded catabolic pathways, host factors and cell metabolism. Environ Microbiol **1:**103-4.
38. **Merrick, M.** 1993. In a class of its own- the RNA polymerase sigma factor s54 (sN). Mol. Microbiol. **10:**903-909.
39. **Merrick, M. J., and J. R. Coppard.** 1989. Mutations in genes downstream of the *rpoN* gene (encoding sigma 54) of *Klebsiella pneumoniae* affect expression from sigma 54-dependent promoters. Mol Microbiol **3:**1765-75.
40. **Miller, J.** 1972. p. 352-355, Experiments in molecular genetics. Cold Spring Harbor, N.Y., USA.
41. **Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Chris Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. A. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummler, and C. M. Fraser.** 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol **4:**799-808.
42. **Neuwald, A. F., L. Aravind, J. L. Spouge, and E. V. Koonin.** 1999. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res **9:**27-43.
43. **Paget, M. S., and J. D. Helmann.** 2003. The sigma70 family of sigma factors. Genome Biol **4:**203.
44. **Petersen, L., and A. Krogh.** 2003. Modelling of Rho dependent transcription termination sites in the bacterium *Helicobacter pylori.* on-line publication.
45. **Powell, B. S., D. L. Court, T. Inada, Y. Nakamura, V. Michotey, X. Cui, A. Reizer, M. H. Saier, Jr., and J. Reizer.** 1995. Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *Escherichia coli*. Enzyme IIANtr affects growth on organic nitrogen and the conditional lethality of an *era*ts mutant. J Biol Chem **270:**4822-39.
46. **Rabus, R., J. Reizer, I. Paulsen, and M. H. Saier, Jr.** 1999. Enzyme I(Ntr) from *Escherichia coli*. A novel enzyme of the phosphoenolpyruvate-dependent phosphotransferase system exhibiting strict specificity for ıts phosphoryl acceptor, NPr. J Biol Chem **274:**26185-91.
47. **Ramos, J. L., S. Marqués, and K. N. Timmis.** 1997. Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators. Annu Rev Microbiol **51:**341-73.
48. **Ramos, J. L., N. Mermod, and K. N. Timmis.** 1987. Regulatory circuits controlling transcription of TOL plasmid operon encoding meta cleavage pathway for degradation of alkylbenzoates by *Pseudomonas*. Mol. Microbiol.**:**293-300.
49. **Ramos-González, M. I.** 1993. Ph.D. degree Thesis. University of Granada, Spain.
50. **Reitzer, L., and B. L. Schneider.** 2001. Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. Microbiol Mol Biol Rev **65:**422-44.
51. **Reizer, J., A. Reizer, M. H. Saier, Jr., and G. R. Jacobson.** 1992. A proposed link between nitrogen and carbon metabolism involving protein phosphorylation in bacteria. Protein Sci **1:**722-6.
52. **Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse.** 1993. A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. Science **262:**1407-13.
53. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold spring Harbor, N.Y., Cold spring Harbor Laboratory., N.Y.
54. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Promoter in the nodulation region of the *Rhizobium leguminosarum* symbiosis plasmid pRL1J1. Plant Mol Biol **9:**27-39.
55. **Wigneshweraraj, S. R., P. C. Burrows, P. Bordes, J. Schumacher, M. Rappas, R. D. Finn, W. V. Cannon, X. Zhang, and M. Buck.** 2005. The second paradigm for activation of transcription. Prog Nucleic Acid Res Mol Biol **79:**339-69.
56. **Zhao, H., X. Li, D. E. Johnson, and H. L. Mobley.** 1999. Identification of protease and rpoN-associated genes of uropathogenic Proteus mirabilis by negative selection in a mouse model of ascending urinary tract infection. Microbiology **145 (Pt 1):**185-95.
57. **Zou, C., N. Fujita, K. Igarashi, and A. Ishihama.** 1992. Mapping the cAMP receptor protein contact site on the alpha subunit of *Escherichia coli* RNA polymerase. Mol Microbiol **6:**2599-605.

IV.4. CAPÍTULO 4.

Secuencias palindrómicas repetitivas (REPs) específicas de especie en *Pseudomonas putida*.

Autores: Isabel Aranda-Olmedo, Raquel Tobes, Maximino Manzanera, Juan Luís Ramos y Silvia Marqués.

Publicado en: Nucleic Acids Res. 2002 Apr 15;30(8):1826-33.

Resumen:

Pseudomonas putida KT2440 es una bacteria de suelo que coloniza eficazmente la raíz de muchas plantas y degrada una amplia variedad de compuestos aromáticos tóxicos. El genoma de esta bacteria ha sido secuenciado. Hemos localizado una secuencia de 35 pb con estructura de palíndromo imperfecto, que originariamente se encontró repetida tres veces corriente abajo del terminador del gen *rpoH*, puede detectarse más de 800 veces en el cromosoma de esta cepa. La estructura de este segmento de DNA es análoga a las llamadas secuencias REP (*repetitive extragenic palindromic elements*) descritas en enterobacterias, si bien su secuencia es diferente. El análisis informático de la presencia y distribución de esta secuencia repetida en el cromosoma de *Pseudomonas putida* reveló que, al menos en un 80% de los casos, la secuencia es extragénica y, en un 82% de los casos, la distancia de este elemento extragénico al final de uno de los genes vecinos es menor de 100 pb. Este fragmento de 35 pb aparece bien como elemento único, bien formando parejas o, en pocos casos, como agrupaciones de hasta cinco elementos de orientación alterna. El estudio mediante PCR de cromosomas de diferentes aislados de especies del género *Pseudomonas*, usando un oligonucleótido complementario a la región más conservada de esta secuencia, puso de manifiesto que el fragmento repetido sólo está presente en la especie *Pseudomonas putida*. Por este motivo sugerimos que el elemento de 35 pb es una secuencia REP característica de *Pseudomonas putida*. Es, por tanto, la primera vez que se describe y caracteriza una secuencia REP en un grupo de no enterobacteriaceas.

1826–1833 *Nucleic Acids Research, 2002, Vol. 30, No. 8*

Species-specific repetitive extragenic palindromic (REP) sequences in *Pseudomonas putida*

Isabel Aranda-Olmedo, Raquel Tobes, Maximino Manzanera, Juan L. Ramos and Silvia Marqués*

Consejo Superior de Investigaciones Científicas, Estación Experimental del Zaidín, Departamento de Bioquímica y Biología Molecular y Celular de Plantas, Apdo. de correos 419, E-18080 Granada, Spain

Received November 9, 2001; Revised and Accepted February 22, 2002

ABSTRACT

***Pseudomonas putida* KT2440 is a soil bacterium that effectively colonises the roots of many plants and degrades a variety of toxic aromatic compounds. Its genome has recently been sequenced. We describe that a 35 bp sequence with the structure of an imperfect palindrome, originally found repeated three times downstream of the *rpoH* gene terminator, is detected more than 800 times in the chromosome of this strain. The structure of this DNA segment is analogous to that of the so-called enterobacteriaceae repetitive extragenic palindromic (REP) sequences, although its sequence is different. Computer-assisted analysis of the presence and distribution of this repeated sequence in the *P.putida* chromosome revealed that in at least 80% of the cases the sequence is extragenic, and in 82% of the cases the distance of this extragenic element to the end of one of the neighbouring genes was <100 bp. This 35 bp element can be found either as a single element, as pairs of elements, or sometimes forming clusters of up to five elements in which they alternate orientation. PCR scanning of chromosomes from different isolates of *Pseudomonas* sp. strains using oligonucleotides complementary to the most conserved region of this sequence shows that it is only present in isolates of the species *P.putida*. For this reason we suggest that the *P.putida* 35 bp element is a distinctive REP sequence in *P.putida*. This is the first time that REP sequences have been described and characterised in a group of non-enterobacteriaceae.**

INTRODUCTION

Pseudomonads are able to metabolise an enormous range of natural and synthetic organic compounds. They play a crucial role in the process of mineralisation and recycling of organic matter in nature (1). Their high adaptation capacity and their catabolic potential have instigated biochemical and genetic studies of different species of the genus *Pseudomonas* over the years (2). Among them, the soil bacterium *Pseudomonas putida* has deserved special attention due to its potential applications in biotechnology for the control of environmental pollution, promotion of plant growth and control of pathogens (3).

Pseudomonas putida mt-2 (ATCC 33015) was first described by Stanier and co-workers (1) and was later shown to carry two different pathways for benzoate metabolism (4). *Pseudomonas putida* KT2440 (5) is a plasmid cured spontaneous *rmo*$^{-}$ derivative of *P.putida* mt-2, isolated to allow genetic analysis and manipulation. This strain has been extensively characterised both physiologically, biochemically and genetically, so that it is currently considered a representative strain of the species *P.putida*. Its genome was the subject of an early sequencing programme, through which the sequence of its chromosome has recently been determined and is currently in the process of annotation (www.tigr.org).

Repetitive extragenic palindromic (REP) elements were first described in *Escherichia coli* as 35 bp sequences composed of a highly conserved inverted repeat with the potential of forming a stem–loop structure (6,7). The sequences have been extensively characterised in *E.coli* and *Salmonella typhimurium* where they are present in the chromosome more than 500 times either as single independent units or as part of different types of clusters, i.e. bacterial interspersed mosaic elements (BIMEs) (8). Similar sequences were found in *Klebsiella pneumoniae* and other enterobacteria (9). To date, REP sequences have not been described in other prokaryotic microorganisms.

We previously characterised in detail the *rpoH* gene of *P.putida*, which is convergent with the *mtgA* gene. Analysis of the 198 bp intergenic gene region revealed the presence of a 35 bp inverted repeat element located 13 bp downstream of the *rho*-independent terminator of the *rpoH* gene. This 35 bp sequence was repeated three times in this intergenic region. In the present study, we have carried out BLAST scans of the available genome sequence of the strain and we have found that this element is repeated more than 800 times in the chromosome, with a high degree of sequence conservation. We have experimentally determined that this specific 35 bp DNA sequence is restricted to strains of the species *P.putida*. The possible function of this type of sequences is discussed.

*To whom correspondence should be addressed. Tel: +34 958 121011; Fax: +34 958 129600; Email: silvia@eez.csic.es

The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors

MATERIALS AND METHODS

Bacterial strains and growth conditions

The following *Pseudomonas* sp. strains were used in this work: *P.putida* KT2440 (5), *P.putida* SMO116 (10), *P.putida* MTB5 (11), *P.putida* MTB6 (11), *P.putida* DOT-T1E (12), *P.putida* F1 (13), *Pseudomonas* sp. JLR11 (14), *Pseudomonas aeruginosa* PAO1162 (15), *P.aeruginosa* SSS1 (15), *P.aeruginosa* 7NSK2 (15), *Pseudomonas fluorescens* (Pseudomonas Reference Culture Collection, CSIC, Granada, Spain), *P.fluorescens* PF11 (16), *Pseudomonas syringae* pv syringae (17), *Pseudomonas stutzeri* (18), *P.stutzeri* EEZ29 (19), *Pseudomonas mendocina* KR1 (20), *Pseudomonas vesicularis* CECT-327 (Spanish Type Culture Collection, University of Valencia, Spain). The *E.coli* strains used in this work were: DH5α (21) and ET8000 (22). *Escherichia coli* strains were routinely grown at 37°C in Luria–Bertani (LB) medium (23). *Pseudomonas* strains were grown at 30°C in LB medium or in minimal medium (basal M9 medium supplemented with Fe-citrate, $MgSO_4$ and trace metals) with glucose (10 mM) as the sole carbon source (24). When required, antibiotics were used at the following final concentrations (μg/ml): tetracycline (10), kanamycin (50), rifampicin (20), chloramphenicol (30) and streptomycin (50 or 100).

Plasmids

Plasmids pSH27, pMPR and pMPRH have been described previously (25). The following plasmids were constructed in this study: pA1D is a pMPR derivative that carries the (5′-GCCGGCCTCTTCGCGGGTAAGCCCGCTCCTACAGG-GCTGCA-3′) REPa1 sequence cloned in the *Pst*I site between $\lambda p'_R$ and *′lacZ* in the same orientation as it is found downstream of *rpoH*, pA1I is similar to pA1D but with the REPa1 sequence cloned in the opposite orientation. Plasmid pMPRH0 is a pMPR derivative carrying the *rpoH* terminator and the REPa1 sequence obtained as a PCR product from pSH27 using the primers 5′-CTGCAGTGTAAAGAAAAGCCGC-3′ and 5′-CTGCAGCAACCGCATCCCC-3′, the amplified product was cloned in the *Pst*I site between $\lambda p'_R$ and *′lacZ*. Plasmid pA12 is a pMPR derivative carrying the complete REPa sequence, which includes REPa1 and REPa2 (the sequence of the linker is 5′-CCGGCCTCTTCGCGGGTAAGCCCGCT-CCTACAGGGGATGCGGTTGCCTGTAGGCGCGGGTTT-GCCCGCGAGGAGGCCGGTGCA-3′), cloned in the same orientation as it is found downstream of *rpoH*, in the *Pst*I site between $\lambda p'_R$ and *′lacZ*; pA21 is similar to pA12, but with the complete REPa sequence cloned in the opposite orientation. Plasmid pMPRA1 is a pMPR derivative carrying a random sequence of the same length as REPa1 (5′-GGCGCGG-CAGCCACTGGGGGTTTAAAGGCGGCCACCCTGCA-3′) cloned between $\lambda p'_R$ and *′lacZ*. Plasmids pMPRA2 and pMPRA3 are equivalent to pMPRA1 but carry two copies of the random sequence cloned in direct (pMPRA2) or inverted (pMPRA3) orientation. All constructions were confirmed by sequencing, and plasmids were introduced into *P.putida* by electroporation and into *E.coli* ET8000 by transformation.

DNA manipulations

Total chromosome or plasmid DNA preparations, digestion of DNA with restriction enzymes, ligations, transformations and agarose or polyacrylamide gel electrophoresis were done according to standard methods (23). Plasmid DNA was isolated by the alkaline lysis method with the QIAprep spin plasmid minipreps kit (Qiagen, Hilden, Germany). DNA cloned in plasmids was sequenced on both strands by using appropriate primers and the dideoxy sequencing termination method with dichlororhodamine (Perkin Elmer, Madrid, Spain). DNA amplification reactions were carried out in a GeneAmp PCR system 2400 with the Expand High Fidelity PCR system (Roche, Barcelona, Spain). For amplification of *Pseudomonas* genomic DNA (0.1 μg/25 μl reaction), the oligonucleotide REPc (5′-GTAGGAGCGGGTTTACCCG-CGAA-3′) (3 μM) was used. The cycling conditions were as follows: 94°C for 3 min, followed by 25 cycles at 94°C for 1 min, 69°C (stringent) or 50°C (relaxed) for 1 min and 70°C for 10 min, followed by a final 10 min step at 70°C.

β-Galactosidase assays

Overnight cultures grown on LB supplemented with the appropriate antibiotics were diluted 1:100 in fresh medium and grown until the exponential phase was reached. Samples were taken to determine β-galactosidase activity as described previously (26). Data are the mean of three independent assays; SD values were <15% of the given values.

Computer-assisted detection of *P.putida* KT2440 palindromic sequences

Stand-alone BLAST was downloaded from the National Center for Biotechnology Information (NCBI) for local use. A database with all the contigs of *P.putida* [The Institute for Genomic Research (TIGR), released November 7, 2000] was screened against the 35 nt sequence 5′-CCGGCCTCTTCGCG-GGTAAGCCCGCTCCTACAGGG-3′, which is found downstream of the *P.putida rpoH* gene. We have developed specific computer programs for assisting in the detection and analysis of REP sequences in the genome of *P.putida*. A set of programs deal with the transformation of the BLAST generated output to an appropriate format for the following. (i) Selection of REP sequences by *E* and length. (ii) Generation of a multiple alignment to derive a consensus sequence for the *P.putida* REP sequence. To this end, all selected REP sequences were transformed to a multifasta format. The multiple sequence alignment software used was Multalin (27). (iii) Study of the distance between two REP sequences and their orientation with respect to each other.

The gene-finding program used to annotate the *P.putida* genome was based on a hidden Markov model (HMM) with modules for coding and non-coding regions, START and STOP codons as well as for ribosome binding sites. The HMM is trained on open reading frames (ORFs) with Swissprot homologues and is then used to identify putative genes which score significantly higher than random ORFs (T.S.Larsen and A.Krogh, manuscript in preparation).

Other series of programs analyse REP sequences with respect to the annotated genome in order to detect (i) extragenic or intragenic location of the REP sequences; (ii) distance between REP sequences restricted to the same intergenic space; (iii) clusters in the same intergenic space; (iv) the size of the intergenic spaces in the *P.putida* genome; (v) the different types of intergenic spaces defined by the different orientation of the limiting genes (genes of equal sense, convergent genes and divergent genes); (vi) the presence of REP sequences in

each type of intergenic space; and (vii) distances between the REP sequence pairs and the end of the convergent genes limiting an intergenic space. The programs are written in BASIC and will be made available by the authors upon request. A version written in C is currently being developed.

RESULTS

Identification of a repetitive element in the *P.putida* KT2440 chromosome

In the genome of *P.putida* KT2440 the *rpoH* gene is followed by a 34 bp inverted repeat sequence that forms a hairpin and has *rho*-independent terminator activity (25). This sequence is within the 198 bp-long intergenic sequence that separates *rpoH* from its neighbouring convergent *mtgA* gene. Detailed analysis of this 198 bp intergenic region revealed that in addition to the hairpin structure, three copies of a well conserved 35 bp sequence located at 13, 57 and 102 bp from the terminator sequence was present: these were organised as a direct unit followed by two copies in the opposite orientation (Fig. 1A). The sequence within each unit was partially palindromic, containing an internal 6 bp inverted repeat. An initial search for homology with the intergenic *rpoH*/*mtgA* region against DNA sequences deposited in GenBank revealed almost 30 sequences with significant hits in which the conserved sequence corresponded to the stretch of 35 bp. All these sequences belonged to strains of the species *P.putida* or to unidentified *Pseudomonas* strains. The alignment of these sequences revealed a conserved internal inverted repeat 5′-GCGGGN$_4$CCCGC-3′. The element seemed to be widespread in sequences deposited in GenBank for genes belonging to bacteria of the genus *Pseudomonas*. Consequently, we decided to analyse in detail the presence of this sequence in the recently completed genome of *P.putida* KT2440 (www.tigr.com).

We selected the 35-base sequence 5′-CCGGCCTCT-TCGCGGGTAAGCCCGCTCCTACAGGG-3′ located downstream of the *rpoH* gene as a query sequence for a BLAST search in the whole *P.putida* KT2440. The BLAST available at TIGR had limited the number of sequences with hits in the output. Hence, we used a locally executable BLAST without this limitation. A preliminary analysis of the detected sequences allowed us to establish their main features: (i) a length of 35 nt; (ii) the presence of the central palindromic motif GCGGGnnnnCCCGC; and (iii) a dispersed similarity along the 35 bases.

The BLAST parameter '*W*' defining the size of word for the initial matching was 11, which is the default value. We realised that it was necessary to set this parameter to 7 to detect a pattern with these characteristics. As only the central motif was totally conserved, the parameter penalising the mismatch (*q*) was also changed from –3 to –2, and since the central palindromic motif did not allow gaps, the BLAST search was done without gaps. From this BLAST search with these parameters in the complete genome of *P.putida* we obtained a set of around 1300 sequences.

Multiple alignment of the sequences was carried out and we established a filter of significance (*E*) and length for this set. We selected sequences longer than 19 bases with an *E*-value <1. This reduced the number of sequences to 804 (Fig. 2 shows the alignment of the best 30 sequences matching the query) and

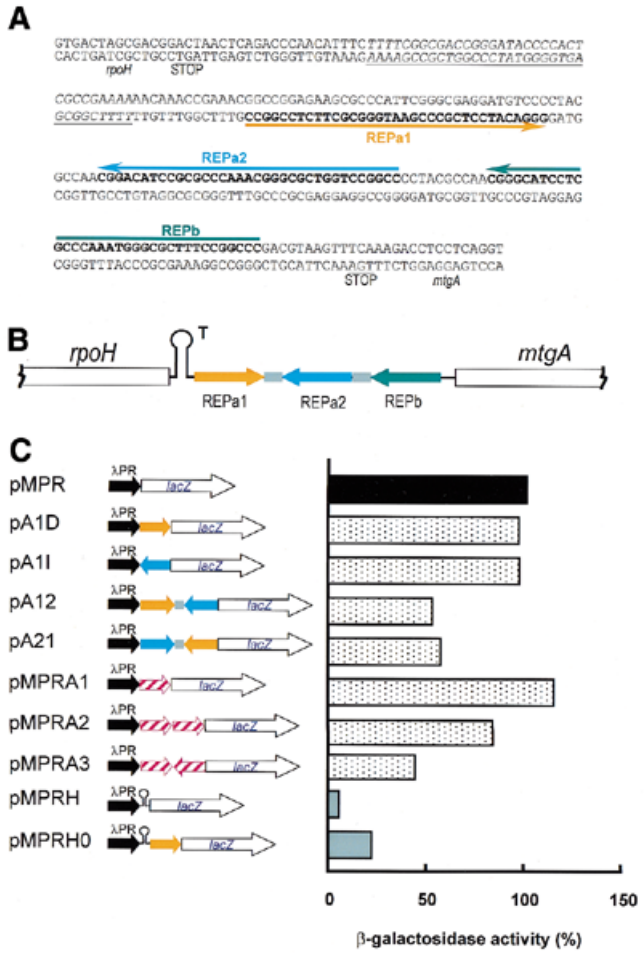

Figure 1. (**A**) DNA sequence and features between the *rpoH* and *mtgA* genes. The STOP codons of *rpoH* and *mtgA* are indicated. The rho-independent terminator sequence is in italics and underlined. Arrows underline the three repetitive sequences found in this intergenic region. The direction of the arrow indicates the orientation of the repetitive sequence. (**B**) Schematic representation of the intergenic region. The orange, blue and green arrows indicate the orientation of the three different REP sequences found downstream of *rpoH*. T indicates the rho-independent terminator. (**C**) Termination activity of sequences located downstream the *rpoH* gene. *Pseudomonas putida* KT2440 carrying the indicated plasmids was grown until exponential phase was reached, and β-galactosidase was determined as described in Materials and Methods. Data are presented as percentage of the activity measured in cells bearing pMPR (400 Miller units). The leftmost arrow (black) depicts the $\lambda p'_R$ promoter, the white arrow indicates the *′lacZ* reporter gene, while the orange, blue and green arrows between the $\lambda p'_R$ promoter and *′lacZ* show the different REP sequences found downstream of *rpoH*. Striped arrows show a random sequence, in either orientation, and the hairpin indicates the *rho*-independent terminator. Data are the mean of three independent assays, with SD <10% of the given value.

allowed for definition of the following consensus sequence: 5′-c-ggcctcTTCGCGGGtaaaCCCGCtCCtaCaggg-3′ (uppercase letters indicate the presence of this base in this position in 90% of the aligned sequences and lowercase letters in 50% of the sequences) with a central palindromic motif (underlined). The central dyad symmetry was maintained in most of the sequences through compensatory base changes. In 98% of the cases, the change GGGt to GGGa was counterbalanced by the change aCCC to tCCC (data not shown). We evaluated the relevance of this palindromic structure with the rationale that,

REP number	SEQUENCE
	1 35
1	CCGGCCCTTTCGCGGGTGAACCCGTTCCTGCAGCG
578	CCGGCCCTTCCGCGGGTGAACCCGCTCCTACAGCG
6	CCGGCCCTTTCGCGGGTGAACCCGCTCCTACGGGC
198	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACGGGC
631	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACGGGC
29	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACGGCC
266	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACGGGG
584	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACAGGC
375	CTGGCCCTTTCGCGGGTAAACCCGCTCCTACGGGC
71	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACACGG
732	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACGCGG
499	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACACAG
633	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACACGC
318	CCGGCCCCTTCGCGGGTAAACCCGCTCCTACACGG
598	CCGGCCCTTTCGCGGGTGAACCCGCTCCTACACGC
343	CGGGCCCTTTCGCGGGTAAACCCGCTCCTACAGGG
774	CGGGCCCTTTCGCGGGTAAACCCGCTCCTACAGGG
365	CCGGCCCATTCGCGGGTAAACCCGCTCCTACACAG
568	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACACAA
15	GCGGCCCATTCGCGGGTAAACCCGCTCCTACAGGG
462	CCGGCCCATTCGCGGGTAAACCCGCTCCTACAGGG
53	GCAGCCCATTCGCGGGTAAACCCGCTCCTACAAGG
279	GCAGCCCATTCGCGGGTGAACCCGCTCCTACAAGG
220	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACAGGT
238	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACAGGT
362	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACAGGT
363	CCGGCCCTTTCGCGGGTAAACCCGCTCCTACAGGT
262	CCGGCGCTTTCGCGGGTAAACCCGCTCCTACAGGT
666	CCGGCCCTTTCGCGGGTGAACCCGCTCCTACAGGT
759	CCGGCCCTTTCGCGGGTGAACCCGCTCCTACAGGT
Consensus	c.GGCCtcTTCGCGGGTAAACCCGCTCCTACAGGg

Figure 2. Sequence alignment of the 30 best *P.putida* REP sequences matching the REPa1 located downstream *rpoH*. Numbers are those assigned to each REP sequence in our database. Letters in red are bases present at this position in 90% of the 804 sequences. Letters in blue are bases present at this position in 50% of the 804 sequences. The palindromic motif is underlined.

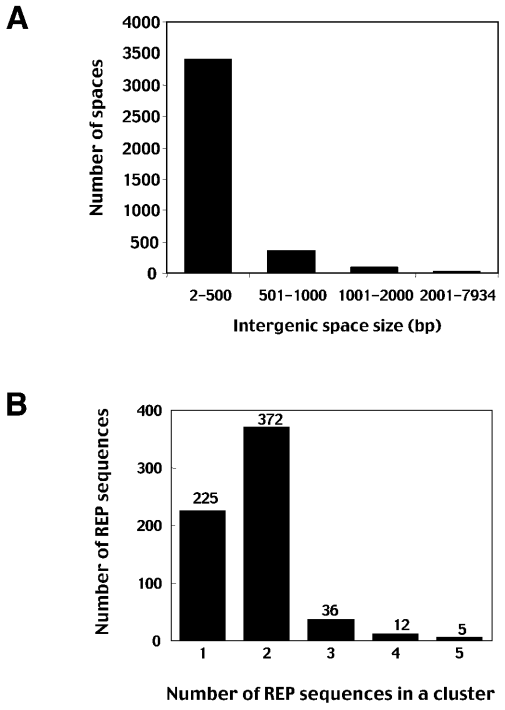

Figure 3. (**A**) Size distribution of intergenic spaces in *P.putida* KT2440. Intergenic spaces were grouped in four intervals, according to their size. The figure shows the frequency for each size interval. (**B**) REP sequence distribution in *P.putida* KT2440. In *P.putida* KT2440 REP sequences are distributed as isolated elements or forming clusters. We have represented the frequency of REP sequences that appear isolated or forming clusters of two, three, four or five REP sequences.

if the secondary structure was maintained through compensatory mutations, these mutations would appear at a higher frequency than expected. When we analysed the 804 REP sequences, we found 85 base changes on the left side of the central palindrome $GCGGGN_4CCCGC$, and 106 changes on the right side. Among these changes, 21 were symmetrical and complementary. Given a mutation on the left side of the palindrome, we estimated the probability of finding a compensatory mutation on the other side of the palindrome (p_{cm}) as a factor of the probability of finding a mutation in the symmetrical position ($106/804 \times 5$), and the probability of the change being complementary (1/3). Therefore, the expected frequency of complementary mutations would be $85 \times p_{cm} = 0.75$. However, the observed frequency of complementary mutation was 21, 28-fold of what was expected. These data reinforce the importance of conserving the palindromic structure, and allow us to suggest that the secondary structure is conserved through the selection of compensatory mutations. Because of the structure, abundance, degree of conservation and parallelism of these sequences with the REP sequences previously described in enterobacteriaceae, we believe we have found for the first time REP sequences in pseudomonadaceae (6,7).

Pseudomonas putida REP sequences are favoured in extragenic spaces between convergent genes

To detect the extragenic or intragenic allocation of the sequences, the distance between two adjacent sequences and the distance to the flanking genes, we developed computer-assisted methods to analyse these sequences in the context of the ORFs in the genome of *P.putida* KT2440. We found that ~80% of these REP sequences were found in extragenic regions. In 89% of cases, the intergenic region was <500 bases in length (Fig. 3A). Taking into account that only 11% of the genome bases of *P.putida* are extragenic (R.Tobes, unpublished data), this location of REP sequences in extragenic spaces is not likely to be random. We performed a chi-square analysis to test the randomness of this distribution. We concluded that for each base, the intra-REP location and the extragenic location were related features with a probability close to 1 (since the *P*-value of no relation is 0.000000), thus suggesting that the presence of REP sequences in intergenic spaces is not random. This could reflect a selection against the appearance of these elements within coding regions, or a positive selection for the presence of REP sequences in extragenic spaces.

We also analysed the distance between REP sequences within the same intergenic space. In all cases where more than one REP sequence was present, they were separated by <51 bases and always appeared in opposite orientation. When there are several REP sequences within an intergenic region, these sequences form a cluster. We detected 225 isolated REP sequences, 372 REP sequences forming pairs, 36 forming clusters of three sequences, 12 in clusters of four sequences and one cluster of five sequences (Fig. 3B). This mode of organisation has also been described in the *E.coli* chromosome where some REP sequences are organised as complex elements called BIMEs (28). In *P.putida*, when clustered as pairs, REP sequences were always found in opposite orientation separated by short stretches of sequence, opening the possibility of

Table 1. Statistical analysis of the organisation of the REP sequences that appear in intergenic spaces

Intergenic space types[a]	NS[b]	RF[c]	EF[d]	RF/EF[e]
→•→	1281	209	219.75	0.95
←•←	1111	155	190.59	0.81
→•← convergent	719	236	123.34	1.91
←•→ divergent	678	50	116.31	0.42
Total	3789	650		

[a]Types of intergenic spaces depending on gene orientation, which is schematised by arrows.
[b]Number of intergenic spaces of each type in the genome of *P.putida*.
[c]REP frequencies: number of REP sequences in each type of intergenic space.
[d]Expected frequencies of REP sequences in each type of intergenic space.
[e]Ratio between REP frequencies and expected frequencies.

Table 2. Statistical analysis of the organisation of the *E.coli* BIME sequences that appear in intergenic spaces

Intergenic space types[a]	NS[b]	BF[c]	EF[d]	BF/EF[e]
→•→	1113	48	74.91	0.64
←•←	1195	56	80.43	0.69
→•← convergent	610	126	41.05	3.06
←•→ divergent	528	2	35.54	0.05
Total	3447	232		

[a]Types of intergenic spaces depending on gene orientation, which is schematised by arrows.
[b]Number of intergenic spaces of each type in the genome of *E.coli*.
[c]BIME frequencies: number of BIME sequences in each type of intergenic space.
[d]Expected frequencies of BIME sequences in each type of intergenic space.
[e]Ratio between BIME frequencies and expected frequencies.

secondary structure formation. This would involve the co-evolution of the sequences in a pair, and would imply that the similarity between two elements in a pair should be higher than the similarity between randomly selected pairs of REP sequences. To test this hypothesis, we performed the following analysis. We selected all the pairs of REP sequences (a pair is defined as two inverted REP sequences located in the same intergenic space and <100 bp apart from each other) and ran a BLAST (Bl2seq) between both elements of each pair. The average *E*-value obtained was 0.00278. Considering the group of 804 REP sequences, there are 322 806 different possible pairs of REP sequences. We randomly selected 120 967 REP pairs of the 322 806 possible different pairs of the 804 REP sequences and ran a BLAST (Bl2seq) as before. The average *E*-value obtained in this case was 0.01179, which is a value 4.241 times higher than the average *E*-value between REP sequence pairs. This suggests a selective pressure favouring similarity between REP sequences in a pair.

With the aim of searching for a relationship between the presence of intergenic REP sequences and the orientation of the genes limiting the intergenic regions, we defined four types of intergenic spaces: (i) between convergent genes; (ii) between divergent genes; (iii) between genes positively oriented (coded by the main DNA chain); and (iv) between genes negatively oriented (coded by the complementary DNA chain) (Table 1). If the REP sequences were randomly allocated in the different types of intergenic spaces, their distribution would depend on the number of intergenic spaces of each type. However, the number of REP sequences between convergent genes is strikingly 2-fold higher than the expected value. Moreover, the presence of REP sequences between divergent genes is less than half of the expected value (Table 1). It is worth noting that very similar results were obtained when only REP sequences that appear as isolated elements were analysed. These data suggest that the REP sequences are preferentially localised in intergenic regions limited by convergent genes, their presence between divergent genes being avoided. Given that this feature seemed to us essential in *P.putida* REP sequences because of putative functional implications, we wondered if other known REP sequences, i.e. *E.coli* REP sequences, would share this characteristic. With this aim, we performed a similar analysis of *E.coli* REP sequences based on data available at

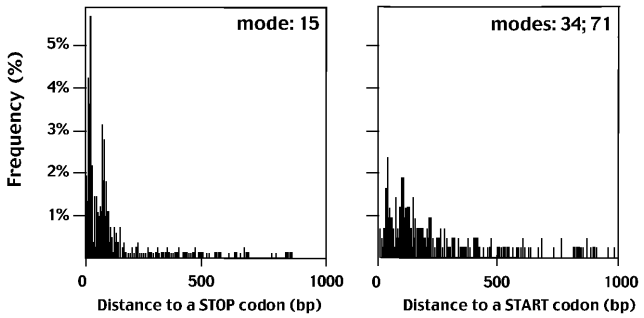

Figure 4. Distance of the intergenic REP sequences to the beginning and to the end of a gene. Each intergenic space is limited by two genes. The orientation of these genes determines a START codon or a STOP codon as the limit of each extreme of the intergenic space. For each intergenic REP sequence we analysed its distance to the limits of the intergenic space to generate two sets of data: (i) distances of REP sequences to STOP codons limiting its intergenic space (829 distances); and (ii) distances of REP sequences to START codons limiting its intergenic space (424 distances). The frequency distribution of both data sets is presented.

www.pasteur.fr/recherche/unites/pmtg/repet/tableauBIMEcoli.html and the *E.coli* genome annotation available at www.ncbi.nlm.nih.gov/genbank/genomes/Bacteria/Escherichia_coli_K12/U00096.gbk. Strikingly, we also found that for *E.coli* the observed frequency of REP sequences between convergent genes was 3-fold the expected value (Table 2).

To search for a putative relationship between every intergenic REP sequence and its two flanking genes, we analysed the distances of each REP sequence to its neighbouring gene considering their START (5′-terminus) or STOP (3′-terminus) codon. Since the frequency of REP sequences between convergent genes was the highest, we could define 829 distances to a STOP codon and 424 distances to a START codon. Figure 4 shows a graphic presentation of the frequency of each distance, ordered by sizes. Clearly, in the set of distances to a STOP codon, the values were grouped around a median value of 56 bp, where the most frequent value (mode) was 15 bp. Of the values, >80% were <92 bp (Fig. 4). In contrast, when we analysed the distances to a START codon, the median was

significantly higher (128 bp). Furthermore, two modes were found, 34 and 71 bp, and only 40% of the distances to a START codon were <100 bp (Fig. 4). These results suggest that the REP elements in *P.putida* KT2440 are probably related to the neighbouring gene(s), and their function would be exerted through their position at the end of a gene.

When we specifically analysed the pairs of REP sequences located between convergent genes, we found that their distances to the ends of these genes seemed to be maintained <30 bp: 84% of the distances to the end of the right gene and 68% of the distances to the end of the left gene were <30 bases. This fact probably has functional implications.

REP sequences are not specific transcription terminators

Because of the partial palindromic structure and the location of these sequences at the end of ORFs, we decided to test whether they played a role in transcription termination. We cloned the 35 bp element and all possible combinations found downstream *rpoH* in different orientations between the $\lambda p'_R$ promoter and *′lacZ*, in the plasmids detailed in Materials and Methods, to determine whether they exhibited transcriptional termination activity. We found that while the true rho-independent terminator of *rpoH* lowered the activity to ~5% (pMPRH), the presence of the 35 bp sequence in either orientation downstream $\lambda p'_R$ had little effect on the β-galactosidase activity, and the two sequences in inverted orientation only reduced 45% of the β-galactosidase with respect to the construction without the insert (Fig. 1C). To test whether this low termination activity was sequence specific (i.e. exclusive of complementary REP pairs) or due to a putative secondary structure formed between two inverted sequences of this length, we constructed pMPRA2 and pMPRA3, similar to pA12 and pA21, but with two identical random sequences in direct or inverted orientation, instead of the REP sequences. Figure 1C shows that two random sequences in inverted orientation produce the same effect as two REP sequences. These results suggest that the 35 bp element by itself is probably not a terminator, and that the ability of the REP sequence pairs to reduce expression of the downstream gene does not depend on their sequence, but rather on their inverted orientation. In any case, the effect produced is very low compared with the termination activity of a true *rho*-independent terminator.

REP sequences specifically identify *P.putida*

Escherichia coli REP sequences have been extensively used in taxonomic studies to determine the diversity of bacterial populations (29). Repetitive element sequence-based PCR (rep-PCR) enables the generation of DNA fingerprint patterns to discriminate bacterial species and strains. The primers more frequently used for rep-PCR-based fingerprinting analysis are REP, ERIC and BOX sequences (30). We decided to test whether the REP elements of *P.putida* KT2440 could be used in a more specific manner to specifically detect and genotype *P.putida*. We selected several strains belonging to eight species of the genus *Pseudomonas* and seven strains of *P.putida* available in our laboratory collection. We isolated chromosomal DNA from each strain and performed PCR using an oligonucleotide with the *P.putida* REP consensus sequence. Figure 5 shows that only *P.putida* strains gave PCR products under these conditions, whereas none of the non-*P.putida* strains gave any signal, even when using low stringency annealing conditions.

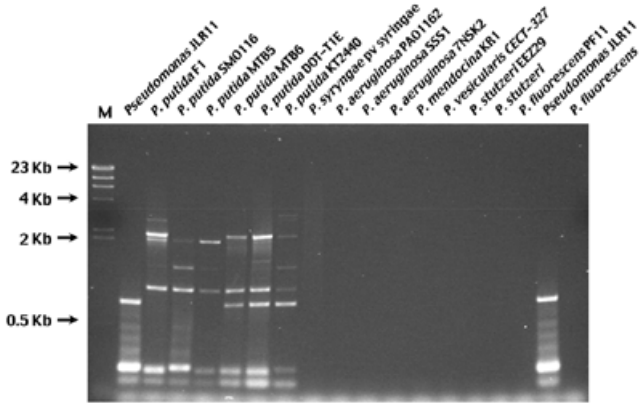

Figure 5. PCR amplification of *Pseudomonas* strains genomic DNA with REPc primer. Equivalent amounts of total chromosomal DNA isolated from each strain was amplified as described in Materials and Methods and the PCR products were subjected to agarose electrophoresis.

These results suggest that the REP sequence of *P.putida* KT2440 identified here allows the identification of *P.putida* strains. The band pattern obtained with all *P.putida* strains revealed common bands, and several strain-specific bands. This probably reflects genome reorganisations, and allows a very specific genotyping of strains. A genotyping method based on species-specific REP sequences would only require the detection of the presence or absence of PCR products, rather than the more complex analysis of band patterns. Such a method would be suitable for the automation of genotyping procedures.

DISCUSSION

The analysis presented in this work shows the presence of highly repetitive extragenic palindromic sequences in the chromosome of *P.putida* KT2440. The structure of the REP sequence detected in *P.putida* is very similar to that described in *E.coli* and several enterobacteriaceae such as *Salmonella* sp. and *Klebsiella* sp., although the DNA sequence is species specific and is dissimilar. In fact, PCR chromosome amplification with an oligonucleotide complementary to the REP element has revealed that this particular sequence can only be detected in strains of the *P.putida* species. The development of methods and conditions for the optimal use of this sequence in the detection, identification and typing of bacterial strains will make it a valuable tool for taxonomic and population analysis, both in the laboratory and in natural environments. In addition, the use of a single primer in the PCR analysis notably increases the specificity of the reaction, which requires the close presence of two similar sequences in opposite orientation.

REP sequences of different enterobacteriaceae described so far share an internal structure: a 35 base sequence with a central palindromic motif and characteristic positions defining a head and a tail. In *P.putida* REP sequences, the most conserved region is 29 bp long and contains an internal 14 bp dyad symmetry whose presence tends to be maintained through compensatory changes. This internal palindrome is shorter than the imperfect dyad symmetry of *E.coli* REP sequences, which is 24 bp long.

In our search, 80% of the REP sequences were found outside ORFs, therefore they did not interfere with gene sequences. This proportion could be even higher since, for the sake of simplicity in our analysis, we have considered as intragenic those REP sequences that overlapped with gene ORFs by only 1 bp. When we analysed the presence of REP sequences close to homologous genes in *E.coli* and *P.putida*, we obtained no significant similarity in the position of REP sequences. This suggests that the function of REP sequences is not related to specific gene functions.

In *E.coli,* only 83 out of 500 REP sequences are present as single units, while the rest are part of more complex mosaic elements, called BIMEs (31). The situation in *P.putida* seems to be different: only eight BIMEs were found and most of the REP sequences were found as single elements or forming pairs. Although lower than in *E.coli*, the presence of REP sequence pairs in *P.putida* is very significant, and strikingly, in these pairs the two REP elements were always found in opposite orientation. This suggests a specific functional role for the pairs (32). The disposition as inverted repeats opens the possibility of stem–loop structure formation although they do not seem to be involved in the transcription termination as determined experimentally in this study. Recently, a role in transcription attenuation has been suggested for *E.coli* BIMEs (33). Espéli *et al.* (33) found a similar level (~50%) of decrease in expression of genes located downstream of a pair of inverted REP sequences. Unfortunately, they did not test the putative effect of a random DNA sequence with a similar structure.

Our finding of REP sequences in the non-enterobacteriaceae *P.putida* supports the hypothesis that these sequences are a more general phenomenon in bacteria. Therefore, it is tempting to speculate that this could mean that they are involved in an important bacterial function(s), though not yet identified. The presence of these sequences in enterobacteriaceae has been related to several functions, such as stabilisation of mRNA (34–36), organisation of the chromosome, insertion of genetic elements, binding site for proteins such as IHF (37), DNA polymerase I (38) and DNA gyrase (39). Thorough studies carried out with this last protein have shown that it is able to bind and cleave REP sequences (40). In addition, HU stimulates high affinity binding of gyrase, but inhibits cleavage of the sequence (41). Yang and Ferro-Luzzi Ames (41) suggested that REP sequences could be the target for gyrase action on the chromosome to maintain the appropriate level of negative supercoiling, and to anchor the chromosome supercoiled domain loops to each other. However, this hypothesis cannot explain why most REP sequences are located at the 3′-terminus of the genes. Changes in DNA supercoiling mediated by DNA gyrase could play a role in the induction of expression of some genes and could provide a general way of increasing expression of genes in the cell in response to environmental stress (42). Initiation of transcription for many genes is sensitive to DNA supercoiling. On the other hand, the situation is especially interesting in the intergenic spaces between convergent genes, where most of the *P.putida* REP pairs are located. In those cases, the distance of the REP pair to the end of the flanking genes is usually very short (<30 bp), thus suggesting that their function is probably related to those genes. It is known that two neighbouring convergent genes simultaneously transcribed generate positive supercoiling in the DNA ahead of the two RNA polymerases moving towards each other, i.e. the intergenic region (43,44). DNA gyrase, which has been shown to bind REP sequences in *E.coli*, is known to relax positively supercoiled domains (44). Therefore, it is tempting to suggest that one main role of REP sequences would actually be to allow DNA gyrase to bind and relax DNA when excessive positive supercoiling is generated, especially between two convergent genes. This would explain our finding that most REP pairs are located between convergent genes in *P.putida*, close to their STOP codon, and why this is also the case in *E.coli*, as shown in Table 2.

Our results show that the REP sequences appear at a very low frequency between divergent genes. The DNA region between the beginning of two genes is a functionally compromised region, because the promoters of at least two genes are allocated in these regions, where the transcriptional machinery needs to bind. If REP sequences were the target of cellular protein, i.e. DNA gyrase, etc., their presence in this region would interfere with the expression of divergent genes. Also if REP sequences were targets for insertion of genetic elements or for DNA rearrangement, as suggested (45,46), they would be expected to be deleterious if found in promoter regions.

In summary, the finding of REP sequences in non-enterobacteriaceae suggests a more general and crucial role for these sequences. Their significant presence as extragenic, in pairs as inverted repeats, at the end of genes and between convergent genes can give clues towards revealing their function. Finally, the expected broad presence of REP sequences in bacteria together with their species specificity opens new ways for easy and precise bacterial genotyping.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

We thank Ana Hurtado for the preparation of chromosomal DNAs and T. S. Larsen and I. Cases for preliminary annotation and suggestions on the handling of the *Pseudomonas* genome sequence available at www.tigr.org. This study was supported by a grant from the European Commission (BIO 2000-3126-CE).

REFERENCES

1. Stanier,R.Y., Palleroni,N.J. and Doudoroff,M. (1966) The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.*, **43**, 159–271.
2. Sokatch,J. (1986) *The Bacteria. Pseudomonas.* Academic Press, New York, Vol. 10.
3. Ramos,J.L., Diaz,E., Dowling,D., de Lorenzo,V., Molin,S., O'Gara,F., Ramos,C. and Timmis,K.N. (1994) The behavior of bacteria designed for biodegradation. *Biotechnology (NY)*, **12**, 1349–1356.
4. Nakazawa,T. and Yokota,T. (1973) Benzoate metabolism in *Pseudomonas putida* (arvilla) mt 2: demonstration of two benzoate pathways. *J. Bacteriol.*, **115**, 262–267.
5. Franklin,F.C.H., Bagdasarian,M., Bagdasarian,M.M. and Timmis,K.N. (1981) Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. *Proc. Natl Acad. Sci. USA*, **78**, 7458–7462.
6. Higgins,C.F., Ames,G.F., Barnes,W.M., Clement,J.M. and Hofnung,M. (1982) A novel intercistronic regulatory element of prokaryotic operons. *Nature*, **298**, 760–762.

7. Stern,M.J., Ames,G.F., Smith,N.H., Robinson,E.C. and Higgins,C.F. (1984) Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell*, **37**, 1015–1026.
8. Bachellier,S., Saurin,W., Perrin,D., Hofnung,M. and Gilson,E. (1994) Structural and functional diversity among bacterial interspersed mosaic elements (BIMEs). *Mol. Microbiol.*, **12**, 61–70.
9. Bachellier,S., Perrin,D., Hofnung,M. and Gilson,E. (1993) Bacterial interspersed mosaic elements (BIMEs) are present in the genome of *Klebsiella*. *Mol. Microbiol.*, **7**, 537–544.
10. Moller,S., Pedersen,A.R., Poulsen,L.K., Arvin,E. and Molin,S. (1996) Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative *in situ* hybridization and scanning confocal laser microscopy. *Appl. Environ. Microbiol.*, **62**, 4632–4640.
11. Huertas,M.J., Duque,E., Molina,L., Rosselló-Mora,R., Mosqueda,G., Godoy,P., Christensen,B., Molin,S. and Ramos,J.L. (2000) Tolerance to sudden organic solvent shocks by soil bacteria and characterisation pf *Pseudomonas putida* strains isolated from toluene polluted sites. *Environ. Sci. Technol.*, **34**, 3395–3400.
12. Ramos,J.L., Duque,E., Huertas,M.J. and Haidour,A. (1995) Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J. Bacteriol.*, **177**, 3911–3916.
13. Gibson,D.T., Hensley,M., Yoshioka,H. and Mabry,T.J. (1970) Formation of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*. *Biochemistry*, **9**, 1626–1630.
14. Esteve-Núñez,A., Lucchesi,G., Philipp,B., Schink,B. and Ramos,J.L. (2000) Respiration of 2,4,6-trinitrotoluene by *Pseudomonas* sp. strain JLR11. *J. Bacteriol.*, **182**, 1352–1355.
15. Hofte,M., Mergeay,M. and Verstraete,W. (1990) Marking the rhizopseudomonas strain 7NSK2 with a Mu d(lac) element for ecological studies. *Appl. Environ. Microbiol.*, **56**, 1046–1052.
16. Aarons,S., Abbas,A., Adams,C., Fenton,A. and O'Gara,F. (2000) A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. *J. Bacteriol.*, **182**, 3913–3919.
17. Collmer,A. and Bauer,D.W. (1994) *Erwinia chrysanthemi* and *Pseudomonas syringae*: plant pathogens trafficking in extracellular virulence proteins. *Curr. Top. Microbiol. Immunol.*, **192**, 43–78.
18. Zumft,W.G., Dohler,K., Korner,H., Lochelt,S., Viebrock,A. and Frunzke,K. (1988) Defects in cytochrome cd1-dependent nitrite respiration of transposon Tn5-induced mutants from *Pseudomonas stutzeri*. *Arch. Microbiol.*, **149**, 492–498.
19. Ramos-González,M.I., Duque,E. and Ramos,J.L. (1991) Conjugational transfer of recombinant DNA in cultures and in soils: host range of *Pseudomonas putida* TOL plasmids. *Appl. Environ. Microbiol.*, **57**, 3020–3027.
20. Whited,G.M. and Gibson,D.T. (1991) Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to p-cresol in *Pseudomonas mendocina* KR1. *J. Bacteriol.*, **173**, 3010–3016.
21. Hanahan,D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, **166**, 557–580.
22. MacNeil,T., Roberts,G.P., MacNeil,D. and Tyler,B. (1982) The products of *glnL* and *glnG* are bifunctional regulatory proteins. *Mol. Gen. Genet.*, **188**, 325–333.
23. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. Abril,M.A., Michán,C., Timmis,K.N. and Ramos,J.L. (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. *J. Bacteriol.*, **171**, 6782–6790.
25. Manzanera,M., Aranda-Olmedo,I., Ramos,J.L. and Marqués,S. (2001) Molecular characterization of *Pseudomonas putida* KT2440 *rpoH* gene regulation. *Microbiology*, **147**, 1323–1330.
26. Miller,J. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Corpet,F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.*, **16**, 10881–10890.
28. Gilson,E., Saurin,W., Perrin,D., Bachellier,S. and Hofnung,M. (1991) Palindromic units are part of a new bacterial interspersed mosaic element (BIME). *Nucleic Acids Res.*, **19**, 1375–1383.
29. Louws,F.J., Fulbright,D.W., Stephens,C.T. and de Bruijn,F.J. (1994) Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl. Environ. Microbiol.*, **60**, 2286–2295.
30. Louws,F.J., Rademaker,J.L.W. and de Bruijn,F.J. (1999) The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection and disease diagnosis. *Ann. Rev. Phytopathol.*, **37**, 81–125.
31. Bachellier,S., Clement,J.M. and Hofnung,M. (1999) Short palindromic repetitive DNA elements in enterobacteria: a survey. *Res. Microbiol.*, **150**, 627–639.
32. Gilson,E., Perrin,D., Clement,J.M., Szmelcman,S., Dassa,E. and Hofnung,M. (1986) Palindromic units from *E. coli* as binding sites for a chromoid-associated protein. *FEBS Lett.*, **206**, 323–328.
33. Espéli,O., Moulin,L. and Boccard,F. (2001) Transcription attenuation associated with bacterial repetitive extragenic BIME elements. *J. Mol. Biol.*, **314**, 375–386.
34. Newbury,S.F., Smith,N.H., Robinson,E.C., Hiles,I.D. and Higgins,C.F. (1987) Stabilization of translationally active mRNA by prokaryotic REP sequences. *Cell*, **48**, 297–310.
35. Newbury,S., Smith,N.H. and Higgins,C.F. (1987) Differential mRNA stability controls relative gene expression within a polycistronic operon. *Cell*, **51**, 1131–1143.
36. Stern,M.J., Prossnitz,E. and Ferro-Luzzi Ames,G. (1988) Role of the intercistronic region in post-transcriptional control of gene expression in the histidine transport operon of *Salmonella typhimurium*: involvement of REP sequences. *Mol. Microbiol.*, **2**, 141–152.
37. Engelhorn,M., Boccard,F., Murtin,C., Prentki,P. and Geiselmann,J. (1995) *In vivo* interaction of the *Escherichia coli* integration host factor with its specific binding sites. *Nucleic Acids Res.*, **23**, 2959–2965.
38. Gilson,E., Perrin,D. and Hofnung,M. (1990) DNA polymerase I and a protein complex bind specifically to *E. coli* palindromic unit highly repetitive DNA: implications for bacterial chromosome organization. *Nucleic Acids Res.*, **18**, 3941–3952.
39. Yang,Y. and Ames,G.F. (1988) DNA gyrase binds to the family of prokaryotic repetitive extragenic palindromic sequences. *Proc. Natl Acad. Sci. USA*, **85**, 8850–8854.
40. Espéli,O. and Boccard,F. (1997) *In vivo* cleavage of *Escherichia coli* BIME-2 repeats by DNA gyrase: genetic characterization of the target and identification of the cut site. *Mol. Microbiol.*, **26**, 767–777.
41. Yang,Y. and Ferro-Luzzi Ames,G. (1990) The family of Repetitive Extragenic Palindromic Sequences: interaction with DNA gyrase and histonelike protein HU. In Drlica,K. and Riley,M. (eds), *The Bacterial Chromosome*. American Society of Microbiology, Washington, DC, pp. 211–225.
42. Ramos,J.L., Gallegos,M.T., Marqués,S., Ramos-González,M., Espinosa-Urgel,M. and Segura,A. (2001) Responses of Gram-negative bacteria to certain environmental stressors. *Curr. Opin. Microbiol.*, **4**, 166–171.
43. Liu,L.F. and Wang,J.C. (1987) Supercoiling of the DNA template during transcription. *Proc. Natl Acad. Sci. USA*, **84**, 7024–7027.
44. Wu,H.Y., Shyy,S.H., Wang,J.C. and Liu,L.F. (1988) Transcription generates positively and negatively supercoiled domains in the template. *Cell*, **53**, 433–440.
45. Bachellier,S., Clement,J.M., Hofnung,M. and Gilson,E. (1997) Bacterial interspersed mosaic elements (BIMEs) are a major source of sequence polymorphism in *Escherichia coli* intergenic regions including specific associations with a new insertion sequence. *Genetics*, **145**, 551–562.
46. Clément,J.M., Wilde,C., Bachellier,S., Lambert,P. and Hofnung,M. (1999) IS1397 is active for transposition into the chromosome of *Escherichia coli* K-12 and inserts specifically into palindromic units of bacterial interspersed mosaic elements. *J. Bacteriol.*, **181**, 6929–6936.

SUPPLEMENTARY MATERIAL

Figure S1. Aligment of *Pseudomonas putida* KT2440 REP sequences. Letters in red are bases present at this position in 90% of the 804 sequences. Letters in blue are bases present at this position in 50% of the 804 sequences. +/- refers to the DNA chain where the sequence is found.

REP N°	Contig	SEQUENCE 1 35
+1	10732	CCGGCCCTTT CGCGGGTGAA CCCGTTCCTG CAGCG
-578	10727	CCGGCCCTTC CGCGGGTGAA CCCGCTCCTA CAGCG
-6	10732	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CGGGC
-198	10704	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGGGC
-631	10851	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGGGC
-29	10734	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGGCC
-266	10724	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGGGG
+584	10754	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAGGC
+375	10726	CTGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGGGC
-71	10708	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CACGG
-732	10773	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGCGG
-499	10752	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CACAG
-633	10851	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CACGC
-318	10713	CCGGCCCCTT CGCGGGTAAA CCCGCTCCTA CACGG
-598	10765	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CACGC
+343	10717	CGGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAGGG
-774	12029	CGGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAGGG
+365	10726	CCGGCCCATT CGCGGGTAAA CCCGCTCCTA CACAG
+568	10727	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CACAA
+15	10734	GCGGCCCATT CGCGGGTAAA CCCGCTCCTA CAGGG
+462	10749	CCGGCCCATT CGCGGGTAAA CCCGCTCCTA CAGGG
-53	10714	GCAGCCCATT CGCGGGTAAA CCCGCTCCTA CAAGG
+279	10721	GCAGCCCATT CGCGGGTGAA CCCGCTCCTA CAAGG
+220	10710	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAGGT
-238	10755	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAGGT
+362	10726	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAGGT
-363	10726	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAGGT
+262	10719	CCGGCGCTTT CGCGGGTAAA CCCGCTCCTA CAGGT
+666	gpp13	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CAGGT
-759	10832	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CAGGT
+645	10789	CGGGCCCTTT CGCGGGTGAA CCCGCTCCTA CGGGT
-119	10708	CCGGCCTTTT CGCGGCTAAA GCCGCTCCTA CAGGG
+615	10725	CCGGCCCTTT CGCGGCTAAA GCCGCTCCTA CAGGG
-616	10725	CCGGCCCTTT CGCGGCTAAA GCCGCTCCTA CAAGG
-337	10785	CCGGCCCTTT CGCGGGTAAA NCCGCTCCTA CAGGA
-37	10734	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CGACG
-550	10720	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CGACG
-562	10748	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CGACG
-285	10757	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGACG
-309	10763	GCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CGACG
+49	10714	GCGGCCCATT CGCGGGTGAA CCCGCTCCTA CGACG
-752	10761	CTGGCCCTTT CGCGGGTGAA CCCGCTCCTA CGACG
-793	10791	CCGGCCCATT CGCGGGTGAA CCCGCTCCTA CGGCG
+322	10760	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAACG
-457	10793	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CAAGG
+506	10752	CGGGCCCTTT CGCGGGTGAA CCCGCTCCTG CGAGG
-117	10708	CCGGCCCATT CGCGGGTGAA CCCGCTCCTA CCGGG
-352	10717	CTGGCCCTTT CGCGGGTAAA CCCGCTCCTA CTACG
-386	10762	CAGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGACG
-735	10773	CAGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGACG
-453	10772	CAGGCCCATT CGCGGGTAAA CCCGCTCCTA CAACG
+110	10708	CTGGCCCTTT CGCGGGTGAA CCCGCTCCTA CACCG

+210	10711	CTGGCCCTTT CGCGGGTAAA CCCACTCCTA CACCG
+157	10739	GCCGCCCTTT CGCGGGTGAA CCCGCTCCTA CGGGC
+3	10732	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGG
+9	10734	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGG
+47	10714	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGG
-206	10711	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGG
-4	10732	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGA
-18	10734	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAAGG
-46	10714	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAAGG
-93	10708	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAAGG
-149	10739	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAGGG
-245	10719	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAGGG
+446	10772	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAGGG
+172	10704	CCGGCCTCTT CGCGGGTAAG CCCGCTCCTA CAGGG
-396	10762	CCGGCCTCTT CGCGGGTAAA CCCGCTCCAA CAGGG
-560	10748	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGGGG
-691	Pseud	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGGGG
-10	10734	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGT
-84	10708	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAGGT
-134	10716	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAAGT
-313	10713	CGGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGT
+19	10734	CGGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGGGC
-669	10728	CGGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGGGG
+81	10708	CCGACCTCTT CGCGGGTAAA CCCGCTCCTA CATGG
+306	10763	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CATGG
-82	10708	CCGGCCTCTT CGCGGGTAAA CCCGATCCTA CAGGG
-124	10716	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAAGG
+572	10727	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAAGG
-746	10774	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAAAG
+553	gpp69	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAAGA
-162	10704	GCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGC
-168	10704	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAACG
-274	10721	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAACG
-331	10760	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAACG
+229	10755	CCGGCCTCTT CGCGGCTAAA CCCGCTCCTA CAACG
-179	10704	CCGGCCTCTT CGCGGCTAAA GCCGCTCCTA CAGGG
-212	10711	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGCGG
-710	10712	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGGGG
-649	10788	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAAGC
-223	10710	CAGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGG
-251	10719	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAAAG
+327	10760	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTG CAGGG
+11	10734	CCGGCCTCTT CGCGGGTAAA TCCGCTCCTA CAGGC
+85	10708	CCGGCCTCTT CGCGGGCAAG CCCGCTCCTA AAGGG
-326	10760	CCGGCCTCTT CGCGGGCAAG CCCGCTCCTA CAGGG
-340	10717	CCGGCCTCTT CGCGGGCAAG CCCGCTCCTA CGGGG
+448	10772	CCGGCCTCTT CGCGGGCAAG CCCGCTCCTA CACGG
-259	10719	CCGGCCTCTT CGCGGGGAAG CCCGCTCCAA CAGGG
-604	10765	CCGGCCTCTT CGCGGGCAAG CCCGCTCCTA CAACG
+341	10717	CCGGCCTCTT CGCGGGCAAG CCCGCTCCTA CAGGT
+385	10762	CCGGCCTCTT CGCGGGCAAG CCCGCTCCTA CACCG
+92	10708	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGA
+133	10716	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGGT
-156	10739	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGGGG
+288	10757	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAGGG
+315	10713	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAGGG
-592	10754	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAGCG
-111	10708	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGGGC
+612	gpp91	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGGGC
-392	10762	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGGGA
+125	10716	CCGGCCTCTT CGCGGGTAAA GCCGCACCTG CAGGG
+617	10725	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CACGA
-221	10710	CCGGCCCCTT CGCGGGTAAA CCCGCTCCTA CAGGA
-230	10755	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAGTC

-329	10760	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGGTC
-688	10723	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGAAC
-234	10755	CCGGCCTCTT CGCGGGTAAA CCCGCGCCTA CAGCG
-712	10712	CCGGCCTCTT CGCGGGTGAA CCCGCGCCTA CAGCG
-12	10734	GCCGGCTCTT CGCGGGTAAA CCCGCTCCTA CGGGG
-14	10734	CCGGCCTCTT CGCGGCTAAA GCCGCTCCTA CGAGG
-493	10752	CCGGCCTCTT CGCGGCTAAA GCCGCTCCTG CAAGG
+17	10734	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CACAG
-729	10709	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CACAG
+151	10739	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CACAG
+24	10734	CCAGCCTCTT CGCGGGTAAA CCCGCTCCCA CAAGG
-39	10734	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGGCG
-41	10734	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACG
-430	10775	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACG
-445	10775	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACG
-531	10735	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACG
-640	gpp98	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACG
+70	10708	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAACG
+123	10716	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAACG
+169	10704	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CCACG
+213	gpp11	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGACG
-272	10721	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGACG
+570	10727	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGACG
-346	10717	CGGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACG
-443	10775	GCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACG
-637	gpp98	GCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACG
+564	10767	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGAGG
-639	gpp98	CCGGCCTCTT CGCGGGTGAA CCCGCTCATA CGACG
+83	10708	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CCGCG
-170	10704	CGGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAACG
+222	10710	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAACA
-264	10719	GCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGACG
-369	10726	GCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGAGG
-399	10718	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTG CGACG
-48	10714	TCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGACG
-202	10711	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGACC
+583	10754	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA TGAGG
-437	10775	CGGGCCTCTT CGCGGGTGAA CCCGCTCCTA CCAGG
+755	10832	CAGGCCTCTT CGCGGGTGAA CCCGCTCCTA CCAGG
-634	10851	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CCAGG
+51	10714	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGACA
+273	10721	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGAGG
-421	10718	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAACG
+730	gpp57	CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CAACG
-548	10720	CTGGCCTCTT CGCGGGTGAA CCCGCTCCTA CAACG
-241	10787	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGCAG
+284	10757	CCGGCCTCTT CGCGGGTGAA CCCGCTCCTA CGCTG
-556	gpp71	CCGGTCTCTT CGCGGGTAAA CCCGCTCCTA CACAG
-75	10708	CCGGCCTCTT CGCGGGTAAA CTCGCTCCTA CGAAG
-384	gpp46	CCGGCCTCTT CGCGGGTAAA CTCGCTCCTA CGAAG
+141	10716	CGGGCCATTT CGCGGGTGAA CCCGCTCCTA CAGGG
-505	10752	CCGGCCTATT CGCGGGTGAA CCCGCTCCTA CGGGG
+610	gpp87	CCGGCCTATT CGCGGGTGAA CCCGCTCCTA CGGGG
+705	10712	CGGGCCTATT CGCGGGTGAA CCCGCTCCTA CGGGA
+167	10704	CCGGCCTATT CGCGGGTGAG CCCGCTCCTA CAGGT
-655	10753	CCGGCCTATT CGCGGGTGAA CCCGCTCCTA CAAAG
+148	10739	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CACCA
-214	gpp11	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CACCT
-603	10765	CCGGCCTCTT CGCGGGTAAA CCCGCTCCTA CACCT
+345	10717	CGGGCTTCTT CGCGGGTGAA CCCGCTCCTA CAACG
+254	10719	CGGGCCTCTT CGCGGGTAAA CCCGTTCCTA CAGGC
+290	10757	CCCGGCGCTT CGCGGGTAAA CCCGCTCCTA CAGGG
+94	10708	CTGGCCCCTT CGCGGGTAAA CCCGCTCCTA CAGAG
+250	10719	CTGGCCCCTT CGCGGGTAAA CCCGCTCCTA CAAGG

```
+260    10719  CTGGGCCGTT CGCGGGTAAA CCCGCTCCTA CAGGG
-693    Pseud  CTGGCCGCTT CGCGGGTAAA CCCGCTCCTA CACCG
-459    10793  TCGGCCTATT CGCGGGTGAA CCCGCTCCTA CGACG
-467    10749  CCGGCCTATT CGCGGGTAAA CCCGCTCCTA CGAGC
-682    10715  GTGGCCTATT CGCGGGTAAA CCCGCTCCTA CGAGG
-766    10729  GTGGCCTATT CGCGGGTAAA CCCGCTCCTA CGACG
+218    10710  CCGGCCTTTT CGCGGGTAAA CCCGCTCCTA CAACG
-463    10749  CCGGCCTATT CGCGGGTAAA CCAGCTCCTA CAACG
-671    10728  CCGGCCTATT CGCGGGTAAA CCCGCGCCTA CAACA
+21     10734  CTGGCCTCTT CGCGGGTAAA CCCGCTCCTA CCAAA
-145    10716  CTGGCCTCTT CGCGGGTAAA CCCGCTCCCA CAAAG
+466    10749  CTGGCCTCTT CGCGGGTAAA CCCGCTCCCA CAGGG
+23     10734  CGGGCCTCTT CGCGGGTAAA CCCGCGCCTA CAGGA
-121    10708  CCGGCCTCTT CGCGGGTAAG CCCGCGCCTA CAGGT
-692    Pseud  CCGGCCTCTT CGCGGGTGAA CCCGCGCCTA CAGGT
+74     10708  CCGGCCCCTT CGCGGGTAAA CCCGCGCCTA CAGGT
+383    gpp46  CCGGCCCCTT CGCGGGTAAA CCCGCGCCTA CAGGT
-249    10719  CCTGCCTCTT CGCGGGTAAA CCCGCGCCTA CAGGT
-270    10724  CCGGCCTCTT CGCGGGTAAA CCCGCGCCTG CAAGT
+248    10719  CCGGCCTTTT CGCGGGTAAA ACCGCGCCTA CAGGT
-143    10716  ACGGCCTCTT CGCGGCTAAA GCCGCTCCTA CGGGG
-334    10760  TCGGCCTCTT CGCGGCTAAA GCCGCTCCTA CGAGC
-428    10775  AGGGCCTCTT CGCGGGTAAA CCCGCTCCTA CGGGC
-653    10753  AGGACCTCTT CGCGGGTAAA CCCGCTCCTA CGCGA
+286    10757  GCGGCCTCTT CGCGGGTAAA CCCGCCCCTA CGGGG
-535    10735  TCGGCCTCTT CGCGGGTAAA CCCGCCCCTA CGCAG
-166    10704  CTGGCCTCTT CGCGGGTAAA CCCGATCCTA CGGCG
-295    10722  CTGGCCTCTT CGCGGCTAAA GCCGTTCCTA CAGGG
+336    10785  CTGGCCTCTT CGCGGGTAAA CCCGMTCCTA CAGGG
+294    10722  CTGGCCTCTT CGCGGCTAAA GCCGCTCCTA CAGGT
-486    10746  CTGGCCTCTT CACGGCTAAA GCCGCACCTA CAGGG
+201    10711  CCGGCCGCTT CGCGGGTGAA CCCGCTCCTA CATTG
+205    10711  ACGGCCTCTT CGCGGGTAAA CCCGCTCCTA CATTG
+5      10732  CCGGCCTCTT CGCGGGCAAG CCCGCTCCTG CCAGA
-554    gpp69  CCGGCCTCTT CGCGGGGGAA CCCGCTCCTG CCAGG
-26     10734  ATATCGCATT CGCGGGTAAA CCCGCTCCTA CAAGG
+601    10765  ATGACGCATT CGCGGGTGAA CCCGCTCCTA CAAGG
+61     10707  CTGACGCATT CGCGGGTAAA CCCGCTCCTA CAGGG
+625    10725  CTGCCGCATT CGCGGGTAAA CCCGCTCCTA CAGGG
+534    10735  CTGGCCCATT CGCGGGTGAA CCCGCTCCTA CGCGG
+536    10735  CTGACGCATT CGCGGGTGAA CCCGCTCCTA CGGGG
-268    10724  CTGGCCCATT CGCGGGTGAA CCCGCTCCTA CAAGT
+507    10752  CTGGCGCATT CGCGGGTGAA CCCGCTCCTA CAAAA
-719    gpp95  CTGGCGCATT CGCGGGTGAA CCCGCTCCTA CAAAA
+795    10777  CTGCCGCATT CGCGGGTGAA CCCGCTCCTA CATAG
+801    gpp65  CTGCCGCATT CGCGGGTGAA CCCGCTCCTA CATAG
-299    10722  CCGACGTATT CGCGGGTAAA CCCGCTCCTA CAGGG
+419    10718  CCGACGCATT CGCGGGTAAA CCCGCTCCTA CAGGG
+440    10775  CCGACGCATT CGCGGGTAAA CCCGCTCCTA CAAGG
+521    10751  CCGACGCATT CGCGGGTAAA CCCGATCCTA CAGGG
-744    10731  CCGACGCATT CGCGGGTAAA CCCGCGCCTA CAGGG
-409    10718  CCCTCGCATT CGCGGGTAAA CCCGCTCCTA CAGGG
-451    10772  CCGACGCATT CGCGGGTGAA CCCGCTCCTG CAGGG
-695    Pseud  CCGACGCCTT CGCGGGTAAA CCCGCTCCTA CAAAA
-54     10714  CTGCCCCATT CGCGGGTAAA CCCGCTCCTA CAACC
-538    10735  CTGCCCCATT CGCGGGTAAA CCCGCTCCTA CAACC
-67     10707  CGGACGCATT CGCGGGTGAA CCCGCTCTTA CATGG
+106    10708  CCTCCGCATT CGCGGGTAAA CCCGCGCCTA CAGGG
-750    10761  CCACCGCATT CGCGGGTGAA CCCGCTCCTA CAGGC
-622    10725  CCATCGCATT CGCGGGTGAA CCCGCTCCCA CAGGG
-796    10777  CCATCGCATT CACAGGTAAA CCCGCGCCTA CAGGG
-247    10719  CCGGTCCTTT CGCGAGTAAG CCCGCGCCTG CAGGG
+402    10718  CCGGCCCATT CGCGGGTAAA CCCGCGCCTA CAGCG
+427    10775  CCGGCCCTTT CGCGGGTAAA CCCGCGCCTA CAGGC
```

-768	10745	CCGGCCCTTT CGCGGGTAAA CCCGCGCCTA CAGGC
-721	10776	CCGGCCCTTT CGCGGGTAAA CCCGCCGCTA CAAGC
-713	10712	CTGGCCCTTT CGCGGGTAAA CCCGCGCCTA CAGCC
-219	10710	CGNGCCTTTT CGCGGGTAAA CCCGCTCCTA CACAG
+7	10732	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CAGAA
+131	10716	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CATGG
-539	10735	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CAAGG
-197	10704	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CAAAG
+559	10748	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CAAAG
-590	10754	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CAGGG
-764	10729	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CAAAT
+632	10851	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CATCC
-8	10732	CTACCGTATT CGCGGGTAAA CCCGCACCTA CACTG
+452	10772	CTACCGTATT CGCGGGTAAA CCCGCTCCTA TGTGG
+38	10734	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CAACG
+444	10775	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CCACG
+530	10735	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CCACG
+638	gpp98	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CCACG
+40	10734	CCACCGCATT CGCGGGTAAA CCCGCTCCTA CCACG
+190	10704	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CCAGA
+442	10775	CCACCGTATT CGCGGGTAAA CCCGCGCCTA CCACG
+636	gpp98	CCACCGTATT CGCGGGTAAA CCCGCGCCTA CCACG
+473	10749	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CCTTG
-99	10708	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CACAC
+792	10791	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CACAC
+602	10765	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CACAG
+579	10727	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CGCGC
-569	10727	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CACAT
+240	10787	CCACCGCATT CGCGGGTAAA CCCGCTCCTA CGCAG
+30	10734	CCACTGTATT CGCGGGTAAA CCCGCTCCTA CGCTG
+108	10708	CTATCGTATT CGCGGGTAAA CCCGCTCCTA CACGT
+751	10761	CTGACGTATT CGCGGGTAAA CCCGCTCCTA CACGT
+233	10755	CTACCCTATT CGCGGGTAAA CCCGCTCCTA CGCCT
+706	10712	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CGCCT
+591	10754	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CACCA
+540	10720	CTACCGTATT CGCGGGTAAA CCCGCTCCTA CGCCC
-372	10726	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CACCT
-423	10718	CGACCGTATT CGCGGGTAAA CCCGCTCCTA CACCT
+504	10752	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CGCCT
+769	10745	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CGCCT
-611	gpp87	CCACCGTATT CGCGGGTAAA CCCGCTCCTA CGCCT
+353	10717	CTACCGTATT CGCGGGTAAA CCCGCTCCAG GGTGT
+34	10734	GCAGCCCATT CGCGGGTAAA CCCGCTCCTA CGAAA
-35	10734	GCGGCCCATT CGCGGGTAAA CCCGCGCCTA CAAAA
+400	10718	CCGGCCTATT CGCGGGTGAA CCCGCGCCTG CGAAA
+398	10718	CCGGCCCCTT CGCGGGTAAA CCCGCGCCTG CAAAG
+261	10719	CCGGCGCATT CGCGGGTGAA CCCGCTCCTA CACCT
-333	10760	ACGGCCCATT CGCGGGTGAA CCCGCTCCTA CACAG
-338	10785	CCGGCCCTTT CGCGGGTAAA CCCGCTCCTA CGCTC
-360	10717	CCGGCCCTTT CGCGGGTGAA CCCGCTCCTA CGCTC
+339	10717	CTGGCCCTTT CGCGGGTAGA CCCGCTCCTA CGCAC
+368	10726	CGGGCCCCTT CGCGGGTGAA CCCGCCCCTA CACTG
+747	gpp88	CCGGCCTATT CACAGGTAAA CCCGCTCCTA CGATA
-122	10708	CGGGCCTCTT CGCGGCTAAA GCCGCTCCTG CACCG
-16	10734	TGCCCCGGTT CGCGGGTGAA CCCGCTCCTA CCAGG
+98	10708	TGCGCCCCTT CGCGGGTAAA CCCGCTCCTA CAAGA
+708	10712	TGCAGCCCTT CGCGGGTGAA CCCGCTCCTA CAACA
+180	10704	TGCAGCCCTT CGCGGGTGAA CCCGCTCCTA CGGGG
-276	10721	TGCAGCCCTT CGCGGGTGAA CCCGCTCCTA CGGGG
+643	10789	TGCAGCCCTT CGCGGGTGAA CCCGCTCCTA CGGGG
+689	10723	TGCAGCCCTT CGCGGGTGAA CCCGCTCCTA CGCGA
-69	10708	TGCGACCATT CGCGGGTAAA TCCGCTCCTA CAGGG
-185	10704	CGCGGTCGTT CGCGGGTAAA CCCGCTCCTA CGGGG
+28	10734	CGCCGGTGTT CGCGGGTGAA CCCGCTCCTA CGGGG

```
+756    10832  CGCCGGTGTT CGCGGGTGAA CCCGCTCCTA CAGGG
-159    10739  CGCCGGTGTT CGCGGGTGAA CCCGCTCCTA CAGGC
+78     10708  CGCCGGTGTT CGCGGGTAAA CCCGCTCCTA CAAGG
-376    10726  CGCCGGTGTT CGCGGGTAAA CCCGCTCCTA CAGAG
+113    10708  CGCCCGTGTT CGCGGGTAAA CCCGCTCCTA CGGGT
-382    10705  CGCCGGTGTT CGCGGGTAAA CCCGCTCCTA CAGGT
-320    10713  CGCCGGTGTT CGCGGATAAA CCCGCTCCTA CGGGG
+417    10718  CGCCGGTGTT CGCGGGTGAA CCCGCCCCTA CAAGG
-142    10716  TGCCGGTGTT CGCGGGTGAA CCCGCTCCTA CAACG
+237    10755  TGCCGGTGTT CGCGGGTGAA CCCGCTCCTA CGACG
-707    10712  TGCCCGTGTT CGCGGGTGAA CCCGCTCCTA CAAGG
-694    Pseud  TGCCGGTGTT CGCGGGTGAA CCCGCTCCTA CAATG
+182    10704  TGCCGGTGTT CGCGGGTAAA CCCGCTCCTA CAAGC
-411    10718  TGCCGGTGTT CGCGGGTAAA CCCGCTCCTA CAGGG
+513    10751  TGCCGGTGTT CGCGGCTGAA GCCGCTCCTA CAGGG
-269    10724  TGCCGGTGTT CGCGGGTAAA CCCGCTCCTA CGGGT
-786    10741  TGCCGGTGTT CGCGGGTAAA CCCGCTCCTA CAAAT
-658    10753  TGCCGGTGTT TGCGGGTAAA CCCGCTCCTA CAAGG
-800    gpp10  TGCCGGTGTT TGCGGGTAAA CCCGCTCCTA CAAGG
+308    10763  TGCCGGTGTT CGCGGGTAAA CCCGCGCCTA CGGGG
+114    10708  TGCCGGTGTT CGCGGGTAAA CCCGTTCCTA CAGCG
-356    10717  TGCCAGTGTT CGCGGGCAAG CCCGCTCCTG CAAGG
-620    10725  AGCCGGTGTT CGCGGGCAAA CCCGCGCCTA CAGGC
+805    10769  TGTCGGTGTT CGCGGCTAAA GCCGCTCCTA CAATC
-22     10734  TTGGCTTCTT CGCGGGCAAG CCCGCTCCTA CATGC
+312    10713  TTGGCCACTT CGCGGGTAAA CCCGCTCCTA CATGC
+498    10752  TTGGCACCTT CGCGGGTAAA CCCGCTCCTA CATGT
-45     10734  CTGGATTCTT CGCGGGTAAA CCCGCACCTA CAAGG
-267    10724  CTGGCTTCTT CGCGGGTGAA CCCGCTCCTA CAGGG
+773    gpp13  CTGGCTTCTT CGCGGGTGAA CCCGCTCCTA CAGGG
-303    10722  GTGGCTTCTT CGCGGGTAAA CCCGCTCCTA CAGGG
-558    gpp31  CTGGCATCTT CGCGGGTAAA CCCGCTCCTA CAGGG
-414    10718  CTGCCTGCTT CGCGGGTGAA CCCGCTCCTA CAGGG
+722    10776  ATGCCTTCTT CGCGGGTGAA CCCGCTCCCA CAGGG
+317    10713  GTGTCATCTT CGCGGGTAAA CCCGCTCCTA CGGGG
+720    10776  GTGTCTTCTT CGCGGGTAAA CCCGATCCTA CAGGG
+737    10768  GTGTCTTCTT CGCGGGTAAA CCCGCTCCTA CAGGC
+496    10752  GTGTCTTCTT CGCGGGTAAA CCCGCTCCTA CAATA
-120    10708  TGGCCTTCTT CGCGGGTGAA CCCGCTCCTA CAGGG
-441    10775  TCGCCTTCTT CGCGGGTAAA CCCGCTCCTA CAAGA
+517    10751  TCGCCTTCTT CGCGGGTAAA CCCGCTCCTA CAGGT
+175    10704  TCGCTTTCTT CGCGGGTAAA CCCGCTCCTA CAGGG
-196    10704  TCGCCTGCTT CGCGGGTGAA CCCGCTCCTA CAGGC
+413    10718  TCGGCTGCTT CGCGGGTAAA CCCGCTCCTA CAGGT
-565    10767  TCGGCTGCTT CGCGGGTAAA CCCGCTCCTA CAGGG
-187    10704  TCGCCTGCTT CGCGGGTAAA CCCGCTCCTA CAAAG
-648    10789  TCGCCTGTTT CGCGGGTAAA CCCGCTCCTA CGGGC
-785    10741  TCGCCCGTTT CGCGGGTAAA CCCGCTCCTA CAGGC
-401    10718  TCGGCTGCTT CGCGGGCAAG CCCGCTCCTG CGGGG
+547    10720  TCGTCTGCTT CGCGGGTAAA CCCGCTCCTA CGGGG
-422    10718  TTGCCTGCTT CGCGGGTGAA CCCGCTCCTA CAACA
+623    10725  TTGCCTGCTT CGCGGGTGAA CCCGCCCCTA CAAGG
-508    10752  GCGCCTGCTT CGCGGGTGAA CCCGCTCCTA CAACG
-152    10739  TCGCGGCCTT CGCGGGTAAA CCCGCTCCTA CAACA
-263    10719  CCATCGTATT CGCGGGTGAA CCCGTTCCTA CACCG
+454    10772  CCCTCGTATT CGCGGGTGAA CCCGCTCCTG CAGGC
+97     10708  TTGGCCTCTT CGCGGGTAAA CCTGCCCCTA CAGGG
-307    10763  TTGGCCTCTT CGCGGGTAAA CCTGCCCCTA CAGGG
+328    10760  CTGGCCTCTT CGCGGGTAAA CCCGCCCCTA CAGCC
-613    gpp91  CTGGCCTCTT CGCGGGTAAA CCCGCCCGTA GGAGC
+387    10762  TCGGGCTCTT CGCGGGTAAA CCCGCCCCTA CAACC
-147    10739  TTGGACCTTT CGCGGGTAAA CCCGCTCCTA CAGGG
+184    10704  AGGGCCCTTT CGCGGGTAAA CCCGCTCCTA CAGGA
-714    10712  ATGGCCCTTT CGCGGGTAAA CCCGCTCCTA CACGA
```

-321	10713	CGGGGCCTTT CGCGGGTAAA CCCGCTCCTG CATGG
+557	gpp31	CCGGCCCTTT AGAGGGTAAA CCCGCTCCTA CAGGA
+13	10734	CCGGCCTCTT CGCGGCTGAT GCCGCTCCTA CCGGA
-211	10711	CYGGGCTCTT CGCGGGTGAA KCCGCTCCTA CAGGC
-255	10719	CGGGCCTCTT CGCGAGTAAA CCCGCGCCTG TAGGA
-388	10762	CGGGCCTCTT CGCGGGTAAA CCCGCGCCTA ATAAG
+36	10734	ACGCTACATT CGCGGGTAAA CCCGCTCCTA CCAGG
+522	10751	AGGGTCCTTT CGCGGGTGAA CCCGCTCCTA CGACG
-664	10737	GCCCTCCCTT CGCGGGTAAA CCCGCTCCTA CAGCG
+155	10739	GCGAGCGCTT CGCGGGTAAA CCCGCTGCTG CAGGT
-715	10712	CCGGCCCTTT CGCGGGTAAA CCTGCCCCTA CGACG
-91	10708	AGGGCCTCTT CGCGGGTGAA CCCGCCCCCA CAGGT
-323	10760	TCGGCCTCTT CGCGGGTGAA CCCGCTCCCA CAGAG
+130	10716	ATGGCCTCTT CGCGGGTGAA CCCGCTCCTA CACTG
-777	10771	ATGGCCTCTT CGCGGGTGAA CCCGCTCCTA CACGG
-239	10755	ATGGCCTCTT CGCGGGTGAA CCCGTTCCTA CACGG
+314	10713	TGGGCCTCTT CGCAGGTGAA CCCGCTCCTA CACTG
+226	10710	ATGGCTTCNT NGCGGGTAAA CCCGCTCCTA CACTG
-280	10721	TTGGATTCTT CGCGGGTGAG CCCGCTCCTA CACAG
+436	10775	CAAAAAGCTT CGCGGGTGAA CCCGCTCCTA CAAGG
-544	10720	CCAAAAGCTA CGCGGGTAAA CCCGCTCCTA CAACG
+545	10720	GTAAACGCTT CGCGGGTAAA CCCGCTCCTA CATCA
-614	10725	CTGGAGGCTT CGCGGGTAAA CCCGCTCCTA CAGTG
+76	10708	CCGGCCCTTT CGCGGGCAAG CCCGCTCCCA CAATG
-86	10708	CCGGCCCTTT CGCGGGCAAG CCCGCTCCTA CAATG
+150	10739	CCGGCCCTTT CGCGGGCAAG CTCGCGCCTA CAATG
+277	10721	CCGCCGCATT CGCGGGCAAG CCCGCTCCCA CAGTG
+754	10832	CCGGCCCATT CGCGGGCAAG CCCACTTCCA CAGAG
+676	10764	GCAGCCCATT CGCGGGCAAG CCCGCTCCGA CATTG
-757	10832	GCAGCCCATT CGCGGGCAAG CCCGCACCCA CAGAG
+215	10710	CCAGCCCTTT CGCGGGCAAG CCCGCTCCTA CAAGC
+474	10749	CCACCGCATT CGCGGGCAAG CCCGCTCCTA CATCG
-677	10764	GCGGCCCATT CGCGGGCAAG CCCGCTCCTA CAACG
+526	10730	CGCGGCCTTT CGCGGGCAAG CCCGCTCCTA CAGGG
-571	10727	CAGGCCCTTT CGCGGGCAAG CCCGGTCCTA CAGGG
+529	10735	CGGACGCATT CGCGGGCAAG CCCGCTCCTA CAAGG
-646	10789	CTGACGCATT CGCGGGCAAG CCCGCTCCTG CAGGG
+586	10754	CCGGCACCTT CGCGGGCAAG CCCGCTCCTA CAAGG
+609	10801	GCAGCCCATT CGCGGGCAAG CCCGCTCCTG CACGT
-711	10712	GCAGCCCCTT CGCGGGCAAG CCCGCTCATA CAGGT
-738	10768	CGGGTGCATT CGCGGGCACG CCCGCTCCCT CAAGG
+405	10718	CTGGCCTTTT CGCGGGCAAG CCCGCTTCGA CAGGG
+410	10718	CTGGCCTATT CGCGGGCAAG CCCGCTCCTA CAATT
+549	10720	CTGGCCCTTT CGCGGGCGAG CCCACGCCTA CAGGC
+271	10721	CCGGCCTCTT CGCGGGTAAC CCCGTTCCTA CAGTG
+696	10712	CCGGCCTCTT CGCGGGGAAA CCCGTTCCAA CAGGT
-780	10853	CCGGCCTCTT CGCGACGAAG GACGTTCCTA CAGGT
+325	10760	GCGCGGGCTT CGCGGGTAAA CCCGCTCCTA CGTTT
-335	10760	CGCGNCGCTT CGCGGGTAAA CCCGCTCCTA CGTTT
+511	10751	CGCGGCGCTT CGCGGGTAAA CCCGCTCCTA CGTTT
+138	10716	GTGCCTGCTT CGCGGGTAAA CCCGCTTGTA TGGTA
+394	10762	CTGGCCTCTT CGCGGGTAAA CCCGCTTGTA TGGTA
-736	10773	CCGGCCTCTT CGCGGGTAAA CCCGCTTGTA TGGTA
-344	10717	AGGGCCTCTT CGCGGGTAAA CCCGCTTCTA TCAAA
+791	10756	CCGGCCTCTT CGCGGGTGAA CCCGCTTCTA TCAAA
+566	10767	CGCCCGTGTT CGCGGGTAAA CCCGCTTCTA TCAAA
+803	gpp66	CGCCCGTGTT CGCGGGTAAA CCCGCTTCTA TCAAA
+588	10754	CCGGCCCTTT CGCGGGTAAA CCCGCTTCTA TCAAA
-667	gpp13	CCGGCCCTTT CGCGGGTAAA CCCGCTTCTA TCAAA
+678	10764	CTGTAGCATT CGCGGGTGAA CCCGCTCCTA CCACG
+475	10746	CCGGCCTTTT CGCGGGTTAG CCCTCCCCTA CATTG
-485	10746	CCGGGCTCTT AKCYGGTAAA CCCGCTCCTA CACAC
-748	gpp88	CCGGCCTATC CTGGGGTAAA CCCGCTCCTA CGCAC
-126	10716	CCCGCCTCTT CGCGGGCCAG CCCGCGCCTG CAAGG

+674	10764	CCCGCCGCTT CGCGGGCAAG CCCGCGCCTA CGGGG
-424	10718	TCGGCCTCTT CGCGGGCACG CCCGCGCCTA CGAGC
+366	10726	CCGGCGTCTT CACGGGCAAG CCCGCACCTG CGACT
+599	10765	GCGGCCTGTT CGCGGGCAAG CCCGCCCTTA CATCG
+555	gpp71	TGGCCTCTGT CGCGGGTAAA CCCGCTCCTA CAAAG
-581	10727	CGGCCTATAT CGCGGGTAAA CCCGCTCCTA CGCAC
-2	10732	CTGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGCG
+515	10751	CTGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGG
-354	10717	CTGGCCTCTT CGCGGGCTCG CCCGCTCCCA CAGGG
-137	10716	CTGGCCTCTT CGCGGGCACG CCCGTTCCCA CAGGG
-225	10710	CCGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGG
-656	10753	CCGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGAG
-62	10707	CCGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGT
-95	10708	CCGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGT
+129	10716	CCGGCCTCTT CGCGGGCTCG CCCGCTCCCA CAGGT
+163	10704	CCGCCCTCTT CGCGGGCACG CCCGCTCCCA CAGGT
+425	10775	CCGGCCTCTT CGCGGGCACG CCCGCTCCCA CGGGT
-472	10749	CCGGCCTCTT CGCGGGCGCG CCCGCTCCCA CAGGT
-88	10708	CCGGCCTCTT CGCGGGCGCG CCCGCTCCCA CAGGA
+605	10801	CCGGCCTCTT CGCGGGCGCG CCCGCTCCCA CAGGA
-377	10726	CTGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGT
-783	10736	CTGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGT
+252	10719	CTGGCCTCTT CGCGGGCGCG CCCGCTCCCA CAGGA
-490	10752	CCGGCCTCTT CGCGGGCTCG CCCGCCCCCA CAGGT
-50	10714	CCGGCCTCTT CGCGGGCATG CCCGCTCCCA CAGGT
+87	10708	CCGCCCTCTT CGCGGGCATG CCCGCTCCCA CAGGT
-324	10760	CCGCCCTCTT CGCGGGCATG CCCGCTCCCA CAGGT
+72	10708	CCGGCCTCTT CGCGGGCAAG CCCGCTCCCA CAGGA
-289	10757	CCGGCCTCTT CGCGGGCAAG CCCGCTCCCA CAGGT
+487	10752	CCGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGA
-518	10751	CCGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGT
-673	10764	CCGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGT
+465	10749	CCGGCCTCTT CGCGGGCTTG CCCGTTCCCA CAGGT
+456	10793	CGGGCCTCTT CGCGGGCATG CCCGCTTCCA CAGGT
-73	10708	CTGGCCTCTT CGCGGGCAAG CCCGCTCCCT CAGGT
+293	10722	CTGGCCTGTT CGCGGGCTTG CCCGCTCCCA CAGGT
-510	10751	CTGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGT
+347	10717	CTGGCCTCTT CGCGGGCATG CCCGCTCCCA CAAGT
+228	10710	CTGGCCTCTT CGCGGGCAAG CCCGCTCCCA CAGGG
-420	10718	CTGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGG
-470	10749	CGGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGG
+489	10752	CCGGCCTCTT CGCGGGCTTG CCCGCTCCCA CTGGG
+132	10716	AGGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGG
-158	10739	AGGGCCTCTT CGCGGGCTCG CCCGCTCCCA CAGGT
+477	10746	AGGGCCTCTT CGCGGGCGCG CCCGCTCCCA CAGGT
+683	10807	ACGGCCTCTT CGCGGGCGCG CCCGCTCCCA CAGGT
-527	10730	AAGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGT
+593	10754	TTGGCCTCTT CGCGGGCACG CCCGCTCCCA CAGGA
-154	10739	CCGACGTCTT CGCGGGCATG CCCGCTCCCA CAGGA
+282	10757	CCGGCCTCTT CGCGGGCTCG CCCGCTCCCA CAGCG
+702	10712	GCGGCCTGTT CGCGGGCAAG CCCGCTCCCG CAGGT
-140	10716	CGGGCCTCTT CGCGGGCATG CCCGCTCCCA CTGAC
+726	gpp11	CGGGCCTCTT CGCGGGCATG CCCGCTCCCA CTGAC
-670	10728	CGGGCCTCTT CGCGGGCAAG CCCGCTCCCA CAGGC
+224	10710	ACGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGT
+672	10764	TGGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAAGT
-794	12083	TGGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGT
-488	10752	TCGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGC
+716	gpp95	TCGGCCTCTT CGCGGGCTTG CCCGCTCCCA CAGGC
-407	10718	GGGGCTTCTT CGCGGGCTCG CCCGCTCCCA CAGGT
+258	10719	GAGGCCTCTT CGCGGGGAAG CCCGCTCCCA CAGGT
+433	10775	TCGCCCTCTT CGCGGGCAAG CCCGCTCACA CAGGT
+244	10719	CCGGCCTTTT CCCGGGCAAG CCCGCTCCCC CAGGT
+127	10716	CCGGCCCATT TGCGGGCTTG CCCGCTCCCA CAGGA

+207	10711	GCGGCCCATT CGCGGGCTTG CCCGCTCCCA CAGGC
-287	10757	CCGGCCCTTT CGCGGGCACG CCCGCTCCCA CAGGT
-606	10801	CCGGCCCTTT CGCGGGCATG CCCGCTCCCA CAGGT
+767	10745	CCGGCCCTTT CGCGGGCTTG CCCGCTCCCA CAGGT
+304	10763	CCGGCCCTTT CGCGGGCTCG CCCGCTCCCA CAGGA
+532	10735	CGGGCCCTTT CGCGGGCACG CCCGCTCCCA CAGGA
+753	10761	CAGGCCCATT CGCGGGCACG CCCGCTCCCA CAGGT
+364	10726	CCGGCCCTTT CGCGGGCACG CCCGCTCCCA CAGTA
-461	10793	CCGGCCCTTT CGCGGGCACG CCCGCCCCCA CAGGG
+431	10775	GCAGCCCCTT CGCGGGCACG CCCGCTCCCA CAGGG
-524	10751	CCAACCGCTT CGCGGGCACG CCCGCTCCCA CAAGG
-278	10721	CGCGCCCCTT CGCGGGCAAG CCCGCTCCTA CAGGT
-350	10717	CGCACCGCTT CGCGGGCAAG CCCGCTCCCA CAGGT
-192	10704	CCGGTCTTTT CGCGGGCACG CCCGCTCCCA CAGGA
+203	10711	CCGGCCTTTT CGCGGGCCCG CCCGCACCCA CAGGG
-204	10711	CCGGCCTCTT CGCGGGCCCG CCCGCTCCCA CAAGG
+699	10712	CCGGCTTCTT CGCCGGCAAG CCCGCGCCCA CAGGA
+789	11050	CCGGCTTCTT CGCCGGCAAG CCCGCGCCCA CAGGA
-189	10704	CTGGAGCCTT CGCGGGCATG CCCGCTCCCA CAGGG
-624	10725	CTGGACTCTT CGCGGGCTTG CCCGCTCCCA CACGG
-717	gpp95	CAGGACTCTT CGCGGGCTTG CCCGCTCCCA CAGGA
+231	10755	CCGGCCTCTT CGCTGGCACG CCCGCTCCCA CAGTC
-232	10755	CCGGCCCCTT CGCGGGCACG CCCGCTCCCA CAGTC
-660	10737	CAAGGCCCTT CGCGGGCGCG CCCGCTCCCA CAGGT
-164	10704	TCGCCCTCTT CGCGGGCACG CCCGCTCCCG CAGGG
+174	10704	TCGACCTGTT CGCGGGCATG CCCGCTCCCG CAGGG
+32	10734	CTGACGCATT CGCGGGCGCG CCCGCTCCCA CAGGG
+740	10768	CTGACGCATT CGCGGGCACG CCCGCTCCCA CAGGT
-116	10708	CTGACGCCTT CGCGGGCACG CCCGCTCCCA CAGGG
-43	10734	CTGGCCCATT CGCGGGCAAG CCCGCTCCCA CAGGG
+188	10704	CTGGCCCTTT CGCGGGCACG CCCGCTCCCA CAGGG
-101	10708	GTGGCGCATT CGCGGGCGCG CCCGCTCCCA CAGGC
+630	10851	CTGACGTATT CGCGGGCATG CCCGCTCCCA CAGGG
-44	10734	CTATCGCATT CGCGGGCGTG CCCGCTCCCA CAGGG
+594	10754	CTGGCCTTTT CGCGGGCGCG CCCGCTGCCA CAGGC
+406	10718	ATGGCCTGTT CGCGGGCTTG CCCGCGCCCA CAGGA
-265	10719	GCGGCCTCTT CGCGGGCAAG CCCGCCCCCG CAGAG
+367	10726	CAGGCCTCTT CGCGGGCAGA CMCGCTCCCA CAGGG
-20	10734	TCGGCCTCTT CGCGGGCAAG CCCGCTCCTG CAGGG
+56	10707	AGGGCCTCTT CGCGGGCAAG CCCGCTCCTA CAGGG
-57	10707	TGGGCCTCTT CGCGGGCAAG CCCGCTCCTA CAGGA
-52	10714	ATGGCCTCTT CGCGGGCAAG CCCGCTCCTA CACGG
+96	10708	AGGGCCTCTT CGCCGGCAAG CCCGCTCCTG CAAGG
+393	10762	CTGGCCTCTT CGCGGGCAAG CCCGCTCCTA CGGGG
-476	10746	CTGGCCTCTT CGCGGGCAAG CCCGCTCCTA CAAAG
-600	10765	CGGGCCTGTT CGCGGGCAAG CCCGCTCCTA CACTG
+704	10712	CGGGCCTCTT CGCGGGCAAG CCCGCTCCTA CACAG
+246	10719	CCGCCCTCTT CGCGGGCAAG CCCGCACCTG CACGG
-434	10775	CGGGCTTCTT CGCGGGCAAG CCCGCTCCTG CATGT
+458	10793	CGGGCCTCTT CGCGGGCAAG CCCGCTCCTA CATCA
+641	gpp63	CTGGCCTCTT CGCGGGCACG CCCGCTCCTA CATGT
-173	10704	CCGGCCTCCT CGCGGGCAAA CCCGCGCCTA CAGGC
+408	10718	CTGGCCTCTT CGCGGGCAAG CCCGCGCCTA CACGC
-342	10717	AGGACCTCTT CGCGGGCAAA CCCGCGCCTA CAAGA
-31	10734	CGCCCGTGTT CGCGGGCAAG CCCGCTCCCA CAGGG
-495	10752	CGCCCGTGTT CGCGGGCAAG CCCGCTCCCA CAGGG
-741	10768	CGCCCGTGTT CGCGGGCAAG CCCGCTCCCA CAGGG
-724	10776	CGCCCGTGTT CGCGGGCAAG CCCGCTCCCA CAGGG
-144	10716	CGCCCGTGTT CGCGGGCAAG CCCGCTCCCA CACGC
-358	10717	CGCCCGTGTT CGCGGGCAAG CCCGCTCCCC CAGGG
+374	10726	CGCCCGTGTT CGCGGGCAAG CCCGCTCCCA CAGTC
-681	10792	CGCCCGTGTT CGCGGGCAAG CCCGCTCCCA CAGTG
+416	10718	CGCCCGTGTT CGCGGGCAAG CCCACTCCCA CAGTG
+723	10776	CGCCGGTGTT YGCGGGCAAG CCCGCTCCCA CAGTC

```
-33     10734  TGCCGGTGTT CGCGGGCGAG CCCGCTCCCA CAGGG
-243    10719  TGCCGGTGTT CGCGGGCAAG CCCGCTCCCA CAGGG
+65     10707  TGCCGGTGTT CGCGGGCATG CCCGCTCCCA CAGGG
+194    10704  TGCCGGTGTT CGCGGGCATG CCCGCTCCCA CAGGG
+743    10731  TGCCGGTGTT CGCGGGCATG CCCGCTCCCA CAGGG
-628    10725  TGCCGGTGTT CGCGGGCACG CCCGCTCCCA CAGGG
+357    10717  TGCCGGTGTT CGCGGGCGTG CCCGCTCCCA CAAGG
+627    10725  TGCCGGTGTT CGCGGGCAAG CCMGCTCCCA CAGGG
-679    10764  TGCCGGTGTT CGCGGGCAAG CCCGCTCCTA CGGGG
+391    10762  TGCCCGGGTT CGCGGGCGTG CCCGCTCCCA CAGGG
+355    10717  CGCCCGTGTT CGCGGGCGCG CCCGCTCCCA CAGTG
+763    10729  TGCCGGTGTT CGCGGGCGCG CCCGCTCCCA CAGTG
+139    10716  CGCCGGTGTT CGCGGGCACG CCCGCTCCCA CAGGT
-727    gpp11  CGCCGGTGTT CGCGGGCACG CCCGCTCCCA CAGGT
+300    10722  TGCCGGTGTT CGCGGGCACG CCCGCTCCCA CAGGT
-181    10704  CGCCGGTGMT CGCGGGCACG CCCGCTCCCA CAAGG
-455    10772  CGCCGGTGTT CGCGGGCACG CCCGCTCCCA CATGG
-762    10729  CGCCCGTGTT CGCAGGCACG CCCGCTCCCA CAAGG
+528    10735  CGCCGGTGTT CGCGGGCACG TCCGCTCCCA CAAGG
+373    10726  CGCCGGTGTT CGCGGGCACG CCCGCTCCCA CGGGG
-675    10764  CGCCGGTGTT CGCGGGCAAG CCCGCTTCTA CAGGG
+685    10807  CGCCGGTGTT CGCGGGCGTG CCCGCTCAAA CAGGG
-301    10722  CGCCCGTGTT CGCGGGCATG CCCGCTCCCG AAAGT
+654    10753  CGCCGGTGWT CGCGGGCACG CCCGCTCCCA CAGTT
-64     10707  TTGGCTTTTT CGCGGGCATG CCCGCTCCCA CAGGA
+298    10722  TTGGCTTTTT CGCGGGCATG CCCGCTCCCA CAGGG
-537    10735  TTGGCTTTTT CGCGGGCATG CCCGCTCCCA CAGGG
+153    10739  CTGGGTTCTT CGCGGGCATG CCCGCTCCCA CAGGT
-195    10704  TTGGCTTCTT CGCGGGCATG CCCGCTCCCA CAGGT
-784    10736  TTGGCTTCTT CGCGGGCTTG CCCGCTCCCA CAGGT
-698    10712  TTGGCTTCTT CGCGGGCACG CCCGCTCCCA CAGGT
-541    10720  TGGCCTTCTT CGCGGGCATG CCCGCTCCCA CAGGT
+471    10749  TTGGTTTCTT CGCGGGCTTG CCCGCTCCCA CAGGC
+731    10773  TTAGATTTTT CGCGGGCACG CCCGCTCCCA CAGGT
-128    10716  GTGGATTATT CGCGGGCTTG CCCGCTCCCA CAGGT
+412    10718  GTGGTTTCTT CGCGGGCATG CCCGCTCCTA CAGGT
+296    10722  TTGTATTCTT CGCGGGCGCG CCCGCTCCCA CAGGG
+728    gpp11  TTGGATTCTT CGCGGGCACG CCCGCTCCCA CAGGG
+567    10740  TTGAATTCTN TGCGGGCACG CCCGCTCCCA CAGGG
+89     10708  CTGGCCTCTW YGCGGGCATG CCCGCTCCCA CAGCG
-642    gpp63  CTGGCCTCTC TCCGGGCAAG CCCGCTCCCA CAGGG
-650    10788  CGCGCCCCTC CGCGGGCTTG CCCGCTCCCA CAGGG
-104    10708  GCGGCCTCTT CGCGGGCAAG CCCGCTCCCA AATGC
+351    10717  AACGGCTGTT CGCGGGCACG CCCGCTCCCA GATGG
+42     10734  AAGGGCCATT CGCGGGCAAG CCCGCTCCCA CAGGA
+371    10726  GAAGGCGATT CGCGGGCAAG CCCGCTCCCA CAGGT
+112    10708  GAGGGCCATT CGCGGGCATG CCCGCTCCCA CCGGT
+63     10707  AGGGCCCTTT CGCGGGCAAG CCCGCTCCCA CGGGT
+191    10704  ATGGCCCTTT CGCGGGCTTG CCCGCTCCCA CAGGT
-595    10754  TCGGCCCTTT CGCGGGCATG CCCGCTCCCA CAGGT
+771    10802  TGGGCTGTTT CGCGGGCATG CCCGCTCCCA CAGGT
-788    gpp22  TGGGCTGTTT CGCGGGCATG CCCGCTCCCA CAGGT
-253    10719  TCGGGGCATT CGTGGGCACG CCCGCTCCCA CAGGT
+58     10707  CGCGGCCCTT CGCGGGCACG CCCGCTCCCA CAGAG
+621    10725  TGCGGCCCTT CGCGGGCTCG CCCGCTCCCA CAGAG
+480    10746  GGCGGCCCTT CGCGGGCAAG CCCGCTCCCA CAGAG
-503    10752  CGCGGCCCTT CGCGGGCTAG CCCGCTCCCA CAGAG
-432    10775  GGCGGCCCTT CGCGGGCGCG CCCGCTCCCA CAGAA
-380    10705  CGCGGCCCTT CGCGGGCACG CCCGCTCTCA CAGGA
-59     10707  TGCAGCCCTT CGCAGGCACG CCCGCTCCCA CAGGT
+379    10705  TGCAGCCCTT CGCGGGCACG CCCGCTCCCA CAGGT
+378    10705  TGCAGCCCTT CGCGGGCACG CCCGCTCCCA CAGGA
-481    10746  TGCAGCCCTT CGCCGGCACG CCCGCTCCCA CAGGT
-596    10754  GGCAGCCCTT CGCGGGCACG CCCGCTCCCA CAGGT
```

```
+589    10754  TGCGGCCCTT CGCGGGCACG CCCGCTCCCA CAGGA
+576    10727  TGCGCCCCTT CGCGGGCACG CCCGCTCCCA CGGGT
-709    10712  TGCAGCCCTT CGCGGGCTTG CCCGCTCCCA CAGGG
+739    10768  TGCGGCCCTT CGCGGGCTTG CCCGCTCCCA CAGGT
-439    10775  TGAGGCCGTT CGCGGGCACG CCCGCTCCCA CAGGT
+659    10737  TCAACCCCTT CGCGGGCACG CCCGCTCCCA CAGGT
-483    10746  TTGAGCCCTT CGCGGGCATG CCCGCTCCCA CAGGT
+597    10765  TGCGGCCATT CGCGGGCAAG CCCGCTCCCA CACGT
-478    10746  TGCGGTCCTT CGCGGGCTTG CCCGCTCCCA CAGGC
-770    10745  TCGCGGCCTT CGCGGGCTTG CCCGCTCCCA CAGGT
-580    10727  CGCAGACCTT CGCGGACACG CCCGCTCCCA CAGGT
+381    10705  AGAAAGCATT CGCGGGCATG CCCGCTCCCA CACGT
-426    10775  TGAAGGCATT CGCGGGCATG CCCGCTCCCA CAGGG
+502    10752  TGCAGCCCTT CGCGGGCATG CCCGCTCCCA CAGTG
-635    10851  TGGTCGCATT CGCGGGCAAG CCCGCTCCCA CAGAG
-734    10773  TGCTCCCATT CGCGGGCATG CCCGCTCCCA CAGTA
-77     10708  TTGGGCCCTT CGCGGGCAAG CCCGCTTCTA CAGGG
+177    10704  TGCGGCCCTT CGCGGGCAAG CCCGCTCCTG CACAG
+500    10752  TGCGGCCCTT CGCGGGCAAG CCCGCTCCTA CAAAG
-501    10752  TGCAGCCCTT CGCGGGCAAG CCCGCTCCTA CAGGG
+27     10734  TTGTCTGCTT CGCGGGCGAG CCCGCTCCCA CAGGG
-684    10807  TCGTCTGCTT CGCGGGCATG CCCGCTCCCA CAGGT
-66     10707  TTGCCTGCTT CACGGGCACG CCCGCTCCCA CAGGG
+171    10704  TTGCCTGCTT CGCGGGCACG CCCGCTCCCA CAGGA
+450    10772  TTGCCTGCTT CGCGGGCGCG CCCGCTCCCA CAAGG
-686    10807  TCGCCTGATT CGCGGGCATG CCCGCTCCCA CAGAG
-758    10832  TCGCCTGATT CGCGGGCACG CCCGCTCCCA CAGGG
+275    10721  TCGCCTTCTT CACGGGCTCG CCCGCTCCCA CAGGA
-390    10762  TGCCTTCATT CGCGGGCGCG CCCGCTCCCA CAGGG
-725    10776  TCGCTTGTTT CGCGGGCACG CCCGCTCCCA CAGGT
+482    10746  CGCCCTCGTT CGCGGGCACG CCCGCTCCCT CAGGT
-514    10751  TGCCCTCGTT CGCGGGCAAG CCCGCTCCCA CAGGT
+103    10708  GCGGCTGCTT CGCGGGCAAG CCCGCTCCCA CACGT
-348    10717  GCGCCTGCTT CGCGGGCACG CCCGCTCCCA CAGGT
+680    10792  GCGCCTGCTT CGCGGGCAAG CCCGCTCCTA CAGGT
-657    10753  GTGCCTGCTT CGCGGGCAAG CCCGCTCCCA CAGAT
-497    10752  GCGCCTGCAT CGCGGGCAAG CCCGCTCCTA CACAG
-236    10755  TCACCTGTTT CGCGGGCATG CCCGCTCCCA CGCGT
+60     10707  CTGACTGCTT CGCGGGCACG CCCGCTCCCA CAGGT
+661    10737  CTGGTTGCTT CGCGGGCCCG CCCGCTCCCA CAGGT
-359    10717  GTGGATGCTT CGCGGGCTCG CCCGCTCCCA CAGGA
-107    10708  GAGCCTTGTT CGCGGGCACG CCCGCTTCCA CAGGA
+435    10775  TACCATGCTT AGCGGGCACG CCCGCTCCCA CAGGA
+217    10710  CCGGCCGCTT CATGGGCAAG CCCGCTCCCT CAGGG
-332    10760  CTGCCTTCTT CGCGGGCGCG CCCGCTCCCA CAGAG
-582    10727  TTGCCGTCTT CGCAGGCGCG CCCGCTCCCA CAGTC
+542    10720  TGACCCTCGT CGCGGGCTCG CCCGCTCCCA TAGGT
+349    10717  GTGTTGGCTT CGCGGGCAAG GCCGCTCCCA CACGG
-626    10725  GTGCTTGCTT CGCGGGCAAG CCCGCTCCCA CAGGG
-587    10754  CTGGTCCCTT CGCGGGCAAG CCCGCTCCTA CGGGG
+135    10716  AGGTCCTTTT CGCGGGCAAG CCCGCTCCTG CAGGT
+438    10775  TCGACAGTTT CGCGGGCAAG CCCGCTCCTG CACGC
+100    10708  TGACCCTTTT CGCGAGCAAG CCCGCCCCTA CAGCA
+186    10704  TGAGGCCATT CGCGGGTAAA CCCGCGCCTA CACCG
+102    10708  TTACCTTCTT CGCGGGCAAG CCCGCTCCTG CAGAT
+193    10704  TTGGCTTGTT CGCGGGTAGA CCCGCTCCTG CATTT
-105    10708  TTGGCCTGTT CGCGGGGGAA TCCGCTCCTG CAACT
+492    10752  TTGCCTTCTT CGCGGGTCAA TCCGCTCCTA CGAGC
+663    10737  GTGGCTGCTT CGCGGGCAAG CCCGCTCCTA CGGCT
-772    10802  TTGGCTGCTT CGCGGGCATG CCCGCTCCAA CAACG
+787    gpp22  TTGGCTGCAT CGCGGGCATG CCCGCTCCAA CAACG
-804    10829  GTGTCTGATT CGCGGGCAAG CCCGCCCCTA TAACG
-227    10710  TTAAGGCATT CGCGGGTAAA CCCGCTCCTA CATTG
+765    10729  ATATCGTATT CGCGGGTGAA CCCGCACCTA CATTG
```

```
+302    10722  ACATCGCATT CGCGGGTAAA CCCGTTCCTA CAGAG
-512    10751  AGATCGCATT CGCGGGTAAA CCCGATCCTA CAGGG
-665    10737  AGCCCGTATT CGCGGGTAAA CCCATTCCTA CAGAA
+718    gpp95  CGGACGCATT CGCGGGTAAA CCCGTTCCTT CGATA
+330    10760  GGGAAACATT CGCGGGTAAA CCCGCTCCTA CAGAC
+319    10713  ATGACGCATN TCCGGGTAAA CCCGCTCCTA CAAAG
-464    10749  CCGACGCATN TCCGGGTAAA CCCGCTCCRA CAAGT
+494    10752  GTTGTTTCTT CGCGGGTAAA CCCGCTCCTA CGGGT
-525    10751  CCAATTGCTT CGCGGGTAAA CCCGCTCCCG CCGGG
-449    10772  TCGGCCTCTT CACGGGTACG CCAGCTCCCA CAATA
-703    10712  AGGGCCCTCT CGCGGGCAAG CCCGCTCTCA CAGGG
-575    10727  CCGGCCTCTT CGTGAGCCAA CCCACTCCTA CACTC
+574    10727  CTGGCCTCCC CGCGGGTCAA CCCGCTCCGG CAAGG
-283    10757  CTGGACTCTT CGCCAGCAGG CCCGCCCCTA CAGCG
-700    10712  CTGGCCTCTT CGCCGGCAAA CCTGCGCCCA CACGG
-790    11050  CTGGCCTCTT CGCCGGCAAA CCTGCGCCCA CACGG
-297    10722  GCGGCCTTTT CGCGCTAAAG GCCGCTCCTG CACAA
-546    10720  AAGGCCTTTT CGCGGGTAAA CCCCCTCCTG ATGTA
+418    10718  CGCCCGTGTT CGCGGGTAAA CCCGCTCAAG GATCG
-799    10706  CACCGGTGTT CGNGGGTAAA CCCGCTCCTA CATTG
+519    10751  CGCGGATGTT CGCGGGTGAC CCCACGCCTA CATAG
-160    10739  TGTGCTTCTT CGCGGGTAAA CCCGCGCCGA CGAAA
-176    10704  CCGGCCTCTT CGCCGGTAAA CCCGTGCCCA AGGTA
+460    10793  CGCCCCTGTT CGCGGGTAAA CCCGTGCCCA CAGGT
+235    10755  GCCGCCCATT CGCGGGTGAG CCCGCGCCTG CAGGT
+292    10757  CCACCCCATT CGCGGGCAAG CCTGTGCCTA CAACT
+745    10731  CCGCCGCATT CGCGGGTAAG CCTGTTCCTA CAGGT
-281    10721  CCATCGTATT CNCGGGCAAG CCTGCTCCCA CAGGA
-778    10771  CGCAGCCCAT CGCAGGCAAG CCAGCTCCTA CACCG
+109    10708  CGCCCTCGTT CGCGGCTGAA GCCGCTCCTA CACAA
-523    10751  TGCCCTTGTT CGCGGGTAAA TCCGCTTCTA CACAA
+178    10704  TTGCTTGCTT CGCGGCTAAA GCCGCTCCTA CACAG
+543    10720  CTCTATTCTT CGCGGGTAAA CCCGCCCCTA CACAC
+165    10704  CCGGCCCTTT CGCGGGTGAA CCCGCTGATG GACAC
-618    10725  CCGGTCCTTT CGCGGGTAAA CCCGCCCCTA TACAC
+760    10729  CCGGCCCTTT CGCGGGTGAA CCCGATTACA AGGGC
-447    10772  CGGGCCCTTT CGCGGGTGAA CCCGCTCTCA TCAAA
+701    10712  CCGGCCCCTT CGCGGGTGAA CCCGCTCTCA TCAAA
+577    10727  CTACCGTATT CGCGGGTAAA CCCGCTCTCA TCAAA
+652    10753  GCGGCCCTTT CGCGGGTAAA CCCGCTCGCG TAGGA
+733    10773  CTACCGCATT CGCGGGTGAA CCCGCTCCTA TGTAT
+647    10789  TCGCCTGTTT CGTGGGTGAC CCCGCTCCTA CGAGA
+775    10771  CGGGCCCCTT CGCGGGCAAG CCCTAACCCA CAAAA
-183    10704  ACCATCGCAT TCCGGGTAAA CCCGCTCCTA CCAGG
+484    10746  TCCGGCCCTT TCGGGGTAAA CCCGCTCCTA CAGTG
+798    10706  TCGCCCGGCT AGCGGGTAAA CCCGGGCCTG CAGGG
-316    10713  CATCGCATTC GNCGGGTGAA CCCGCTCCTA CAGGG
+520    10751  GCCGGTATTT CGCGGGCAAA CCCGAGCCTA CAAAG
-305    10763  CGACTTCATT CGCGGGCAAG CCCGCTCCTG CACCA
-573    10727  TGGGTTCATT CGCGGGCAAG CCCGCTGCTA CAGGG
-397    10762  CGGCATCTTT GCGGGGCAAG CCCGCTGCCA CAGGT
-516    10751  ATGGCCTCTT CGCCGGCACG CCCATTCCTA TTGGT
+25     10734  CTATATTCTT CGCGGGCATG CCCGCTCCCA CAGCA
+469    10749  ATGTACTCTT CGCGGGCTTG CCCGCTCTCC CAAGG
+619    10725  TTGCATTCTT CACGGGCATG CCCGCTCCCC CAAAG
+533    10735  TATGTGTGTT CGCGGGCAAG CCCGCTCCTA TGTAT
-662    10737  TGGGTTTCTT CGCGGGCCTG CCCGCTCCCA CAYAT
-55     10714  CCGGCCTCTT CGCGGCTAAA GCCGGAAGAG GCCGG
+146    10739  CCGGCCTCTT CGCGGATAAA CCCACAGGTC CCCCA
+90     10708  ATGGCCTCTT CGCGGGTAAA CCCGCTACAG GGCCT
+68     10708  CCGGCCTCTT TGCGGGCACG CCCGCGAAGA GGCCG
-361    10717  CCGGCCTCTT TGCGGGCACG CCCGCGAAGA GGCCG
+782    gpp32  CCGGCCTCTT TGCGGGCACG CCCGCGAAGA GGCCG
-781    gpp11  CCGGCCTCTT TGCGGGCACG CCCGCGAAGA GGCCG
```

```
+242    10719  CCGGCCTCTT CGCGGGCAAG CCCGCGAAGA CGCCA
-256    10719  CCGGCCTTTT CGCGGGCAAG CCCGCGAAGA GGCCA
+479    10746  CTGGCCTCTT CGCGGGCAAG CCCGCGAAGA GGCCA
-200    10704  GGGGCCTCTT CGCGGGCGAG CCCGCGAAGA GGCCT
+370    10726  AGGGCCTCTT CGCGGGCACG CCCGCGAAGA GGCCT
-551    10720  CCAGCCTCTT CGCGGGCAAG CCCGCGAAAG GGTCG
-651    10788  AGGGCCTCTT CGCGGGCAAG CCCGCGAAAG GGCCG
-552    10720  CCGGCCCCTT CGCGGGCACG CCCGCGAAGA AGCAG
+776    10771  CCTGCCTCTT CGCGAGCATG CCCGCGAAGA GGCAG
-415    10718  GGGGCATCTT CGCGGGCAAG CCCGCGAAGA TGCCA
+404    10718  TCGGCCTCAT CGCGGTCAAG CCCGCGATGA GGCCG
+687    10723  TGCGNCTCTT CGCGGGCAAG GCCGCGAAGA GGCCG
+216    10710  CCGGCCTCTT CTCGGGTGAA CCCGCGAAGA GGCCG
+311    10713  CCGGCCTCTT CGCGGGTAAA CCCGCGAAGA GGCCA
-491    10752  CCGGCCCCTT CGCGGGTAAA CCCGCGAAGG GGCCA
-563    10748  CCGGCCTCTT CGCGGGTAAA GCCGCGAAAG GGCCG
+802    10810  CCGGCCGCTT CGCGGATCAA CCCGCGAAGC GGCCA
+429    10775  CGGGCCTCTT CGCGGGTAAA CCCGCTAATG GGCCA
+257    10719  TTGGCCTCTT CGCGGATAAA CCCGCGAATA GGCCA
-468    10749  TTGGCCTCTT CGCGGGTAAC CCCGCGAAGA GGCGG
+80     10708  CCGGCCCTTT CGCGGGCAAG CCCGCGAAGA AGCCG
+749    10761  ACGGCCCTTT CGCGGGCAAG CCCGCGAAGA GGCCG
+209    10711  ACGGCCCTTT CGCGGGCAAG CCCGCGAAAA GGGCC
+389    10762  GCGGCCCTTT CGCGGGCATG CCCGCGAAAG GGCCG
+403    10718  CCGGCCCTTT CGCGGGTAAA CCCGCGAAAG GGCCG
+509    10751  CCGGCCCTTT CGCGGATAAC CCCGCGAAAG GGCCG
-629    10725  GCGGCCCATT CGCGGGTGAA CCCGCGAAGA GGCCG
-79     10708  CGGGCTTCTT CGCGGTTAAG CCCGCTCTCC AGGCA
+161    10704  CCGGCCTCTT CGCGGGTAAA CCCTGCACGG TCGCC
+607    10801  CCGGCCTCTT CGCGGGTAAA CCCGCCTACC AGGCA
-644    10789  CCGGCCTCTT CGCGGGTAAC CCCGCCCCCC TGGCG
+697    10712  CCGGCCTCTT CGCGGGCATG CCCCACAGGG ACAGT
-291    10757  TGGGCCACTT CGCGGGTAAA CCCGCACAGC TCCAG
+668    10728  CCGGCCTATT CGCGGGTAAA CCCCTCCTAC AACGG
+310    10713  CCGGCCCTTT CGCGGGCAAG CACAGGCAAC CACAC
-199    10704  GAGGCCGCTG CGCCGGTAAG CTCGCTGCAG CGCAG
+115    10708  GCCTAGCGAT CGCGGGCACG CCCGCTCCCA CAGGT
-118    10708  CCCTTGCAGG AGCGGGCACG TCCGCTCCCA CAGGG
+136    10716  ACCCTGTGGG AGCGGGCACG CCCGCTCCCA CAGGG
-585    10754  TCCCTGTGGG AGCGGGCAAG CCCGCTCCCA CAGGT
+742    10731  ACCCTGTGGG AGCGGGCGAG CCCGCTCCCA CAGTG
+761    10729  CGGGCTGCTT CGTGGGCAAG CCCGCCCCTG ACAAA
+395    10762  TGCCGGCTCT CKSGGGTAAA CCCGCTCCTA CCTCG
-208    10711  GTGTTGGCTT CGCGGGCAAG CCCGCTTGTG TCGCG
-797    10777  CCGGCCTCTT CGGGGGTAAT GCACAGCACT CAGAG
+779    10853  CCGGCCTCTT CGCGGCTAAC AACCGTTCCT ACAGG
-561    10748  ATGGCCTCTT CGGGGGTGAG CACGTCGGTG ACGTA
Consensus      c.ggcctcTT CGCGGGtaaa CCCGCtCCta Caggg
```

V. DISCUSIÓN GENERAL

Muchos microorganismos procariotas son de interés medioambiental por sus particulares propiedades metabólicas. Sus genomas codifican numerosos elementos implicados en transducción de señales y en regulación de genes, reflejo de su capacidad para detectar y responder a las distintas señales medioambientales. El conocimiento de la regulación de estos sistemas metabólicos es pieza clave e indispensable para el diseño de protocolos de aplicación ambiental (105).

Las diferentes rutas aerobias de degradación de compuestos aromáticos descritas en bacterias comprenden una serie limitada de tipos de reacciones enzimáticas que acaban convergiendo en unos pocos intermediarios comunes (catecol, protocatecuato, gentisato o hidroquinona) que son incorporados al metabolismo central (43, 85). Sin embargo, los mecanismos empleados para regular el funcionamiento de estas rutas son complejos. Se han descrito sistemas de regulación controlados por proteínas pertenecientes a casi todas las familias de reguladores descritas en bacterias: LysR (como los reguladores CatR y ClcR), AraC (como el regulador XylS del promotor Pm del plásmido TOL), NtrC (como los reguladores XylR, DmpR o HbpR), etc (36, 60, 201). Sin embargo, es posible distinguir entre esta regulación específica de ruta y una regulación global que controla la expresión de las distintas rutas de forma coordinada con el conjunto de genes de la bacteria en respuesta a su estado fisiológico y energético (112, 130, 184).

La ruta de degradación de tolueno del plásmido pWW0 de *P. putida* mt-2 está regulada a estos dos niveles. La regulación específica de las rutas *upper* y *meta* se lleva a cabo por activación de las proteínas reguladoras XylR y XylS en presencia de sustratos aromáticos de la ruta. La forma activa de XylR y XylS promueve la transcripción desde los promotores Pu y P_{S1} en el caso de XylR y Pm en el caso de XylS (110, 172). En los promotores Pu y P_{S1}, este tipo de modulación mediada por reguladores específicos está subordinada a una regulación global compleja, que sigue siendo objeto de estudio en la actualidad. Los dos promotores dependen de la RNAP asociada al factor σ^{54} ($E\sigma^{54}$), el único factor sigma alternativo en procariotas que no pertenece a la familia de σ^{70}. El factor σ^{54} está codificado por el gen *rpoN*, que en bacterias se encuentra generalmente agrupado con un conjunto de ORFs de secuencia conservada y de función hasta el momento desconocida.

El trabajo desarrollado en esta tesis doctoral amplia el conocimiento de los recursos genéticos empleados por *P. putida* mt-2 para el control global de la ruta *upper* codificada en el plásmido pWW0 y profundiza en la caracterización de la agrupación génica *rpoN*.

V.1. Factores genéticos con un papel en la regulación global de la ruta *upper*

En los últimos 15 años se han reunido una serie de indicios que sugieren que determinadas condiciones de crecimiento inhiben la expresión de la ruta *upper* del plásmido pWW0 (45, 46, 81, 107). Esta inhibición se produce aun estando presentes todos los elementos necesarios para su inducción, es decir la RNAP, el promotor, el regulador y el efector. De este modo, se observó que la inducción de los operones catabólicos era débil al inicio de la fase exponencial de crecimiento pero fuerte al final de esta fase (81), que este silenciamiento de la ruta *upper* en fase exponencial se producía cuando las células se cultivaban en medio rico (22) y que la ruta también se

inhibía en situaciones en las que la fuente de carbono estaba en exceso en el medio (45, 46, 78). A pesar de no existir un modelo claro de qué procesos conducen a la represión de esta ruta catabólica, parece evidente que *P. putida* cuenta con más de un mecanismo para reducir su expresión.

Al inicio de este trabajo, se realizaron una serie de ensayos para determinar si los productos de algunos genes del cromosoma de *P. putida* KT2440 que juegan un papel en la regulación global de otros sistemas estaban o no involucrados en la regulación global de la ruta *upper*. También se analizaron los genes del entorno cromosómico de *rpoN*. Nuestros resultados mostraron que *vfr*, homólogo del gen *crp* de enterobacteriaceas en *Pseudomonas* (16, 68, 191), no influía en la regulación de la ruta *upper*. Tampoco observamos cambios analizando mutantes en los genes *relA* y *spoT*, que codifican las proteínas responsables de la síntesis de (p)ppGpp (20, 81) y Capítulo 1). La concentración de esta molécula efectora juega un papel importante en la supervivencia de la bacteria ante condiciones desfavorables para el crecimiento. El ejemplo mejor conocido es sin duda el incremento de (p)ppGpp ante la escasez de aminoácidos, siendo la unión de tRNAs no cargados al ribosoma la señal que estimula la síntesis (84). Este resultado que descarta a (p)ppGpp como señal en el fenómeno de represión de la ruta *upper* no sorprende, ya que concuerda con el estudio previo que compara el efecto de esta molécula efectora sobre los sistemas regulador/promotor DmpR/Po (de la ruta catabólica de (metil)fenol de *Pseudomonas* sp. CF600) y XylR/Pu (de la ruta *upper* de pWW0, en *P. putida* KT2440), en el que se concluyó que la influencia de (p)ppGpp sobre Pu era poco significativa (193). Entre los genes de proteínas relacionadas con el nivel energético de la bacteria, se ensayaron los mutantes carentes de Aer (que parece capaz de detectar cambios redox del sistema de transporte electrónico) (11, 12) o de CyoB (subunidad mayor de la oxidasa terminal de la cadena de transporte de electrones), que participa en la regulación de la ruta de alcanos del plásmido OCT de *P. putida* GPo1 (38, 39) y de la ruta de degradación de cresoles del plásmido pPGH1 de *P. putida* H (150). Sólo en el caso de CyoB se detectó una ligera liberación de la represión que el YE ejerce sobre Pu y P_{S1}. Sin embargo, observamos que los niveles de mRNA de las subunidades de la oxidasa terminal son mayores en presencia de YE (Capítulo 1). Este aumento puede explicarse por un incremento en la tasa respiratoria, según lo observado en cultivos de *E. coli* a los que se les ha añadido casaminoácidos (122). Las cepas mutadas en los dos marcos de lectura abierta conocidos como *orf102* y *orf284*, del entorno cromosómico de *rpoN*, no veían disminuida la represión de Pu y P_{S1} por YE. Concluimos que sus productos génicos no participaban en la represión de estos promotores en medio rico (Capítulo 1). Sólo se obtuvo una liberación de la represión en los mutantes de los genes *ptsN* y *crc*, que pasamos a analizar en mayor profundidad.

Las diversas condiciones de cultivo en las que se ha observado inhibición de los promotores dependientes de σ^{54} de la ruta TOL y del sistema formado por el promotor Po y el regulador DmpR en *Pseudomonas* CF600 se recogen en una serie de publicaciones procedentes de laboratorios independientes (22, 31, 45, 46, 78, 81, 107, 194). Los primeros trabajos con σ^{54} sugerían que los genes en 3' con respect a *rpoN* jugaban un papel represor sobre la actividad de ciertos promotores dependientes de σ^{54} (117). Estudios posteriores mostraron que la proteína IIA^{Ntr}, producto del gen

cromosómico *ptsN*, era uno de los factores involucrados en la represión de la ruta *upper* cuando hay un exceso de glucosa en el medio de cultivo. El efecto no era consecuencia de una diferencia en el consumo de glucosa con respecto a la cepa silvestre, por lo que se interpretó como la interferencia en un sistema de transducción de una señal en el que IIA^{Ntr} debía estar participando (24). La fosforilación del residuo His^{68} de IIA^{Ntr}, muy conservado en la mayoría de los miembros de la familia, parecía ser clave para la inhibición de la ruta *upper* por carbono (24). Sin embargo, IIA^{Ntr} no participaba en la represión observada durante crecimiento exponencial en LB, ya que la ruta *upper* seguía reprimida en un mutante *ptsN* hasta entrar en fase estacionaria (21).

En nuestra aproximación hemos utilizado dos modelos experimentales para analizar el fenómeno de represión catabólica sobre la ruta *upper* de *P. putida* KT2440. El primer modelo utiliza cultivos estanco en medio definido con adición de YE como agente represor, condición que reproduce la represión observada en medio rico durante crecimiento exponencial. El segundo modelo estudia cultivos continuos a tasa máxima de dilución, condición en la que la represión es consecuencia de un exceso de carbono en el medio y no depende de la velocidad de crecimiento de la población, ya que es posible observar represión de la ruta a diferentes velocidades de crecimiento siempre que el carbono esté en exceso (46). Con estas dos aproximaciones, hemos puesto de manifiesto que la proteína IIA^{Ntr} participa en la represión de la ruta *upper* cuando *P. putida* se cultiva en medio rico (Capítulo 1, figura 3; Capítulo 2, figura 2). Efectivamente, la adición de YE a células cultivadas en cultivo estanco en un medio definido, que supone un cambio inmediato a condiciones de crecimiento a velocidad máxima (Duetz, comunicación personal), reprodujo la represión descrita en LB (Capítulo 1, figura 2). Bajo esta condición, la inducción de la ruta *upper* se inhibía en un 80-90%. Por el contrario, la expresión inducida de la ruta *upper* en un mutante *ptsN* sólo se reprimía parcialmente. Como en los casos descritos anteriormente, es decir en un medio con exceso de glucosa o en medio LB, las dianas de la represión provocada por la adición de YE fueron los promotores dependientes de σ^{54}, Pu y P_{S1}. Debido a la particular disposición de los genes reguladores *xylR* y *xylS* (106) (Introducción General página 6: figura 3B), es posible estimar el grado de ocupación de la maquinaria de transcripción en función de los niveles de expresión del gen *xylR* desde sus promotores P_{R1} y P_{R2} (106). La unión del regulador XylR a sus UAS para la transcripción desde el promotor P_{S1} impide el acceso de la $RNAP\sigma^{70}$ a los promotores P_{R1} y P_{R2} para transcribir *xylR* (106). De este modo, un descenso en la transcripción de *xylR* informa indirectamente de una mayor ocupación de las UAS para transcribir P_{S1}. Los resultados obtenidos cuando analizamos esta región en condiciones de represión por YE mostraban que la expresión inducida de P_{S1} disminuye en presencia de YE y esto se refleja en un aumento de la transcripción desde P_{R1} y P_{R2} (Capítulo 1, figura 4). De algún modo, la adición de YE parece influenciar la ocupación de las UAS por XylR. Dado que los promotores Pu y P_{S1} presentan esencialmente la misma estructura típica de promotores σ^{54} (Introducción General, página 6: figura 3), están controlados por el mismo regulador XylR, y se comportan de modo semejante en respuesta a un inductor y/o represor, los resultados deducidos para P_{S1} se pueden extrapolar a Pu. Como en un mutante carente de IIA^{Ntr} la represión observada en P_{R1} y P_{R2} en presencia de

inductor es igual en presencia y ausencia de YE, probablemente por permanencia de XylR en las UAS (ya que hay un incremento de expresión de P_{S1}) (Capítulo 1, figura 4), podemos sugerir que el efecto represor de IIA^{Ntr} podría ser consecuencia de la interferencia directa con el complejo transcripcional, impidiendo la correcta unión del regulador XylR a sus sitios en la secuencia de DNA, o la interacción del regulador con la RNAP, o incluso la actividad de la RNAP. En un mutante *ptsN* la adición de YE no provocaba el mismo efecto: la transcripción desde los promotores de *xylR* sigue reprimida cuando IIA^{Ntr} no ejerce su papel (Capítulo 1, figura 4).

Por otra parte, los resultados obtenidos utilizando la segunda aproximación, represión catabólica por exceso de fuente de carbono en cultivo continuo, nos ha permitido concluir que IIA^{Ntr} juega, además, un papel en la represión de la ruta durante crecimiento a tasa de dilución alta (μ_{max}) (Capítulo 2). En condiciones de cultivo continuo a μ_{max}, la fuente de carbono (succinato) está en exceso en el medio y la expresión de Pu y P_{S1} inducida por *o*-xileno está inhibida en la cepa silvestre (45). La ausencia de IIA^{Ntr} en el mutante *ptsN* libera parcialmente la represión de ambos promotores (Capítulo 2, figura 2). Paralelamente, la transcripción desde P_{R1} y P_{R2} a tasa de dilución alta (μ_{max}) es menor en el mutante *ptsN* que en la cepa silvestre, lo que concuerda con una mayor ocupación de las UAS por XylR para la transcripción desde P_{S1}, favorecida en este mutante (Capítulo 2, figura 3). Parece por tanto que el mecanismo de actuación de IIA^{Ntr} en la represión de la ruta *upper* y del gen *xylS* por exceso de carbono en cultivo continuo es semejante a la represión en presencia de YE en cultivo estanco. El hecho de que en los dos tipos de aproximación la fuente de carbono utilizada como agente represor no sea la misma sugiere que la inhibición no es efecto de un compuesto concreto, sino de un conjunto de ellos capaces de disparar un mecanismo de respuesta común.

En resumen, podría decirse que IIA^{Ntr} participa en los diferentes fenómenos de represión de la ruta *upper* provocados por exceso de fuente de carbono en el medio, ya sea glucosa o succinato en cultivo continuo, o medio rico en cultivo estanco. En todos los casos, IIA^{Ntr} parece seguir un mismo modo de actuación. Por una parte, la localización conservada del gen que codifica a IIA^{Ntr} en el entorno cromosómico de *rpoN* en un grupo numeroso de bacterias concuerda con su papel en la represión catabólica de sistemas dependientes de σ^{54}. Por otra parte, su parecido con elementos de os sistemas PTS y la posibilidad de recibir y de transferir un grupo fosfato podrían revelar una relación entre el estado energético de la célula y la competencia de IIA^{Ntr} para actuar sobre tales sistemas. Se ha sugerido que IIA^{Ntr} se encuentra fosforilada en su forma activa (24). Podría deducirse que las condiciones en las que se ha observado represión de la ruta *upper* (crecimiento exponencial en medio rico o a altas concentraciones de compuestos carbonados, que al ser metabolizados son fuente de energía para la célula) son condiciones favorables para la fosforilación de IIA^{Ntr}, que se presentaría por tanto en su forma activa. Por el contrario, las condiciones que permiten la expresión de la ruta *upper* no son condiciones energéticamente favorables. En este caso es fácil pensar que IIA^{Ntr} podría no estar fosforilada, lo que explicaría su incapacidad para reprimir la transcripción de Pu y P_{S1}. Los detalles del modo de actuación de IIA^{Ntr} son actualmente objeto de estudio en nuestro grupo.

En el año 1991 se describió por primera vez en *P. aeruginosa* la existencia de un nuevo gen involucrado en la represión catabólica de varias rutas independientes de esta cepa, al que se denominó *crc* (217). Estudios posteriores han puesto de manifiesto que este gen es ubicuo en *Pseudomonas*, donde participa en la represión de distintas rutas metabólicas (39, 72, 123, 208, 217, 227) y Capítulo 1). Hemos estudiado el posible papel de *crc* en la represión de la ruta *upper* por YE. Nuestro trabajo aporta datos que implican por primera vez al regulador global Crc en la inhibición de la ruta. En cultivo estanco en medio definido, la represión de la transcripción desde Pu y P_{S1} por YE en presencia de inductor es sólo parcial en un mutante *crc* (Capítulo 1, figura 3). Sin embargo, a diferencia de lo observado para *ptsN*, el efecto de esta mutación no afecta a la actividad de los promotores P_{R1} y P_{R2} sino que ésta muestra un comportamiento semejante al de la cepa silvestre en presencia del represor YE (Capítulo 1, figura 4). Esto sugiere que la diana del mecanismo de acción de la proteína Crc difiere de la propuesta para la proteína IIA^{Ntr} y probablemente no está relacionada con cambios a nivel del complejo transcripcional responsable de la actividad de Pu y P_{S1}. Dadas las características de la proteína Crc, que presenta homología con nucleasas de DNA aunque no tiene una actividad ni exo- ni endonucleasa demostrada, podría actuar a nivel post-transcripcional o podría ser un elemento del sistema de detección de señales ambientales que conducen a la inhibición de la ruta *upper*. Adyacente y en orientación opuesta al gen *crc* se encuentra el gen *pyrE* que codifica la orotato fosforibosiltransferasa implicada en biosíntesis de uridina-5'-monofosfato (UMP) (103). Este nucleótido recibe un grupo fosfato por acción de una quinasa y se transforma en uridina-5'-trifosfato (UTP), que a su vez es el precursor de la citidina-5'-trifosfato (CTP) adquiriendo un grupo amino. La conservación de la asociación *crc-pyrE* en el genoma de diferentes especies de *Pseudomonas* sugiere una posible relación entre ambos genes. En *P. putida* KT2440, la distancia entre *crc* y *pyrE* es de 80 pb, de modo que la unión de la maquinaria transcripcional para la transcripción de uno de los genes podría influenciar la transcripción del otro. Algunos trabajos iniciales demostraron que la expresión de *pyrE* se reprime cuando existe una concentración alta de UTP o CTP en el medio (91, 152, 182). De este modo, la expresión de *crc* podría estar modulada por cambios que afecten a la expresión de *pyrE*, muy relacionados con el estado energético de la célula. El papel de Crc como represor de Pu y P_{S1}, a través de una señal disparada por la presencia de YE en el medio, concuerda con el nivel de expresión de Crc. Los elementos que participan en la regulación específica de TOL no modifican su concentración de mRNA en función de la presencia o ausencia de YE. Sin embargo los niveles de mRNA de *crc* aumentan al añadir YE al medio de cultivo, aunque no podemos discernir si se debe a un aumento en la transcripción o a una mayor estabilidad del mRNA en esta condición, en la que Pu y P_{S1} están reprimidos (Capítulo 1, figura 5). Este aumento se observa también en LB durante la fase exponencial (176). Recientemente se ha descrito que los niveles de mRNA de *crc* disminuyen en fase estacionaria, etapa del crecimiento en la que Pu y P_{S1} no están reprimidos (176, 226). Parece por tanto que existe una correlación entre condiciones de represión y niveles de expresión de Crc. Teniendo en cuenta que Crc no interviene en la represión de la ruta *upper* por un exceso de glucosa o succinato en el medio, cabría esperar que

la expresión del gen *crc* en estas condiciones fuera baja. La elucidación de la función molecular de Crc ayudará a entender su papel en la represión de Pu y $P_{S1.}$

Sorprendentemente, cuando analizamos el efecto de una mutación en *crc* utilizando nuestra segunda aproximación experimental, es decir en cultivo continuo creciendo a μ_{max}, observamos que la represión ejercida era semejante a la de la cepa silvestre (Capítulo 2, figura 2). Este resultado se corroboró en ensayos que reproducían las condiciones de represión por exceso de glucosa en medio estanco utilizados por otros grupos (24) (Capítulo 2, figura 4). En todos los casos, el comportamiento de la cepa mutante *crc* fue comparable al de la cepa silvestre: el exceso de carbono produjo la represión de los promotores de TOL dependientes de σ^{54}, y tampoco se observaron diferencias en la transcripción desde los promotores de *xylR* con respecto a la cepa silvestre (Capítulo 2, figura 3). Estos resultados ponen de manifiesto la probable existencia en *P. putida* de varios modos de detectar un exceso de fuente de carbono o una situación energética favorable en la célula, ya que existe una diferencia en los factores genéticos responsables de la inhibición de la ruta *upper* y del gen *xylS* en función de cual sea la señal represora.

Finalmente se analizó la transcripción de los promotores de la ruta *upper* en un doble mutante, incapaz de producir las proteínas IIA^{Ntr} y Crc. La disminución en la represión por YE de Pu y P_{S1} en el doble mutante *crc ptsN* no equivale a la suma de los efectos de cada una de las mutaciones individuales, sino que el grado de liberación de Pu y P_{S1} es similar al obtenido en los mutantes simples (Capítulo 1, figura 3). Este resultado estaría indicando que IIA^{Ntr} y Crc son componentes de una misma ruta de transmisión de señal y que la eliminación de uno o más componentes de esta cascada de regulación daría como resultado el mismo fenotipo. En cualquier caso, la relación entre los reguladores IIA^{Ntr} y Crc estaría limitada a la actuación en medio rico ya que Crc no controla la expresión de la ruta *upper* en un medio con exceso de glucosa o succinato (Capítulo 2).

Paralelamente a la caracterización de la represión de los niveles inducidos de Pu y P_{S1}, llevamos a cabo un análisis de los niveles de transcripción basal de estos promotores. Observamos que estos niveles dependían también de la presencia de XylR y estaban reprimidos por YE. A diferencia del nivel inducido por *o*-xileno, el nivel basal no se liberó de la represión en los mutantes *crc* y *ptsN* (resultados no mostrados). Esto nos lleva a concluir que la regulación mediada por IIA^{Ntr} y Crc debe estar relacionada de algún modo con el mecanismo de inducción del regulador XylR y/o de interacción de éste con el promotor o la maquinaria transcripcional (Capítulo 1). Sin embargo, el hecho de que la represión ejercida por IIA^{Ntr} también ocurra cuando XylR se sustituye por un mutante constitutivo carente del dominio de unión al efector (21, 143) sugiere que el mecanismo de inducción de la proteína no es la diana del regulador global.

V.2. Estudio del entorno cromosómico del gen *rpoN* de *P. putida* KT2440

El factor σ^{54} de la RNAP confiere especificidad en el reconocimiento de una secuencia promotora en la posición -12/-24 con respecto al punto de inicio de la transcripción y es esencial para el inicio de la transcripción en estos promotores (18, 75, 124). Como hemos discutido anteriormente, la regulación global que controla la

expresión de las rutas catabólicas del plásmido TOL se ejerce a nivel de dos promotores dependientes de σ^{54}, Pu y P_{S1}. Por este motivo, decidimos estudiar la regulación de la expresión de *rpoN*, el gen que lo codifica. Generalmente en bacterias, el gen *rpoN* se agrupa de forma conservada con una serie de ORFs formando lo que se ha llamado agrupación génica *rpoN*. Salvo en el caso de *rpoN*, la función de los genes de la agrupación se desconoce.

En este trabajo definimos con exactitud el punto de inicio de la transcripción de *rpoN*, que ya había sido delimitado anteriormente en un fragmento de 86 pb, y confirmamos que el gen se reprime por su producto σ^{54}, tanto en medio rico (LB) como en medio mínimo (con glucosa como fuente de carbono) (97) y Capítulo 3, figura 2), de manera que la concentración de σ^{54} está modulada en función de las necesidades de la célula. Un análisis transcripcional exhaustivo de la región puso de manifiesto la presencia de otro promotor, al que llamamos P_{102}, que da lugar a transcritos que solapan con las 91 últimas bases del extremo 3' de *rpoN* (Capítulo 3, figura 3). No se detectó ningún otro promotor en la región de DNA posterior a *orf102*, por lo que dedujimos que P_{102} dirigía la expresión de cuatro genes: *orf102*, *ptsN*, *orf284* y *ptsO*. En la región intergénica que separa al gen *rpoN* de la *orf102* existen dos posibles señales de terminación de la transcripción, semejantes a las descritas para otros genes (Capítulo 3, figura 7). Uno de estos potenciales terminadores presenta las características propias de los llamados terminadores independientes de Rho, que interrumpen la transcripción formando una estructura secundaria en forma de horquilla que detiene la progresión de la RNAP, que acaba liberándose del DNA (29). Además, en esta región puede observarse una secuencia similar a las descritas en *Helicobacter pylori* como sitios de terminación de la transcripción en los que interviene la proteína Rho. Estos sitios presentan una secuencia de Cs por la que Rho muestra afinidad y, a poca distancia, una secuencia de Ts que favorece la inestabilidad en la unión de la RNAP y su liberación (149). Es de esperar que una parte de los transcritos iniciados en P_{102} terminen en la región intergénica *rpoN-orf102*. Sin embargo, según nuestros resultados (Capítulo 3), la RNAP puede también superar las señales de terminación y dar lugar a un transcrito de mayor tamaño que abarca los genes posteriores a *rpoN*. Esto es aplicable tanto a los transcritos originados en P_{102}, que serían abortados por funcionamiento del terminador, como a los transcritos originados desde el promotor de *rpoN*, todo en función de la capacidad de la RNAP para superar o no las señales de terminación (Capítulo 3, figura 7).

El análisis del promotor P_{102} revela la presencia de una secuencia -10/-35 que se ajusta al consenso de secuencias -10/-35 reconocidas por la RNAP en *P. putida* (TTGACC-N_{17}-TATACT) . Además, antes de la posición -35 se detectó una región rica en adeninas, semejante al llamado elemento "UP" de ciertos promotores de *E. coli* (Capítulo 3, figura 3). La función de estos elementos es favorecer la interacción con la RNAP mediante contactos con el dominio C-terminal de la subunidad α (α-CTD) para incrementar la transcripción (4, 49, 50, 167-169). La eliminación de este elemento redujo de 5 a 6 veces la actividad de P_{102}, poniendo de manifiesto su funcionalidad (Capítulo 3, figura 6), que se confirmó ensayando P_{102} en cepas de *E. coli* que sobreproducen una subunidad α delecionada en el dominio C-terminal, esencial para la interacción con el elemento UP (Capítulo 3, tabla 3). En general, el mayor número

de elementos reguladores se encuentra en la región de P_{102}. Si, como se ha propuesto, ORF102 y las demás proteínas de la agrupación modulan la expresión de *rpoN*, las consecuencias de la regulación en P_{102} se extenderían a todos aquellos sistemas dependientes del factor σ^{54}.

Además del promotor de *rpoN* y del promotor P_{102}, hemos hallado otros dos promotores nuevos al analizar la cadena complementaria a la que codifica la agrupación génica *rpoN* (Capítulo 3, figuras 4 y 5). Los transcritos obtenidos desde estos dos promotores antisentido no parecen codificar proteínas. Uno de estos transcritos antisentido (antiptsN) comienza en el extremo 3' de *ptsN* y el otro (antirpoN) en el extremo 3' de *rpoN* (Capítulo 3, figuras 4 y 5). Este hallazgo apunta a un nuevo nivel de regulación de la expresión de *rpoN* y *ptsN*. Ambos podrían unirse a su correspondiente RNA complementario para regular aspectos como la vida media del mRNA o el acceso de la maquinaria de traducción. Pero no sólo la existencia de estos transcritos antisentido son indicio de una regulación post-transcripcional en esta región del cromosoma. El hecho de que el producto de la *orf102* sea semejante a ciertas proteínas de unión a ribosoma y la presencia de una serie de repeticiones invertidas en la secuencia líder del mRNA que precede a las regiones codificantes de *rpoN* y *orf102* son factiblemente otros elementos de este nivel de regulación aún por explicar.

Un último punto a esclarecer es la presencia de cinco sitios de reconocimiento del factor σ^{54} en la región promotora de P_{102} y del promotor $P_{anti\textit{rpoN}}$, que solapan con secuencias clave para la transcripción de estos dos promotores divergentes (Capítulo 3, figura 3E). Aunque no hemos determinado si los sitios pueden ser ocupados por σ^{54} o por la holoenzima ($E\sigma^{54}$), en ambos casos la unión afectaría a la transcipción en los dos sentidos. Esta cuestión está siendo investigada actualmente en nuestro laboratorio.

V.3. Elementos repetidos en el cromosoma de *P. putida* KT2440

Las regiones extragénicas de un genoma son de crucial interés. En ellas se concentra una amplia variedad de señales necesarias para la regulación de la transcripción de las secuencias codificantes. Incluyen secuencias reconocidas por la maquinaria transcripcional, terminadores y sitios de unión de reguladores. En *P. putida* KT2440, las regiones extragénicas suponen un 10-12% del genoma. En este trabajo se ha presentado y caracterizado un elemento de 35 pb, semejante a las secuencias REP de enterobacterias, muy representado en el cromosoma de *P. putida* KT2440. Se localizaron más de 800 copias de este elemento a lo largo de todo el cromosoma y su situación en el 80% de los casos fue extragénica (Capítulo 4). Las secuencias REP de *P. putida* KT2440 son parcialmente palindrómicas e incluyen una repetición invertida interna de 6 pb.

Desde su descubrimiento en enterobacteriaceas (37, 74), se han atribuido diferentes funciones a las secuencias REP. Se ha sugerido que podrían ser sitios de unión de proteínas como IHF, DNA polimerasa I o girasa de DNA (13, 47, 61, 225), o actuar como atenuadores de la transcripción (48). La localización de las REP de *P. putida* en regiones extragénicas del cromosoma nos llevó a analizar el posible papel de estas secuencias en la terminación de la transcripción. Utilizando un plásmido

diseñado para la detección de terminadores, comprobamos que no participan en la terminación de la transcripción, sea cual sea su orientación (Capítulo 4, figura 1).

Las REP han sido consideradas también un elemento más de la regulación génica global (198). Se sabe que cuando se transcriben pueden estabilizar el mRNA, impidiendo su degradación por exonucleasas y determinando el grado de expresión del gen que las precede (26, 94, 127, 187). En general, su expresión bajo ciertas condiciones ambientales podría conducir a cambios en el patrón de expresión de determinados grupos de genes en la bacteria. Igualmente se han relacionado con mecanismos de recombinación (96, 98) y se sabe que son diana de secuencias de inserción (27, 160, 197). Por ello se ha sugerido que a largo plazo participan en mecanismos de adaptación a condiciones ambientales, facilitando el reordenamiento de genes.

El análisis de su distribución en *P. putida* KT2440 mostró que las secuencias REP se situaban preferentemente entre genes convergentes (Capítulo 4, tabla 1). En bacterias, el grado de enrollamiento del DNA influencia el nivel de transcripción de muchos genes (200, 219). La transcripción de dos genes convergentes produce un alto grado de superenrollamiento en su región intergénica, que recupera su estado relajado por acción de una DNA girasa (28, 59). Ya hemos comentado la afinidad de la DNA girasa por las secuencias REPs (Introducción general, página 16) (47, 225). La unión de la girasa a estas secuencias podría garantizar la actuación de esta proteína en la región intergénica de genes convergentes, liberando a la molécula de DNA de la torsión generada.

Aunque los genes de algunas rutas catabólicas están bien conservados entre las distintas cepas de *P. putida*, las secuencias REP parecen contribuir significativamente a la diversidad entre especies (43). La inserción de uno o más elementos REP entre dos genes de una ruta podría modificar su mecanismo de regulación con respecto a otra especie en la que no haya ocurrido la inserción. Ejemplos de diferencias en la inserción de REPs se han descrito en la agrupación de los genes *pha*, para la degradación de fenilacetato (mientras que la cepa KT2440 tiene una sola REP detrás del gen *phaL*, la cepa U tiene dos REPs convergentes en la región intergénica *phaI-phaJ*), en el conjunto de genes *ben* y *cat* para degradación de benzoato y catecol (las dos REP invertidas anteriores al gen *benE* de KT2440 no están en la cepa PRS2000, y esta última cepa tiene a su vez una REP detrás del gen *catR* que no está en KT2440. La cepa RB1 tiene una REP en la región intergénica *catB-catC*) (85, 156).

Finalmente, se ha sugerido que las secuencias REPs podrían servir como señales que permiten a la bacteria reconocer su DNA como propio y diferenciarlo de un DNA extraño. Este mecanismo de defensa podría conferirles una ventaja evolutiva frente a otros microorganismos (198)

Son varias las aplicaciones diseñadas a raíz del análisis de la estructura y distribución de estas secuencias. En este aspecto, como se ha señalado en el capítulo 4, las secuencias REPs permiten la identificación fácil de bacterias hasta nivel de especie e incluso de cepa (Capítulo 4; (30, 210). Esto está en consonancia con el hallazgo reciente de secuencias REPs en otras especies del género *Pseudomonas*

que presentan una secuencia de nucleótidos particular y diferente a la de *P. putida* (51, 139, 198). Igualmente, las características de las secuencias REP han permitido hacer correcciones en la anotación del genoma. Dado que estos elementos son en su gran mayoría extragénicos, su localización dentro de posibles regiones codificantes podía ser debida a un error en la anotación de éstas. Efectivamente, se observó que las secuencias REP detectadas como no-extragénicas se encontraban incluidas mayoritariamente en ORFs que no presentaban homología con ninguna proteína conocida y que, en ocasiones, eran de pequeño tamaño (Capítulo 4; (198). Por otra parte, la ausencia de secuencias REPs en una determinada región del cromosoma podría indicar un origen diferente de esta región. De hecho, las REPs están ausentes en las islas genómicas, lo que refleja su diferente procedencia con respecto al resto del genoma. La distribución de las secuencias REPs puede ser útil, por tanto, para localizar grupos de genes que hayan sido adquiridos por transferencia horizontal y para el diseño de modelos evolutivos.

El actual trabajo ha servido como base para un conjunto de estudios posteriores que han ampliado el conocimiento de las regiones cromosómicas extragénicas en *Pseudomonas* y en otros genomas bacterianos conocidos (136, 160, 197-199). Además, se ha promovido el interés por estas regiones extragénicas en trabajos que describen el contenido de genomas bacterianos (51, 139).

De todo lo expuesto en esta tesis doctoral se puede concluir que el control del metabolismo en *Pseudomonas putida* KT2440 es un claro ejemplo de la compleja red de regulación desplegada por muchas bacterias para adaptarse a los recursos presentes en el medio y reducir el coste energético de su actividad.

VI. CONCLUSIONES

1) La interrupción de los genes *crc* o *ptsN* elimina parcialmente la represión de la actividad de los promotores Pu y P_{S1} por extracto de levadura. En estas condiciones, la transcripción del gen *crc* incrementa.

2) La proteína IIA^{Ntr}, pero no Crc, parece interferir de forma directa con el mecanismo de activación de la transcripción mediada por el regulador XylR en sus promotores diana Pu y P_{S1}

3) Sólo la proteína IIA^{Ntr} está implicada en la inhibición de ambos promotores por exceso de fuente de carbono en cultivo continuo. Esto implica que la bacteria cuenta con más de una estrategia para responder a diferentes cambios ambientales que requieran que la ruta de degradación de tolueno esté apagada.

4) Dos promotores dirigen la transcripción de los genes de la agrupación génica *rpoN*. Uno de ellos está situado delante de *rpoN* y se autorregula. El otro se localiza en el extremo 3' dentro del gen *rpoN* y dirige la transcripción del resto de genes. Se ha detectado en esta región la presencia de dos transcritos antisentido que darían lugar a mRNAs complementarios a los transcritos de *ptsN* y *rpoN*.

5) Más de 800 copias de un palíndromo imperfecto de 35 pb semejante a las REPs de enterobacteriaceas se distribuyen en las regiones extragénicas del cromosoma de *Pseudomonas putida* KT2440 preferentemente entre genes convergentes. Son secuencias específicas de especie y permiten la discriminación a nivel de cepa. No funcionan como terminadores de la transcripción.

VII. BIBLIOGRAFÍA

1. **Abril, M. A., M. Buck, and J. L. Ramos.** 1991. Activation of the Pseudomonas TOL plasmid upper pathway operon. Identification of binding sites for the positive regulator XylR and for integration host factor protein. J Biol Chem **266:**15832-8.
2. **Aiba, H.** 1983. Autoregulation of the Escherichia coli crp gene: CRP is a transcriptional repressor for its own gene. Cell **32:**141-9.
3. **Aiba, H.** 1985. Transcription of the Escherichia coli adenylate cyclase gene is negatively regulated by cAMP-cAMP receptor protein. J Biol Chem **260:**3063-70.
4. **Aiyar, S. E., R. L. Gourse, and W. Ross.** 1998. Upstream A-tracts increase bacterial promoter activity through interactions with the RNA polymerase alpha subunit. Proc Natl Acad Sci U S A **95:**14652-7.
5. **Arfin, S. M., A. D. Long, E. T. Ito, L. Tolleri, M. M. Riehle, E. S. Paegle, and G. W. Hatfield.** 2000. Global gene expression profiling in Escherichia coli K12. The effects of integration host factor. J Biol Chem **275:**29672-84.
6. **Aviv, M., H. Giladi, G. Schreiber, A. B. Oppenheim, and G. Glaser.** 1994. Expression of the genes coding for the Escherichia coli integration host factor are controlled by growth phase, rpoS, ppGpp and by autoregulation. Mol Microbiol **14:**1021-31.
7. **Ball, C. A., R. Osuna, K. C. Ferguson, and R. C. Johnson.** 1992. Dramatic changes in Fis levels upon nutrient upshift in Escherichia coli. J Bacteriol **174:**8043-56.
8. **Baysse, C., M. Cullinane, V. Denervaud, E. Burrowes, J. M. Dow, J. P. Morrissey, L. Tam, J. T. Trevors, and F. O'Gara.** 2005. Modulation of quorum sensing in Pseudomonas aeruginosa through alteration of membrane properties. Microbiology **151:**2529-42.
9. **Bertoni, G., N. Fujita, A. Ishihama, and V. de Lorenzo.** 1998. Active recruitment of sigma54-RNA polymerase to the Pu promoter of Pseudomonas putida: role of IHF and alphaCTD. Embo J **17:**5120-8.
10. **Bertoni, G., S. Marqués, and V. de Lorenzo.** 1998. Activation of the toluene-responsive regulator XylR causes a transcriptional switch between sigma54 and sigma70 promoters at the divergent Pr/Ps region of the TOL plasmid. Mol. Microbiol. **27:**651-659.
11. **Bibikov, S. I., L. A. Barnes, Y. Gitin, and J. S. Parkinson.** 2000. Domain organization and flavin adenine dinucleotide-binding determinants in the aerotaxis signal transducer Aer of Escherichia coli. Proc Natl Acad Sci U S A **97:**5830-5.
12. **Bibikov, S. I., R. Biran, K. E. Rudd, and J. S. Parkinson.** 1997. A signal transducer for aerotaxis in Escherichia coli. J Bacteriol **179:**4075-9.
13. **Boccard, F., and P. Prentki.** 1993. Specific interaction of IHF with RIBs, a class of bacterial repetitive DNA elements located at the 3' end of transcription units. Embo J **12:**5019-27.
14. **Bordo, D., R. L. van Monfort, T. Pijning, K. H. Kalk, J. Reizer, M. H. Saier, Jr., and B. W. Dijkstra.** 1998. The three-dimensional structure of the nitrogen regulatory protein IIANtr from Escherichia coli. J Mol Biol **279:**245-55.
15. **Bosch, L., L. Nilsson, E. Vijgenboom, and H. Verbeek.** 1990. FIS-dependent trans-activation of tRNA and rRNA operons of Escherichia coli. Biochim Biophys Acta **1050:**293-301.
16. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol Rev **56:**100-22.
17. **Braeken, K., M. Moris, R. Daniels, J. Vanderleyden, and J. Michiels.** 2006. New horizons for (p)ppGpp in bacterial and plant physiology. Trends Microbiol **14:**45-54.
18. **Cannon, W., M. T. Gallegos, and M. Buck.** 2001. DNA melting within a binary sigma(54)-promoter DNA complex. J Biol Chem **276:**386-94.

19. **Carmona, M., V. de Lorenzo, and G. Bertoni.** 1999. Recruitment of RNA polymerase is a rate-limiting step for the activation of the sigma(54) promoter Pu of Pseudomonas putida. J Biol Chem **274:**33790-4.
20. **Carmona, M., M. J. Rodriguez, O. Martínez-Costa, and V. De Lorenzo.** 2000. In vivo and in vitro effects of (p)ppGpp on the sigma(54) promoter Pu of the TOL plasmid of Pseudomonas putida. J Bacteriol **182:**4711-8.
21. **Cases, I., and V. de Lorenzo.** 2000. Genetic evidence of distinct physiological regulation mechanisms in the sigma(54) Pu promoter of Pseudomonas putida. J Bacteriol **182:**956-60.
22. **Cases, I., V. de Lorenzo, and J. Pérez-Martín.** 1996. Involvement of sigma factor σ^{54} in exponential silencing of the *Pseudomonas putida* TOL plasmid Pu promoter. Mol. Microbiol.
23. **Cases, I., J. A. López, J. P. Albar, and V. De Lorenzo.** 2001. Evidence of multiple regulatory functions for the PtsN (IIA(Ntr)) protein of *Pseudomonas putida*. J. Bacteriol. **183:**1032-1037.
24. **Cases, I., J. Pérez-Martín, and V. de Lorenzo.** 1999. The IIANtr (PtsN) protein of *Pseudomonas putida* mediates the C source inhibition of the sigma54-dependent Pu promoter of the TOL plasmid. J Biol Chem **274:**15562-8.
25. **Cashel, M.** 1975. Regulation of bacterial ppGpp and pppGpp. Annu Rev Microbiol **29:**301-18.
26. **Cheng, Z. F., and M. P. Deutscher.** 2005. An important role for RNase R in mRNA decay. Mol Cell **17:**313-8.
27. **Choi, S., S. Ohta, and E. Ohtsubo.** 2003. A novel IS element, IS621, of the IS110/IS492 family transposes to a specific site in repetitive extragenic palindromic sequences in Escherichia coli. J Bacteriol **185:**4891-900.
28. **Cozzarelli, N. R.** 1980. DNA gyrase and the supercoiling of DNA. Science **207:**953-60.
29. **d'Aubenton Carafa, Y., E. Brody, and C. Thermes.** 1990. Prediction of rho-independent Escherichia coli transcription terminators. A statistical analysis of their RNA stem-loop structures. J Mol Biol **216:**835-58.
30. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of Rhizobium meliloti isolates and other soil bacteria. Appl Environ Microbiol **58:**2180-7.
31. **de Lorenzo, V., I. Cases, M. Herrero, and K. Timmis.** 1993. Early and late responses of TOL promoters to pathway inducers: identification of growth-phase-dependent promoters in *Pseudomonas putida* with *lacZ-tet* bicistronic reporters. J. Bacteriol. **175:**6902.
32. **de Lorenzo, V., M. Herrero, M. Metzke, and K. N. Timmis.** 1991. An upstream XylR and IHF induced nucleoprotein complex regulates the σ54 dependent Pu promotor of TOL plasmid. EMBO J. **10:**1159-1167.
33. **Delgado, A., and J. L. Ramos.** 1994. Genetic evidence for activation of the positive transcriptional regulator Xy1R, a member of the NtrC family of regulators, by effector binding. J Biol Chem **269:**8059-62.
34. **Delgado, A., R. Salto, S. Marqués, and J. L. Ramos.** 1995. Single amino acids changes in the signal receptor domain of XylR resulted in mutants that stimulate transcription in the absence of effectors. J Biol Chem **270:**5144-50.
35. **Devos, D., J. Garmendia, V. de Lorenzo, and A. Valencia.** 2002. Deciphering the action of aromatic effectors on the prokaryotic enhancer-binding protein XylR: a structural model of its N-terminal domain. Environ Microbiol **4:**29-41.
36. **Díaz, E., and M. A. Prieto.** 2000. Bacterial promoters triggering biodegradation of aromatic pollutants. Curr Opin Biotechnol **11:**467-75.
37. **Dimri, G. P., K. E. Rudd, M. K. Morgan, H. Bayat, and G. F. Ames.** 1992. Physical mapping of repetitive extragenic palindromic sequences in Escherichia

coli and phylogenetic distribution among Escherichia coli strains and other enteric bacteria. J Bacteriol **174:**4583-93.

38. **Dinamarca, M. A., I. Aranda-Olmedo, A. Puyet, and F. Rojo.** 2003. Expression of the Pseudomonas putida OCT plasmid alkane degradation pathway is modulated by two different global control signals: evidence from continuous cultures. J Bacteriol **185:**4772-8.
39. **Dinamarca, M. A., A. Ruíz-Manzano, and F. Rojo.** 2002. Inactivation of cytochrome o ubiquinol oxidase relieves catabolic repression of the Pseudomonas putida GPo1 alkane degradation pathway. J Bacteriol **184:**3785-93.
40. **Ditto, M. D., D. Roberts, and R. A. Weisberg.** 1994. Growth phase variation of integration host factor level in Escherichia coli. J Bacteriol **176:**3738-48.
41. **Dixon, R.** 1986. The *xylABC* promoter from the *Pseudomonas putida* TOL plasmid is activated by nitrogen regulatory genes in *Escherichia coli*. Mol. Gen. Genet**:**129-136.
42. **Domínguez-Cuevas, P., P. Marín, J. L. Ramos, and S. Marqués.** 2005. RNA polymerase holoenzymes can share a single transcription start site for the Pm promoter. Critical nucleotides in the -7 to -18 region are needed to select between RNA polymerase with sigma38 or sigma32. J Biol Chem **280:**41315-23.
43. **Dos Santos, V. A.** 2004. Genomic Insights in the metabolism of aromatic compounds in Pseudomonas. *In* R. J. (ed.), Pseudomonas, vol. 3. Kluwer Academic/Plenum Publishers.
44. **Dos Santos, V. A., S. Heim, E. R. Moore, M. Stratz, and K. N. Timmis.** 2004. Insights into the genomic basis of niche specificity of Pseudomonas putida KT2440. Environ Microbiol **6:**1264-86.
45. **Duetz, W. A., S. Marqués, C. de Jong, J. L. Ramos, and J. G. van Andel.** 1994. Inducibility of the TOL catabolic pathway in *Pseudomonas putida* (pWW0) growing on succinate in continuous culture: evidence of carbon catabolite repression control. J Bacteriol **176:**2354-61.
46. **Duetz, W. A., S. Marqués, B. Wind, J. L. Ramos, and J. G. van Andel.** 1996. Catabolite repression of the toluene degradation pathway in *Pseudomonas putida* harboring pWW0 under various conditions of nutrient limitation in chemostat culture. Appl Environ Microbiol **62:**601-6.
47. **Espeli, O., and F. Boccard.** 1997. In vivo cleavage of Escherichia coli BIME-2 repeats by DNA gyrase: genetic characterization of the target and identification of the cut site. Mol Microbiol **26:**767-77.
48. **Espeli, O., L. Moulin, and F. Boccard.** 2001. Transcription attenuation associated with bacterial repetitive extragenic BIME elements. J Mol Biol **314:**375-86.
49. **Estrem, S. T., T. Gaal, W. Ross, and R. L. Gourse.** 1998. Identification of an UP element consensus sequence for bacterial promoters. Proc Natl Acad Sci U S A **95:**9761-6.
50. **Estrem, S. T., W. Ross, T. Gaal, Z. W. Chen, W. Niu, R. H. Ebright, and R. L. Gourse.** 1999. Bacterial promoter architecture: subsite structure of UP elements and interactions with the carboxy-terminal domain of the RNA polymerase alpha subunit. Genes Dev **13:**2134-47.
51. **Feil, H., W. S. Feil, P. Chain, F. Larimer, G. DiBartolo, A. Copeland, A. Lykidis, S. Trong, M. Nolan, E. Goltsman, J. Thiel, S. Malfatti, J. E. Loper, A. Lapidus, J. C. Detter, M. Land, P. M. Richardson, N. C. Kyrpides, N. Ivanova, and S. E. Lindow.** 2005. Comparison of the complete genome sequences of Pseudomonas syringae pv. syringae B728a and pv. tomato DC3000. Proc Natl Acad Sci U S A **102:**11064-9.

52. **Fernández, S., V. de Lorenzo, and J. Pérez-Martín.** 1995. Activation of the transcriptional regulator XylR of Pseudomonas putida by release of repression between functional domains. Mol Microbiol **16:**205-13.
53. **Fraile, S., F. Roncal, L. A. Fernández, and V. de Lorenzo.** 2001. Monitoring intracellular levels of XylR in Pseudomonas putida with a single-chain antibody specific for aromatic-responsive enhancer-binding proteins. J Bacteriol **183:**5571-9.
54. **Gallegos, M. T., S. Marqués, and J. L. Ramos.** 1996. Expression of the TOL plasmid xylS gene in Pseudomonas putida occurs from a alpha 70-dependent promoter or from alpha 70- and alpha 54- dependent tandem promoters according to the compound used for growth. J Bacteriol **178:**2356-61.
55. **Gang, J. B., and J. G. Harman.** 1997. CRP:cAMP complex binding to the lac operator region induces a structural change in lac DNA. Mol Cells **7:**444-7.
56. **Garmendia, J., and V. de Lorenzo.** 2000. The role of the interdomain B linker in the activation of the XylR protein of *Pseudomonas putida*. Mol. Microbiol. **38:**401-410.
57. **Garmendia, J., and V. de Lorenzo.** 2000. Visualization of DNA-protein intermediates during activation of the Pu promoter of the TOL plasmid of Pseudomonas putida. Microbiology **146 (Pt 10):**2555-63.
58. **Gaston, K., A. Bell, A. Kolb, H. Buc, and S. Busby.** 1990. Stringent spacing requirements for transcription activation by CRP. Cell **62:**733-43.
59. **Gellert, M., K. Mizuuchi, M. H. O'Dea, H. Ohmori, and J. Tomizawa.** 1979. DNA gyrase and DNA supercoiling. Cold Spring Harb Symp Quant Biol **43 Pt 1:**35-40.
60. **Gerischer, U.** 2002. Specific and global regulation of genes associated with the degradation of aromatic compounds in bacteria. J Mol Microbiol Biotechnol **4:**111-21.
61. **Gilson, E., D. Perrin, and M. Hofnung.** 1990. DNA polymerase I and a protein complex bind specifically to E. coli palindromic unit highly repetitive DNA: implications for bacterial chromosome organization. Nucleic Acids Res **18:**3941-52.
62. **Gomada, M., H. Imaishi, K. Miura, S. Inouye, T. Nakazawa, and A. Nakazawa.** 1994. Analysis of DNA bend structure of promoter regulatory regions of xylene-metabolizing genes on the Pseudomonas TOL plasmid. J Biochem (Tokyo) **116:**1096-104.
63. **Gomada, M., S. Inouye, H. Imaishi, A. Nakazawa, and T. Nakazawa.** 1992. Analysis of an upstream regulatory sequence required for activation of the regulatory gene xylS in xylene metabolism directed by the TOL plasmid of Pseudomonas putida. Mol Gen Genet **233:**419-26.
64. **González-Gil, G., P. Bringmann, and R. Kahmann.** 1996. FIS is a regulator of metabolism in Escherichia coli. Mol Microbiol **22:**21-9.
65. **González-Pérez, M. M., J. L. Ramos, M. T. Gallegos, and S. Marqués.** 1999. Critical nucleotides in the upstream region of the XylS-dependent TOL meta-cleavage pathway operon promoter as deduced from analysis of mutants. J Biol Chem **274:**2286-90.
66. **Good, L.** 2003. Translation repression by antisense sequences. Cell Mol Life Sci **60:**854-61.
67. **Gottesman, S.** 1984. Bacterial regulation: global regulatory networks. Annu Rev Genet **18:**415-41.
68. **Grainger, D. C., D. Hurd, M. Harrison, J. Holdstock, and S. J. Busby.** 2005. Studies of the distribution of Escherichia coli cAMP-receptor protein and RNA polymerase along the E. coli chromosome. Proc Natl Acad Sci U S A **102:**17693-8.

69. **Greated, A., L. Lambertsen, P. A. Williams, and C. M. Thomas.** 2002. Complete sequence of the IncP-9 TOL plasmid pWW0 from Pseudomonas putida. Environ Microbiol **4:**856-71.
70. **Hanamura, A., and H. Aiba.** 1991. Molecular mechanism of negative autoregulation of Escherichia coli crp gene. Nucleic Acids Res **19:**4413-9.
71. **Hatfield, G. W., and C. J. Benham.** 2002. DNA topology-mediated control of global gene expression in Escherichia coli. Annu Rev Genet **36:**175-203.
72. **Hester, K. L., J. Lehman, F. Najar, L. Song, B. A. Roe, C. H. MacGregor, P. W. Hager, P. V. Phibbs, Jr., and J. R. Sokatch.** 2000. Crc is involved in catabolite repression control of the bkd operons of *Pseudomonas putida* and *Pseudomonas aeruginosa*. J Bacteriol **182:**1144-9.
73. **Hester, K. L., K. T. Madhusudhan, and J. R. Sokatch.** 2000. Catabolite repression control by *crc* in 2xYT medium is mediated by posttranscriptional regulation of *bkdR* expression in *Pseudomonas putida*. J Bacteriol **182:**1150-3.
74. **Higgins, C. F., G. F. Ames, W. M. Barnes, J. M. Clement, and M. Hofnung.** 1982. A novel intercistronic regulatory element of prokaryotic operons. Nature **298:**760-2.
75. **Hirschman, J., P. K. Wong, K. Sei, J. Keener, and S. Kustu.** 1985. Products of nitrogen regulatory genes ntrA and ntrC of enteric bacteria activate glnA transcription in vitro: evidence that the ntrA product is a sigma factor. Proc Natl Acad Sci U S A **82:**7525-9.
76. **Holtel, A., M. A. Abril, S. Marqués, K. N. Timmis, and J. L. Ramos.** 1990. Promoter upstream activator sequences are required for expression of the *xylS* gene and upper pathway operon on the *Pseudomonas* TOL plasmid. Mol. Microbiol. **4:**1551-1556.
77. **Holtel, A., D. Goldenberg, H. Giladi, A. B. Oppenheim, and K. N. Timmis.** 1995. Involvement of IHF protein in expression of the Ps promoter of the Pseudomonas putida TOL plasmid. J Bacteriol **177:**3312-5.
78. **Holtel, A., S. Marqués, I. Möhler, U. Jakubzik, and K. N. Timmis.** 1994. Carbon source-dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. J. Bacteriol. **176:**1773-1776.
79. **Holtel, A., K. N. Timmis, and J. L. Ramos.** 1992. Upstream binding sequences of the XylR activator protein and integration host factor in the xylS gene promoter region of the Pseudomonas TOL plasmid. Nucleic Acids Res **20:**1755-62.
80. **Hommais, F., E. Krin, C. Laurent-Winter, O. Soutourina, A. Malpertuy, J. P. Le Caer, A. Danchin, and P. Bertin.** 2001. Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. Mol Microbiol **40:**20-36.
81. **Hugouvieux-Cotte-Pattat, N., T. Köhler, M. Rekik, and S. Harayama.** 1990. Growth-phase-dependent expression of the *Pseudomonas putida* TOL plasmid pWW0 catabolic genes. J Bacteriol **172:**6651-60.
82. **Huo, Y. X., Z. X. Tian, M. Rappas, J. Wen, Y. C. Chen, C. H. You, X. Zhang, M. Buck, Y. P. Wang, and A. Kolb.** 2006. Protein-induced DNA bending clarifies the architectural organization of the sigma54-dependent glnAp2 promoter. Mol Microbiol **59:**168-80.
83. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1987. Overproduction of the xylS gene product and activation of the xylDLEGF operon on the TOL plasmid. J Bacteriol **169:**3587-92.
84. **Jain, V., M. Kumar, and D. Chatterji.** 2006. ppGpp: stringent response and survival. J Microbiol **44:**1-10.
85. **Jiménez, J. I., B. Minambres, J. L. Garcia, and E. Díaz.** 2002. Genomic analysis of the aromatic catabolic pathways from Pseudomonas putida KT2440. Environ Microbiol **4:**824-41.

86. **Jishage, M., K. Kvint, V. Shingler, and T. Nystrom.** 2002. Regulation of sigma factor competition by the alarmone ppGpp. Genes Dev. **16:**1260-1270.
87. **Johansson, J., C. Balsalobre, S. Y. Wang, J. Urbonaviciene, D. J. Jin, B. Sonden, and B. E. Uhlin.** 2000. Nucleoid proteins stimulate stringently controlled bacterial promoters: a link between the cAMP-CRP and the (p)ppGpp regulons in Escherichia coli. Cell **102:**475-85.
88. **Kaldalu, N., T. Mandel, and M. Ustav.** 1996. TOL plasmid transcription factor XylS binds specifically to the Pm operator sequence. Mol Microbiol **20:**569-79.
89. **Kaldalu, N., U. Toots, V. de Lorenzo, and M. Ustav.** 2000. Functional domains of the TOL plasmid transcription factor XylS. J Bacteriol **182:**1118-26.
90. **Kar, S., R. Edgar, and S. Adhya.** 2005. Nucleoid remodeling by an altered HU protein: reorganization of the transcription program. Proc Natl Acad Sci U S A **102:**16397-402.
91. **Kelln, R. A., J. J. Kinahan, K. F. Foltermann, and G. A. O'Donovan.** 1975. Pyrimidine biosynthetic enzymes of Salmonella typhimurium, repressed specifically by growth in the presence of cytidine. J Bacteriol **124:**764-74.
92. **Kessler, B., V. de Lorenzo, and K. N. Timmis.** 1993. Identification of a cis-acting sequence within the Pm promoter of the TOL plasmid which confers XylS-mediated responsiveness to substituted benzoates. J Mol Biol **230:**699-703.
93. **Kessler, B., S. Marqués, T. Köhler, J. L. Ramos, K. N. Timmis, and V. de Lorenzo.** 1994. Cross talk between catabolic pathways in Pseudomonas putida: XylS-dependent and -independent activation of the TOL meta operon requires the same cis-acting sequences within the Pm promoter. J Bacteriol **176:**5578-82.
94. **Khemici, V., and A. J. Carpousis.** 2004. The RNA degradosome and poly(A) polymerase of Escherichia coli are required in vivo for the degradation of small mRNA decay intermediates containing REP-stabilizers. Mol Microbiol **51:**777-90.
95. **King, N. D., and M. R. O'Brian.** 2001. Evidence for direct interaction between enzyme I(Ntr) and aspartokinase to regulate bacterial oligopeptide transport. J Biol Chem **276:**21311-6.
96. **Kofoid, E., U. Bergthorsson, E. S. Slechta, and J. R. Roth.** 2003. Formation of an F' plasmid by recombination between imperfectly repeated chromosomal Rep sequences: a closer look at an old friend (F'(128) pro lac). J Bacteriol **185:**660-3.
97. **Köhler, T., J. F. Alvarez, and S. Harayama.** 1994. Regulation of the rpoN, ORF102 and ORF154 genes in Pseudomonas putida. FEMS Microbiol Lett **115:**177-84.
98. **Kumagai, M., and H. Ikeda.** 1991. Molecular analysis of the recombination junctions of lambda bio transducing phages. Mol Gen Genet **230:**60-4.
99. **Kustu, S., A. K. North, and D. S. Weiss.** 1991. Prokaryotic transcriptional enhancers and enhancer-binding proteins. Trends Biochem Sci **16:**397-402.
100. **Laurie, A. D., L. M. Bernardo, C. C. Sze, E. Skarfstad, A. Szalewska-Palasz, T. Nystrom, and V. Shingler.** 2003. The role of the alarmone (p)ppGpp in sigma N competition for core RNA polymerase. J Biol Chem **278:**1494-503.
101. **Lee, C. R., B. M. Koo, S. H. Cho, Y. J. Kim, M. J. Yoon, A. Peterkofsky, and Y. J. Seok.** 2005. Requirement of the dephospho-form of enzyme IIA for derepression of *Escherichia coli* K-12 *ilvBN* expression. Mol Microbiol **58:**334-44.
102. **Macchi, R., L. Montesissa, K. Murakami, A. Ishihama, V. De Lorenzo, and G. Bertoni.** 2003. Recruitment of sigma54-RNA polymerase to the Pu promoter of Pseudomonas putida through integration host factor-mediated positioning switch of alpha subunit carboxyl-terminal domain on an UP-like element. J Biol Chem **278:**27695-702.

103. **MacGregor, C. H., S. K. Arora, P. W. Hager, M. B. Dail, and P. V. Phibbs, Jr.** 1996. The nucleotide sequence of the Pseudomonas aeruginosa pyrE-crc-rph region and the purification of the crc gene product. J Bacteriol **178:**5627-35.
104. **MacGregor, C. H., J. A. Wolff, S. K. Arora, and P. V. Phibbs, Jr.** 1991. Cloning of a catabolite repression control (crc) gene from *Pseudomonas aeruginosa,* expression of the gene in *Escherichia coli*, and identification of the gene product in *Pseudomonas aeruginosa*. J Bacteriol **173:**7204-12.
105. **Marqués, S., I. Aranda-Olmedo, and J. L. Ramos.** 2006. Controlling bacterial physiology for optimal expression of gene reporter constructs. Curr Opin Biotechnol **17:**50-6.
106. **Marqués, S., M. T. Gallegos, M. Manzanera, A. Holtel, K. N. Timmis, and J. L. Ramos.** 1998. Activation and repression of transcription at the double tandem divergent promoters for the xylR and xylS genes of the TOL plasmid of Pseudomonas putida. J Bacteriol **180:**2889-94.
107. **Marqués, S., A. Holtel, K. N. Timmis, and J. L. Ramos.** 1994. Transcriptional induction kinetics from the promoters of the catabolic pathways of TOL plasmid pWW0 of *Pseudomonas putida* for metabolism of aromatics. J Bacteriol **176:**2517-24.
108. **Marqués, S., M. Manzanera, M. M. González-Pérez, M. T. Gallegos, and J. L. Ramos.** 1999. The XylS-dependent Pm promoter is transcribed in vivo by RNA polymerase with sigma 32 or sigma 38 depending on the growth phase. Mol Microbiol **31:**1105-13.
109. **Marqués, S., M. Manzanera, M. M. González-Pérez, R. Ruíz, and J. L. Ramos.** 1999. Biodegradation, plasmid-encoded catabolic pathways, host factors and cell metabolism. Environ Microbiol **1:**103-4.
110. **Marqués, S., and J. L. Ramos.** 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Mol Microbiol **9:**923-9.
111. **Martín, R. G., and J. L. Rosner.** 1997. Fis, an accessorial factor for transcriptional activation of the mar (multiple antibiotic resistance) promoter of Escherichia coli in the presence of the activator MarA, SoxS, or Rob. J Bacteriol **179:**7410-9.
112. **Martínez-Antonio, A., and J. Collado-Vides.** 2003. Identifying global regulators in transcriptional regulatory networks in bacteria. Curr Opin Microbiol **6:**482-9.
113. **Martínez-Bueno, M., A. J. Molina-Henares, E. Pareja, J. L. Ramos, and R. Tobes.** 2004. BacTregulators: a database of transcriptional regulators in bacteria and archaea. Bioinformatics **20:**2787-91.
114. **Mermod, N., J. L. Ramos, A. Bairoch, and K. N. Timmis.** 1987. The xylS gene positive regulator of TOL plasmid pWWO: identification, sequence analysis and overproduction leading to constitutive expression of meta cleavage operon. Mol Gen Genet **207:**349-54.
115. **Merrick, M.** 1993. In a class of its own- the RNA polymerase sigma factor s54 (sN). Mol. Microbiol. **10:**903-909.
116. **Merrick, M., M. Taylor, M. J. Saier, and J. Reizer.** 1995. The role of the genes downstream of the N structural gene *rpoN* in *Klebsiella pneumoniae*. Nitrogen fixation: Fundamentals and applications., p. 189-194. Kluwer Academic Publishers, St. Petersburg, Russia.
117. **Merrick, M. J., and J. R. Coppard.** 1989. Mutations in genes downstream of the rpoN gene (encoding sigma 54) of Klebsiella pneumoniae affect expression from sigma 54-dependent promoters. Mol Microbiol **3:**1765-75.
118. **Metzger, S., I. B. Dror, E. Aizenman, G. Schreiber, M. Toone, J. D. Friesen, M. Cashel, and G. Glaser.** 1988. The nucleotide sequence and characterization of the relA gene of Escherichia coli. J Biol Chem **263:**15699-704.

119. **Metzger, S., E. Sarubbi, G. Glaser, and M. Cashel.** 1989. Protein sequences encoded by the relA and the spoT genes of Escherichia coli are interrelated. J Biol Chem **264:**9122-5.
120. **Michan, C., L. Zhou, M. T. Gallegos, K. N. Timmis, and J. L. Ramos.** 1992. Identification of critical amino-terminal regions of XylS. The positive regulator encoded by the TOL plasmid. J Biol Chem **267:**22897-901.
121. **Michiels, J., T. Van Soom, I. D'Hooghe, B. Dombrecht, T. Benhassine, P. de Wilde, and J. Vanderleyden.** 1998. The Rhizobium etli rpoN locus: DNA sequence analysis and phenotypical characterization of rpoN, ptsN, and ptsA mutants. J Bacteriol **180:**1729-40.
122. **Minagawa, J., H. Nakamura, I. Yamato, T. Mogi, and Y. Anraku.** 1990. Transcriptional regulation of the cytochrome b562-o complex in Escherichia coli. Gene expression and molecular characterization of the promoter. J Biol Chem **265:**11198-203.
123. **Morales, G., J. F. Linares, A. Beloso, J. P. Albar, J. L. Martínez, and F. Rojo.** 2004. The Pseudomonas putida Crc global regulator controls the expression of genes from several chromosomal catabolic pathways for aromatic compounds. J Bacteriol **186:**1337-44.
124. **Morett, E., and M. Buck.** 1989. In vivo studies on the interaction of RNA polymerase-sigma 54 with the Klebsiella pneumoniae and Rhizobium meliloti nifH promoters. The role of NifA in the formation of an open promoter complex. J Mol Biol **210:**65-77.
125. **Morfeldt, E., D. Taylor, A. von Gabain, and S. Arvidson.** 1995. Activation of alpha-toxin translation in Staphylococcus aureus by the trans-encoded antisense RNA, RNAIII. Embo J **14:**4569-77.
126. **Nakazawa, T.** 2002. Travels of a Pseudomonas, from Japan around the world. Environ Microbiol **4:**782-6.
127. **Newbury, S. F., N. H. Smith, E. C. Robinson, I. D. Hiles, and C. F. Higgins.** 1987. Stabilization of translationally active mRNA by prokaryotic REP sequences. Cell **48:**297-310.
128. **Nilsson, L., H. Verbeek, E. Vijgenboom, C. van Drunen, A. Vanet, and L. Bosch.** 1992. FIS-dependent trans activation of stable RNA operons of Escherichia coli under various growth conditions. J Bacteriol **174:**921-9.
129. **Ninnemann, O., C. Koch, and R. Kahmann.** 1992. The E.coli fis promoter is subject to stringent control and autoregulation. Embo J **11:**1075-83.
130. **Nogueira, T., and M. Springer.** 2000. Post-transcriptional control by global regulators of gene expression in bacteria. Curr Opin Microbiol **3:**154-8.
131. **Nojiri, H., M. Shintani, and T. Omori.** 2004. Divergence of mobile genetic elements involved in the distribution of xenobiotic-catabolic capacity. Appl Microbiol Biotechnol **64:**154-74.
132. **O'Neill, E., P. Wikstrom, and V. Shingler.** 2001. An active role for a structured B-linker in effector control of the sigma54-dependent regulator DmpR. Embo J **20:**819-27.
133. **Opel, M. L., K. A. Aeling, W. M. Holmes, R. C. Johnson, C. J. Benham, and G. W. Hatfield.** 2004. Activation of transcription initiation from a stable RNA promoter by a Fis protein-mediated DNA structural transmission mechanism. Mol Microbiol **53:**665-74.
134. **Oshima, T., C. Wada, Y. Kawagoe, T. Ara, M. Maeda, Y. Masuda, S. Hiraga, and H. Mori.** 2002. Genome-wide analysis of deoxyadenosine methyltransferase-mediated control of gene expression in Escherichia coli. Mol Microbiol **45:**673-95.
135. **O'Toole, G. A., K. A. Gibbs, P. W. Hager, P. V. Phibbs, Jr., and R. Kolter.** 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by Pseudomonas aeruginosa. J Bacteriol **182:**425-31.

136. **Pareja, E., P. Pareja-Tobes, M. Manrique, E. Pareja-Tobes, J. Bonal, and R. Tobes.** 2006. ExtraTrain: a database of Extragenic regions and Transcriptional information in prokaryotic organisms. BMC Microbiol **6:**29.
137. **Parekh, B. S., and G. W. Hatfield.** 1996. Transcriptional activation by protein-induced DNA bending: evidence for a DNA structural transmission model. Proc Natl Acad Sci U S A **93:**1173-7.
138. **Parvatiyar, K., E. M. Alsabbagh, U. A. Ochsner, M. A. Stegemeyer, A. G. Smulian, S. H. Hwang, C. R. Jackson, T. R. McDermott, and D. J. Hassett.** 2005. Global analysis of cellular factors and responses involved in *Pseudomonas aeruginosa* resistance to arsenite. J Bacteriol **187:**4853-64.
139. **Paulsen, I. T., C. M. Press, J. Ravel, D. Y. Kobayashi, G. S. Myers, D. V. Mavrodi, R. T. DeBoy, R. Seshadri, Q. Ren, R. Madupu, R. J. Dodson, A. S. Durkin, L. M. Brinkac, S. C. Daugherty, S. A. Sullivan, M. J. Rosovitz, M. L. Gwinn, L. Zhou, D. J. Schneider, S. W. Cartinhour, W. C. Nelson, J. Weidman, K. Watkins, K. Tran, H. Khouri, E. A. Pierson, L. S. Pierson, 3rd, L. S. Thomashow, and J. E. Loper.** 2005. Complete genome sequence of the plant commensal Pseudomonas fluorescens Pf-5. Nat Biotechnol **23:**873-8.
140. **Pérez-Martín, J., and V. De Lorenzo.** 1995. The amino-terminal domain of the prokaryotic enhancer-binding protein XylR is a specific intramolecular repressor. Proc. Natl. Acad. Sci. USA **92:**9392-9396.
141. **Pérez-Martín, J., and V. de Lorenzo.** 1996. ATP binding to the sigma 54-dependent activator XylR triggers a protein multimerization cycle catalyzed by UAS DNA. Cell **86:**331-339.
142. **Pérez-Martín, J., and V. de Lorenzo.** 1996. Identification of the repressor subdomain within the signal reception module of the prokaryotic enhancer-binding protein XylR of Pseudomonas putida. J Biol Chem **271:**7899-902.
143. **Pérez-Martín, J., and V. de Lorenzo.** 1996. In vitro activities of an N-terminal truncated form of XylR, a sigma 54-dependent transcriptional activator of Pseudomonas putida. J Mol Biol **258:**575-87.
144. **Pérez-Martín, J., and V. De Lorenzo.** 1995. Integration host factor suppresses promiscuous activation of the sigma 54-dependent promoter Pu of Pseudomonas putida. Proc Natl Acad Sci U S A **92:**7277-81.
145. **Pérez-Martín, J., and V. de Lorenzo.** 1996. Physical and functional analysis of the prokaryotic enhancer of the sigma 54-promoters of the TOL plasmid of Pseudomonas putida. J Mol Biol **258:**562-74.
146. **Pérez-Martín, J., and V. de Lorenzo.** 1995. The sigma 54-dependent promoter Ps of the TOL plasmid of Pseudomonas putida requires HU for transcriptional activation in vivo by XylR. J Bacteriol **177:**3758-63.
147. **Pérez-Martín, J., K. N. Timmis, and V. de Lorenzo.** 1994. Co-regulation by bent DNA. Functional substitutions of the integration host factor site at sigma 54-dependent promoter Pu of the upper-TOL operon by intrinsically curved sequences. J Biol Chem **269:**22657-62.
148. **Peter, B. J., J. Arsuaga, A. M. Breier, A. B. Khodursky, P. O. Brown, and N. R. Cozzarelli.** 2004. Genomic transcriptional response to loss of chromosomal supercoiling in Escherichia coli. Genome Biol **5:**R87.
149. **Petersen, L., and A. Krogh.** 2003. Modelling of Rho dependent transcription termination sites in the bacterium *Helicobacter pylori.* on-line publication.
150. **Petruschka, L., G. Burchhardt, C. Muller, C. Weihe, and H. Herrmann.** 2001. The cyo operon of Pseudomonas putida is involved in carbon catabolite repression of phenol degradation. Mol Genet Genomics **266:**199-206.
151. **Phillips, A. T., and L. M. Mulfinger.** 1981. Cyclic adenosine 3',5'-monophosphate levels in Pseudomonas putida and Pseudomonas aeruginosa during induction and carbon catabolite repression of histidase synthesis. J Bacteriol **145:**1286-92.

152. **Pierard, A., N. Glansdorff, D. Gigot, M. Crabeel, P. Halleux, and L. Thiry.** 1976. Repression of Escherichia coli carbamoylphosphate synthase: relationships with enzyme synthesis in the arginine and pyrimidine pathways. J Bacteriol **127:**291-301.
153. **Polayes, D. A., P. W. Rice, M. M. Garner, and J. E. Dahlberg.** 1988. Cyclic AMP-cyclic AMP receptor protein as a repressor of transcription of the spf gene of Escherichia coli. J Bacteriol **170:**3110-4.
154. **Poole, R. K., and G. M. Cook.** 2000. Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation. Adv Microb Physiol **43:**165-224.
155. **Powell, B. S., D. L. Court, T. Inada, Y. Nakamura, V. Michotey, X. Cui, A. Reizer, M. H. Saier, Jr., and J. Reizer.** 1995. Novel proteins of the phosphotransferase system encoded within the rpoN operon of Escherichia coli. Enzyme IIANtr affects growth on organic nitrogen and the conditional lethality of an erats mutant. J Biol Chem **270:**4822-39.
156. **Prieto, M. A., E. Díaz, and J. L. Garcia.** 1996. Molecular characterization of the 4-hydroxyphenylacetate catabolic pathway of Escherichia coli W: engineering a mobile aromatic degradative cluster. J Bacteriol **178:**111-20.
157. **Ramos, J. L., S. Marqués, and K. N. Timmis.** 1997. Transcriptional control of the Pseudomonas TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid- encoded regulators. Annu Rev Microbiol **51:**341-73.
158. **Ramos, J. L., C. Michan, F. Rojo, D. Dwyer, and K. Timmis.** 1990. Signal-regulator interactions. Genetic analysis of the effector binding site of xylS, the benzoate-activated positive regulator of Pseudomonas TOL plasmid meta-cleavage pathway operon. J Mol Biol **211:**373-82.
159. **Ramos, J. L., A. Stolz, W. Reineke, and K. N. Timmis.** 1986. Altered effector specificities in regulators of gene expression: TOL plasmid xylS mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA **83:**8467-8471.
160. **Ramos-González, M. I., M. J. Campos, J. L. Ramos, and M. Espinosa-Úrgel.** 2006. Characterization of the Pseudomonas putida mobile genetic element ISPpu10: an occupant of repetitive extragenic palindromic sequences. J Bacteriol **188:**37-44.
161. **Rappas, M., J. Schumacher, F. Beuron, H. Niwa, P. Bordes, S. Wigneshweraraj, C. A. Keetch, C. V. Robinson, M. Buck, and X. Zhang.** 2005. Structural insights into the activity of enhancer-binding proteins. Science **307:**1972-5.
162. **Rebbapragada, A., M. S. Johnson, G. P. Harding, A. J. Zuccarelli, H. M. Fletcher, I. B. Zhulin, and B. L. Taylor.** 1997. The Aer protein and the serine chemoreceptor Tsr independently sense intracellular energy levels and transduce oxygen, redox, and energy signals for Escherichia coli behavior. Proc Natl Acad Sci U S A **94:**10541-6.
163. **Reizer, J., A. Reizer, M. H. Saier, Jr., and G. R. Jacobson.** 1992. A proposed link between nitrogen and carbon metabolism involving protein phosphorylation in bacteria. Protein Sci **1:**722-6.
164. **Repik, A., A. Rebbapragada, M. S. Johnson, J. O. Haznedar, I. B. Zhulin, and B. L. Taylor.** 2000. PAS domain residues involved in signal transduction by the Aer redox sensor of Escherichia coli. Mol Microbiol **36:**806-16.
165. **Rescalli, E., S. Saini, C. Bartocci, L. Rychlewski, V. De Lorenzo, and G. Bertoni.** 2004. Novel physiological modulation of the Pu promoter of TOL plasmid: negative regulatory role of the TurA protein of *Pseudomonas putida* in the response to suboptimal growth temperatures. J Biol Chem **279:**7777-84.
166. **Rippe, K., N. Mucke, and A. Schulz.** 1998. Association states of the transcription activator protein NtrC from E. coli determined by analytical ultracentrifugation. J Mol Biol **278:**915-33.

167. **Ross, W., S. E. Aiyar, J. Salomon, and R. L. Gourse.** 1998. Escherichia coli promoters with UP elements of different strengths: modular structure of bacterial promoters. J Bacteriol **180:**5375-83.
168. **Ross, W., A. Ernst, and R. L. Gourse.** 2001. Fine structure of E. coli RNA polymerase-promoter interactions: alpha subunit binding to the UP element minor groove. Genes Dev **15:**491-506.
169. **Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse.** 1993. A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. Science **262:**1407-13.
170. **Ross, W., J. F. Thompson, J. T. Newlands, and R. L. Gourse.** 1990. E.coli Fis protein activates ribosomal RNA transcription in vitro and in vivo. Embo J **9:**3733-42.
171. **Rui, S., and Y. C. Tse-Dinh.** 2003. Topoisomerase function during bacterial responses to environmental challenge. Front Biosci **8:**d256-63.
172. **Ruíz, R., M. I. Aranda-Olmedo, P. Domínguez-Cuevas, M. I. Ramos-González, and S. Marqués.** 2004. Transcriptional regulation of the toluene catabolic pathways., p. 509-537. *In* R. JL (ed.), Pseudomonas, vol. 2. Kluwer Academic/Plenum Publishers, London.
173. **Ruíz, R., S. Marqués, and J. L. Ramos.** 2003. Leucines 193 and 194 at the N-terminal domain of the XylS protein, the positive transcriptional regulator of the TOL meta-cleavage pathway, are involved in dimerization. J Bacteriol **185:**3036-41.
174. **Ruíz, R., and J. L. Ramos.** 2002. Residues 137 and 153 at the N terminus of the XylS protein influence the effector profile of this transcriptional regulator and the sigma factor used by RNA polymerase to stimulate transcription from its cognate promoter. J Biol Chem **277:**7282-6.
175. **Ruíz, R., J. L. Ramos, and S. M. Egan.** 2001. Interactions of the XylS regulators with the C-terminal domain of the RNA polymerase alpha subunit influence the expression level from the cognate Pm promoter. FEBS Lett **491:**207-11.
176. **Ruíz-Manzano, A., L. Yuste, and F. Rojo.** 2005. Levels and activity of the Pseudomonas putida global regulatory protein Crc vary according to growth conditions. J Bacteriol **187:**3678-86.
177. **Ryu, S.** 1998. CRP.cAMP-dependent transcription activation of the Escherichia coli pts Po promoter by the heat shock RNA polymerase (Esigma32) in vitro. Mol Cells **8:**614-7.
178. **Saier, M. H., Jr.** 1989. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. Microbiol Rev **53:**109-20.
179. **Sarubbi, E., K. E. Rudd, H. Xiao, K. Ikehara, M. Kalman, and M. Cashel.** 1989. Characterization of the spoT gene of Escherichia coli. J Biol Chem **264:**15074-82.
180. **Schneider, R., A. Travers, and G. Muskhelishvili.** 2000. The expression of the Escherichia coli fis gene is strongly dependent on the superhelical density of DNA. Mol Microbiol **38:**167-75.
181. **Schroder, O., and R. Wagner.** 2002. The bacterial regulatory protein H-NS--a versatile modulator of nucleic acid structures. Biol Chem **383:**945-60.
182. **Seong, G. H., E. Kobatake, K. Miura, A. Nakazawa, and M. Aizawa.** 2002. Direct atomic force microscopy visualization of integration host factor-induced DNA bending structure of the promoter regulatory region on the Pseudomonas TOL plasmid. Biochem Biophys Res Commun **291:**361-6.
183. **Sheridan, S. D., C. J. Benham, and G. W. Hatfield.** 1998. Activation of gene expression by a novel DNA structural transmission mechanism that requires

supercoiling-induced DNA duplex destabilization in an upstream activating sequence. J Biol Chem **273:**21298-308.

184. **Shingler, V.** 2003. Integrated regulation in response to aromatic compounds: from signal sensing to attractive behaviour. Environ Microbiol **5:**1226-41.
185. **Shingler, V.** 1996. Signal sensing by sigma 54-dependent regulators: derepression as a control mechanism. Mol Microbiol **19:**409-16.
186. **Siegel, L. S., P. B. Hylemon, and P. V. J. Phibbs.** 1977. Cyclic adenosine 3',5'-monophosphate levels and activities of adenylate cyclase and cyclic adenosine 3',5'-monophosphate phosphodiesterase in Pseudomonas and Bacteroides. J Bacteriol **129:**87-96.
187. **Stern, M. J., E. Prossnitz, and G. F. Ames.** 1988. Role of the intercistronic region in post-transcriptional control of gene expression in the histidine transport operon of Salmonella typhimurium: involvement of REP sequences. Mol Microbiol **2:**141-52.
188. **Storz, G.** 2002. An expanding universe of noncoding RNAs. Science **296:**1260-3.
189. **Storz, G., J. A. Opdyke, and A. Zhang.** 2004. Controlling mRNA stability and translation with small, noncoding RNAs. Curr Opin Microbiol **7:**140-4.
190. **Su, W., S. Porter, S. Kustu, and H. Echols.** 1990. DNA-looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the bacterial glnA promoter. Proc Natl Acad Sci U S A **87:**5504-8.
191. **Suh, S. J., L. J. Runyen-Janecky, T. C. Maleniak, P. Hager, C. H. MacGregor, N. A. Zielinski-Mozny, P. V. Phibbs, Jr., and S. E. West.** 2002. Effect of vfr mutation on global gene expression and catabolite repression control of Pseudomonas aeruginosa. Microbiology **148:**1561-9.
192. **Swinger, K. K., and P. A. Rice.** 2004. IHF and HU: flexible architects of bent DNA. Curr Opin Struct Biol **14:**28-35.
193. **Sze, C. C., L. M. Bernardo, and V. Shingler.** 2002. Integration of global regulation of two aromatic-responsive sigma(54)-dependent systems: a common phenotype by different mechanisms. J Bacteriol **184:**760-70.
194. **Sze, C. C., T. Moore, and V. Shingler.** 1996. Growth phase-dependent transcription of the sigma(54)-dependent Po promoter controlling the Pseudomonas-derived (methyl)phenol dmp operon of pVI150. J Bacteriol **178:**3727-35.
195. **Taylor, B. L., and I. B. Zhulin.** 1998. In search of higher energy: metabolism-dependent behaviour in bacteria. Mol Microbiol **28:**683-90.
196. **Tendeng, C., and P. N. Bertin.** 2003. H-NS in Gram-negative bacteria: a family of multifaceted proteins. Trends Microbiol **11:**511-8.
197. **Tobes, R., and E. Pareja.** 2006. Bacterial repetitive extragenic palindromic sequences are DNA targets for Insertion Sequence elements. BMC Genomics **7:**62.
198. **Tobes, R., and E. Pareja.** 2005. Repetitive extragenic palindromic sequences in the Pseudomonas syringae pv. tomato DC3000 genome: extragenic signals for genome reannotation. Res Microbiol **156:**424-33.
199. **Tobes, R., and J. L. Ramos.** 2005. REP code: defining bacterial identity in extragenic space. Environ Microbiol **7:**225-8.
200. **Travers, A., and G. Muskhelishvili.** 2005. DNA supercoiling - a global transcriptional regulator for enterobacterial growth? Nat Rev Microbiol **3:**157-69.
201. **Tropel, D., and J. R. van der Meer.** 2004. Bacterial transcriptional regulators for degradation pathways of aromatic compounds. Microbiol Mol Biol Rev **68:**474-500.
202. **Tsuda, M., and T. Iino.** 1987. Genetic analysis of a transposon carrying toluene degrading genes on a TOL plasmid pWW0. Mol Gen Genet **210:**270-6.
203. **Tsuda, M., K. Minegishi, and T. Iino.** 1989. Toluene transposons Tn4651 and Tn4653 are class II transposons. J Bacteriol **171:**1386-93.

204. **Valls, M., M. Buckle, and V. de Lorenzo.** 2002. In vivo UV laser footprinting of the Pseudomonas putidasigma 54Pu promoter reveals that integration host factor couples transcriptional activity to growth phase. J Biol Chem **277:**2169-75.
205. **Valls, M., and V. de Lorenzo.** 2003. Transient XylR binding to the UAS of the Pseudomonas putida sigma54 promoter Pu revealed with high intensity UV footprinting in vivo. Nucleic Acids Res **31:**6926-34.
206. **van der Meer, J. R., and V. Sentchilo.** 2003. Genomic islands and the evolution of catabolic pathways in bacteria. Curr Opin Biotechnol **14:**248-54.
207. **van Montfort, R. L., T. Pijning, K. H. Kalk, I. Hangyi, M. L. Kouwijzer, G. T. Robillard, and B. W. Dijkstra.** 1998. The structure of the *Escherichia coli* phosphotransferase IIAmannitol reveals a novel fold with two conformations of the active site. Structure **6:**377-88.
208. **Velázquez, F., I. di Bartolo, and V. de Lorenzo.** 2004. Genetic evidence that catabolites of the Entner-Doudoroff pathway signal C source repression of the sigma54 Pu promoter of *Pseudomonas putida*. J Bacteriol **186:**8267-75.
209. **Velázquez, F., S. Fernández, and V. de Lorenzo.** 2006. The upstream-activating sequences of the sigma54 promoter Pu of Pseudomonas putida filter transcription readthrough from upstream genes. J Biol Chem **281:**11940-8.
210. **Versalovic, J., T. Koeuth, and J. R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res **19:**6823-31.
211. **Wagner.** 2003. Noncoding RNAs, p. pp 243-258, Edited by Jan Barciszewski and Volker A. Erdmann ed, vol. Chapter 17.
212. **Wagner, E. G., and K. Flardh.** 2002. Antisense RNAs everywhere? Trends Genet **18:**223-6.
213. **Wang, G., A. Peterkofsky, P. A. Keifer, and X. Li.** 2005. NMR characterization of the *Escherichia coli* nitrogen regulatory protein IIANtr in solution and interaction with its partner protein, NPr. Protein Sci **14:**1082-90.
214. **West, S. E., A. K. Sample, and L. J. Runyen-Janecky.** 1994. The vfr gene product, required for Pseudomonas aeruginosa exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. J Bacteriol **176:**7532-42.
215. **Wikstrom, P., E. O'Neill, L. C. Ng, and V. Shingler.** 2001. The regulatory N-terminal region of the aromatic-responsive transcriptional activator DmpR constrains nucleotide-triggered multimerisation. J Mol Biol **314:**971-84.
216. **Williams, P. A., R. M. Jones, and L. E. Shaw.** 2002. A third transposable element, ISPpu12, from the toluene-xylene catabolic plasmid pWW0 of Pseudomonas putida mt-2. J Bacteriol **184:**6572-80.
217. **Wolff, J. A., C. H. MacGregor, R. C. Eisenberg, and P. V. Phibbs, Jr.** 1991. Isolation and characterization of catabolite repression control mutants of Pseudomonas aeruginosa PAO. J Bacteriol **173:**4700-6.
218. **Wootton, J. C., and M. H. Drummond.** 1989. The Q-linker: a class of interdomain sequences found in bacterial multidomain regulatory proteins. Protein Eng **2:**535-43.
219. **Wu, H. Y., and M. Fang.** 2003. DNA supercoiling and transcription control: a model from the study of suppression of the leu-500 mutation in Salmonella typhimurium topA- strains. Prog Nucleic Acid Res Mol Biol **73:**43-68.
220. **Xu, J., and R. C. Johnson.** 1997. Cyclic AMP receptor protein functions as a repressor of the osmotically inducible promoter proP P1 in Escherichia coli. J Bacteriol **179:**2410-7.
221. **Xu, J., and R. C. Johnson.** 1995. Fis activates the RpoS-dependent stationary-phase expression of proP in Escherichia coli. J Bacteriol **177:**5222-31.

222. **Xu, J., and R. C. Johnson.** 1995. Identification of genes negatively regulated by Fis: Fis and RpoS comodulate growth-phase-dependent gene expression in Escherichia coli. J Bacteriol **177:**938-47.
223. **Yamada, H., S. Muramatsu, and T. Mizuno.** 1990. An Escherichia coli protein that preferentially binds to sharply curved DNA. J Biochem (Tokyo) **108:**420-5.
224. **Yang, C. C., and H. A. Nash.** 1989. The interaction of E. coli IHF protein with its specific binding sites. Cell **57:**869-80.
225. **Yang, Y., and G. F. Ames.** 1988. DNA gyrase binds to the family of prokaryotic repetitive extragenic palindromic sequences. Proc Natl Acad Sci U S A **85:**8850-4.
226. **Yuste, L., A. B. Hervas, I. Canosa, R. Tobes, J. I. Jiménez, J. Nogales, M. M. Pérez-Pérez, E. Santero, E. Díaz, J. L. Ramos, V. de Lorenzo, and F. Rojo.** 2006. Growth phase-dependent expression of the Pseudomonas putida KT2440 transcriptional machinery analysed with a genome-wide DNA microarray. Environ Microbiol **8:**165-77.
227. **Yuste, L., and F. Rojo.** 2001. Role of the *crc* gene in catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. J Bacteriol **183:**6197-206.
228. **Zheng, D., C. Constantinidou, J. L. Hobman, and S. D. Minchin.** 2004. Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. Nucleic Acids Res **32:**5874-93.
229. **Zhou, L. M., K. N. Timmis, and J. L. Ramos.** 1990. Mutations leading to constitutive expression from the TOL plasmid meta-cleavage pathway operon are located at the C-terminal end of the positive regulator protein XylS. J Bacteriol **172:**3707-10.