CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
ESTACIÓN EXPERIMENTAL DEL ZAIDÍN

UNIVERSIDAD DE GRANADA
FACULTAD DE CIENCIAS

ANÁLISIS GENÓMICO, TRANSCRIPCIONAL Y DE FLUJO DEL METABOLISMO DE LA GLUCOSA EN *Pseudomonas putida* KT2440.

Tesis Doctoral

Teresa del Castillo Santaella

2008

Editor: Editorial de la Universidad de Granada
Autor: Teresa del Castillo Santaella
D.L.: Gr. 2668 - 2007
ISBN: 978-84-338-4671-6

ANÁLISIS GENÓMICO, TRANSCRIPCIONAL Y DE FLUJO DEL METABOLISMO DE LA GLUCOSA EN *Pseudomonas putida* KT2440.

Memoria que presenta la licenciada
en Farmacia, Teresa del Castillo Santaella,
para aspirar al Título de Doctor

Fdo.: Teresa del Castillo Santaella

Vº Bº del Director

Fdo.: Juan Luis Ramos Martín
Doctor en Biología
Profesor de investigación del C.S.I.C

Universidad de Granada
2008

Esta Tesis Doctoral ha sido realizada en el
Departamento de Protección Ambiental de la
Estación Experimental del Zaidín (C.S.I.C.), Granada.

A mi familia
A Samuel

Cuando creas que lo que haces
es una gota en un océano,
piensa que sería menos el océano
sin esa gota.

Madre Teresa de Calcuta

AGRADECIMIENTOS

Quiero agradecerle a mi director de Tesis Doctoral el Dr. Juan Luis Ramos, la oportunidad que me dio hace cuatro años para unirme a su grupo de investigación y por todo el esfuerzo que ha puesto en guiarme a lo largo de estos años.

Darle las gracias al Dr. Uwe Sauer por aceptarme en su grupo, por la experiencia que viví en mis dos estancias en Zürich y todo lo que aprendí. A Simon, Sophi, Beat, Annik, por ser unos maravillosos compañeros y por supuesto a Tobias por ayudarme a sentirme como en casa en Suiza. De la segunda estancia, no puedo olvidar a; Roeland y Domonika, y en especial de Stephanie, por su gran optimismo y simpatía.

A mis padres y mis hermanas, mi maravillosa y gran familia que siempre han confiado en mi y me han dado todo su apoyo.

A Patri M., a Paola, a Águeda, por siempre tener una sonrisa, una frase de ánimo y un halago a mi bizcocho. A Patri D. y Chechu por compartir como suyas mis alegrías. A Toñi, por esa paciencia infinita que le permite arreglar un gel de mil pedazos o arrugado como una pasa y por poner orden para que todos lo disfrutemos. A Jesús de la Torre y Nené por ser unos estupendos compañeros de laboratorio y a nuestros nuevos fichajes Alí, Adela y Paloma. A Jesús Lacal por esa paciencia infinita que transmite que me ha servido de ejemplo para frenar mis nervios.

A Silvia Marqués por ser un ejemplo de mujer trabajadora, a Ana Segura, Maribel y Manolo, Tino, MªTrini, Peter, Rolf, Estrella… os admiro a todos.

Darles las gracias a Mai y a Carmen por su ayuda en el trabajo y a MªAngustias por hacer que funcione siempre la cocina.

No puedo olvidar a todos los que en estos años de una u otra manera me han ayudado para que todo resultase más fácil, gracias de corazón; Alejo, África, Sandy, Craig, Rene, Chema, Patri Godoy, Miguel, Amalia, María, Regina, Fátima, Vanina, Cecilia, Sara, Isabel, Lázaro, etc…

A Samuel, por mantenerme en pie cuando creía que no podía más, ofrecerme tu apoyo y consuelo en los momentos difíciles, por creerme capaz de hacerlo y y ayudarme a que se hiciese realidad, por tener siempre una frase que me saque una sonrisa, por demostrarme que la vida tiene cosas maravillosas y que el trabajo no debe consumirnos. Gracias bimbo por estar siempre a mi lado, eres mi vida.

ÍNDICE

Páginas

ABREVIATURAS **xxi**

ÍNDICE DE FIGURAS **xxiii**

ÍNDICE DE TABLAS **xxv**

I. INTRODUCCIÓN GENERAL **1**

1. El género *Pseudomonas* 3
    1.1. *Pseudomonas putida* 5

2. Metabolismo de carbohidratos por *Pseudomonas* 8
    2.1. Ruta de Entner-Doudoroff 8
    2.2. Ciclo de los intermediarios del gliceraldehído-3-fosfato 10
    2.3. Catabolismo de la glucosa 11
        2.3.1. Entrada de la glucosa al periplasma 11
        2.3.2. Vía fosforilativa 12
            a) Transporte 12
            b) Reacción para convertir la glucosa en 6-fosfogluconato 12
        2.3.3. Vía oxidativa 13
            a) Reacción que tiene lugar en el periplasma 13
            b) Transporte y metabolismo 13
        2.3.4. Patrones de inducción de los genes del catabolismo de glucosa 14
    2.4. Metabolismo de la fructosa y hexitoles 15
        2.4.1. Metabolismo de la fructosa 15
        2.4.2. Catabolismo de hexitoles 16
    2.5. Regulación de las enzimas degradativas del metabolismo de carbohidratos en *Pseudomonas* 16

3. El plásmido TOL: Modelo de control transcripcional de las rutas catabólicas 18
        3.1. Lazo *meta* 20
        3.2. Lazo *upper* 21

II. OBJETIVOS 23

III. RESULTADOS 27

III. CAPÍTULO 1. Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: Genomic and Flux analysis. **29**

RESUMEN 29
SUMMARY AND INTRODUCTION 31
MATERIALS AND METHODS 32
RESULTS 34
Isolation and characterization of mutants deficient in glucose metabolism in KT2440 34
KT2440 exhibits inducible glucokinase and gluconokinase activity 35
Net fluxes from glucose in the wild type and mutants in the early steps of glucose metabolism 35
Global analysis of the genes involved in glucose metabolism 36
(i) Gene organization and transcriptionalarchitecture of the peripheral glucose catabolism genes 36
(ii) Global transcriptomic analysis of KT2440 in response to glucose 37
DISCUSSION 39
Multiple peripheral and convergent pathways in the initial steps of glucose metabolism in *P. putida* 39
Glucose catabolism enzymes in all peripheral pathways are inducible 39
How are the glucose catabolic genes organized in other *Pseudomonas* species? 40
REFERENCES 40
SUPPLEMETAL MATERIAL 43

III. CAPÍTULO 2. A set of activators and repressors control peripheral glucosa pathways in *Pseudomonas putida* to yield a common central intermediate. **45**

RESUMEN 45
SUMMARY AND INTRODUCTION 47

MATERIALS AND METHODS 50
RESULTS 52
Construction of mutants in genes putatively involved in the regulation of glucosa metabolism and their characterization 52
Fusion of the glucosa catabolic operon promoter regions to *´lacZ* to validate the arrays 54
Identification of the transcription start point (tsp) of the glucose catabolism operon 54
DISCUSSION 55
REFERENCES 58
MATERIAL SUPLEMENTARIO 61

III. CAPÍTULO 3. Simultaneous catabolite repression between glucose and toluene metabolism in *Pseudomonas putida* is channeled through different signaling pathways. 63

RESUMEN 63
SUMMARY AND INTRODUCTION 65
MATERIALS AND METHODS 66
RESULTS 67
Simultaneous utilization of glucose and toluene by P. putida KT2440 and isogenic mutants of this strain 67
Results of the flux analyses support the simultaneous use of glucose and toluene by *P. putida* 67
Global transcriptomic response of P. putida KT2440 cells growing with glucose and toluene 69
Toluene affects the level of glucokinase activity in cells growing on glucose and toluene 70
Identification of the glucose metabolite(s) involved in toluene repression 70
Identification of master regulators involved in crossed repression of the toluene and glucose degradation genes 71
DISCUSSION 71
REFERENCES 72
SUPPLEMETAL MATERIAL 75

IV. DISCUSIÓN GENERAL **77**

1. Estudio del metabolismo de la glucosa en *Pseudomonas putida* 79

2. Estudio del metabolismo de la glucosa en bacterias del género *Pseudomonas* 82
El regulón *hex* y su entormo genético 83
El operón del metabolismo del gluconato 85
El operón del metabolismo del 2-cetogluconato 87

3. Reguladores del metabolismo de la glucosa en bacterias del género *Pseudomonas* 88
El regulador GltR 88
El regulador GnuR 89
El regulador PtxS 89
El regulador HexR 90

ANEXOS 93

V. CONCLUSIONES **101**

VI. BIBLIOGRAFÍA **105**

ADN Ácido desoxirribonucleico

ADP Adenosín difosfato

ARN Ácido ribonucleico

ARNP RNA polimerasa

ARNr Ácido ribonucleico ribosómico

AMP Adenosín monofosfato

AMPc Adenosín monofosfato cíclico

ATP Adenosín trifosfato

ARN Ácido ribonucleico

C Citosina

Eda 2-ceto-3-deoxy-6-fosfogluconato aldolasa

Edd 6-fosfogluconato dehidratasa

Fbp Fructosa-1,6-bifosfatasa

Fda Fructosa-1,3-bifosfato aldolasa

FU Sistema de transporte de fructosa

G Guanina

Gap Gliceraldehído-3-fosfato deshidrogenasa

Gcd Glucosa deshidrogenasa

GGU Sistema de transporte de glucosa y gluconato

Glk Glucoquinasa

H_2O Agua

HU Heat-unstable nucleoid protein

IHF Factor de integración (integration host factor)

Kb Kilobase

KDGP 2-ceto-3-deoxi-6-fosfogluconato

Mb millón de pares de bases

$NAD(P)^+$ Nicotinamida Adenina dinucleótido (fosfato)

OAA Oxalacetato

OP Operador

ORF Marco abierto de lectura

pb o **bp** Par(es) de bases

PEP Fosfoenolpiruvato

6PG 6-fosfogluconato

Pgi Glucosa-6-fosfato isomerasa

Pgl 6-fosfogluconolactonasa

PYR Piruvato

PTS Sistema de fosfotransferasa

REP Secuencias repetidas extragénicas palíndromicas

TCA Ciclo de los ácidos tricarboxílicos

TCAi Intermediarios del TCA

TNT 2,4,6-trinitrotolueno

μ_{max} Tasa máxima de crecimiento

UAS Secuencias activadoras (upstream activator sequence)

Zwf Glucosa-6-fosfato deshidrogenasa

ÍNDICE DE FIGURAS

INTRODUCCIÓN **Página**

Figura 1. Metabolismo de la glucosa, gluconato, 2-cetogluconato, manitol, sorbitol y fructosa en bacterias del género *Pseudomonas*. 9

Figura 2. Ciclo de los intermediarios del gliceraldehído-3-fosfato. 11

Figura 3. Catabolismo del tolueno o hidrocarburos similares hasta intermediarios del ciclo de Krebs. 19

Figura 4. Los promotores del plásmido TOL bajo el control de la proteína XylR y XylS. 20

CAPÍTULO I

Figure 1. Glucose catabolism in *P. putida* as deduced from gene annotations. 32

Figure 2. *In vivo* carbon flux distribution in *P. putida* KT2440 obtained from METAFoR and net-flux analyses. 35

Figure 3. Organization of the peripheral glucose catabolic genes. 37

Figure 4. Transcriptional pattern of the *gap-1, glk* and *edd* region involved in glucose catabolism. 38

CAPÍTULO II

Figure 1. Glucose catabolism in *P. putida* as deduced from gene annotations. 48

Figure 2. Organization of the peripheral glucose catabolic genes. 49

Figure 3. Transcription start point of the promoters in front of the *ptxS, kguE, gnuK, gnuR* and *gap-1* genes. 56

Supplementary Figure 1. Transcription start point of the promoters in front of the *edd, zwf-1, hexR* and *gstA* genes. 61

CAPÍTULO III

Figure 1. TOL plasmid promoters under the control of the XylR protein. 66

Figure 2. Integration of glucose and toluene metabolism into central metabolism in *P. putida* KT2440. 68

Figure 3. ^{13}C-labeling patterns in different amino acids derived from [^{13}C]-glucose.68

Figure 4. Induction of Pu in parental strain *P. putida* KT2440 and *edd* and *eda* mutants of this strain. 70

DISCUSIÓN

Figura 1. Organización de los genes del regulón *hex* y región adyacente. 84

Figura 2. Representación de los genes que codifican el operón del gluconato. 86

Figura 3. Representación de los genes del metabolismo del 2-cetogluconato. 88

ÍNDICE DE TABLAS

Páginas

CAPÍTULO I

Table 1. Strains and plasmid used in this study. 33

Table 2. Growth characteristics of *P. putida* KT2440 and isogenic mutants with mutations in the glucose metabolism network. 34

Table 3. Levels of Glk and GnuK in KT2440 and its isogenic mutants growing with different carbon sources. 35

Table 4. Selected flux rates obtained from net-flux analysis. 36

Table 5. Genes up-regulated in wild-type *P. putida* KT2440 cells growing on glucose versus cells growing on citrate. 38

Supplementary Table 1. Down-regulated genes. 43

CAPÍTULO II

Table 1. Strains and plasmids used in this study. 50

Table 2. Genes differentially up-regulated in the GnuR mutant background with respect to the genes expressed in the parental strain. 52

Table 3. Genes differentially up-regulated in the HexR mutant background with respect to the genes expressed in the parental strain. 53

Table 4. Genes differentially up-regulated in the PtxS mutant background with respect to the genes expressed in the parental strain. 53

Table 5. Genes differentially up-regulated in the GltR-2 mutant background with respect to the genes expressed in the parental strain. 54

Table 6. Expression from P_{edd} and P_{zwf} in KT2440 and the *hexR* mutant strain. 55

Supplementary Table 1. Primers used in primer extension analyses. 61

CAPÍTULO III

Table 1. Strains and plasmids used in this study. 66

Table 2. Growth rates and physiological parameters of *P. putida* KT2440(pWW0) and isogenic mutants of this strain growing with different carbon sources. 67

Table 3. *P. putida* KT2440 upregulated genes in cells growing on glucose plus toluene compared to cells growing on glucose alone. 69

Table 4. Glucokinase activities in wild-type and mutant cells. 70

Table 5. Expression of the Pu promoter fused to *lac*Z in the wild type and glucose mutants growing under different conditions. 70

Suppl. Table 1. *Pseudomonas putida* downregulated genes in cells growing on glucose plus toluene compared to cells grown on glucose alone. 75

Suppl. Table 2. Fold-change of genes encoding proteins for glucose metabolism in cells growing on glucose plus toluene and glucose alone. 76

ANEXOS

Anexo 1. Regulón *hex* y su entorno genético. 95

Anexo 2. Glucosa-6-fosfato deshidrogenasa: Zwf-2 y Zwf-3. 98

Anexo 3. Operón del metabolismo del gluconato. 98

Anexo 4. Operón del metabolismo del 2-cetogluconato. 99

INTRODUCCIÓN GENERAL

1. El género *Pseudomonas*

El género *Pseudomonas* fue descrito por Migula (1894) como "células con estructuras polares móviles. La formación de esporas se da en algunas especies, pero es un fenómeno raro". Esta definición tan genérica, fue aceptada durante mucho tiempo, dando origen a que numerosas bacterias originalmente adscritas como especies del género *Pseudomonas* fueran asignadas a otros géneros. Hasta los años 60 los intentos de clasificación de las bacterias se centraban en las características morfológicas o fisiológicas (Palleroni, 2003). Según la clasificación del manual de Bergey de Bacteriología Sistemática, las *Pseudomonas* son "células en forma de bastón, curvadas o derechas, pero no helicoidales, de entre 0.5 – 1 μm de diámetro por 1.5 – 5 μm de longitud". Sin embargo, el desarrollo de técnicas como la hibridación ADN/ADN o ARNr/ADN permitió una clara división de las especies de *Pseudomonas* (Palleroni *et al.,* 1972). Siguiendo esta última clasificación, Palleroni propuso cinco grupos taxonómicos (ARN-I a ARN-V). Más tarde, la secuenciación del ARNr 16S reflejó la diversidad de estos grupos, siendo finalmente el grupo ARN-I, dentro de la subclase-γ de Proteobacterias, el que a día de hoy se reconoce como el que engloba a las verdaderas *Pseudomonas* (Bergey *et al*., 2005).

Las *Pseudomonas* son Gram-negativas, generalmente móviles y presentan uno o varios flagelos polares, aunque también se han descrito flagelos laterales más cortos. Son aerobias, con un tipo de metabolismo respiratorio estricto con oxígeno como aceptor terminal de electrones, aunque en algunos casos el nitrato puede ser usado como aceptor alternativo de electrones, permitiendo el crecimiento anaerobio. Se ha descrito que la cepa JLR11 de la especie *P. putida*, puede utilizar el 2,4,6-trinitrotolueno (TNT) como aceptor alternativo de electrones (Esteve-Nuñez *et al.*, 2000). La mayoría de las especies son organotróficas y se cultivan a pH neutro, a temperaturas de la zona mesófila y no requieren factores de crecimiento. Son catalasas positivas y pueden ser oxidasas positivas o negativas. La mayoría de las especies del género *Pseudomonas* no acumulan gránulos de poli-β-hidroxibutirato pero cuando se cultivan en alcanos o gluconato pueden formar poli-hidroxialcanoatos (Haywood *et al.,* 1990). No se conocen estados de células viables no cultivables.

El espectro nutricional de cada especie del género *Pseudomonas* es característico presentando una menor variabilidad entre cepas de una misma especie. Este grupo de bacterias se caracteriza por su extrema versatilidad metabólica (Palleroni, 1993; Stainer *et al.,* 1966). Entre los compuestos órganicos que son capaces de metabolizar se encuentran hidratos de carbono, ácidos alifáticos, aminas, amidas, aminoácidos, compuestos aromáticos y alcoholes.

Las bacterias del género *Pseudomonas* del que se han descrito alrededor de 60 especies, además de su extraordinaria versatilidad metabólica, se caracterizan por su amplia distribución en el medio ambiente, habiéndose aislado de plantas a partir de suelos limpios y contaminados, y aguas continentales y marinas. El genoma de cinco especies diferentes de *Pseudomonas* se ha secuenciado recientemente y han revelado que todas ellas comparten un conjunto de 2000 genes de un total de 5000 a 6000 genes presentes en sus correspondientes genomas.

Entre las bacterias cuyo genoma se ha secuenciado, existen patógenas oportunistas como *P. aeruginosa* PAO1 (Stover *et al.,* 2000) y *P. entomophila* L48 (Vodovar *et al.*, 2006), un patógeno vegetal como *P. syringae* pv. tomato (Buell *et al.,* 2003) y dos bacterias inocuas como *P. putida* KT2440 (Nelson *et al.,* 2002) y *P. fluorescens* Pf-5 (Paulsen *et al.,* 2005). La comparación genómica entre cepas patógenas y cepas inocuas reveló que en las cepas no virulentas no portaban los genes que codifican exotoxinas, enzimas hidrolíticas específicas, factores de respuesta hipersensitiva y sistemas de secreción tipo III. En cambio, cepas virulentas y no virulentas comparten otras propiedades como adhesión y biosíntesis de polímero, pili tipo IV, adhesinas, proteínas relacionadas con estrés, etc (Nelson *et al.,* 2002). A continuación se describen algunas propiedades de estas especies. La especie *Pseudomonas putida* objeto de este estudio, se describirá más adelante.

Pseudomonas aeruginosa es una bacteria ubicua del medio ambiente que causa infecciones en una amplia variedad de hospedadores, incluyendo insectos, plantas y animales. En humanos, este organismo puede colonizar algunas superficies mucosas y puede invadir los tejidos y la sangre. Las infecciones pueden ser de corta duración y superficiales, bacteremias o infecciones crónicas como ocurre en pacientes con fibrosis quística. El genoma de las cepas PAO1 y UCBPP-PA14 (Lee *et al*., 2006)

se han secuenciado recientemente, habiendo sido el genoma de PAO1 el primer genoma secuenciado completo del género *Pseudomonas* (Stover *et al.,* 2000).

Pseudomonas syringae es un organismo fitopatógeno. Las cepas de esta especie se caracterizan por su diversidad y su interacción específica con diferentes especies de plantas, habiéndose descrito cerca de 50 patovares en base a la capacidad de la cepa de infectar diferentes especies de plantas (Jamir *et al.,* 2004). La cepa DC3000 que secuenció el grupo de A. Collmer es un fitopatógeno de plantas de tomate y *Arabidopsis* (Buell *et al.,* 2003).

Pseudomonas fluorescens es una rizobacteria que promueve el crecimiento de plantas. Además tiene propiedades de biocontrol, ya que protege las raíces de algunas especies de plantas contra hongos parásitos como *Fusarium* o *Pythium*, así como algunos nematodos (Paulsen *et al.,* 2005).

Las cepas pertenecientes al género *Pseudomonas* son a menudo tolerantes y/o resistentes a agentes nocivos presentes en suelos, incluyendo antibióticos, desinfectantes, detergentes, metales pesados y disolventes orgánicos. Por lo general, las *Pseudomonas* poseen múltiples plásmidos y transposones conjugativos o transmisibles que codifican los genes necesarios para el metabolismo de hidrocarburos y la resistencia a agentes nocivos. Existe un gran interés en explotar las características de estas bacterias por sus posibles aplicaciones en biotecnología, incluyendo biorremediación de sitios contaminados (Dejonghe *et al.,* 2001; Timmis *et al.,* 1994), mejorar la calidad de combustibles, por ejemplo mediante desulfurización (Galán *et al.,* 2000), biocatálisis para la producción de sustancias químicas de valor añadido (Rojas *et al.,* 2004; Schmid *et al.,* 2001; Wubbolts & Timmis, 1990; Zeyer *et al.,* 1985), la producción de bioplásticos (Olivera *et al.,* 2001) y agentes que fomentan el crecimiento de plantas y el control de plagas en plantas (Walsh *et al.*, 2001).

1.1. *Pseudomonas putida*

Las bacterias de la especie *Pseudomonas putida* pertenecen al grupo de *Pseudomonas* "fluorescentes", denominadas así por la producción de pigmentos que

emiten fluorescencia. Son bacterias quimiorganotróficas, muy versátiles metabólicamente, capaces de adaptarse a vivir en diferentes hábitas. Se han aislado a partir de suelos limpios, suelos agrícolas y suelos industriales contaminados, así como de aguas continentales. Este grupo de bacterias tienen un gran potencial en biorremediación de suelos, además su capacidad de colonizar la rizosfera de plantas le ha permitido que sea objeto de estudio para el desarrollo de nuevos biopesticidas (Espinosa-Urgel *et al.,* 2002).

La cepa *P. putida* KT2440 utilizada en este estudio deriva de la cepa *P. putida* mt-2 aislada en 1963 en Japón por Hosakawa. La cepa mt-2 se identificó como una cepa capaz de degradar *meta*-toluato, de donde derivó el nombre mt. En principio fue incluida dentro de la especie *Pseudomonas arvilla* y no fue hasta 1974 cuando se introdujo dentro de la especie *Pseudomonas putida* (Williams & Murray, 1974). En 1975 se comprobó que la cepa mt-2 portaba el plásmido TOL, el cual contiene los genes que codifican las enzimas necesarias para la degradación de toluenos y xilenos (Worsey & Williams, 1975). En 1981 se construyó *Pseudomonas putida* KT2440 como un derivado de mt-2 curado del plásmido TOL (Bagdasarian *et al.,* 1981) y presuntamente deficiente en el mecanismo de restricción de ADN exógeno, lo que hace que esta cepa sea ampliamente utilizada para la expansión de rutas catabólicas con fines degradativos (Bagdasarian & Timmis, 1982; Ramos *et al.,* 1994) y como hospedador en la clonación y expresión de genes heterólogos para su utilización en procesos de biotransformación de compuestos químicos con valor añadido (Delgado *et al.*, 1992; Kellerhals *et al.*, 1999; Kraak *et al.*, 1997) o de interés farmacológico (Tan *et al*., 1997).

Recientemente, se ha secuenciado el genoma de KT2440 (Nelson *et al.,* 2002), el cual tiene 6,18 Mb (Weinel *et al.,* 2002), con un contenido en G+C de 61,6%. En el genoma de esta cepa se han descrito 105 islas genómicas con un contenido distinto en GC (Weinel *et al.,* 2002). Estas islas genómicas están principalmente implicadas en la captación y degradación de compuestos orgánicos, transporte de iones, síntesis y secreción de metabolitos secundarios, siendo las principales responsables de la elevada versatilidad metabólica presente en *P. putida*. En numerosas ocasiones dicha versatilidad metabólica se encuentra ligada a plásmidos, que portan genes cuyos productos se encargan de canalizar diversos

sustratos hacia el metabolismo central. Por ejemplo, el plásmido TOL (Williams & Murray, 1974), el plásmido NAH7 (Dunn & Gunsalus, 1973) y el plásmido CAM (Rheinwald *et al.,* 1973) portan los genes implicados en el catabolismo de tolueno/xilenos, naftaleno y alcanfor, respectivamente. En el cromosoma de KT2440 se han encontrado genes que codifican rutas de degradación de compuestos orgánicos que en ocasiones son productos naturales como los derivados de la degradación de la lignina (Jiménez *et al.,* 2002) o xenobióticos como haloaromáticos, nitroaromáticos, etc… Todas estas características contribuyen al espectro nutricional de esta especie, permitiéndole reproducirse en distintos ambientes y metabolizar una amplia gama de compuestos naturales y sintéticos.

La anotación inicial del genoma de la cepa KT2440 sugería 5420 marcos de lectura abiertos. Dentro de estos, 80 genes aparecen truncados o alterados como consecuencia de la inserción de transposones o elementos móviles. A su vez, se han identificado 804 copias de una secuencia palindrómica repetida y extragénica de 35 pb conocidas como REP (Aranda-Olmedo *et al.,* 2002). Se han identificado en el cromosoma de KT2440 tres genomas de bacteriófagos. Dentro de los 5420 marcos de lectura abiertos predichos, aparecen 600 de éstos anotados como proteínas hipotéticas, es decir, proteínas sin función conocida. En muchos de los casos puede tratarse de artefactos producto de los algoritmos utilizados en la predicción de genes. Aproximadamente el 12% del genoma codifica sistemas de transporte, como por ejemplo, los transportadores de tipo ABC que constituyen la principal familia de transportadores presentes en KT2440. Además, esta cepa es capaz de protegerse frente a compuestos tóxicos exportándolos a través de bombas de extrusión como las bombas RND y los transportadores MFS (Ramos *et al.,* 2002). También ha adquirido sistemas mono- y di-oxigenasas, oxidorreductasas, ferredoxinas y citocromos, dehidrogenasas, proteínas del metabolismo del azufre y glutation-S-transferasas, factores sigmas alternativos, reguladores y sistemas de respuesta a estrés (Nelson *et al.,* 2002). KT2440 tiene 330 genes asociados a la biosíntesis y degradación de componentes de la superficie celular. Posee genes involucrados en la interacción planta-bacteria en la rizosfera, un conjunto de genes de biogénesis de fimbrias tipo IV, al menos cuatro operones de la biosíntesis del flagelo (Rodríguez-Hervás *et al.,* comunicación personal), de fibras rizadas y pilis tipo I, que le confiere a *P. putida* la característica de adhesión a superficies en general (Espinosa-Urgel *et al.*, 2002).

Dentro de la especie *P. putida* no se conoce ninguna cepa que sea patógena de plantas o animales. Esto hace que se haya explotado en el desarrollo de numerosas técnicas biotecnológicas, como por ejemplo, el diseño de nuevas rutas catabólicas destinadas a la degradación de compuestos contaminantes (Erb *et al.,* 1997; Ramos *et al.,* 1986; Ramos *et al.,* 1987a; Rojo *et al.,* 1987), producción de intermediarios en la síntesis química de moléculas complejas (Wubbolts & Timmis, 1990). A su vez, es un buen colonizador de rizosfera de maíz, trigo, fresa, caña de azúcar y espinacas (Espinosa-Urgel *et al.,* 2002). Hoy día, se están usando en el desarrollo de biopesticidas y como promotoras del crecimiento de plantas. La ubicuidad de *P. putida* refleja su elevada capacidad para adaptarse a una enorme variedad de condiciones físico-químicas presentes en los distintos hábitats donde vive, reflejando la capacidad que tiene la cepa de integrar las señales recibidas del medio externo con el estado fisiológico celular, llevando a la activación de una red compleja de regulación que controla el metabolismo celular (Regenhardt *et al.,* 2002).

2. Metabolismo de carbohidratos por *Pseudomonas*

Desde hace varias décadas se ha estudiado la importancia de las diferentes rutas de utilización de carbohidratos en el género *Pseudomonas*, basándose principalmente en el comportamiento de mutantes deficientes en enzimas de la ruta y en datos de experimentos de respirometría. En general, las bacterias del género *Pseudomonas* utilizan pocos carbohidratos como fuente de carbono, entre éstos se encuentra; glucosa, gluconato, 2-cetogluconato, fructosa, sorbitol y manitol (Lessie & Phibbs Jr, 1984) lo que indica un espectro muy reducido cuando se compara con otras especies, como *Escherichia coli* que utilizan glucosa, gluconato, fructosa, galactosa, sacarosa, maltosa, lactosa, arabinosa, trehalosa, sorbitol, manitol, galactitol, etc… Además la información sobre el metabolismo de carbohidratos se reducía al de la glucosa fundamentalmente en las cepas patógenas de la especie *P. aeruginosa* (Lessie & Phibbs Jr, 1984).

2.1. Ruta de Entner – Doudoroff

El catabolismo de carbohidratos en *Pseudomonas*, se caracteriza por no utilizar la ruta de Embden-Meyerhof debido a la falta del gen que codifica la 6-fosfofructokinasa (Raps & De Mosss, 1962; Schleissner *et al.,* 1997; Tiwari & Campbell, 1969; Wood & Schwerdt, 1954). En su lugar, utiliza la ruta de Entner-Doudoroff que se descubrió en *Pseudomonas saccharophila* (De Ley, 1960; Entner & Doudoroff, 1952), en la que se obtiene gliceraldehído-3-fosfato y piruvato a partir de 6-fosfogluconato que es el intermediario clave (Figura 1) (Blevins *et al.,* 1975; Entner & Doudoroff, 1952; Stern *et al.,* 1960). Posteriormente, experimentos de respirometría mostraron que esta ruta es la mayoritaria en otras *Pseudomonas* (Palumbo & Witter, 1969; Spangler & Gilmour, 1966).

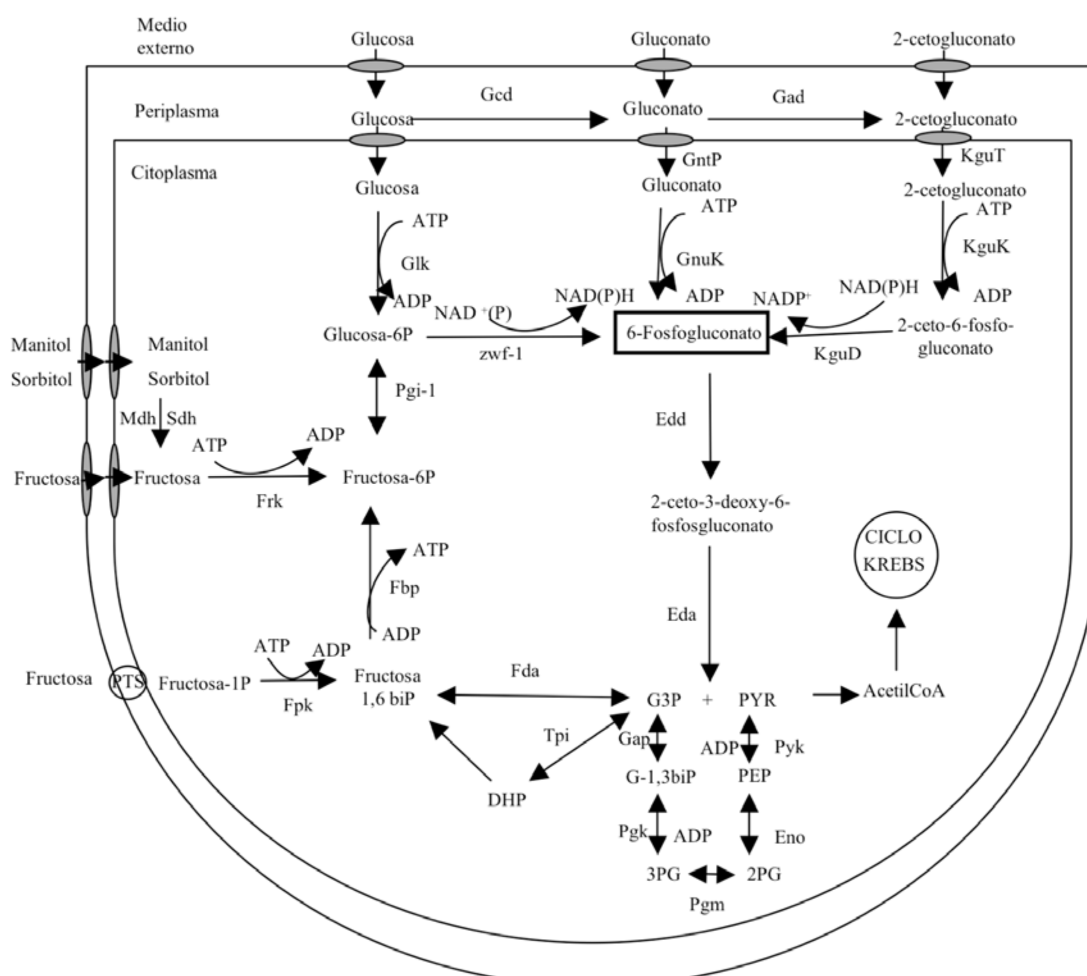


Figura 1. Metabolismo de la glucosa, gluconato, 2-cetogluconato, manitol, sorbitol y fructosa en bacterias del género *Pseudomonas*. Abreviaciones: Gcd, glucosa deshidrogenasa; Gad, gluconato deshidrogenasa; Mdh, manitol deshidrogenasa; Sdh, sorbitol deshidrogenasa; Glk, glucoquinasa; Zwf, glucosa-6-fosfato deshidrogenasa; GnuK, gluconoquinasa; KguK, 2-cetogluconato quinasa; KguD, 2-cetogluconato-6-fosfato reductasa; Edd, 6-fosfogluconato dehidratasa; Eda, 2-ceto-3-deoxi-6-fosfogluconato aldolasa; Pgi, glucosa-6-fosfato isomerasa; Fbp, fructosa-1,6-bifosfatasa; Fda, fructosa-1,3-bifosfato aldolasa; Fpk, fructosa-1-fosfato quinasa; Frk, fructoquinasa; PTS, sistema de transporte fosfotransferasa de fructosa; GntP, transportador de gluconato; KguT, transportador de 2-cetogluconato; Tpi, triosa fosfoato isomerasa; Gap, gliceraldehído-3-fosfato deshidrogenasa; Pgk, 3-fosfoglicerato quinasa; Pgm, fosfoglicerato mutasa; Eno, enolasa; Pyk, piruvato quinasa; PYR, piruvato; PEP, fosfoenolpiruvato; G3P, gliceraldehído-3-fosfato; G1,3biP, glicerato-1,3-bifosfato; 3PG, 3-fosfoglicerato; 2PG, 2-fosfoglicerato; DHP, dihidroxiacetona fosfato.

La ruta de Entner-Doudoroff parte del 6-fosfogluconato que se convierte por la 6-fosfogluconato dehidratasa (Edd) en 2-ceto-3-deoxi-6-fosfogluconato (KDGP), el cual y por la acción de la 2-ceto-3-deoxi-6-fosfogluconato aldolasa (Eda) da lugar a piruvato y gliceraldehído-3-fosfato (Lessie & Phibbs Jr, 1984) que se convierten posteriormente en intermediarios del ciclo de Krebs (Figura 1). En bacterias de las especies *P. aeruginosa* y *P. putida,* mutantes en la 6-fosfogluconato dehidratasa (Edd) son incapaces de crecer en glucosa, gluconato, 2-cetogluconato o manitol, pero crecen igual que la cepa silvestre en citrato, succinato o acetato (Blevins *et al.,* 1975; Vicente & Cánovas, 1973a; Willard *et al.,*1975). Esto contrasta con el comportamiento de *E. coli* que crecería utilizando la ruta de Embner-Meyerhof y las pentosas fosfato.

El gliceraldehído-3-fosfato puede rendir fructosa 1,6-bifosfato por acción de la fructosa-1,3-bifosfato aldolasa (Fda), y ésta por acción de la fructosa-1,6-bifosfatasa (Fbp) rinde fructosa-6-fosfato que tras isomerización por acción de la glucosa-6-fosfato isomerasa (Pgi) da lugar 6-fosfogluconato (Figura 1). El papel de la gluconeogésis no es muy importante en *P. putida,* debido a que un mutante en la fosfogliceratoquinasa (Pgk) y por tanto, deficiente en la gluconeogénesis muestra un crecimiento similar al de la cepa silvestre (Aparicio *et al.,* 1971).

2.2. Ciclo de los intermediarios del gliceraldehído-3-fosfato

El gliceraldehído-3-fosfato está en equilibrio con la dihidroxiacetona fosfato por la acción de triosa fosfato isomerasa (Tpi) (Hochster & Katznelson, 1958) (Figura 1). Además el gliceraldehído-3-fosfato por la acción de la gliceraldehído-3-fosfato deshidrogenasa (Gap) dependiente de NAD^+ da lugar al glicerato-1,3-bifosfato que por acción de la 3-fosfoglicerato quinasa (Pgk) dependiente de ADP forma el glicerato-3-fosfato. Este último a través de la fosfoglicerato mutasa (Pgm) da lugar al glicerato-2-fosfato y por acción de la enolasa (Eno) rinde fosfoenolpiruvato (PEP). Las reacciones mediadas por la triosa fosfato isomerasa, la gliceraldehído 3-fosfato deshidrogenasa, la 3-fosfoglicerato quinasa, la fosfoglicerato mutasa y la enolasa son reversibles (Figura 1). El PEP se convierte en piruvato (PYR) gracias a la piruvato quinasa (Pyk) dependiente de ADP (Figura 2). La PEP sintasa (PEPs) permite el paso de piruvato a PEP que se inhibe por la presencia de AMP y PEP, en cambio la Pyk se

activa en presencia de AMP y se inhibe por ATP. El piruvato puede entrar directamente a nivel del oxalacetato (OAA) en el ciclo de Krebs por la acción de la piruvato carboxilasa (Pyc), que es una enzima inducible en bacterias cultivadas en glucosa o lactato (O`Brien *et al.,* 1977). Y el OAA puede convertirse en piruvato por la acción de la OAA descarboxilasa (OAAd). Para cerrar este ciclo, el PEP puede transformarse en OAA por la PEP carboxilasa (PEPc) que es activada por acetil-CoA, ADP e inhibida por aspartato y viceversa por acción de la PEP carboxiquinasa (PEP ck) en *P. aeruginosa* PAO1, *P. syringae* DC3000 y *P. entomophila* L48 (O`Brien *et al.,* 1977). En las demás *Pseudomonas* cuyo genoma se ha secuenciado no existe la PEP carboxiquinasa (Figura 2).

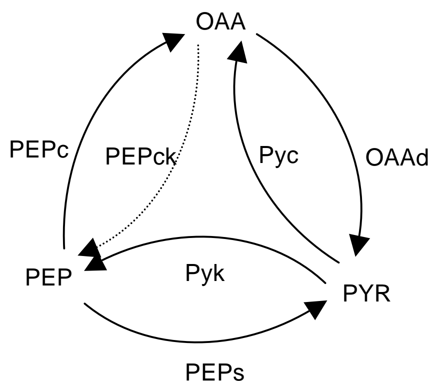


Figura 2. Ciclo de los intermediarios del gliceraldehído-3-fosfato. OAA, oxalacetato; PEP, fosfoenopiruvato; PYR, piruvato; OAAd, oxalacetato descarboxilasa; Pyc, piruvato carboxilasa; PEPc, fosfoenolpiruvato carboxilasa; PEPck fosfoenolpiruvato carboxiquinasa; Pyk, piruvato quinasa; PEPs , fosfoenolpiruvato sintasa.

2.3. Catabolismo de la glucosa

2.3.1. Entrada de la glucosa al periplasma

Las bacterias Gram-negativas tienen dos membranas que separan el citoplasma del medio externo; la membrana externa y la membrana interna. En la membrana externa existen porinas que permiten el paso de pequeñas moléculas por difusión (Nikaido, 1992), sin embargo el paso al citoplasma requiere, en general, de proteínas de membrana interna que suelen utilizar energía para el transporte de los solutos (Braun, 1995).

La glucosa atraviesa preferentemente la membrana externa a través de la porina OprB que es inducida por éste azúcar, por el que posee alta afinidad y especificidad y es reprimida por ácidos orgánicos. La porina OprB de *P. putida* es funcionalmente homóloga a la porina OprB de *P. aeruginosa* (Saravolac *et al.,*1991).

Estudios realizados en *P. aeruginosa* con la porina OprB demostraron que permite el paso de disacáridos y que posee preferencia por la glucosa y monosacáridos relacionados (Trias *et al.,* 1988).

En *Pseudomonas*, la glucosa puede seguir dos vías en el periplasma para dar lugar al 6-fosfogluconato: la fosforilativa, en la cual la glucosa es transportada al citoplasma y fosforilada a glucosa-6-fosfato (Eisenberg *et al.*, 1974; Haas & Holloway, 1978; Hunt & Phibbs Jr, 1983; Lessie & Neidhardt, 1967; Phibbs Jr & Eagon, 1970; Phibbs Jr *et al.,* 1978; Tiwari & Campbell, 1969; Whitfield *et al.*, 1982) y la oxidativa, que tiene lugar en el periplasma (Hunt y Phibbs Jr, 1983; Midgley & Dawes, 1973; Quay *et al.,* 1972; Roberts *et al.*, 1973; Vicente & Cánovas, 1973a; Wood & Schwerdt, 1954) oxidando la glucosa a gluconato y 2-cetogluconato (Figura 1).

2.3.2. Vía fosforilativa

a) Transporte

Las bacterias del género *Pseudomonas* pueden transportar la glucosa al interior de la célula, atravesando la membrana interna, por un sistema de transporte activo que requiere una proteína de unión a la glucosa sugiriendo que forma parte de un sistema de tipo ABC (Cuskey *et al.,* 1985; Higgins *et al.* 1990), en el que no está implicado el sistema fosfoenolpiruvato fosfotransferasa (PTS) (Midgley & Dawes, 1973). Este transporte de glucosa que requiere energía metabólica, está sujeto a inducción (Basu *et al.,* 2006), represión e inhibición competitiva, lo que muestra la estereoespecificidad del sistema. Además se reprime fuertemente por acetato, citrato, succinato, fumarato y malato, e incluso en presencia de un exceso de glucosa en el medio (Hylemon & Phibbs, 1972; Mukkada *et al.,* 1973).

b) Reacción para convertir la glucosa en 6-fosfogluconato

La glucosa una vez en el citoplasma es fosforilada por la glucoquinasa dependiente de ATP (Glk) dando lugar a glucosa-6-fosfato, la cual se oxida por la glucosa-6-fosfato deshidrogenada (Zw*f*) dependiente de $NAD(P)^+$ y rinde 6-

fosfogluconato (Coffee & Hu, 1970; Coffee & Hu, 1972; Eisenberg *et al.*, 1974; Guymon & Eagon, 1974; Hunt y Phibbs Jr, 1983; Lessie y Neidhardt, 1967; Lynch & Franklin, 1978; Midgley & Dawes, 1973; Tiwari & Campbell, 1969; Whiting *et al.,* 1976b; Wood & Schwerdt, 1953). La glucosa-6-fosfato deshidrogenasa (Zwf) de las bacterias de las especies *P. aeruginosa* y *P. fluorescens* puede utilizar como aceptor de electrones tanto $NADP^+$ como NAD^+, además ambas son inducibles y sensibles a control por represión catabólica (Lessmann *et al.,* 1975; Phibbs *et al.,* 1978). Se han descrito y purificado dos isoenzimas glucosa-6-fosfato deshidrogenasa en las bacterias de la especie *P. fluorescens,* siendo una de las enzimas inhibida por ATP (Lessmann *et al.,* 1975).

2.3.3. Vía oxidativa

a) Reacción que tiene lugar en el periplasma

Los primeros estudios en *Pseudomonas* (De Ley, 1960; Lockwood *et al.,* 1941; Stokes & Campbell, 1951) identificaron la ruta oxidativa con la conversión de glucosa en 6-fosfogluconato en la que el gluconato y el 2-cetogluconato eran los intermediarios. Ello ocurre en dos oxidaciones sucesivas en el periplasma e independientes de piridín nucleótido (Midgley & Dawes, 1973; Narrod & Wood, 1956; Roberts *et al.*, 1973) en las que la glucosa rinde gluconato por la acción de la glucosa deshidrogenasa (Gcd) y el gluconato rinde 2-cetogluconato por acción de la gluconato deshidrogenasa (Gad) (Matsushita & Ameyama, 1982; Matsushita *et al.,* 1982). La ventaja que tiene *Pseudomonas* de convertir la glucosa rápidamente a gluconato y/o 2-cetogluconato podría deberse a la competición entre bacterias por la fuente de carbono (Whiting *et al.,* 1976a; 1976b). En algunas cepas de *Pseudomonas* cuando se cultivan en glucosa acumulan gluconato y 2-cetogluconato en el medio, y en el caso concreto de la especie *P. fluorescens* se ha cuantificado (15.21 mmol $g^{-1}h^{-1}$) (Fuhrer *et al.,* 2005).

b) Transporte y metabolismo

El gluconato en el periplasma puede ser transportado al interior de la célula, por un transportador específico de gluconato (GntP), y fosforilado por la

gluconokinasa dependiente de ATP (GnuK) dando lugar al 6-fosfogluconato (Figura 1). Igualmente el 2-cetogluconato, resultante de la oxidación del gluconato en el periplasma, puede ser transportado al interior de la célula, por un transportador específico de cetogluconato (KguT) (Lynch & Franklin, 1978; Torrontegui *et al.,* 1976; Whiting *et al.,* 1976a; Whiting *et al.,* 1976b). Una vez en el citoplasma el 2-cetogluconato es fosforilado por la 2-cetogluconato kinasa dependiente de ATP (KguK) dando lugar a 2-ceto-6-fosfogluconato (Mukadda *et al.,* 1973; Narrod & Wood, 1956; Roberts *et al*., 1973), el cual es posteriormente reducido por la 2-cetogluconato-6-fosfato reductasa dependiente de NAD(P)H (KguD) dando lugar al 6-fosfogluconato (Frampton & Wood, 1961; Nandadasa *et al.,* 1974; Roberts *et al*., 1973) (Figura 1). Vicente & Cánovas (1973a) demostraron que la glucosa se cataboliza principalmente, pero no completamente, por oxidación a gluconato en *Pseudomonas putida* ATCC 12633. Además describieron que dentro de la vía oxidativa la minoritaria era la fosforilación del gluconato a 6-fosfogluconato y la mayoritaria la vía del 2-cetogluconato.

Aunque las vías fosforilativa y oxidativa están presentes en *P. aeruginosa* PAO1, se ha propuesto que las bacterias de esta cepa utilizan fundamentalmente la vía oxidativa en condiciones de aerobiosis y la vía fosforilativa cuando se cultivan bajo condiciones de anaerobiosis (Hunt & Phibbs Jr*,* 1981; Hunt & Phibbs Jr*,* 1983).

2.3.4. Patrones de inducción de los genes del catabolismo de glucosa

Vicente & Cánovas (1973b) demostraron que la glucosa, el gluconato y el 2-cetogluconato inducían la síntesis de la glucosa deshidrogenasa, gluconato deshidrogenasa, 6-fosfogluconato dehidratasa y KDGP aldolasa en *P. putida* ATCC 12633. La fructosa y el glicerol también las inducían pero los niveles de las enzimas fueron más bajos. No obstante, la gliceraldehído-3-fosfato deshidrogenasa estaba derreprimida con estos sustratos y solo se indujo en presencia de compuestos de tres carbonos como el glicerol o el glicerato. Mientras que los niveles de la actividad gluconokinasa incrementaron con glucosa y gluconato, la actividad glucosa-6-fosfato deshidrogenasa y de la 2-cetogluconato quinasa aumentaron en presencia de glucosa, gluconato y 2-cetogluconato, y los niveles de inducción de KDGP aldolasa fueron mayores en presencia de su sustrato, KDGP (Vicente & Cánovas., 1973b).

2.4. Metabolismo de la fructosa y hexitoles

2.4.1. Metabolismo de la fructosa

Una de las vías de entrada y metabolismo de la fructosa en *P. putida* es a través del sistema acoplado de la actividad fosfotransferasa dependiente de PEP (PTS). En el sistema PTS se transfiere el fosfato del PEP a tres componentes: EI, HPr y EII para finalmente cederlo al azúcar (Velázquez *et al.,* 2007). El componente EII es específico del azúcar, y es un polipéptido que consta de tres subdominios (EIIA, EIIB y EIIC). En el cromosoma de *P. putida* KT2440, el componente EI::HPr::EIIA^{Fru} está codificado por *fruB*, y EIIB::EIIC^{Fru} por *fruA*. Cuando se aislaron mutantes en el gen *fruB* de la cepa *P. putida* KT2440, éstos fueron incapaces de crecer en fructosa deduciéndose que era el principal sistema de transporte de fructosa, pero estos resultados no coinciden con las predicciones genómicas (Velázquez *et al.,* 2007). En el genoma de KT2440 hay genes que permitirían que la fructosa entrase en la célula y se fosforilara a fructosa-1-fosfato, la cual a través de la 1-fosfofructoquinasa (Fpk) daría lugar, a la fructosa-1,6-bifosfato (Figura 1) (Phibbs *et al.,* 1978; Sawyer *et al.,* 1977). La fructosa-1,6-bifosfato tiene dos vías para rendir piruvato. La vía minoritaria donde la fructosa-1,6-bifosfato rinde gliceraldehído-3-fosfato y piruvato, y la mayoritaria, la vía Entner-Doudoroff, donde la fructosa-1,6-bifosfato se convierte en 6-fosfogluconato por la acción de la glucosa-6-fosfato isomerasa (Pgi) y la glucosa-6-fosfato deshidrogenasa (Zwf), ya que un mutante en uno de estos dos genes presentan una tasa de crecimiento extremadamente baja cuando se cultivaron en fructosa como única fuente de carbono (Phibbs *et al.,* 1978). Tanto el sistema PTS como la 1-fosfofructoquinasa se inducen en presencia de fructosa (Phibbs *et al.,* 1978).

Otro sistema de transporte de fructosa en *Pseudomonas putida* es el denominado FU que puede transportar tanto glucosa como fructosa, aunque sólo la fructosa lo induce, y el succinato lo reprime (Vicente, 1975). Existe un sistema de transporte de glucosa-gluconato (GGU) que también puede utilizarlo la fructosa para entrar en la célula (Vicente, 1975). En la bacterias de la especie *P. aeruginosa*, la fructosa es transportada al citoplasma por transporte activo y es convertida en fructosa-6-fosfato por la fructoquinasa (Frk) dependiente de ATP (Figura 1) (Phibbs *et al.,* 1978).

2.4.2. Catabolismo de hexitoles

El catabolismo de otros hexitoles como el manitol o el sorbitol se ha estudiado en *P. aeruginosa* (Phibbs *et al.,* 1978). El manitol y el sorbitol se convierten en fructosa, fructosa-6-fosfato, glucosa-6-fosfato y 6-fosfogluconato, que entra en la ruta de Entner-Doudoroff (Figura 1). El crecimiento en presencia de manitol/sorbitol indujo el sistema de transporte activo del manitol/sorbitol respectivamente, la manitol/sorbitol deshidrogenasa (Mdh/Sdh) dependiente de NAD^+, la fructoquinasa, la glucosa-6-fosfato deshidrogenasa, la 6-fosfogluconato dehidratasa y la KDPG aldolasa (Figura 1) (Phibbs *et al.,* 1978). En *P. aeruginosa* PAO1, la utilización de manitol/sorbitol se bloquea en mutantes de la glucosa-6-fosfato deshidrogenasa (Zwf) y de la glucosa-6-fosfato isomerasa (Pgi) (Banerjee *et al.,* 1983, Phibbs *et al.,* 1978). Mutantes aislados en el gen que codifica la fructoquinasa (Frk) no crecen en manitol ni en sorbitol, lo que indica que la fructosa-6-fosfato es un intermediario clave en el metabolismo de ambos hexitoles (Phibbs *et al.,* 1978). Mutantes deficientes en Edd o Eda en *Pseudomonas aeruginosa* tampoco utilizan manitol o sorbitol como fuente de carbono (Blevins *et al.,* 1975; Phibbs *et al.,* 1978).

2.5. Regulación de las enzimas degradativas del metabolismo de carbohidratos en *Pseudomonas*

Pseudomonas crece rápidamente en ácidos tricarboxílicos como citrato, succinato y malato como única fuente de carbono y energía. Cuando las bacterias crecen en una mezcla de ácidos tricarboxílicos y carbohidratos, experimentan un crecimiento diáuxico, consumiendo primero los ácidos tricarboxílicos y después los carbohidratos (Anderson & Wood, 1969; Blevins *et al*., 1975; Hylemon & Phibbs, 1972; Lynch & Franklin, 1978; Tiwari & Campbell, 1969). Este crecimiento diáuxico se produce como consecuencia de la represión de las enzimas de utilización de los carbohidratos y de los sistemas de transporte de éstos (Lynch y Franklin, 1978; Midgley & Dawes, 1973; Ng & Dawes, 1973; Tiwari & Campbell, 1969; Whiting *et al.,* 1976a). En Enterobacterias el contenido en AMPc es el que ajusta la represión catabólica (Bostford, 1981), pero en los estudios realizados en *Pseudomonas* no se encontraron diferencias en los niveles intracelulares de AMPc cuando las células se cultivaron en azúcares o ácidos orgánicos (Siegel *et al.,* 1977). Un ejemplo de

represión catabólica es el que se descubrió cuando se cultivaban células de *P. aeruginosa* en un quimiostato con citrato y glucosa. Se observó que en esas condiciones se afectaba de forma directa el nivel de las enzimas de la vía oxidativa, la vía fosforilativa y de la ruta Entner-Doudoroff y además disminuía la capacidad de transportar glucosa (Ng & Dawes, 1973; Whiting *et al.,* 1976a). Cuando se estudió la vía oxidativa y fosforilativa en *P. aeruginosa,* se observó que el gluconato jugaba un papel crítico en la vía fosforilativa, ya que inhibía el transporte de glucosa y reprimía la formación del sistema de transporte de éste azúcar, favoreciendo así la vía oxidativa (Midgley & Dawes, 1973; Whiting *et al*., 1976a; 1976b). Por otro lado, en las bacterias de la especie *P. fluorescens*, el malato bloquea el transporte de gluconato y 2-cetogluconato pero no afecta a la conversión de glucosa a 2-cetogluconato (Lynch & Franklin, 1978). Esto sugería que en *Pseudomonas fluorescens* no se reprimía la síntesis de la glucosa deshidrogenasa y la gluconato deshidrogenasa.

En las bacterias de la especie *P. aeruginosa* las enzimas Zwf, Eda, Glk y Edd son estrictamente coinducibles en presencia de glucosa, gluconato, manitol, fructosa, glicerol o glicerol-3-fosfato y están sujetas a represión catabólica por intermediarios del ciclo del ciclo de Krebs (Temple *et al.,* 1990). Sin embargo, cuando Petruschka *et al.* (2002) estudiaron la represión catabólica del promotor del gen *zwf* (P_{zwf}), en *P. putida* H y KT2440, observaron que el efecto del succinato y el lactato en la inducción de la transcripción de *zwf* y la actividad glucosa-6-fosfato deshidrogenasa era mucho más bajo que el encontrado en *P. aeruginosa*. Estos resultados, sugerían que la expresión de P_{zwf} no estaba sujeta a represión catabólica por succinato en ambas cepas de *P. putida.*

Además, la actividad de la glucosa-6-fosfato deshidrogenasa (Zwf) de la vía fosforilativa se inhibe por ATP (Lessie & Neidhardt, 1967; Lessmann *et al*., 1975; Maurer *et al*., 1982; Schindler & Schlegel, 1969), la cual provoca una disminución en su afinidad por NAD^+ y/o por la D-glucosa-6-fosfato. Las bacterias de la especie *P. fluorescens* tienen dos enzimas Zwf; una sensible a ATP y activa con NAD^+ o $NADP^+$ y otra específica de $NADP^+$. En cambio, *P. aeruginosa* sólo presenta una glucosa-6-fosfato deshidrogenasa, que es inhibida fuertemente por ATP (Lessie & Neidhardt, 1967). Se ha sugerido que la fuerte inhibición de Zwf por ATP ocurre para prevenir la degradación de ATP bajo condiciones de gluconeogénesis, donde las hexosas fosfato

son esenciales para la biosíntesis del material de la pared celular y otras macromoléculas precursoras (Lessie & Neidhardt, 1967).

3. El plásmido TOL: Modelo de control transcripcional de las rutas catabólicas

Se han aislado plásmidos que degradan tolueno y xilenos, de diferentes localizaciones alrededor del mundo. Estos plásmidos tienen diferentes tamaños, incompatibilidades de grupo, organización de genes catabólicos, pero normalmente codifican rutas enzimáticas similares (Assinder & Williams, 1990).

El plásmido TOL pWW0 (117 kb) de *P. putida* es el plásmido catabólico mejor estudiado y fue aislado por Peter Williams a principios de los años 70. El plásmido permite a *P. putida* degradar tolueno y xilenos, *m*-etiltolueno y 1,3,4-trimetilbenceno (Assinder & Williams, 1990; Kunz & Chapman,1981; Worsey & Williams, 1975). La transferencia del plásmido a otras *Pseudomonas* les confiere la capacidad de utilizar estos hidrocarburos (Ramos-González *et al.,* 1991). La información genética para el metabolismo coordinado de estos hidrocarburos está codificada por los operones *xyl* del plásmido TOL, los cuales se localizan en dos transposones Tn*4651* (con un tamaño de 56 kb) y Tn*4653* (con un tamaño de 70 kb) (Shingler & Pavel, 1995). Posee 148 marcos de lectura abiertos (ORFs) (Greated *et al.,* 2002) y se ha localizado la zona del origen de replicación y la región implicada en las funciones de transferencia (Franklin *et al.,* 1981).

Los genes *xyl* se agrupan en dos bloques diferenciados que se transcriben en la misma dirección; el operón *upper*, que codifica las enzimas para oxidar tolueno/xilenos hasta ácido benzoico/toluatos y el operón *meta,* en el que las enzimas oxidan el ácido benzoico/toluatos hasta los intermediarios del ciclo de Krebs. La degradación de tolueno e hidrocarburos similares hasta los intermediarios del ciclo de Krebs comienza con la oxidación del grupo metilo del carbono 1 del anillo aromático por acción de una monooxigenasa para producir el correspondiente alcohol bencílico (Figura 3). La oxidación posterior de la cadena lateral se lleva a cabo por dos deshidrogenasas dando lugar al benzaldehído, y éste al benzoato. Este conjunto de reacciones constituye la ruta *upper*. Los ácidos carboxílicos son metabolizados a

través de la ruta *meta*, en la cual el benzoato es oxidado y descarboxilado en el catecol correspondiente. Estos compuestos sufren una fisión en *meta* para dar lugar al ácido 2-hidroximucónico semialdehído. El metabolismo del semialdehído rinde 2-oxopenta-4-enoato que es canalizado hasta los intermediarios del ciclo de Krebs (Figura 3) (Harayama *et al.,* 1989; Ramos *et al.,* 1997).

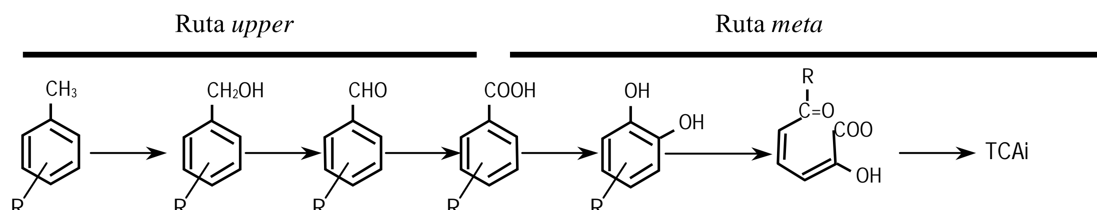


Figura 3. Catabolismo del tolueno o hidrocarburos similares hasta intermediarios del ciclo de Krebs. El tolueno se metaboliza hasta ácido benzoico a través de la ruta *upper* y este ácido es catabolizado hasta los intermediarios del ciclo de Krebs (TCAi) a través de la ruta *meta*.

La organización y la función de los genes catabólicos de los operones *upper* y *meta* se determinó mediante mutagénesis con transposones, clonación y secuenciación del ADN, así como por estudios de expresión de los mismos. La ruta *upper* codifica siete genes *xylUWCMABN* (Harayama *et al.*, 1984; Williams *et al.*, 1997), y los enzimas implicados en la degradación de benzoatos hasta intermediarios del ciclo de Krebs están codificados por la ruta *meta*. El orden de los genes, *xylXYZLTEGFJQKIH*, lo establecieron Haramaya & Rekik (1990). Los trece genes se extienden sobre 11 kb y constituyen uno de los operones más largos de procariotas (Haramaya *et al.,* 1984; Marqués *et al.,* 1993).

Adyacente al extremo 3´ corriente abajo del operón *meta* se localizan dos genes reguladores, *xylS* y *xylR*, que se transcriben desde promotores divergentes (Figura 4) (Inouye *et al.,* 1987b; Marqués *et al.,* 1994; Ramos *et al.,* 1987a). El gen *xylR* codifica la proteína XylR, que pertenece a la familia de reguladores de NtrC/NifA (Morett & Segovia, 1993). El gen *xylS* codifica la proteína XylS que pertenece a la familia de reguladores transcripcionales de AraC/XylS (Gallegos *et al.,* 1993; Gallegos *et al.,*1997; Ramos *et al.,* 1990b).

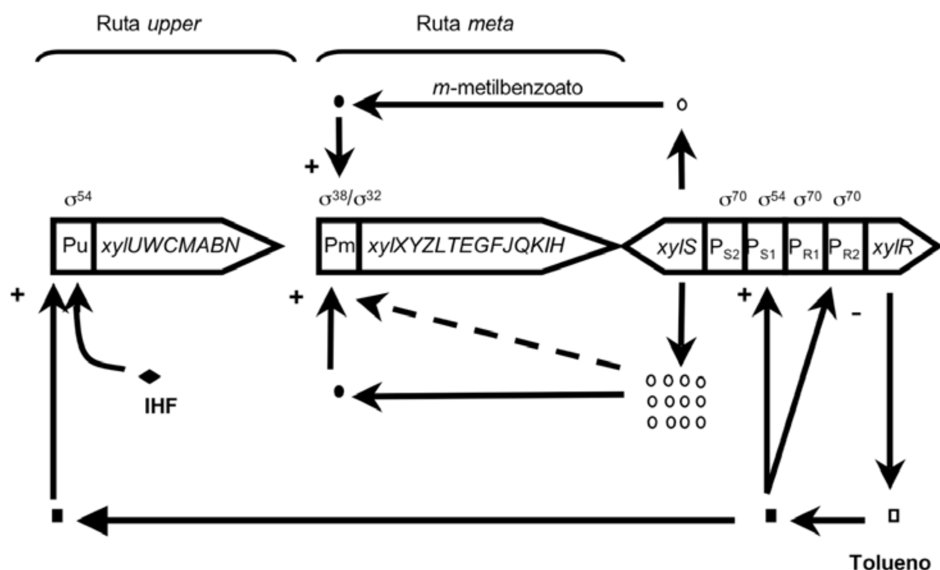


Figura 4. Los promotores del plásmido TOL bajo el control de la proteína XylR y XylS. La región intergénica de 300 pb entre los genes reguladores *xylS* y *xylR*, tiene cuatro promotores: dos promotores de *xylR* dependientes de σ^{70}, P_{R1} y P_{R2} y divergentes a éstos, los promotores de *xylS* P_{S1} y P_{S2}, dependientes de σ^{54} y de σ^{70} respectivamente. El gen *xylR* se expresa a partir de los promotores P_R, transcribiendo la proteína XylR inactiva ($XylR_i$) (□) la cual, en presencia de tolueno, se activa ($XylR_a$) (■) y estimula la transcripción (+) desde Pu y P_{S1} y reprime su propia síntesis (-). El factor σ^{54} participa en al transcripción de Pu y P_{S1}. La proteína IHF tiene un papel positivo en la transcripción del promotor Pu. El gen *xylS* se expresa a partir del promotor P_{S2} dependiente de σ^{70}, transcribiendo la proteína XylS inactiva ($XylS_i$) (O), la cual en presencia de m-metilbenzoato se activa ($XylR_a$) (●) y estimula la transcripción de la ruta *meta* desde Pm. (Ramos *et al.,* 1997).

El modelo de control de la expresión de las rutas catabólicas de los operones del plásmido TOL, explica la inducción de enzimas de cada operón en *P. putida* (pWW0) en células cultivadas en fase exponencial en xilenos o toluatos (Marqués & Ramos, 1993). El control genético de los operones refleja su organización bioquímica. Existen dos lazos reguladores; uno, funciona en células cultivadas en toluatos (lazo *meta*) y el otro sistema (lazo *upper*) funciona creciendo con xilenos, pero la expresión de ambos está coordinada.

3.1. Lazo *meta*

El lazo *meta* funciona de tal manera, que en células cultivadas en ausencia de efectores, el gen *xylS* se expresa constitutivamente a niveles bajos a partir del promotor P_{S2} dependiente de σ^{70} y se produce una pequeña cantidad de proteína inactiva ($XylS_i$) (Gallegos *et al.,* 1996; González-Perez *et al.,* 1999) (Figura 4).

Cuando el efector se añade al medio de cultivo, la proteína $XylS_i$ interacciona con el efector y es activada ($XylS_a$) para estimular la transcripción del promotor Pm de la ruta *meta* (Franklin *et al.*, 1981; Franklin *et al.*, 1983; Inouye *et al.,* 1984; Marqués *et al.,* 1994; Ramos *et al.,* 1986). La presencia de *m*-metilbenzoato es detectado por la célula como compuesto tóxico y desencadena una respuesta a estrés que lleva a un aumento de la concentración de σ^{32}, por otro lado necesario para la transcripción desde Pm durante el crecimiento en fase exponencial (Domínguez-Cuevas *et al.,* 2005; Marqués *et al.,* 1999; Ramos *et al.,* 1997). En ausencia de XylS el promotor Pm responde a benzoato pero no a *m*-metilbenzoato. Kessler *et al.* (1994) propusieron que ello podía deberse a la regulación cruzada que existe entre la ruta *meta* de TOL y el regulador BenR del cromosoma, ya que tanto XylS como BenR reconocen la misma secuencia en la región promotora de Pm.

3.2. Lazo *upper*

Cuando el efector en el medio es tolueno/xilenos, *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. El regulador del lazo *upper*, *xylR* controla la transcripción de la ruta catabólica en células cultivadas en tolueno/xilenos, es dependiente de dos promotores que se transcriben por la ARN polimerasa con σ^{70} y además controla su propia síntesis (Figura 4). La proteína inactiva ($XylR_i$) interacciona directamente con la molécula efectora (Delgado & Ramos, 1994), cambia su conformación y adquiere una forma activa ($XylR_a$) y así estimula la transcripción del operón de la ruta *upper* a partir del promotor Pu, que requiere la presencia de σ^{54} y de la proteína IHF (Abril *et al.,* 1991; Bertoni *et al.*, 1998; Gomada *et al.,* 1994). La unión de IHF produce una curvatura en el ADN, facilitando el contacto entre el activador y la ARNPσ^{54} unidos a distancia (Seong *et al.,* 2002) necesaria para la formación del complejo abierto y el comienzo de la transcripción. La proteína $XylR_a$ también estimula la expresión del gen *xylS*, induciendo la transcripción a partir del promotor P_{S1} dependiente de σ^{54} y de la proteína HU. Como resultado, en las células cultivadas en xilenos, *xylS* se expresa de dos promotores; el promotor P_{S2} que es constitutivo e independiente de XylR y el promotor P_{S1} que es dependiente de σ^{54} y XylR. El gen *xylS* es sobreexpresado y la proteína $XylS_i$ se activa bajo estas condiciones, y así, es capaz de activar la

transcripción de la ruta *meta* a partir del promotor Pm, incluso en ausencia del efector de la ruta *meta* (Inouye *et al.,* 1987a; Mermod *et al.,* 1987; Ramos *et al.,* 1987b). La proteína IHF ejerce un efecto represor en P_{S1}, y la proteína HU ejerce un efecto positivo. La curvatura del ADN en P_{S1} parece ser insuficiente y HU aumenta la flexibilidad de la región facilitando el contacto de XylR con las UAS y la ARNPσ^{54} (Pérez-Martín & de Lorenzo, 1995).

En este trabajo de Tesis Doctoral se ha avanzado en la elucidación de las rutas de degradación de glucosa en *P. putida*, y el mecanismo de regulación cruzada en el metabolismo de glucosa y tolueno cuando *P. putida* (pWW0) se cultiva en presencia de estos compuestos como fuente de carbono.

OBJETIVOS

A lo largo de esta Tesis Doctoral hemos estudiado el metabolismo de la glucosa en *Pseudomonas putida* partiendo de trabajos anteriores en esta especie (Vicente & Cánovas, 1973a), en los cuales se describía que el metabolismo de la glucosa en *Pseudomonas putida* ocurría a través de la vía oxidativa hasta 6PG, el cual rinde piruvato y gliceraldehído-3-fosfato a través de la ruta de Entner-Doudoroff. Al comparar estos estudios con la información obtenida a partir de la secuenciación del genoma de *P. putida* KT2440 (Nelson *et al.,* 2002), vimos que no coincidían y quisimos esclarecer las vías que esta cepa utiliza para metabolizar éste azúcar. También nos interesó la distribución de los genes, así como los reguladores implicados en el metabolismo de la glucosa. Por otro lado, muchos autores han estudiado el efecto de represión catabólica de la glucosa sobre el metabolismo de aromáticos (Cases *et al.,* 1999; Aranda-Olmedo *et al.,* 2005) y nosotros quisimos observar este efecto cuando las células crecían en presencia de dos fuentes de carbono metabolizables por *Pseudomonas putida,* glucosa y tolueno. Con el fin de esclarecer las vías operativas en el metabolismo de la glucosa en las bacterias de la especie *P. putida* KT2440 y el efecto de represión catabólica que ejercía la glucosa sobre el metabolismo del tolueno y/o viceversa, se plantearon los siguientes objetivos:

1. Estudio del metabolismo de la glucosa de *P. putida* KT2440. Análisis de la organización global de los genes. Estudio transcriptómico en respuesta a glucosa.

2. Análisis del flujo de la glucosa en el metabolismo global de *P. putida* KT2440.

3. Identificación de los posibles reguladores de los operones del metabolismo de la glucosa.

4. Utilización simultánea de glucosa y tolueno en *P. putida* KT2440 y sus mutantes. Estudio del de flujo metabólico y análisis transcriptómico. Identificación de los metabolitos y de los reguladores implicados en la represión catabólica.

RESULTADOS

III. CAPÍTULO 1. RESUMEN.

Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: Genomic and Flux analysis.

Teresa del Castillo, Juan L. Ramos, José J. Rodriguez-Herva, Tobias Fuhrer, Uwe Sauer, y Estrella Duque

En este estudio, mostramos que el catabolismo de la glucosa en *Pseudomonas putida* ocurre a través de tres rutas que operan simultáneamente y que convergen a nivel del 6-fosfogluconato, el cual se metaboliza a compuestos intermedios centrales por acción de las enzimas Edd y Eda, de la ruta de Entner/Doudoroff. Cuando la glucosa entra en el espacio periplásmico a través de porinas específicas como OprB, puede pasar al citoplasma o ser oxidada a gluconato. La glucosa es transportada al interior del citoplasma en un proceso mediado por un sistema de transporte tipo ABC codificado por los marcos de lectura abiertos PP1015 al PP1018 y ser entonces fosforilada por la glucoquinasa (codificada por el gen *glk*) y convertida por la glucosa-6-fosfato deshidrogenasa (codificada por el gen *zwf*) a 6-fosfogluconato. El gluconato en el periplasma puede ser transportado al citoplasma y posteriormente fosforilado por la gluconoquinasa a 6-fosfogluconato u oxidado a 2-cetogluconato, el cual es transportado al citoplasma y posteriormente fosforilado y reducido a 6-fosfogluconato. En la cepa silvestre, el consumo de glucosa fue de alrededor de 6 mmol g^{-1} h^{-1}, el cual permitió una velocidad de crecimiento de 0,58 h^{-1} y un rendimiento de 0,44g de biomasa/g carbono utilizado. Análisis de flujo con glucosa marcada con ^{13}C reveló que, en el ciclo de Krebs, la fracción mayoritaria de oxalacetato se produjo por la vía del piruvato, más que de la oxidación directa de malato por la malato deshidrogenasa. Las enzimas, los reguladores y los sistemas de transporte de las tres rutas se indujeron en respuesta a glucosa en el medio externo como revelaron ensayos enzimáticos y *microarrays*. Generamos una serie de mutantes isogénicos en uno o más de los pasos de las tres rutas y encontramos que, aunque los tres funcionan simultáneamente, la ruta de la glucoquinasa y el bucle del 2-cetogluconato fueron cuantitativamente más importantes que la fosforilación directa a gluconato. En términos de organización física, los genes del catabolismo de la glucosa están organizados en una serie de grupos dispersos a lo largo del cromosoma. Dentro de cada uno de los grupos, los genes que codifican las porinas, transportadores, enzimas, y reguladores forman operones, sugiriendo que los genes de cada grupo coevolucionaron. El gen *glk* que codifica la glucoquinasa se localizó en un operón con el gen *edd*, mientras que el gen *zwf*-1, que codifica la glucosa-6-fosfato deshidrogenasa, forma un operón con el gen *eda*. Por tanto, los genes de la ruta de la glucoquinasa y los de la ruta de Entner-Doudoroff están físicamente ligados y se inducen simultáneamente. Por eso, podemos concluir que la ruta de la glucoquinasa es *sine qua non* para que *P. putida* crezca en glucosa.

Journal Bacteriology
Vol.189, Nº. 14, pp. 5142-5152, Julio de 2007

Journal of Bacteriology, July 2007, p. 5142–5152 Vol. 189, No. 14
0021-9193/07/$08.00+0 doi:10.1128/JB.00203-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Convergent Peripheral Pathways Catalyze Initial Glucose Catabolism in *Pseudomonas putida*: Genomic and Flux Analysis▽†

Teresa del Castillo,[1] Juan L. Ramos,[1]* José J. Rodríguez-Herva,[1] Tobias Fuhrer,[2] Uwe Sauer,[2] and Estrella Duque[1]

Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, C/ Prof. Albareda, 1, E-18008 Granada, Spain,[1] and Institute for Molecular Systems Biology, ETH, CH-8093 Zurich, Switzerland[2]

Received 7 February 2007/Accepted 29 April 2007

In this study, we show that glucose catabolism in *Pseudomonas putida* occurs through the simultaneous operation of three pathways that converge at the level of 6-phosphogluconate, which is metabolized by the Edd and Eda Entner/Doudoroff enzymes to central metabolites. When glucose enters the periplasmic space through specific OprB porins, it can either be internalized into the cytoplasm or be oxidized to gluconate. Glucose is transported to the cytoplasm in a process mediated by an ABC uptake system encoded by open reading frames PP1015 to PP1018 and is then phosphorylated by glucokinase (encoded by the *glk* gene) and converted by glucose-6-phosphate dehydrogenase (encoded by the *zwf* genes) to 6-phosphogluconate. Gluconate in the periplasm can be transported into the cytoplasm and subsequently phosphorylated by gluconokinase to 6-phosphogluconate or oxidized to 2-ketogluconate, which is transported to the cytoplasm, and subsequently phosphorylated and reduced to 6-phosphogluconate. In the wild-type strain, glucose was consumed at a rate of around 6 mmol g^{-1} h^{-1}, which allowed a growth rate of 0.58 h^{-1} and a biomass yield of 0.44 g/g carbon used. Flux analysis of ^{13}C-labeled glucose revealed that, in the Krebs cycle, most of the oxalacetate fraction was produced by the pyruvate shunt rather than by the direct oxidation of malate by malate dehydrogenase. Enzymatic and microarray assays revealed that the enzymes, regulators, and transport systems of the three peripheral glucose pathways were induced in response to glucose in the outer medium. We generated a series of isogenic mutants in one or more of the steps of all three pathways and found that, although all three functioned simultaneously, the glucokinase pathway and the 2-ketogluconate loop were quantitatively more important than the direct phosphorylation of gluconate. In physical terms, glucose catabolism genes were organized in a series of clusters scattered along the chromosome. Within each of the clusters, genes encoding porins, transporters, enzymes, and regulators formed operons, suggesting that genes in each cluster coevolved. The *glk* gene encoding glucokinase was located in an operon with the *edd* gene, whereas the *zwf-1* gene, encoding glucose-6-phosphate dehydrogenase, formed an operon with the *eda* gene. Therefore, the enzymes of the glucokinase pathway and those of the Entner-Doudoroff pathway are physically linked and induced simultaneously. It can therefore be concluded that the glucokinase pathway is a sine qua non condition for *P. putida* to grow with glucose.

It is well established that bacteria of the genus *Pseudomonas* metabolize glucose exclusively by the Entner-Doudoroff (ED) pathway (9, 48, 49, 50, 52), in which 6-phosphogluconate is the key intermediate. The initial breakdown of glucose to 6-phosphogluconate in *Pseudomonas putida* was proposed to be catalyzed primarily by two consecutive periplasmic oxidation reactions mediated by glucose dehydrogenase and gluconate dehydrogenase. The resulting 2-ketogluconate is then transported into the cell and converted into 2-keto-6-phosphogluconate, which is later reduced to 6-phosphogluconate (Fig. 1) (21, 28). The above proposal was supported by the fact that mutants from *P. putida* Y-71.4 that were unable to synthesize 6-phosphogluconate from 2-ketogluconate grew very slowly on glucose or gluconate. In the ED pathway (9), 6-phosphogluconate is converted into 2-keto-3-deoxy-6-phosphogluconate by the EDD enzyme. This product is in turn split into pyruvate and glyceraldehyde-3-phosphate by EDA, which yields central metabolism compounds.

In *P. putida* ATCC 12633, Vicente and Cánovas (48, 49) were only able to obtain mutants unable to grow on glucose blocked in the *edd* and *eda* genes. Failure to obtain mutants in the steps from glucose to 6-phosphogluconate, which apparently seemed to contradict the suggestion that glucose can be degraded only via gluconate, as in *P. putida* Y-71.4, was explained by arguing that *P. putida* strain ATCC 12633 might have two distinct glucose dehydrogenase proteins, although no direct experimental support was available.

In addition to the above-mentioned pathway for the conversion of glucose into 6-phosphogluconate, another pathway has been described in other species of the genus *Pseudomonas. Pseudomonas aeruginosa* can use the glucokinase pathway, in which glucose is phosphorylated to glucose-6-phosphate, which is subsequently oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (8, 16, 18, 19, 20, 30, 39, 40, 45). Failure to detect glucokinase activity in several *P. putida* strains growing on glucose led several groups to propose that the glucose-6-phosphate pathway was nonfunctional in *P. putida* (12, 23, 27, 36, 37, 48, 49).

* Corresponding author. Mailing address: Consejo Superior de Investigaciones, Biochemistry and Molecular and Cell, Calle Profesor Albareda 1, Granada E-18008, Spain. Phone: 34 958 181600, ext. 289. Fax: 34 958 135740. E-mail: jlramos@eez.csic.es.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 4 May 2007.

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

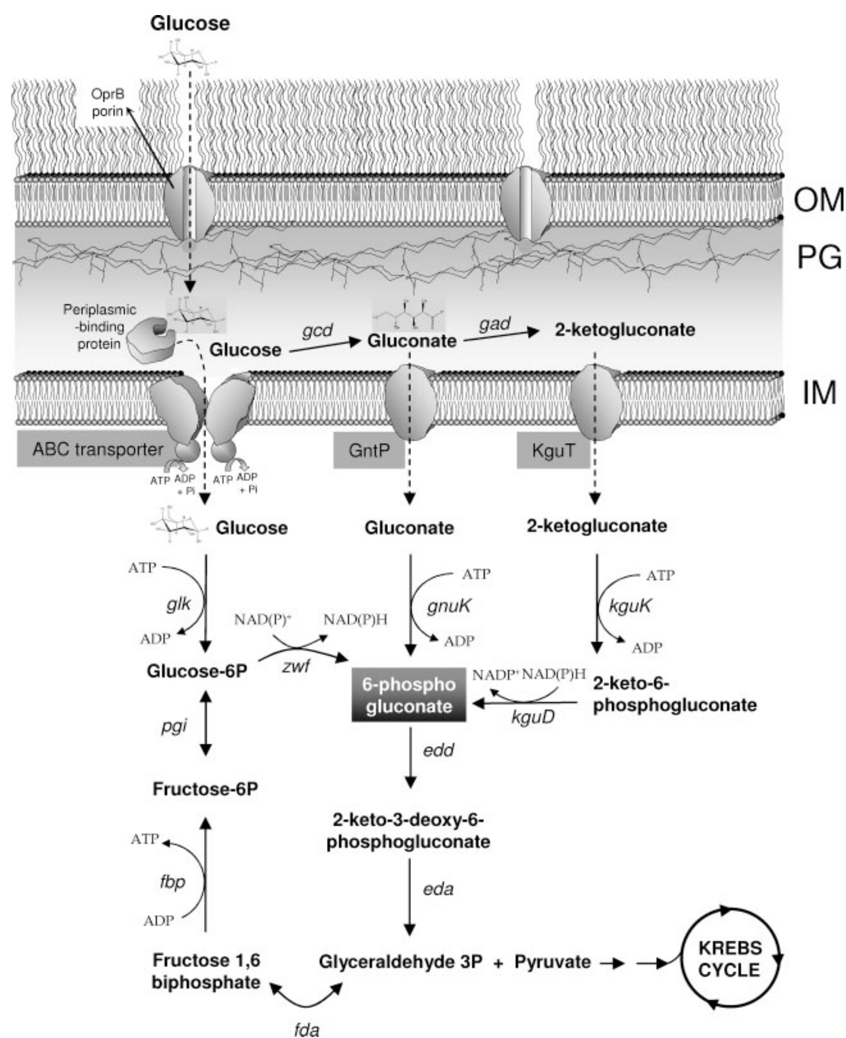


FIG. 1. Glucose catabolism in *P. putida* as deduced from gene annotations. At the top are the events that occur in the outer membrane and the reactions that take place in the periplasmic space. Also shown are the transport of glucose, gluconate, and 2-ketogluconate into the cell. The set of catabolic reactions that take place in the cytoplasm is depicted. The genes encoding the enzymes involved are indicated for all of the steps. OM, outer membrane; PG, periplasmic space; IM, inner membrane.

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

In different species of the genera *Bacillus*, *Oceanobacillus*, *Staphylococcus*, *Geobacillus*, and others (Swiss Prot/ErEMBL), the synthesis of 6-phosphogluconate has been shown to also occur through direct phosphorylation of the gluconate formed by glucose oxidation in a reaction catalyzed by gluconokinase (14, 38, 41). This reaction has been proposed to be of minor, if any, importance in *P. putida* KT2440 (47).

Based on the genome annotation of *P. putida* KT2440 (29, 32, 51), Velázquez et al. (47) proposed the metabolic pathways potentially available for the use of glucose, identifying a single open reading frame (ORF) in the database of The Institute of Genomic Research (TIGR) (http://www.tigr.org) for each of the reactions summarized in Fig. 1. The first and most remarkable feature of the network of transformations that results from projecting the genomic data of KT2440 is that, in principle, it does not fit with the earlier proposal for glucose metabolism in different strains of *P. putida* (15, 21, 28, 36, 48, 49), because up to three pathways to transform glucose into 6-phosphogluconate were identified in silico: (i) the glucose kinase pathway, in which glucose-6-phosphate is the intermediate (Fig. 1); (ii) the direct phosphorylation of gluconate mediated by gluconokinase (Fig. 1); and (iii) the ketogluconate loop, which involves the oxidation of gluconate to 2-ketogluconate (Fig. 1).

This study was undertaken to shed light on the initial steps of glucose metabolism in the soil bacterium *P. putida* KT2440 (25) by generating and characterizing mutants in each of the potential branches leading to 6-phosphogluconate. Growth analysis, metabolic fluxes from glucose in wild-type and isogenic mutant strains, and biochemical and gene expression analysis led us to conclude that *P. putida* KT2440 uses multiple peripheral pathways to convert glucose into the key pathway intermediate, 6-phosphogluconate.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *P. putida* KT2440 and a series of isogenic mutant strains with insertions in genes encoding the ED pathway or in the peripheral pathways leading to 6-phosphogluconate are shown

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Reference
Strains		
P. putida		
KT2440	Wild type, prototroph; Cm^r Rif^r	This laboratory
M1044[b]	*edd*::mini-Tn*5*-Km; Km^r Rif^r	7
M1128[b]	*eda*::mini-Tn*5*-Km; Km^r Rif^r	7
M438[b]	*gcd*::mini-Tn*5*-Km; Km^r Rif^r	7
M580[b]	*gnuK*::mini-Tn*5*-Km; Km^r Rif^r	7
M348[b]	*kguD*::mini-Tn*5*-Km; Km^r Rif^r	7
PSC278[c]	*glk*::pCHESIΩ-Km; Rif^r	This study
E. coli		
CC118λpir	Host to replicate suicide plasmids based on R6K origin	17
HB101 (pRK600)	Host used for cloning assays	17
Plasmids		
pUT-Km	Source of mini-Tn*5*; Km^r	17
pRK600	Helper plasmid; Tra$^+$ *mob*$^+$ Cm^r	17
pCHESIΩKm	Km^r	22

[a] Cm^r, Km^r, and Rif^r stand for resistance to chloramphenicol, kanamycin, and rifampin, respectively.
[b] Collection of KT2440 mutants available at the Consejo Superior de Investigaciones Científicas, Granada, Spain.
[c] *Pseudomonas* Stock Center.

in Table 1, as well as the *Escherichia coli* strains used in this study. Bacterial strains were grown at 30°C in either Luria-Bertani or modified M9 minimal medium (1, 6) with benzoate (15 mM), glucose (0.3% [wt/vol]), or sodium citrate (16 mM) as a carbon source. Liquid cultures were agitated on a gyratory shaker operated at 200 rpm. When appropriate, antibiotics were added at the following concentrations: kanamycin (Km), 25 μg/ml, and rifampin (Rif), 10 μg/ml.

For ^{13}C-labeling experiments, cells were grown in 250-ml baffled shake flasks containing 20 ml M9 medium at 30°C and 200 rpm. Glucose was added entirely in the form of the 1-^{13}C-labeled isotope isomer (99% pure; Omicron Biochemicals, Inc., South Bend, IN) or in the form of a mixture of 20% (wt/wt) [U-^{13}C]glucose (>99% pure; Martek Biosciences Corporation, Columbia, MD) and 80% (wt/wt) natural glucose.

Analytical procedures and physiological parameters. Cell growth was monitored by measuring the turbidity of the cultures at 660 nm. Glucose and acetate concentrations were determined enzymatically with commercially available kits (Boehringer Mannheim). Gluconate and 2-ketogluconate were determined at 210 nm after separation of products by high-pressure liquid chromatography (Perkin-Elmer) using a Supercogel C_8 (4.6 × 250 mm) high-pressure liquid chromatography column. The liquid phase was 5 mM sulfuric acid, and the flow rate was 0.6 ml/min at 60°C. To determine the cell dry weight (CDW), 10-ml cell suspensions were harvested by centrifugation at 15,800 × *g* in a centrifuge using predried and weighed 10-ml tubes. The pellets were washed with 0.9% (wt/vol) NaCl and dried at 105°C for 24 h to a constant weight.

The following physiological parameters were determined by regression analysis during the exponential growth phase in batch cultures (35): maximum specific growth rate, biomass yield on glucose, specific glucose consumption, and by-product formation rates.

Sample preparation and gas chromatography-mass spectrometry analysis. Cell aliquots were harvested in precooled tubes (−4°C) during mid-exponential growth by centrifugation of 7 to 10 ml of the culture broth at 1,200 × *g* and 4°C for 20 min. The pellet was washed twice with 1 ml of 0.9% (wt/vol) NaCl, hydrolyzed in 1.5 ml 6 M HCl for 24 h at 110°C in sealed 2-ml Eppendorf tubes, and desiccated overnight in a heating block at 85°C under a constant air stream. The hydrolysate was dissolved in 50 μl 99.8% pure dimethyl formamide and derivatized with *N*-methyl-*N*-(tert-butyldimethylsilyl)-trifluoroacetamide as described previously (5, 10, 26). One microliter of the derivatized sample was injected into a Hewlett-Packard series 8000 gas chromatographer combined with a model MD 800 mass spectrometer (Fisons Instruments) and analyzed as described previously (5, 10, 26).

METAFoR analysis. For metabolic-flux ratio (METAFoR) analysis, the mass spectra of the derivatized amino acids alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartate, glutamate, histidine, and tyrosine were corrected for the natural abundances of all stable isotopes and unlabeled biomass from the inoculum. Lysine and methionine are not required for the METAFoR analysis used in this study, whereas arginine, asparagine, cysteine, glutamine, and tryptophan are not detectable (42). The amino acids are synthesized from one or more metabolic intermediates, and the mass isotopomer distribution vector of these metabolites was derived from the mass isotopomer distribution vector of the amino acids and used to calculate the fractional contribution of a given pathway or reaction to a target metabolite pool (metabolic-flux ratios) by using a set of algebraic equations implemented in the MATLAB-based program Fiat Flux (26, 55).

Net-flux analysis and master reaction network. The metabolic models used for net-flux analysis were based on the master reaction network, which included 45 reactions and 33 metabolites. Respiration, biomass formation, and a transhydrogenase reaction were included as additional reactions, and ATP and the cofactors NADH and NADPH were included as additional metabolites. Net fluxes were then calculated using (i) the stoichiometric reaction matrix, (ii) the METAFoR analysis-derived flux ratios, (iii) physiological data, and (iv) precursor requirements for biomass synthesis, as described previously (11, 13, 26). Specifically, the following flux ratios were used: pyruvate derived through the ED pathway, oxaloacetate (OAA) originating from phosphoenolpyruvate or pyruvate, the lower and upper bounds of pyruvate originating from malate, and the upper bound of phosphoenolpyruvate derived through the pentose phosphate pathway. The stoichiometric matrix was then solved with the MATLAB-based program by minimizing the sum of the weighted-square residuals of the constraints from both metabolite balances and flux ratios to obtain estimated net fluxes (55).

DNA techniques. Preparation of plasmid and chromosomal DNA, digestion with restriction enzymes, ligation, electrophoresis, and Southern blotting were done by standard methods (2).

Preparation of RNA and reverse transcriptase (RT)-PCR. *P. putida* KT2440 cells were grown overnight in M9 medium with glucose. The cells were then diluted to reach a turbidity at 660 nm of 0.05 in fresh M9 medium with glucose as the sole carbon source and were incubated until the culture reached a turbidity of 1.0 at 660 nm. Then, 15 ml of the cultures was harvested by centrifugation at 7,000 × *g* for 5 min. Total bacterial RNA was isolated as described by Marqués et al. (24). Extracts were treated with RNase-free DNase I (10 U/μl) in the presence of an RNase inhibitor cocktail (RNaseOUT; 40 U/μl).

RT-PCR was done with 0.5 μg RNA in a final volume of 20 μl using the Titan OneTube RT-PCR system according to the manufacturer's instructions (Roche Laboratories). The annealing temperature used for RT-PCR was between 50°C and 58°C, and the cycling conditions were as follows: 94°C for 10 s, 50 to 58°C for 30 s, and 68°C for 30 s. Positive controls (with DNA as the template and *Taq* polymerase) and negative controls (with RNA as the template and *Taq* polymerase) were included in all the assays.

Mini-Tn*5* mutagenesis of *P. putida* strain KT2440. Mini-Tn*5* transposon mutagenesis was carried out by performing triparental mating between *P. putida* KT2440R (Rif^r), *E. coli* CC118λ*pir* (pUT-Km), and the helper strain *E. coli* HB101(pRK600), as described by Duque et al. (7). After 4 h of conjugation, the transconjugants were selected as Rif^r Km^r clones on M9 medium with citrate as a carbon source. Among the set of transconjugants, we searched for mutants unable to grow on M9 medium with glucose as a carbon source. This allowed us to identify mutants with insertions in the *edd* and *eda* genes (see below). Other mutants, with insertions in the *gcd*, *gnuK*, *kguK*, and *kguD* genes, were obtained from the *Pseudomonas* Reference Culture Collection (7). The transposon insertion site in each of the mutants was confirmed by DNA sequencing (7).

Site-specific homologous inactivation of *glk*. To construct a mutant strain bearing an inactivated chromosomal version of the *glk* gene, we generated the corresponding knockout using the appropriate derivative of pCHESIΩKm. Plasmid pCHESIΩKm is based on pUC18 and bears the origin of transfer *oriT* of RP4 and the Ω-Km interposon of plasmid pHP45ΩKm cloned as a HindIII fragment (22). To generate the desired mutation, an internal fragment (540 bp) of the target gene was amplified by PCR with primers provided with EcoRI and BamHI sites and subsequently cloned between the EcoRI and BamHI sites of pCHESIΩKm in the same transcriptional direction as the *lacp* promoter. The recombinant plasmid was mobilized from *E. coli* CC118λ*pir* into *P. putida* KT2440 by triparental mating with the *E. coli* HB101(pRK600) helper strain (33). *P. putida* KT2440 transconjugants bearing a cointegrate of the plasmid in the host chromosome were selected on M9 minimal medium with benzoate (15 mM) as the sole carbon source and kanamycin (33). The nature of the mutation in a few randomly chosen Km^r clones was confirmed by PCR using a primer based on the Km^r gene and another primer that was complementary to the *glk* gene. One correct clone was randomly chosen, and the nature of the mutation was confirmed by Southern blotting (not shown).

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

TABLE 2. Growth characteristics of *P. putida* KT2440 and isogenic mutants with mutations in the glucose metabolism network[a]

Strain	μ_{max} (h^{-1})	Gluconate[b] (mM)	2-Ketogluconate[b] (mM)	Biomass yield (CDW [g]/g of C used)[c]	Carbon uptake (mmol g^{-1} h^{-1})
KT24420	0.56 ± 0.02	1.75 ± 0.36	0.53 ± 0.02	0.44 ± 0.01	6.31 ± 0.05
Gcd	0.45 ± 0.03	ND[e]	ND	0.37 ± 0.01	5.50 ± 0.01
Glk	0.41 ± 0.01	0.61 ± 0.06	0.49 ± 0.14	0.35 ± 0.02	5.44 ± 0.10
GnuK	0.54 ± 0.02	1.7 ± 0.33	3.1 ± 1.35	0.46 ± 0.06	5.31 ± 0.07
KguD	0.44 ± 0.02	2.0 ± 0.14	3.46 ± 1.36	0.44 ± 0.02	5.30 ± 0.10
Edd	NG[d]	ND	ND		
Eda	NG	ND	ND		

[a] Data are the averages and standard deviation of the results for at least four independent cultures, each of them analyzed in duplicate.
[b] Maximal concentration in the culture medium.
[c] Total carbon uptake was calculated from the sum of all three extracellular concentrations of glucose, gluconate, and 2-ketogluconate.
[d] NG, no growth.
[e] ND, not detected.

Preparation of cell extracts. Cells were grown in batch in M9 medium with glucose or citrate as the carbon source until the culture reached a turbidity of around 0.8 at 660 nm. The cells were then harvested by centrifugation at 8,000 × *g* for 7 min, washed twice in 50 mM phosphate buffer (pH 7.0), and frozen at −20°C. To prepare cell extracts, cells were resuspended in 1 ml buffer (100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, and protease inhibitor Cocktail Tabs Complete, EDTA free). The cells were disrupted in a French press at 120 MPa. Whole cells and debris were removed by centrifugation at 11,180 × *g* (45 min; 4°C). The clear supernatant was considered the cell extract. The protein concentration in the cell extract was determined by the Bradford method, using bovine serum albumin as the standard.

Enzyme assays. Enzyme assays were performed at 30°C, and the formation of NAD(P)H was monitored at 340 nm in a Shimadzu UV-160A spectrophotometer. The reaction assays for glucokinase and gluconokinase were as described earlier (18, 44). Specific activities were calculated based on an NAD(P)H extinction coefficient of 6.3 $mM^{-1} \cdot cm^{-1}$.

***P. putida* microarrays.** The genome-wide DNA chip used in this work (printed by Progenika Biopharma) was described in detail previously (54). It consists of an array of 5,539 oligonucleotides (50-mer) spotted in duplicate onto γ-aminosilane-treated slides and covalently linked by UV light and heat. The oligonucleotides represent 5,350 of the 5,421 predicted ORFs annotated in the *P. putida* KT2440 genome (29, 54). The chips are also endowed with homogeneity controls consisting of oligonucleotides for the *rpoD* and *rpoN* genes spotted at 20 different positions, as well as duplicate negative controls at 203 predefined positions.

Microarray hybridization and data analysis. RNA isolation and preparation of labeled cDNA were done exactly as described by Duque et al. (7). To this end, *P. putida* KT2440 cells were grown on M9 medium with glucose or citrate as the carbon source. Prior to the hybridization process, the microarray slides were blocked by immersion in 5× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7), 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 1% (wt/vol) bovine serum albumin for 1 h at 42°C. Then, the slides were washed by two successive immersions in MilliQ water at room temperature, followed by a final wash with isopropanol. The slides were spin dried by centrifugation at 1,500 × *g* for 5 min and used within the next hour. Equal amounts of Cy3- and Cy5-labeled cDNAs, one of them corresponding to the control and the other one to the problem to be analyzed, were mixed, dried in a Speed-Vac, and reconstituted in 35 μl hybridization buffer (5× SSC, 25% [vol/vol] formamide, 0.5% [wt/vol] SDS, 5× Denhardt's solution, 5% [wt/vol] dextran sulfate) preheated to 42°C. The labeled probe was denatured at 98°C for 3 min, applied to the microarray slide, and covered with a glass coverslip. The slide was then placed in a humidified hybridization chamber (AHC ArrayIt hybridization cassette; Telechem International, Inc.) and incubated for 18 to 20 h in a water bath at 42°C in the dark. After hybridization, the microarrays were washed by gentle agitation in 2× SSC, 0.1% [wt/vol] SDS at 42°C for 5 min, followed by a 5-min wash at room temperature in 1× SSC, two 5-min washes in 0.2× SSC, and a final 5-min wash in 0.1× SSC. Finally, the slides were spin dried in a centrifuge at 1,500 × *g* for 5 min and scanned on a GenePix 4100A scanner (Axon Instruments, Inc.). Images were acquired at a 10-μm resolution, and the background-subtracted median spot intensities were determined with GenePix Pro 5.1 image analysis software (Axon Instruments, Inc.). The signal intensities were normalized by the LOWESS intensity-dependent normalization method and analyzed with Almazen System statistical software (Alma Bioinformatics S. L.). To ensure appropriate statistical analysis of the results, RNA preparations from at least four independent cultures were tested for each strain (4). *P* values were calculated by Student's *t* test. A particular ORF was considered differentially expressed if (i) the change was ≥2.0-fold and (ii) the *P* value was 0.05 or lower.

RESULTS

Isolation and characterization of mutants deficient in glucose metabolism in KT2440. We first subjected KT2440 to massive mutagenesis with mini-Tn*5* and selected mutants unable to use glucose as the sole carbon source. We found mutants with insertions at several locations in the *edd* and *eda* genes. These mutants failed to grow on glucose (Table 2) and were also unable to use gluconate as the sole carbon source (not shown). Because the annotation of TIGR pointed toward the existence of single ORFs encoding glucose dehydrogenase (*gcd*; PP1444), glucokinase (*glk*; PP1011), gluconokinase (*gnuK*; PP3416), and 2-ketogluconate-6-phosphate reductase (*kguD*; PP3376), we hypothesized that the inability to obtain mutants in a step above 6-phosphogluconate might be related to the simultaneous functioning of several peripheral pathways leading to 6-phosphogluconate from glucose (Fig. 1).

The *P. putida* Reference Culture Collection has generated a bank of random mini-Tn*5* insertions (7). Inspection of the mutant collection revealed that a number of clones with insertions in genes encoding peripheral-pathway enzymes—namely, mutants defective in *gcd* and unable to produce gluconate, as well as *kguD* and *gnuK* mutants that exhibited deficiencies in gluconate metabolism—were available (Fig. 1). A *glk*-deficient mutant unable to synthesize glucokinase, not available in the collection, was generated by site-directed mutagenesis as described in Materials and Methods.

To further investigate glucose catabolism in *P. putida* KT2440, we decided to compare the growth rates in M9 minimal medium with 16 mM glucose as the sole carbon source of the wild-type strain and mutants in each of three potential peripheral pathways that yield 6-phosphogluconate. Mutants deficient in either of the initial enzymes (Glk or Gcd) exhibited significantly decreased growth rates (0.41 ± 0.01 h^{-1} and 0.45 ± 0.01 h^{-1}) compared to that in the wild-type strain (0.56 ± 0.02 h^{-1}) (Table 2). This indicated that 6-phosphogluconate was produced simultaneously from glucose-6-phosphate and gluconate. The reduced growth rate could not be due only to initially reduced catabolism but also to the metabolic readjustment to produce fructose-6 phosphate. Gluconate can also be directly phosphorylated to 6-phosphogluconate or converted

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

TABLE 3. Levels of Glk and GnuK in KT2440 and its isogenic mutants growing with different carbon sources[a]

Strain or mutants	C source	Glk	GnuK
KT2440	Citrate	1.8 ± 0.7	1.7 ± 0.1
	Glucose	21.1 ± 2.8	18.1 ± 0.8
gcd	Citrate	1.4 ± 0.5	1.4 ± 0.1
	Glucose	31 ± 7.0	1.7 ± 0.4
glk	Citrate	0	1.5 ± 0.1
	Glucose	0	21.6 ± 3.6
gnuK	Citrate	1.9 ± 0.3	0
	Glucose	17.6 ± 0.5	0
kguD	Citrate	0.7 ± 0.4	1.1 ± 0.1
	Glucose	22.8 ± 1.3	10.8 ± 0.8

[a] Activities are given as nmol/mg protein · min. The data are the averages and standard deviations of two to five independent assays run in duplicate. Induced and uninduced values were in all cases statistically different, except for GnuK activity in the *gcd* mutant.

into this compound via the 2-ketogluconate loop. A mutant deficient in 2-ketogluconate metabolism, i.e., with *kguD* knocked out, grew at a lower rate (0.44 ± 0.01 h^{-1}) than a mutant deficient in GnuK (0.54 ± 0.02 h^{-1}). A mutant deficient in *kguK* also grew more slowly than the parental strain and the *gnuK* mutant. This was interpreted as evidence that metabolism via 2-ketogluconate is more efficient than the direct phosphorylation of gluconate.

During growth on glucose, *P. putida* KT2440 accumulated only relatively low extracellular concentrations of gluconate (the maximal accumulation was 1.75 ± 0.36 mM) and 2-ketogluconate (the maximal accumulation was 0.53 ± 0.02 mM), which were eventually consumed. As expected, the mutant deficient in glucose dehydrogenase (Gcd) did not accumulate gluconate (Table 2). The KguD mutant accumulated the highest concentrations of 2-ketogluconate (3.46 ± 1.36 mM) and gluconate (2.0 ± 0.14 mM), as expected from the limited operation of the 2-ketogluconate loop, whereas the inactivation of GnuK led to gluconate accumulation at levels similar to those found with the parental strain (Table 2). This set of results supports the notion that KT2440 can assimilate gluconate both via direct phosphorylation to 6-phosphogluconate and through the 2-ketogluconate loop (Fig. 1).

KT2440 exhibits inducible glucokinase and gluconokinase activity. To obtain unequivocal support for the notion that 6-phosphogluconate can be simultaneously synthesized from both glucose-6-phosphate and gluconate, we decided to determine the levels of glucokinase activity, involved in the direct phosphorylation of glucose, and gluconokinase activity, involved in the direct phosphorylation of gluconate. To this end, wild-type cells were grown with citrate or with glucose as the sole source of carbon, and enzymatic activities were determined in cell extracts. Relatively low levels of glucokinase and gluconokinase activity were detected in cells growing on citrate, which increased around 10-fold in cells growing on glucose (Table 3). This indicated that both enzymes were induced in response to glucose in KT2440.

We also measured these activities in the series of mutants shown in Table 3 in cells grown with citrate or with the sugar. On either substrate, all mutants exhibited wild-type-like levels of glucokinase activity (Table 3), which corroborates the inducible nature of glucokinase (note that no glucokinase activity was found in the *glk* mutant, as expected).

In the *gcd* mutant, gluconokinase activities were equally low in cells growing on citrate and glucose. However, in the rest of the mutant strains, we found that gluconokinase activity was induced when cells were grown on glucose (Table 3). These results supported the idea that GnuK is an inducible activity.

Net fluxes from glucose in the wild type and mutants in the early steps of glucose metabolism. The above-mentioned results suggested that KT2440 metabolizes glucose through three initial pathways that converge at the level of 6-phosphogluconate and that their simultaneous operation is necessary to achieve maximal growth rates. To assess whether the use of different combinations of these initial pathways further impacts on the central carbon metabolism, we used metabolic-flux analysis based on ^{13}C-labeling experiments (10, 26, 42). For this purpose, batch cultures were initiated with mid-exponential-phase cultures at a low inoculum density (less than 1% of the final culture volume) in medium containing 80% natural-abundance glucose and 20% [U-$^{13}C_6$]glucose and, in another series, with 100% [1-^{13}C]glucose. Because we could not resolve fluxes through the three initial pathways, they were lumped for *P. putida* KT2440 as the total carbon uptake rate. The rate of carbon uptake by the wild-type strain was estimated to be 6.31 ± 0.05 mmol g^{-1} h^{-1} for the parental strain, with a biomass yield of 0.44 g biomass/g carbon used (Table 2). A complete net-flux distribution for the wild-type strain is depicted in Fig. 2, and the relevant fluxes for the wild-type anaplerosis and gluconogenesis in *P. putida* KT2440 and its isogenic mutants are given in Table 4. As expected, we found that most pyruvate was produced primarily via the ED pathway. Flux data also revealed that over 80% of the tricarboxylic acid (TCA) cycle carbon flux occurs through the so-called pyruvate shunt, which bypasses malate dehydrogenase in the TCA cycle. This was

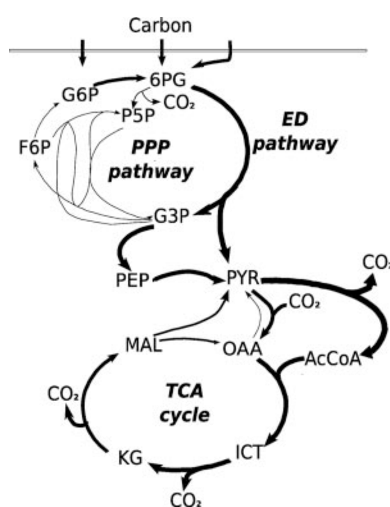


FIG. 2. In vivo carbon flux distribution in *P. putida* KT2440 obtained from METAFoR and net-flux analyses. All fluxes were normalized to the glucose uptake rate (6.31 nmol g $[CDW]^{-1}$ h^{-1}), and the thicknesses of the arrows are scaled to the relative percentages of flux. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; G3P, glyceraldeydehyde-3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; ICT, isocitrate; KG, α-ketoglutarate; MAL, malate.

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

TABLE 4. Selected flux rates obtained from net-flux analysis

Compound[a]	Flux rate (mol g_{CDW}^{-1} h^{-1})[b]				
	WT	Gcd	Glk	KguD	GnuK
ICT from OAA + AcCoA	6.31	5.51	5.72	6.18	6.01
Pyruvate from malate	2.57	1.93	2.65	4.27	3.31
OAA from pyruvate	4.92	4.45	4.81	6.58	5.81
OAA from malate	2.54	2.41	3.10	0.70	1.64

[a] ICT, isocitrate; AcCoA, acetyl-CoA.

[b] Standard deviations were below 5% of the given values. The strains used were either wild-type (WT) or mutants deficient in Gcd, Glk, KguD, or GnuK, as indicated.

deduced from the higher fraction of OAA originating from pyruvate than from malate (Table 4) using the mathematical formula q(6-phosphogluconate $\rightarrow$ glyceraldehyde-3-phosphate + pyruvate)/q(total carbon uptake), where q is rate. Consequently, *P. putida* should exhibit an as-yet-unidentified malic enzyme that converts malate into pyruvate. These results are in agreement with a proposal made by Fuhrer et al. (13) for *Pseudomonas fluorescens*.

The *gcd* and *glk* mutants exhibited a lower total carbon consumption rate than the parental strain: 5.50 ± 0.1 mmol g^{-1} h^{-1} for the *gcd* mutant, with a yield of 0.37 ± 0.01 g biomass/g carbon consumed, and 5.44 ± 0.1 mmol g^{-1} h^{-1} for the *glk* mutant, with a yield of 0.35 ± 0.02 g biomass/g carbon consumed. The intracellular fluxes were very similar to those determined in the parental strain (Table 4).

Somewhat surprising results were obtained for yields and metabolic fluxes with the *kguD* and *gnuK* mutants. Both mutants exhibited lower carbon uptake rates (around 5.3 mmol g^{-1} h^{-1}) than the parental strain; however, yields (around 0.45 g biomass/g carbon used) were similar to those of the parental strain: in both mutants, the pyruvate shunt was enhanced, since the total amount of OAA from pyruvate represented almost 90% of the total flux. The higher yield in the *kguD* and *gnuK* mutants versus the *gcd* and *glk* mutants might be due to redirection of the excess of OAA and NADPH to biomass biosynthesis.

Global analysis of the genes involved in glucose metabolism. (i) Gene organization and transcriptional architecture of the peripheral glucose catabolic genes. We have analyzed the annotated sequence of KT2440 to study in greater detail the physical organization of the genes of the glucose peripheral and convergent pathways to gain further insights into the metabolism of the sugar. We have also determined the operon organization of the genes with an RT-PCR approach. The *gcd* gene corresponded to ORF PP1444, and it was found to be in a cluster of five genes that are transcribed in the same direction (Fig. 3B). The long intergenic distance (295 bp) between the end of ORF PP1446 and the start of the second ORF, encoding a putative porin B (PP1445), suggested that transcription of the ORF encoding PP1446 occurs independently. The distance (203 nucleotides) between *gcd* (the third gene) and the fourth ORF also suggested that the ORF encoding PP1443 is not transcribed with *gcd*. The cluster is therefore likely to be made up of three transcriptional units. It should be noted that the gene in 5′ with respect to *gcd* encodes a porin B family protein that might be involved in the entry of glucose into the periplasmic space (34, 43). Distal with respect to *gcd* are the genes encoding the subunits of gluconate dehydrogenase (PP3382 and PP3383), involved in the conversion of gluconate into 2-ketogluconate. These two genes form an operon (Fig. 3C).

The other gene encoding an enzyme that initiates glucose metabolism, *glk*, was in a cluster with four genes transcribed in the same direction (Fig. 3A). Immediately upstream from *glk* was the *edd* gene, which encodes 6-phosphogluconate dehydratase (PP1010), one of the key enzymes in the ED pathway (Fig. 1). Sequence analysis indicated that the *glk* and *edd* genes not only overlapped, but were transcriptionally coupled. Divergent with respect to the *edd* gene was the *gap-1* gene, which encodes glyceraldehyde 3-phosphate-dehydrogenase (PP1009). This enzyme is involved in the conversion of glyceraldehyde-3-phosphate down to pyruvate. Downstream from the *glk* gene were two genes encoding a two-component regulatory system consisting of a sensor histidine kinase (ORF PP1013) and a DNA-binding protein annotated as GltR (PP1012).

The gene encoding gluconokinase (*gnuK*) is adjacent to a gene involved in the transport of gluconate (PP3417). Divergent with respect to *gnuK* is a gene encoding a transcriptional repressor that belongs to the LacI family (Fig. 3D). We found that the *kguK* and *kguD* genes were located in the same operon with *kguE* and a gene encoding a putative transporter for 2-ketogluconate (Fig. 3C).

Another interesting finding was that one of the alleles encoding glucose-6-phosphate dehydrogenase (*zwf-1*) was in a cluster with the *eda* gene. Upstream from *zwf-1* and transcribed in the opposite direction was a gene encoding a transcriptional regulator called HexR (Fig. 3A). It is worth noting that the *edd*/*glk* and *zwf-1*/*eda* gene clusters are vicinal and that between them lies a set of genes that encode a porin-like protein (PP1019) and an ABC transport system involved in glucose uptake, based on glucose uptake assays (T. del Castillo, E. Duque, and J. L. Ramos, unpublished results).

The physical organization of the genes involved in glucose metabolism suggested that some of them might form operons. This was tested by isolating total RNA from cells growing with glucose in the mid-exponential growth phase and by carrying out RT-PCR analysis. The *edd* and *glk* genes are part of an operon that was transcribed with the *gltR2* gene (Fig. 4). The overlapping gene encoding the sensor kinase PP1013 was also part of this operon (not shown). The following gene, whose function is unknown; the set of genes encoding porin OprB-1; and the ABC transport system made up of PP1015 through PP1019 are also part of this long operon (not shown). The *zwf-1*, *pgl*, and *eda* genes form another operon. Therefore, a relevant feature is the cotranscription of the *glk* and *edd* genes and of the *zwf-1* and *eda* genes, which indicates that the glucose-6-phosphate pathway is induced simultaneously with the ED enzymes.

Our results also showed that *gcd* (ORF PP1444) and porin B2 (ORF PP1445) did not form an operon and that the *gcd* gene was transcribed independently of the other, surrounding genes (not shown). We found that the set of genes from PP3376 to PP3384 is organized in two operons, one that included the *kgu* genes (*kguE* to *kguT*) and the *ptxS* repressor gene plus another that contained the two ORFs encoding the Gad enzyme (PP3382 and PP3383), which also formed an operon with a third ORF, which encodes PP3384 (Fig. 3C).

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

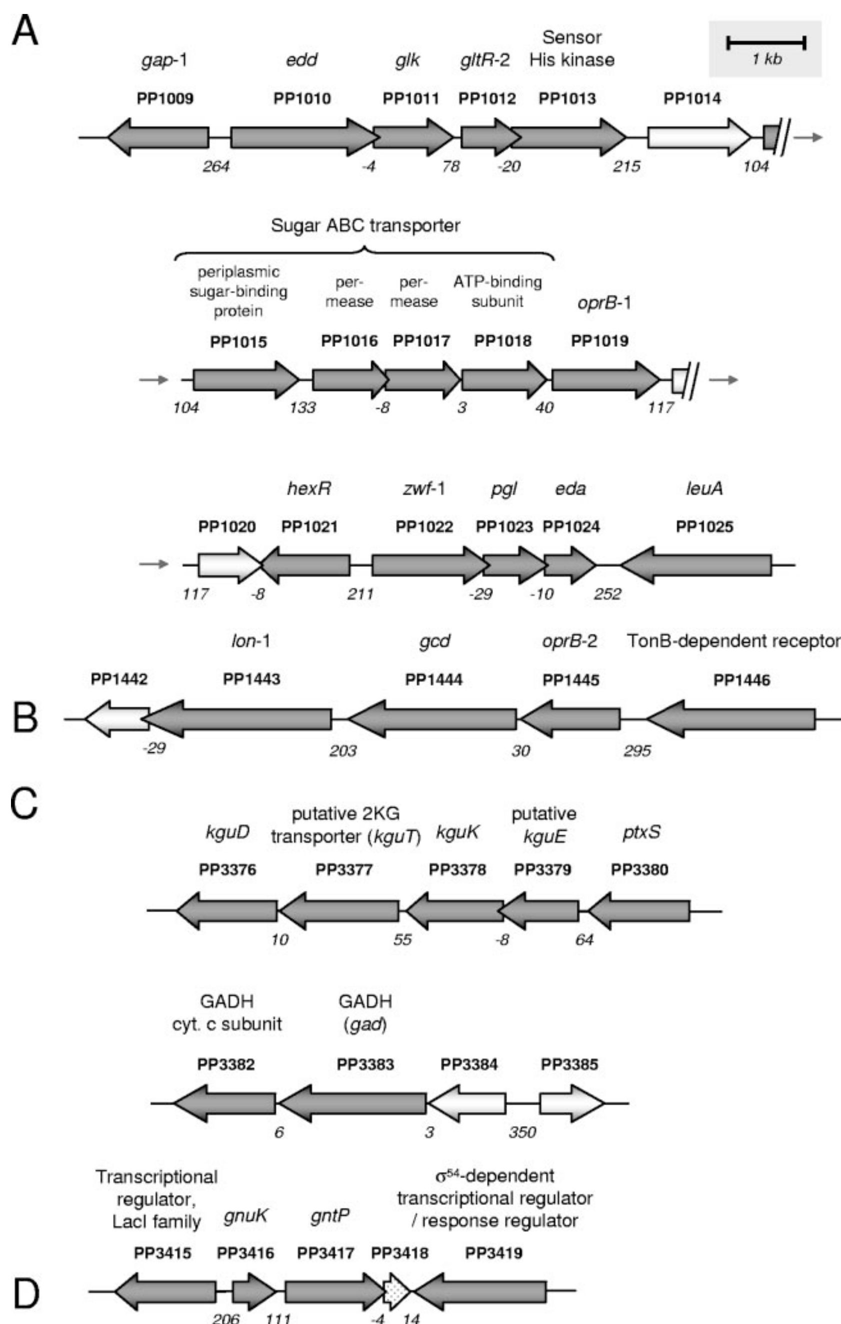


FIG. 3. Organization of the peripheral glucose catabolic genes. The gene numbering and organization are derived from the annotation of the complete genome sequence of *P. putida* KT2440 in the TIGR database. (A) Set of 16 genes, most of which have been assigned a function based on enzymatic assays. (C) Set of nine genes, most of which have a specific role in glucose catabolism. (B and D) Physical organizations of the other genes involved in glucose catabolism in *P. putida* KT2440.

(ii) Global transcriptomic analysis of KT2440 in response to glucose. Because the number of straightforward enzymatic assays for peripheral glucose catabolic-pathway enzymes is limited and because the organization of glucose pathway genes is relatively complex (with clustering of certain genes and scattering over the chromosome of other sets of genes), we decided to study the global response of strain KT2440 to glucose by comparing glucose-grown cells versus citrate-grown cells at the transcriptomic level. As indicated in Materials and Methods, we centered our attention on ≥2.0-fold changes and *P* values of ≤0.05.

Interestingly, we observed a significant induction of two outer membrane porins, OprB1 (PP1019) and OprB2 (PP1445), that have been proposed to be necessary for glucose to enter the periplasmic space. This finding agrees with previous reports that in *P. aeruginosa* and *P. putida*, *oprB* mutants grow deficiently in minimal medium with glucose (53). All genes involved in the oxidation of glucose to gluconate

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

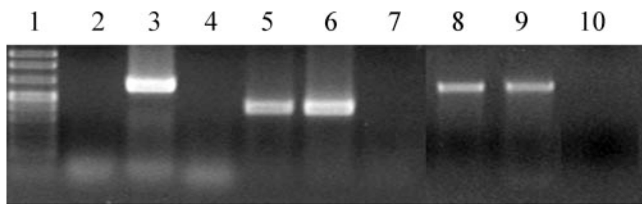


FIG. 4. Transcriptional pattern of the *gap-1*, *glk*, and *edd* region involved in glucose catabolism. Lane 1, DNA size marker (Roche); lane 2, RT-PCR using primers based on the adjacent *gap-1* and *edd* genes; lane 3, positive control for lane 2 with the same oligonucleotides but with DNA as a template; lane 4, negative control for lane 2 with the same oligonucleotides but without retrotranscriptase; lane 5, RT-PCR using primers complementary to RNA of the *edd*/*glk* genes; lane 6, positive control with the same primers as in lane 5 but with DNA as a template; lane 7, negative controls for the assay in lane 5 with the same primers but without retrotranscriptase; lane 8, RT-PCR using primers based on *glk* and *gltR2*; lane 9, positive control with the same oligonucleotides but with DNA as a template; lane 10, negative control for the assay in lane 8 but without retrotranscriptase.

(PP3382, PP3383, PP3376, and PP3378) were induced to significant levels (Table 5). Gluconokinase involved in the phosphorylation of gluconate (PP3416) was induced almost fourfold. The reactions from glucose to gluconate and from gluconate to 2-ketogluconate are known to take place in the periplasm, and entry into the cytoplasm of the oxidation products requires a gluconate-specific transport system (PP3417), as well as a 2-ketogluconate transporter (PP3377). These two transporters were also induced slightly more than twofold (Table 5). In addition, since pseudomonads use the *glk* pathway, glucose itself has to be transported to the cytoplasm. In connection with this, we found that the operon containing ORFs PP1015, PP1016, PP1017, PP1018, and PP1019, which encode a putative ABC glucose uptake system, was also induced at a high level. Therefore, the corresponding gene products most likely constitute a glucose transport system. We found that expression of the *glk* (PP1011) gene increased around 2.0-fold concomitantly with the induction of *zwf-1*, which encodes the

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

TABLE 5. Genes up-regulated in wild-type *P. putida* KT2440 cells growing on glucose versus cells growing on citrate

ORF no.	Gene	Function or family	Induction (*n*-fold)
Pathway genes encoding porins, transporters, and enzymes			
PP1444	*gcd*	Glucose dehydrogenase	2.18
PP1445	*oprB-2*	Porin B	1.96
PP1009	*gap-1*	Glyceraldehyde-3-*P*-dehydrogenase	7.08
PP1010	*edd*		3.46
PP1011	*glk*	Glucokinase	2.12
PP1022	*zwf-1*	Glucose-6-*P*-dehydrogenase	2.25
PP1024	*eda*		8.35
PP3376	*kguD*	Ketogluconate-6-*P*-reductase	3.23
PP3377	*kguT*	2-Ketogluconate transporter	2.18
PP3378	*kguK*	2-Ketogluconate kinase	2.31
PP3379	*kguE*	2-Ketogluconate epimerase	5.80
PP3382	*gadC*	Gluconate dehydrogenase *citC sub*	2.70
PP3383	*gad*	Gluconate dehydrogenase	2.00
PP3384		Hypothetical protein	3.90
PP3416	*gnuK*	Gluconokinase	3.99
PP3417	*gntP*	Gluconate transporter	2.10
PP3418		Hypothetical protein	2.08
PP1015	*gtsA*	ABC transport system	2.06
PP1016	*gtsB*	ABC transport system	2.47
PP1017	*gtsC*	ABC transport system	4.00
PP1018	*gtsD*	ABC transport system	4.14
PP1019	*oprB-1*	Porin B	4.23
Regulatory genes			
PP1012	*gltR*	GltR family	2.50[a]
PP1013	*gltS*	Sensor histidine kinase	2.01[a]
PP3380	*ptxS*	PtxS family	2.93[a]
PP3415	*lacI*	LacI family	2.45[a]
Other functions			
PP1014		Unknown	4.00[a]
PP2968		Putative membrane protein	2.35[a]
PP3384		Hypothetical protein	3.90[a]
PP3418		Hypothetical protein	2.11[a]
PP3555		Methyl-accepting chemotaxis receptor	2.05[a]
PP4073		Hypothetical protein	2.40[a]
PP4488		Hypothetical protein	2.60[a]
PP5346	*oadA*	Oxaloacetate decarboxylase	2.14[a]
PP5347	*accC*	Acetyl-CoA carboxylase	3.17[a]

[a] Change (*n*-fold).

most active glucose-6-*P*-dehydrogenase (PP1022). This enzyme converts glucose-6-phosphate into 6-phosphogluconate.

The key ED genes *edd* (PP1010) and *eda* (PP1024), as well as the gene encoding glyceraldehyde-3-*P*-dehydrogenase (PP1009), were induced in the response to glucose. In particular, *eda* mRNA levels were up to eightfold higher.

Of the five regulators in the vicinity of the catabolic enzymes, *hexR*, the *lacI* family, *ptxS*, *gltR-2*, and ORF PP1013 encoding a sensor kinase, we found that three, namely, GltR (PP1012), PtxS (PP3380), and a LacI-like regulator (PP3415), also increased their expression in response to glucose (Table 5). This suggests that these regulators control their own expression, which is well documented for repressors belonging to different families (31).

In addition to the genes that are directly involved in glucose catabolism, a limited number of genes that in principle have no obvious connection with glucose metabolism were also induced. No annotation is available for six of these genes; for the other three, the annotation suggests that the corresponding gene products encode an OAA decarboxylase (PP5346; *oadA*), an acetyl-coenzyme A (CoA) carboxylase, and a methyl-accepting chemotaxis receptor (PP3555). Induction of *oadA* coincides with the functioning of the pyruvate shunt reported above. This suggests that OAA in the wild-type strain growing on glucose may be produced in excess in the TCA cycle and scavenged via OAA decarboxylase.

To test the potential roles of the other gene products in glucose metabolism, we rescued mutants from the *Pseudomonas* Reference Culture Collection and tested growth on M9 minimal medium with glucose. All mutants, except for PP5347, exhibited growth characteristics similar to those of the wild-type strain in terms of growth kinetics, growth rates, and yields (not shown). The exception was mutant PP5347, which grew at a relatively low rate (growth rate [μ] = $0.22 \pm 0.02\ h^{-1}$), exhibited low yields (0.21 ± 0.01 g/g glucose), and accumulated large amounts of gluconate and 2-ketogluconate.

The set of downregulated genes is provided as supplemental material (see Table S1 in the supplemental material). Among the downregulated genes was, as expected, a set of genes involved in citrate metabolism, including two citrate uptake systems (PP0147 and PP0171), the TCA transport TctC protein (PP1418), the outer membrane copper receptor PP4838, and a series of transporters, porins, and metabolic enzymes probably involved in citrate, but not glucose, metabolism (see Table S1 in the supplemental material).

DISCUSSION

Multiple peripheral and convergent pathways in the initial steps of glucose metabolism in *P. putida*. The projection of the bioinformatic information derived from analysis of the *P. putida* KT2440 genome was not consistent with previous studies of glucose metabolism in other *P. putida* strains. To solve this apparent paradox, we adopted several complementary approaches. On the one hand, we took advantage of the already-available collection of mutants of *P. putida* KT2440 to investigate the potential use of more than one peripheral pathway in the initial step of glucose metabolism. Our results suggest that *P. putida* simultaneously uses all of the peripheral pathways to drive 6-phosphogluconate synthesis, since the inactivation of any of them had marked effects on the growth rates (Table 2). Based on the maximum growth rates (μ_{max}) of the wild type and strains with mutations in key enzymes of the three initial glucose pathways, our results show that glucose 6-kinase and the 2-ketogluconate loop are quantitatively more important pathways than the direct phosphorylation of gluconate by gluconokinase (GnuK) (Table 2). Indeed, the mutant deficient in GnuK activity grew at a higher rate than mutants in any of the other pathways. The presence of convergent pathways guarantees the utilization by this microorganism of a carbon source that is abundant in its natural niche.

Glucose catabolism enzymes in all peripheral pathways are inducible. Enzymatic determinations and microarray analyses showed that enzymes involved in glucose metabolism via gluconate and glucose-6-phosphate were inducible. Although glucose is probably the true inducer of *gcd*, gluconate seems to be the inducer of gluconokinase activity, since, in a *gcd* mutant growing on glucose, the level of this enzyme was 1/10 of that in the wild type (Table 3). Glucokinase and gluconokinase activities were expressed at a low basal level in cells grown with citrate, and these activities increased about 10-fold when the cells were grown on glucose. This is in agreement with the increase in *glk*, *gcd*, and *gnuK* expression observed in microarrays. Therefore, the transcriptional and enzymatic assays indicated that the glucokinase pathway and the enzymes for the direct phosphorylation of gluconate and the 2-ketogluconate loop enzymes are all inducible.

Further insights helping to understand the pattern of gene expression of these convergent pathways were obtained from the bioinformatic analysis of the physical localization of the genes, which showed that glucose catabolism genes in *P. putida* are organized in a series of clusters, that the *glk* gene forms an operon with the *edd* gene, and that the *zwf-1* gene forms an operon with the *eda* gene. Because the *edd* and *eda* gene products are the sine qua non for 6-phosphogluconate metabolism, the physical and genetic organization of the *edd*/*glk* and *eda*/*zwf-1* genes explains why this pathway is operational in *P. putida* KT2440 (46).

On the other hand, it is notable that the physical and transcriptional organization of genes for the metabolism of gluconate indicated that the catabolic enzymes, along with the set of specific porins and transporters, form single transcriptional units (Fig. 3). This structure points toward coevolution of the uptake system and the catabolic enzyme, which seems to guarantee the efficient entry and metabolism of the corresponding metabolites.

Our results also indicate that OprB1 could be a specific porin for the entry of glucose into the periplasmic space and to transport it to the cytoplasm via an ABC transport system made up of PP1015, PP1016, PP1017, and PP1108 (Fig. 3). This set of genes forms an operon independent of that in which the glucokinase gene (*glk*) is present. Therefore, although the genes for the uptake of glucose and its metabolism to 6-phosphogluconate are clustered, up to three different operons can be distinguished.

Global metabolic changes. ^{13}C-based flux analysis was used to identify network operation and responses as a consequence of different entry pathways for glucose. These analyses revealed some novel insights into the global metabolic networks that operate in *P. putida* during glucose metabolism. Our find-

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

ings showed that in the wild-type KT2440 strain, a major fraction of the TCA flux occurred through the pyruvate shunt instead of the malate dehydrogenase, which is similar to other *Pseudomonas* species but contrasts with net TCA fluxes in *E. coli* and *Bacillus subtilis* (13). This set of results indicates that in *P. putida*, excess C is removed via this shunt.

Fluxome analysis revealed that the operation of the TCA cycle was similar in the *glk*- or *gcd*-deficient mutants and in the parental strain, although the total amount of carbon taken up by the mutant strains was lower than that consumed by the parental strain, which probably results in a lower μ_{max} for the mutant strains. In these mutants, the pyruvate shunt operated at a level similar to that in the wild-type strain (Table 4). Net-flux analysis also confirmed the results of previous studies that showed that the pentose phosphate pathway does not contribute to glucose catabolism in *Pseudomonas*.

How are the glucose catabolic genes organized in other *Pseudomonas* species? Our unexpected results regarding glucose metabolism and the bioinformatics projection in *P. putida* led us to examine the gene organization for glucose catabolism in other *Pseudomonas* species for which the complete genome is available. We found that all key enzymes for the synthesis of 6-phosphogluconate from glucose via glucose-6-phosphate or via gluconate are present in *P. fluorescens* Pf-5, *Pseudomonas syringae* DC3000, and *P. aeruginosa* PAO1. BLAST analysis revealed a high degree of sequence conservation for all gene products, ranging from 70% to 95% similarity. The organization shown for porin B and the *gcd* gene in Fig. 3B is conserved in *P. aeruginosa* and *P. fluorescens*, but not in *P. syringae*.

The organization of the *edd/glk* operon and the divergent *gap-1* gene of *P. putida* (Fig. 3A) is also conserved in the other three *Pseudomonas* species. We propose that glucose-6-phosphate represents a compulsory pathway for glucose metabolism, regardless of the functioning of the gluconate pathways, in all of these strains. The organization of *gnuK* and the corresponding gluconate transporter of *P. putida* are also maintained in all three species. The *kgu* cluster is very similarly organized in *P. aeruginosa* and *P. fluorescens*, whereas in *P. syringae*, the genes are not present. Finally, the *gad* genes (PP3383 and PP3384) involved in the oxidation of gluconate to 2-ketogluconate have highly homologous genes in the genomes of *P. fluorescens* and *P. aeruginosa*, with very similar patterns of gene organization. In contrast, the gene homologs are not present in *P. syringae*. The bioinformatics analysis suggests that different species of the genus *Pseudomonas* use the ED pathway for glucose metabolism and that, in principle, all can synthesize the key intermediate 6-phosphogluconate through a series of converging peripheral pathways.

In summary, our study revealed that initial glucose—a source of carbon in the plant rhizosphere (3)—catabolism in the genus *Pseudomonas* occurs through a series of pathways that converge at the level of 6-phosphogluconate and that function simultaneously. This guarantees the efficient assimilation of a carbon source that is abundant in niches occupied by pseudomonads. Moreover, we have shown that, with glucose as a carbon source, malate produced in the TCA cycle is channeled mainly to OAA via the pyruvate shunt.

ACKNOWLEDGMENTS

This study was supported by grant BIO2006-05668 and by Grant to Group CVI191 from the Junta de Andalucía.

We thank Ana Hurtado for DNA sequencing and Jesus de la Torre for help in the selection of mutants. We also thank C. Lorente and M. Fandila for secretarial assistance and K. Shashok for improving the use of 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. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.)** 2006. Current protocols in molecular biology. John Wiley & Sons, New York, NY.
3. **Barber, D. A., and J. K. Martin.** 1976. The release of organic substances by cereal roots into the soil. New Phytol. **76:**68–80.
4. **Brazma, A., P. Hingamp, J. Quackenbush, G. Sherlock, P. Spellman, et al.** 2001. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. Nat. Genet. **29:**365–371.
5. **Dauner, M., and U. Sauer.** 2000. GC-MS analysis of amino acids rapidly provides rich information for isotopomer balancing. Biotechnol. Prog. **16:** 642–649.
6. **Duque, E., A. Haïdour, F. Godoy, and J. L. Ramos.** 1993. Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. J. Bacteriol. **175:**2278–2283.
7. **Duque, E., A. J. Molina-Henares, J. de la Torre, M. A. Molina-Henares, T. del Castillo, J. Lam, and J. L. Ramos.** 2007. Towards a genome-wide mutant library of *Pseudomonas putida* strains KT2440, p. 227–251. *In* J. L. Ramos (ed.), *Pseudomonas*, vol. V. Kluwer, London, United Kingdom.
8. **Duque, E., J.-J. Rodríguez-Herva, J. de la Torre, P. Domínguez-Cuevas, J. Múñoz-Rojas, and J. L. Ramos.** 2007. The RpoT regulon of *Pseudomonas putida* DOT-T1E and its role in stress endurance against solvents. J. Bacteriol. **189:**207–219.
9. **Entner, N., and M. Doudoroff.** 1952. Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*. J. Biol. Chem. **196:**853–862.
10. **Fischer, E., and U. Sauer.** 2003. Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS. Eur. J. Biochem. **270:** 880–891.
11. **Fischer, E., N. Zamboni, and U. Sauer.** 2004. High-throughput metabolic flux analysis based on gas chromatography-mass spectrometry derived ^{13}C constraints. Anal. Biochem. **325:**308–316.
12. **Frampton, E. W., and W. A. Wood.** 1961. Carbohydrate oxidation by *Pseudomonas* fluorescens VI. Conversion of 2-keto-6-phosphogluconate to pyruvate. J. Biol. Chem. **236:**2571–2577.
13. **Fuhrer, T., E. Fischer, and U. Sauer.** 2005. Experimental identification and quantification of glucose metabolism in seven bacterial species. J. Bacteriol. **187:**1581–1590.
14. **Fujita, Y., and T. Fujita.** 1987. The gluconate operon *gnt* of *Bacillus subtilis* encodes its own transcriptional negative regulator. Proc. Natl. Acad. Sci. USA **84:**4524–4528.
15. **Gottschalk, G.** 1986. Bacterial metabolism, 2nd ed. Springer-Verlag, New York, NY.
16. **Guymon, L. F., and R. G. Eagon.** 1974. Transport of glucose, gluconate, and methyl α-D-glucoside by *Pseudomonas aeruginosa*. J. Bacteriol. **117:**1261–1269.
17. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
18. **Hunt, J. C., and P. V. Phibbs.** 1983. Regulation of alternate peripheral pathways of glucose catabolism during aerobic and anaerobic growth of *Pseudomonas aeruginosa*. J. Bacteriol. **154:**793–802.
19. **Hylemon, P. B., and P. V. Phibbs, Jr.** 1972. Independent regulation of hexose catabolizing enzymes and glucose transport activity in *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. **48:**1041–1048.
20. **Lessie, T., and F. C. Neidhardt.** 1967. Adenosine triphosphate-linked control of *Pseudomonas aeruginosa* glucose-6-phosphate dehydrogenase. J. Bacteriol. **93:**1337–1345.
21. **Lessie, T. G., and P. V. Phibbs, Jr.** 1984. Alternate pathways of carbohydrate utilization in pseudomonads. Annu. Rev. Microbiol. **38:**359–387.
22. **Llamas, M. A., J. J. Rodríguez-Herva, R. E. Hancock, W. Bitter, J. Tommassen, and J. L. Ramos.** 2003. Role of *Pseudomonas putida tol-oprL* gene products in uptake of solutes through the cytoplasmic membrane. J. Bacteriol. **185:**4707–4716.
23. **Ma, J.-F., P. W. Hager, M. L. Howell, P. V. Phibbs, and D. J. Hassett.** 1998. Cloning and characterization of the *Pseudomonas aeruginosa zwf* gene encoding glucose-6-phosphate dehydrogenase, an enzyme important in resistance to methyl viologen (paraquat). J. Bacteriol. **180:**1741–1749.

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

24. **Marqués, S., J. L. Ramos, and K. N. Timmis.** 1993. Analysis of the mRNA structure of the *Pseudomonas putida* TOL meta fission pathway operon around the transcription initiation point, the *xylTE* and the *xylFJ* regions. Biochim. Biophys. Acta **1216:**227–236.
25. **Molina, M. A., C. Ramos, E. Duque, M. C. Ronchel, J. M. García, L. Wyke, and J. L. Ramos.** 2000. Survival of *Pseudomonas putida* KT240 in soil and in the rhizosphere of plants under greenhouse and environmental conditions. Soil Biol. Biochem. **32:**315–321.
26. **Nanchen, A., T. Fuhrer, and U. Sauer.** 2006. Determination of metabolic flux ratios from ^{13}C-experiments and gas chromatography-mass spectrometry data: protocol and principles. Methods Mol. Biol. **358:**177–198.
27. **Narrod, S. A., and W. A. Wood.** 1956. Carbohydrate oxidation by *Pseudomonas fluorescens*. V. Evidence of gluconokinase and 2-ketogluconokinase. J. Biol. Chem. **220:**45–55.
28. **Neidhart, F. C., J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.).** 1987. *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, DC.
29. **Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, et al.** 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. **4:**799–808.
30. **Phibbs, P. V., Jr., and R. G. Eagon.** 1970. Transport and phosphorylation of glucose, fructose and mannitol by *Pseudomonas aeruginosa*. Arch. Biochem. Biophys. **138:**470–482.
31. **Ramos, J. L., M. Martínez-Bueno, A. J. Molina-Henares, W. Terán, et al.** 2005. The TetR family of transcriptional repressors. Microbiol. Mol. Biol. Rev. **69:**326–356.
32. **Regenhardt, D., H. Heuer, S. Heim, D. U. Fernandez, C. Strompl, E. R. Moore, and K. N. Timmis.** 2002. Pedigree and taxonomic credentials of *Pseudomonas putida* strain KT2440. Environ. Microbiol. **4:**912–915.
33. **Rodríguez-Herva, J. J., and J. L. Ramos.** 1996. Characterization of an OprL null mutant of *Pseudomonas putida*. J. Bacteriol. **178:**5836–5840.
34. **Saravolac, E. G., N. F. Taylor, R. Benz, and R. E. Hancock.** 1991. Purification of glucose-inducible outer membrane protein OprB of *Pseudomonas putida* and reconstitution of glucose-specific pores. J. Bacteriol. **173:**4970–4976.
35. **Sauer, U., V. Hatzimanikatis, J. E. Bailey, M. Hochuli, T. Szyperski, and K. Wüthrich.** 1997. Metabolic fluxes in riboflavin-producing *Bacillus subtilis*. Nat. Biotechnol. **15:**448–452.
36. **Sawyer, M. H., L. Baumann, S. M. Berman, J. L. Canovas, and R. H. Berman.** 1977. Pathways of D-fructose catabolism in species of *Pseudomonas*. Arch. Microbiol. **112:**49–55.
37. **Schleissner, C., A. Reglero, and J. M. Luengo.** 1997. Catabolism of D-glucose by *Pseudomonas putida* U occurs via extracellular transformation into D-gluconic acid and induction of a specific gluconate transport system. Microbiology **143:**1595–1603.
38. **Seonghun, K., and L. Sun Bok.** 2006. Characterization of *Sulfolobus solfataricus* 2-keto-3-dioxy-D-gluconate kinase in the modified Entner-Doudoroff pathway. Biosci. Biotechnol. Biochem. **70:**1038–1316.
39. **Stern, I. J., C. H. Wang, and C. M. Gilmour.** 1960. Comparative catabolism of carbohydrates in *Pseudomonas species*. J. Bacteriol. **79:**601–611.
40. **Stokes, F. N., and J. J. R. Campbell.** 1951. The oxidation of glucose and gluconic acid by dried cell suspensions of *Pseudomonas aeruginosa*. Arch. Biochem. **30:**121–125.
41. **Swanson, B. L., P. Hager, P. Phibbs, Jr., U. Ochsner, M. L. Vasil, and A. N. Hamood.** 2000. Characterization of the 2-ketogluconate utilization operon in *Pseudomonas aeruginosa* PAO1. Mol. Microbiol. **37:**561–573.
42. **Szyperski, T., J. E. Bailey, and K. Wüthrich.** 1996. Detecting and dissecting metabolic fluxes using biosynthetic fractional ^{13}C labeling and two-dimensional NMR spectroscopy. Trends Biotechnol. **14:**453–459.
43. **Tamber, S., and R. E. W. Hancock.** 2004. The outer membranes of pseudomonads, p. 575–601. *In* J. L. Ramos (ed.), *Pseudomonas*, vol. I. Genomics, life style and molecular architecture. Kluwer, New York, NY.
44. **Temple, L., A. Sage, G. E. Christie, and P. Phibbs, Jr.** 1994. Two genes for carbohydrate catabolism are divergently transcribed from a region of DNA containing the *hexC* locus in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **176:**4700–4709.
45. **Tiwari, N. P., and J. J. Campbell.** 1969. Enzymatic control of the metabolic activity of *Pseudomonas aeruginosa* grown in glucose or succinate media. Biochim. Biophys. Acta **192:**395–401.
46. **van der Werf, M. J., B. Pieterse, N. Van Luijk, F. Schuren, B. van der Werff-van der Vat, K. Overkamp, and R. H. Jellema.** 2006. Multivariate analysis of microarray data by principal component discriminant analysis: prioritizing relevant transcripts linked to the degradation of different carbohydrates in *Pseudomononas putida* S12. Microbiology **152:**257–272.
47. **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.
48. **Vicente, M., and J. L. Cánovas.** 1973. Glucolysis in *Pseudomonas putida*: physiological role of alternative routes from the analysis of defective mutants. J. Bacteriol. **116:**908–914.
49. **Vicente, M., and J. L. Cánovas.** 1973. Regulation of the glucolytic enzymes in *Pseudomonas putida*. Arch. Mikrobiol. **93:**53–64.
50. **Wang, C. H., I. J. Stern, and C. M. Gilmour.** 1959. The catabolism of glucose and gluconate in *Pseudomonas* species. Arch. Biochem. Biophys. **81:**489–492.
51. **Weinel, C., K. E. Nelson, and B. Tummler.** 2002. Global features of the *Pseudomonas putida* KT2440 genome sequence. Environ. Microbiol. **4:**809–818.
52. **Wood, W. A., and R. F. Schwerdt.** 1954. Carbohydrate oxidation by *Pseudomonas fluorescens*. H. Mechanism of hexose phosphate oxidation. J. Biol. Chem. **206:**625–635.
53. **Wylie, J. L., and E. A. Worobec.** 1995. The OprB porin plays a central role in carbohydrate uptake in *Pseudomonas aeruginosa*. J. Bacteriol. **177:**3021–3026.
54. **Yuste, L., A. B. Hervás, I. Canosa, et al.** 2006. Growth phase-dependent expression of the *Pseudomonas putida* KT2440 transcriptional machinery analysed with a genome-wide DNA microarray. Environ. Microbiol. **8:**166–177.
55. **Zamboni, N., E. Fischer, and U. Saüer.** 2005. FiatFlux—a software for metabolic flux analysis from ^{13}C-glucose experiments. BCM Bioinformatics **6:**209.

Downloaded from jb.asm.org by Juan Ramos on July 2, 2007

Supplementary Table 1. Down-regulated genes.

A) Pathway genes encoding porins, transporters, regulators and enzymes

ORF	Gen	Function	Fold change
PP0145	--	Na+/Pi cotransporter family protein	-1.86
PP0146	--	Hypothetical protein	-2.22
PP0147	--	Citrate transporter	-4.12
PP0171	--	ABC transporter ATP-binding protein	-2.06
PP0213	*davD*	Succinate-semialdehyde dehydrogenase	-1.82
PP0214	*davT*	Aminobutyrate aminovalerate	-2.07
PP1157	--	Acetolactate synthase	-3.49
PP1186	*phoP*	Transcriptional regulatory protein PhoP	-2.79
PP1209	--	Cold-shock domain family protein	-2.4
PP1418	--	Tricarboxylic acid transport protein TctC putative	-2.17
PP1419	--	Porin putative	-3.25
PP1696	--	Membrane protein putative	-1.89
PP3252	--	Modification methylase HemK family	-2.05
PP3365	--	Acetolactate synthase	-3.01
PP3390	--	Porin putative	-2.39
PP3437	--	CBS domain protein	-1.92
PP4527	--	Membrane protein putative	-2.17
PP4683	*mrcB*	Penicillin-binding protein	-2.05
PP4838	*oprC*	Outer membrane copper receptor OprC	-1.84
PP4920	--	Lipoprotein putative	-1.9
PP4993	*gshB*	glutathione synthetase	-1.86
PP5245	--	Transcriptional regulator AraC family	-3.15

Other functions

ORF	Gen	Function	Fold change
PP0203	--	Tabtoxinine-beta-lactam dipeptidase, putative	-1.85
PP0672	--	Hypothetical protein	-1.81
PP0801	--	Hypothetical protein	-2.16
PP1051	*xcpV*	Type II secretion pathway protein XcpV	-2.18
PP1233	--	Conserved hypothetical protein	-2.06
PP1415	--	Conserved hypothetical protein	-1.94
PP3480	--	Hypothetical protein	-1.98
PP3631	--	Conserved hypothetical protein	-1.82
PP3672	--	Conserved hypothetical protein	-2.85

III. CAPÍTULO 2. RESUMEN.

A set of activators and repressors control peripheral glucose pathways in *Pseudomonas putida* to yield a common central intermediate

Teresa del Castillo, Estrella Duque and Juan Luis Ramos

Pseudomonas putida KT2440 canaliza la glucosa a partir de tres rutas que convergen en el intermediario 6-fosfogluconato y a través de la ruta central de Entner-Doudoroff. Los genes de estas rutas que convergen están agrupados en tres regiones independientes del cromosoma. Dentro de cada una de estas agrupaciones coexisten un número de unidades monocistrónicas y operones favoreciendo la co-expresión de enzimas catabólicas y sistemas de transporte. La expresión de las tres rutas es mediada por tres represores transcripcionales, HexR, GnuR y PtxS y por un regulador transcripcional positivo, GltR2. En este estudio, hemos generado mutantes en cada uno de los reguladores y llevado ensayos transcripcionales utilizando *microarrays* y fusiones transcripcionales. Estos estudios revelaron que HexR controla los genes de la glucoquinasa/glucosa-6-fosfato deshidrogenasa que rinden 6-fosfogluconato, las enzimas de Entner-Doudoroff que rinden gliceraldehído-3-fosfato y piruvato, y *gap-1* que codifica la gliceraldehído-3-fosfato deshidrogenasa. GltR2 es el regulador transcripcional que controla una porina específica para la entrada de glucosa al interior del espacio periplásmico, así como el operón *gtsABCD* para el transporte de glucosa a través de la membrana interna. GnuR es el represor del transporte del gluconato y de la gluconoquinasa responsable de la conversión del gluconato a 6-fosfogluconato. PtxS, no obstante, controla las enzimas de la oxidación de gluconato a 2-cetogluconato, su transporte y metabolismo, y un conjunto de genes no relacionados con el metabolismo de la glucosa.

A set of Activators and Repressors control Peripheral Glucose Pathways in *Pseudomonas putida* to yield a Common Central Intermediate

Teresa del Castillo[1], Estrella Duque[1] and Juan L. Ramos[1,2*]
[1]Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, E-18008 Granada, Spain and [2]Unidad Asociada de Contaminación Atmosférica, CSIC-Universidad de Huelva, Huelva, Spain

***Pseudomonas putida* KT2440 channels glucose to the central Entner-Doudoroff intermediate 6-phosphogluconate through three convergent pathways. The genes for these convergent pathways are clustered in three independent regions on the host chromosome. A number of monocistronic units and operons coexist within each of these clusters, favouring co-expression of catabolic enzymes and transport systems. Expression of the three pathways is mediated by three transcriptional repressors, HexR, GnuR and PtxS, and by a positive transcriptional regulator, GltR2. In this study we generated mutants in each of the regulators and carried out transcriptional assays using microarrays and transcriptional fusions. These studies revealed that HexR controls the glucokinase/glucose-6-phosphate dehydrogenase genes that yield 6-phosphogluconate, the genes for Entner Doudoroff enzymes that yield glyceraldehyde-3-phosphate and pyruvate, and *gap-1*, which encodes glyceraldehyde-3-phosphate dehydrogenase. GltR2 is the transcriptional regulator that controls specific porins for the entry of glucose into the periplasmic space, as well as the *gtsABCD* operon for glucose transport through the inner membrane. GnuR is the repressor of gluconate transport and gluconokinase responsible for the conversion of gluconate into 6-phosphogluconate. PtxS, however, controls the enzymes for oxidation of gluconate to 2-ketogluconate, its transport and metabolism, and a set of genes unrelated to glucose metabolism.**

Pseudomonas putida mt-2 was isolated from soils in Japan and identified as able to grow on *meta*-toluate as the sole C-source. Sequencing of its genome revealed that it possesses many genes encoding proteins involved in the degradation of plant-derived chemicals including a variety of methoxylated aromatic acids, hydroxylated aromatic acids and other compounds (6, 12, 23). The strain was also found to possess information for the degradation of fructose (39) and glucose (4, 39), two of the most abundant sugars present in plant root exudates (14).

del Castillo *et al.* (4) showed that glucose catabolism in this strain occurred through the simultaneous operation of three peripheral pathways that converge at the level of 6-phosphogluconate (6PG) (Figure 1). This compound is further metabolized by the Entner-Doudoroff pathway to yield glyceraldehyde-3-phosphate and pyruvate. Glucose enters the periplasmic space via the OprB outer membrane porin(s) (16, 33, 37). Once in the periplasm, glucose can be either transported to the cytoplasm via an ABC glucose transport system (2, 11) or oxidized to gluconate. In the cytoplasm glucose is phosphorylated via a glucokinase, and the resulting glucose-6-phosphate is converted to 6PG by the concerted action of the glucose-6-phosphate dehydrogenase and 6-phosphoglucolactonase. Gluconate in the periplasm can be either transported into the cytoplasm via the GntP protein and subsequently phosphorylated to 6PG, or oxidized to 2-ketogluconate, which is transported to the cytoplasm and, upon phosphorylation, is reduced to 6PG by the concerted action of the *kguK/kguD* gene products (Figure 1).

Genome analysis of the set of genes involved in glucose metabolism revealed that they were organized in sets of clusters scattered along the chromosome (4 and Figure 2). Transcriptional analysis revealed a complex organization in operons and monocistronic units within each of these clusters (Figure 2). One of the most relevant features this study disclosed was that the *oprB1* gene, encoding the OprB1 porin involved in the

Corresponding author. Mailing address:EEZ-CSIC, C/Prof. Albareda, 1, E-18008 Granada, Spain. Phone:34 958181600. fax: 34 958 135740. E-mail: jlramos@eez.csic.es.

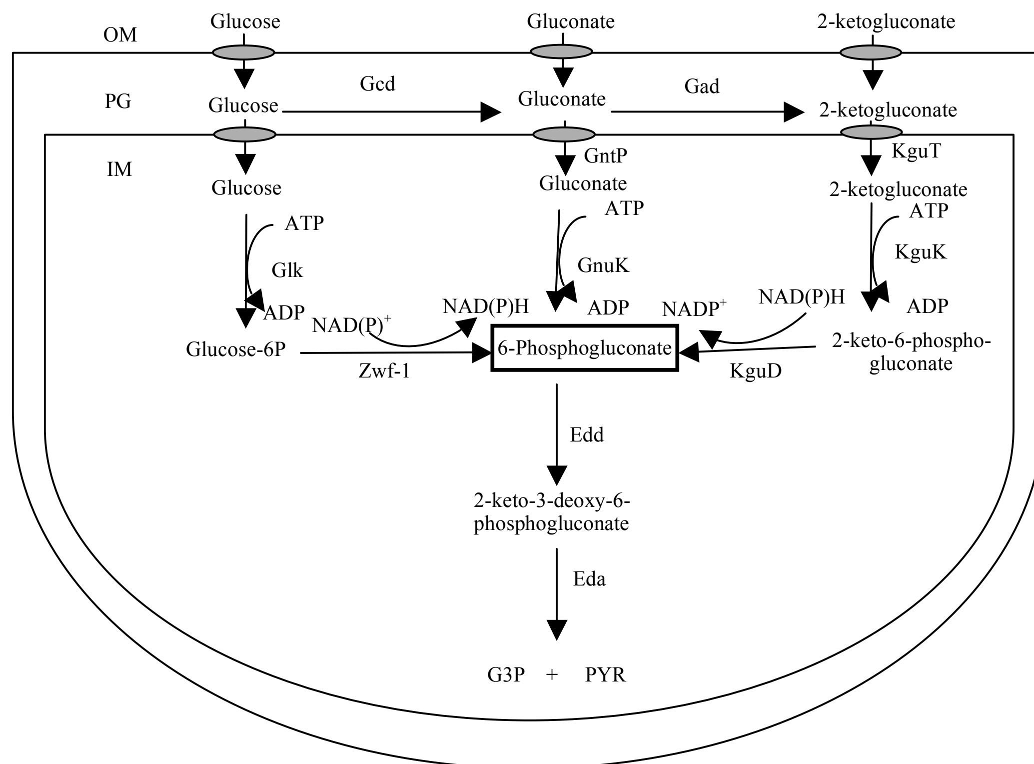


Figure 1. Glucose catabolism in *P. putida* as deduced from gene annotations. At the top are the events that occur in the outer membrane and the reactions that take place in the periplasmic space. Also shown are the transport of glucose, gluconate, and 2-ketogluconate into the cell. The set of catabolic reactions that take place in the cytoplasm is depicted. The genes that encode the enzymes involved are indicated for all steps. OM, outer membrane; PG, periplasmic space; IM, inner membrane.

entry of glucose into the periplasmic space, was in an operon made up of open reading frames (ORFs) PP1015 to PP1019, which encodes the glucose transport system (Figure 2A). Another relevant finding from this study was that the *zwf-1* allele encoding glucose-6-phosphate dehydrogenase (18) was in an operon within the *eda* gene that encodes one of the Entner-Doudoroff enzymes, whereas the gene encoding glucokinase (*glk*) formed an operon with the *edd* gene. Transcribed in the opposite direction to the *zwf-1* promoter was the *hexR* gene, encoding a putative repressor (Figure 2A).

The gene that encodes the gluconate transporter gene (*gntP*) and the gene encoding the gluconokinase (*gnuK*) were contiguous on the chromosome, but did not form an operon (4; Figure 2). Transcribed divergently with respect to the *gnuK* gene was the *gnuR* gene, which encodes a repressor assigned to the LacI family (Figure 2C). The gluconate dehydrogenase genes (PP3384 and PP3382) and the genes encoding the 2-ketogluconate transporter and its subsequent metabolism are within a cluster in the chromosome that covers from PP3376 to PP3384. A regulator of the PtxS family of repressors is found within this cluster of genes, which encode enzymes for gluconate oxidation to 2-ketogluconate (2KG) and its subsequent metabolism (Figure 2B).

Global transcriptomic analysis revealed that all of the catabolic genes and regulators mentioned above were up-regulated in response to glucose, except for the *hexR* gene (4). It was also shown that when KT2440 bearing the TOL plasmid was grown simultaneously in the presence of glucose and toluene, the *glk* branch and the glucose transport system were under catabolite repression control, which was not the case for the set of genes involved in gluconate metabolism. This pointed towards independent evolutionary acquisition of genes for glucose metabolism and differential gene regulation.

The present study was undertaken to further define the regulatory circuits that allow *P. putida* KT2440 to use glucose as the sole C-source. To this end mutants in each of the four potential regulators within the cluster of genes for glucose catabolism were generated. Growth characteristics, global transcriptomic analysis and transcriptional fusions revealed that the GltR2 protein is a transcriptional activator involved in the induction of the glucose transport system. HexR and GnuR are local repressors; the latter controls the expression of gluconate transport and its phosphorylation system, whereas the former controls the three operons involved in glucose metabolism via glucokinase. The regulator PtxS acted as the local repressor of

the 2-KG genes, although surprisingly, in a *ptxS*-deficient mutant, 10 genes with no relation to glucose metabolism were up- or down-regulated, indicating that this regulator exerts a more global type of control.

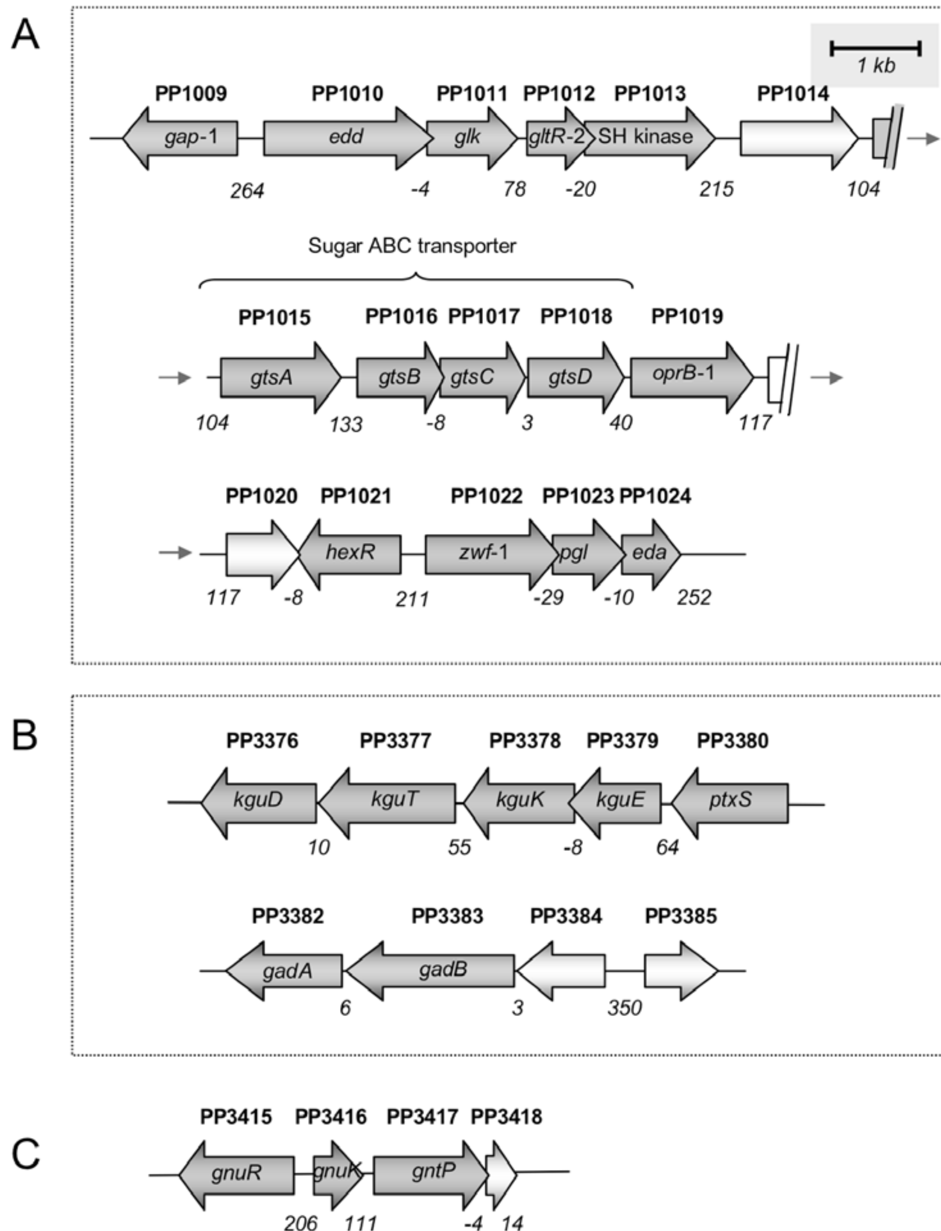


Figure 2. Organization of the peripheral glucose catabolic genes. Gene numbering and organization are derived from the annotation of the complete genome sequence of *P. putida* KT2440 in the TIGR database. (A) Set of 16 genes, most of which have been assigned a function based on enzymatic assays. (B) Set of 8 genes, most of which have a specific role in gluconate catabolism through 2KG. (C) Set of 4 genes involved in gluconate catabolism in *P. putida* KT2440.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Pseudomonas putida* KT2440 and a series of isogenic mutant strains with insertions in the *gltR*2, *hexR, ptxS* and *gnuR* genes are shown in Table 1. The GltR2 and HexR mutant strains were identified by Duque *et al.* (6) in a general screening after random mutagenesis of *P. putida* with mini-Tn*5*-Km. The *ptxS* and *gnuR* mutants were generated in this study, and are described below. *Pseudomonas putida* strains were grown at 30 ºC in M9 minimal medium with glucose (0.3% [wt/vol]) or sodium citrate (16 mM) as a carbon source. When appropriate, antibiotics were added at the following concentration: kanamycin (Km), 25 µg/ml and tetracycline (Tc), 10 µg/ml.

DNA techniques. Preparation of plasmid and chromosomal DNA, digestion with restriction enzymes, ligation, electrophoresis, and Southern blotting were as described before (28, 30).

Site-specific homologous inactivation of *gnuR* and *ptxS* . To construct mutant strains bearing an inactivated chromosomal version of the *gnuR* and *ptxS* genes, we generated the corresponding knock-out using the appropriate derivatives of pCHESIΩKm (17). Plasmid pCHESIΩKm is based on pUC18 and bears the origin of transfer *oriT* of RP4 and the Ω-Km interposon of plasmid pHP45ΩKm cloned as a *Hin*dIII fragment. To generate the desired mutation an internal fragment between 400-500 bp of the target gene was amplified by PCR with primers containing the *Eco*RI and *Bam*HI sites to amplify an internal part of the *gnuR* gene, and with primers containing the *Eco*RI and *Xba*I sites to amplify the *ptxS* gene. The amplified fragments were subsequently cloned between the *Eco*RI and *Bam*HI sites of pCHESIΩ Km (Table 1) in the case of *gnuR*, and *Eco*RI and *Xba*I in the case of the *ptxS* gene (Table 1). In both cases the fragments were cloned in the same transcriptional direction as the P_{lac} promoter. The recombinant plasmids were introduced into *P. putida* KT2440 by electroporation, and transformants bearing a cointegrate of the plasmid in the host chromosome were selected on M9 minimal medium with citrate as a carbon source and Km. The correct insertion of the mutant allele was confirmed by colony-screening PCR using a primer based on the Km marker gene and another primer that was annealed to the sequence complementary to the cloned gene fragment. The correctness of the construction was confirmed by Southern blotting using the target gene as a probe (not shown).

TABLE1. Strains and plasmids used in this study

Strain	Genotype or relevant characteristics	Reference
P. putida KT2440	Wild type, prototroph, Cm^R, Rif^R	This laboratory
P. putida M2052[b]	*gltR*-2*:*mini-Tn*5*-Km, Km^R, Rif^R	6
P. putida M1881[b]	*hexR:*mini-Tn*5*-Km, Km^R, Rif^R	6
P. putida PSC303[c]	*ptxS::*pCHESIΩ-Km, Rif^R	This study
P. putida PSC304[c]	*gnuR::*pCHESIΩ-Km, Rif^R	This study
Plasmids		
pMP220	Tc^R, '*lacZ* promoter probe plasmid	9
pCHESIΩKm	Km^R	17
pTC1	pCHESIΩ-Km bearing *'ptxS'*	This study
pTC2	pCHESIΩ-Km bearing *'gnuR'*	This study
pBedd	Tc^R, pM220 bearing the promoter region of the *edd* gene	This study
pPP1015	Tc^R, pM220 bearing the region upstream of ORF PP1015.	This study
pBhex	Tc^R, pM220 bearing the promoter region of the *hexR* gene	This study
pBzwf	Tc^R, pM220 bearing the promoter region of the *zwf* gene	This study
pBgnuK	Tc^R, pM220 bearing the promoter region of the *gnuK* gene	This study
pPP3384	Tc^R, pM220 bearing the promoter region of ORF PP3384	This study

[a]Cm^R, Km^R and Rif^r stand for resistance to chloramphenicol, kanamycin, and rifampin, respectively.
[b]Collection of KT2440 mutants available at the Consejo Superior de Investigaciones Científicas, Granada, Spain.
[c]*Pseudomonas* Stock Center.

Construction of ‘*lacZ* transcriptional fusions and determination of β-galactosidase assays. Transcriptional fusions were constructed by cloning the putative promoters in 5′ with respect to ‘*lacZ* in pMP220 (9). To this end the sequence upstream of th*e edd,* PP1015, *zwf-1* and *hexR* genes was amplified by PCR with primers containing the *Eco*RI-*Pst*I sites at 5′ and 3′ of the amplified fragment. These fragments were cloned into the *Eco*RI-*Pst*I sites of pMP220. All fusion constructs (pBedd, pPP1015, pBhex and pBzwf) were confirmed by DNA sequencing (Table 1). Transcriptional fusion constructs were assayed in *P. putida* KT2440 and its isogenic mutant background.

The pBedd, pPP1015, pBzwf and pBhex plasmids were introduced into *Pseudomonas putida* KT2440 or its isogenic mutants by electroporation. Transformants were grown overnight on minimal medium with citrate as the sole carbon source in the presence of Tc, and then the cultures were diluted to reach a turbidity (OD_{660}) of 0.05 in the same medium. After 1 h, the cultures were supplemented or not with 5 mM glucose, gluconate, 2KG or fructose and incubation continued at 30 ºC with shaking for 6 h. β-Galactosidase activity was assayed in permeabilized whole cells according to Miller’s method (1972). Three independent assays were run in triplicate.

***Pseudomonas putida* microarrays.** The genome-wide DNA chip used in this work (printed by Progenika Biopharma) was described in detail previously (7, 44). It consists of an array of 5539 oligonucleotides (50-mer) spotted in duplicate onto γ–aminosilane-treated slides, and covalently linked with UV light and heat. The oligonucleotides represent 5350 of the 5421 predicted ORFs annotated in the *P. putida* KT2440 genome (23). The chips are also endowed with homogeneity controls consisting of oligonucleotides for the *rpoD* and *rpoN* genes spotted at 20 different positions, as well as duplicate negative controls at 203 predefined positions.

RNA isolation, preparation of labelled cDNA, microarray hybridization and data analysis. *Pseudomonas putida* KT2440 and mutant cells were grown in minimal medium with citrate as a carbon source to an OD_{660} of about 0.5. Cells from 12-ml culture samples were harvested by centrifugation (7000 × *g*) at 4 °C in tubes pre-cooled in liquid nitrogen. Total RNA was isolated with TRI Reagent (Ambion, ref. 9738), as recommended by the manufacturer, and then subjected to DNase treatment followed by purification with RNeasy columns (Qiagen, cat. no. 74104). RNA concentration was determined spectrophotometrically and its integrity was assessed by agarose gel electrophoresis.

To prepare fluorescently labelled cDNA we primed 25 µg of RNA with 7.5 µg of $pd(N)_6$ random hexamers (Amersham, Cat. No. 27-2166-01). Probes were synthesized at 42 °C for 2 h exactly as described before (7). Labelling efficiency was checked with a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). Prior to the hybridization process, the microarray slides were blocked by immersion in 5× SSC (1× SSC is 0.15 M NaCl; 15 mM sodium citrate, pH 7), 0.1% (wt/vol) SDS, and 1% (wt/vol) bovine serum albumin for 1 h at 42 ºC. Then the slides were washed by two successive immersions in MilliQ water at room temperature, followed by a final wash with isopropanol. The slides were spin-dried by centrifugation at 1500 × g for 5 min and used within the next hour. Equal amounts of Cy3- and Cy5-labelled cDNA, one of them corresponding to the control and the other one to the problem under study, were mixed, dried in a Speed-Vac and reconstituted in 35 µl hybridization buffer (5 × SSC, 25% [vol/vol] formamide, 0.5% [wt/vol] SDS, 5 × Denhardt’s solution, 5% [wt/vol] dextran sulfate) preheated to 42 ºC. The labelled probe was denatured at 98 °C for 3 min, applied to the microarray slide and covered with a glass coverslip. The slide was then placed in a humidified hybridization chamber (AHC ArrayIt Hybridization Cassette; Telechem International, Inc.) and incubated for 18 to 20 h in a water bath away from light at 42 °C. Following hybridization, the microarrays were washed by gentle agitation in 2× SSC, 0.1% [wt/vol] SDS at 42 ºC for 5 min, followed by a 5-min wash at RT in 1× SSC, two 5-min washes in 0.2× SSC, and a final 5-min wash in 0.1× SSC. Finally, the slides were spin-dried in a centrifuge at 1500 × g for 5 min and scanned on a GenePix 4100A scanner (Axon Instruments, Inc.). Images were acquired at 10-µm resolution, and the background-subtracted median spot intensities were determined with GenePix Pro 5.1 image analysis software (Axon Instruments, Inc.). Signal intensities were normalized by applying the LOWESS intensity-dependent normalization method (42), and statistically analysed with Almazen System software (Alma Bioinformatics S.L.). For appropriate statistical analysis of the results, RNA preparations from at least four independent cultures were tested for each strain (1). *P* values were calculated with Student’s *t* test. A particular ORF was considered differentially expressed if (i) the fold change was at least 1.8 and (ii) the *P* value was 0.05 or lower.

Primer extension analysis. For primer extension analyses we isolated RNA as above, and the process was carried out as described by Marqués *et al.* (19). The amount of total RNA template used in each reaction varied between 90 and 100 µg. About 10^5 cpm of ^{32}P-labelled 5′-end oligonucleotides was used as a primer in extension reactions (See Supplementary Table 1). To improve electrophoresis quality, the cDNA products obtained after reverse transcriptase were treated with DNAse-free RNAse (Roche, Ref. number 1119915). The cDNA products were separated

and analysed in urea-polyacrylamide sequencing gels which were exposed to a phosphorscreen (Fuji Photo Film Co; LTD) for 5 to 12 h. Phosphorscreens were scanned with a phosphorimaging instrument (Molecular imager FX, Bio-Rad). Data were quantified with Quantity One software (Bio-Rad)

RESULTS

Construction of mutants in genes putatively involved in the regulation of glucose metabolism and their characterization. *Pseudomonas putida* KT2440 and the four isogenic knock-out mutants in *gltR2, hexR, ptxS* and *gnuR* were grown overnight on M9 minimal medium with glucose as the sole C-source. Then they were washed and suspended in the same medium to reach an initial cell density of 0.05 units at 660 nm, and growth was monitored. We found that after an initial 2-h lag phase the wild-type strain grew exponentially with a μ_{max} of 0.59 ± 0.02 h^{-1} and consumed glucose at a rate of 7.60 ± 0.19 mmol glucose/g cell biomass × h^{-1}. The mutants deficient in *gnuR, hexR* and *ptxS* grew promptly, with growth rates (0.57 ± 0.01 h^{-1} to 0.60 ± 0.02 h^{-1}) and glucose consumption rates (6.84 ± 0.8 to 9.7 ± 0.5 mmol glucose/g cell biomass × h^{-1}) similar to those of the parental strain. In the *gltR2* mutant the lag phase was much longer (about 5 h), but once the culture began to grow the growth and glucose consumption rates were almost identical to those of the parental strain.

TABLE 2. Genes differentially up-regulated in the GnuR mutant background with respect to the genes expressed in the parental strain.

ORF number	*Gene*	Function	Fold induction
PP1023	*pgl*	6-phosphogluconolactonase	1.86
PP1516	--	RND efflux pump	1.91
PP2333	--	GntR family of regulators	1.82
PP2741	--	Hypothetical protein	1.96
PP3415	*gnuR*	Transcriptional repressor	2.85
PP3416	*gnuK*	Gluconate kinase	2.60
PP3417	*gntP*	Gluconate transporter	1.82
PP3418	--	Hypothetical protein	1.80
PP3642	--	Hypothetical protein	1.93

These results suggested that in general, no major effect on growth rate and glucose uptake rate was associated with deficiency in any of the potential regulatory genes for glucose catabolism. However, the above results provided no indication of the specific effects of the mutations on the different catabolic segments involved in glucose degradation. To gain insights into local and global effects, the parental strain and each of the mutant strains were grown on M9 minimal medium with citrate, and total mRNA was prepared and labelled as indicated in Materials and Methods. The expression level was then compared between the mutant strain and the parental one (Tables 2 to 5).

The divergent organization of *gnuR* and the *gnuK gntP* genes (Figure 2) suggested that GnuR might regulate these genes. Indeed we found that in the *gnuR* mutant background the *gnuK* gene was expressed at a level 2.6-fold higher than in the parental strain, as was also the case for *gnuR*, indicating that GnuR controls its own expression. RT-PCR confirmed that the ORFs encoding PP3417 and PP3418 formed an operon. In agreement with this observation was the fact that expression of both genes increased, and that of the ORF encoding the PP3418 hypothetical protein was 1.8-fold times higher in the mutant strain than in the parental one. We found that expression of the *pgl* gene was induced about 1.9-fold, whereas expression of the *zwf-1* gene upstream of *pgl* was not induced. This was somewhat surprising since our previous RT-PCR assays showed that they were part of the *hex* regulon (see below). The results also revealed that the level of four other genes varied ≥1.8-fold. This was found for a set of genes that were not related to gluconate metabolism, among which were PP2333, which encoded a regulator; PP1516, which encoded an RND membrane protein; and the hypothetical proteins PP2741 and PP3642 (Table 2). RT-PCR assays revealed that all of these genes were monocistronic and did not form operons with adjacent genes (not shown).

In the *hexR* mutant background some of the genes in the PP1009 to PP1024 segment of the chromosome changed their expression, whereas another set did not do so (Table 3). In the *hexR* mutant background expression of PP1009, which encodes *gap-1*, and the operon PP1010 to PP1012 increased between 4.89- and 1.94-fold. PP1022 through to PP1024 also increased its expression between 2.04- and 6.07-fold. Only one gene outside the cluster also exhibited significant increase in expression: the ORF encoding the PP4488 hypothetical protein. Surprisingly, the expression level of PP1015 to PP1018, which encodes the glucose ABC transport systems, did not change. This result suggested that its regulation is not under the control of HexR. The level of the *hexR* gene did not change either, which suggests that the protein does not regulate its own expression. Therefore, HexR seems to control the expression of the glucokinase and Entner-Doudoroff pathway enzymes, together with glyceraldehyde-3-phosphate dehydrogenase, the enzyme that acts on the final product of glucose metabolism and helps to channel glucose to Krebs cycle intermediates (Figure 1).

TABLE 3. Genes differentially up-regulated in the HexR mutant background with respect to the genes expressed in the parental strain.

ORF number	Gene	Function	Fold induction
PP1009	*gap-1*	Glyceraldehyde-3-P-dehydrogenase	4.89
PP1010	*edd*	6-Phosphogluconate dehydratase	4.06
PP1011	*glk*	Glucokinase	2.21
PP1012	*gltR-2*	DNA-binding response regulator GltR	1.94
PP1022	*zwf*-1	Glucose-6-P-dehydrogenase	2.04
PP1023	*pgl*	6-Phosphogluconolactonase	6.07
PP1024	*eda*	2-Dehydro-3-deoxyphosphogluconate aldolase	4.77
PP4488	--	Conserved hypothetical protein	3.38

The *ptxS* gene is located within a set of genes that includes the gluconate dehydrogenase operon (PP3384 to PP3382) and the 2-ketogluconate metabolism operon (PP3379 to PP3376). We found that in the *ptxS* mutant background, expression of the first gene in these two operons and that of *ptxS* itself increased between 2.06- and 3.23-fold (Table 4). We also found that the genes for two hypothetical proteins (PP2741 and PP2984) also increased in this mutant background (Table 4). A number of genes were repressed in this mutant background by 1.93- to 3.67-fold, and included 8 ORFs, 4 of which encoded proteins of unknown function, and the other 4 encoding proteins unrelated to glucose metabolism (PP3064, PP3087, PP3095 and PP3252).

TABLE 4. Genes differentially up-regulated in the PtxS mutant background with respect to the genes expressed in the parental strain.

ORF	**Gene**	**Function**	**Fold change**
PP3064		gpX-pyocin R2 tail component protein	−2.37
PP3087		Exonuclease ABC A subunit putative	−1.81
PP3088		Conserved hypothetical protein	−3.06
PP3089		Conserved hypothetical protein	−3.67
PP3095		Chaperone-associated ATPase putative	−2.21
PP3099		Conserved hypothetical protein	−1.94
PP3100		Conserved hypothetical protein	−1.93
PP3252		Modification methylase HemK family	−2.80
PP2741	-	Conserved hypothetical protein	1.92
PP2984	-	Hypothetical protein	1.97
PP3379	*kgu*E	Epimerase KguE putative	2.20
PP3380	*ptxS*	PtxS family	3.23
PP3384	-	Conserved hypothetical protein	2.06

A *gltR2* mutant was generated as described in Materials and Methods. When the transcriptional pattern in cells grown on citrate was compared to that of the parental strain, we found a significant increase in expression (almost 2-fold) for a set of 5 ORFs that encoded proteins of unknown function, which were scattered along the chromosome and had no links with the glucose catabolism gene cluster (Table 5). These results suggested that GltR2 does not function as a repressor for glucose metabolism.

TABLE 5. Genes differentially up-regulated in the GltR2 mutant background with respect to the genes expressed in the parental strain.

ORF	Gene	Function	Fold change
PP0299	-	Conserved hypothetical protein	1.90
PP1516	-	RND efflux pump	1.95
PP1545	-	Hypothetical protein	1.81
PP2059	-	Conserved hypothetical protein	1.86
PP2722	-	Conserved hypothetical protein	1.97

To test whether GltR2 could act as an activator of the glucose transport system as occurs in *P. aeruginosa* (10), we fused the putative promoter region of ORF PP1015 to ‘*lacZ* in pMP220 to yield P_{gstA} and assayed the fusion both in the wild type and in the set of mutant regulators of the glucose degradation pathway. We found that the in parental strain, in the *hexR*, *gnuR* and *ptxS*-mutant backgrounds, β-galactosidase activity increased from negligible levels in the absence of glucose to 630-790 Miller Units in the presence of this sugar. In the *gltR2* mutant background expression was null even in the presence of glucose. This set of results suggests that GltR2 is the positive transcriptional regulator of the glucose transport system.

Fusion of the glucose catabolic operon promoter regions to *'lacZ* to validate the arrays. To verify the set of results presented above we also generated fusions of the promoter of the *edd/glk* operon to ‘*lacZ*(Pedd), as well as to the promoter region of the *zwf-1/pgl/eda* operon (Pzwf). The resulting plasmids, pBedd and pBzwf (Table 1), were transformed in the wild-type strain and the *hexR* mutant, and β-galactosidase activity was determined in the absence and in the presence of glucose (Table 6). P_{edd} and P_{zwf} expression in the absence of glucose was lower in the parental strain than in the *hexR* mutant, which confirmed that HexR represses expression of these two promoters. However, in the presence of glucose the level of expression from these promoters was equally high in both backgrounds (Table 6). We also fused the *hexR* promoter P_{hexR} to ‘*lacZ* and tested its expression. As shown in Table 6, the level of expression was equally high in both backgrounds regardless of the presence of glucose. Hence, these results confirm the expression level determined in microarrays.

We also used fusions of P_{gnuK} and $P_{orfPP3384}$ to *'lacZ* and measured their expression in GnuR and PtxS mutant backgrounds. In the *gnuR* mutant background expression from P_{gnuK} was about 4-fold higher than in the parental strain in the absence of glucose. Expression from $P_{orfPP3384}$ was 2-fold higher in the PtxS-deficient background than in the parental strain in the absence of glucose (not shown). Therefore, this set of results also confirmed our transcriptomic results.

Identification of the transcription start point (tsp) of the glucose catabolism operon. To learn more about expression of the operons regulated by the three transcriptional repressors (PtxS, GnuR, HexR) and the activator (GltR2) in glucose metabolism, we decided to determine the tsp of a number of well-established operons.

a) HexR-regulated genes. For the HexR-regulated genes we determined the tsp of *hexR*, *gap-1*, the *edd/glk* operon and the *zwf-1/eda* operon (See Figure 3 for *gap-1* and Supplementary Figure 1 for the others). In all cases a single tsp was found. For *hexR* the transcription start point was a C located 67 bp upstream of the first G of the first potential GTG codon. We found −10 (−12 TACGAT−7) and −35 (−35, TGGTAC, −30) hexamers similar to those recognized by sigma-70 in *Pseudomonas* (5). No indicative feature of inverted or direct repeats was found between −143 and +67, in accordance with the constitutive expression of this gene.

For *gap-1, edd* and *zwf* a single tsp was also identified, and −10/ −35 hexamers similar to those recognized by RNA polymerase with sigma-70 were found. These three promoters are under HexR repressor control. Repressors have often been

found to exert their role by binding to inverted repeats that partly or fully overlap the RNA-polymerase binding site (31). For this reason we searched for potential inverted repeats between −50 and +50. We found a $GntTtTaN_{12}TAAAAnC$ inverted motif that was located between −21 and +8 in the *zwf*-1 promoter, between −16 and +13 in the *edd* promoter, and between the −17 and +15 in the *gap*-1 promoter. The potential role of this sequence as the true site of recognition by HexR awaits further experimental studies. In accordance with HexR recognizing the above inverted motif is that it was also found in the promoter region of the ORF PP4488 whose level also increased in the *hexR* mutant background.

TABLE 6. Expression from P_{edd} and P_{zwf} in KT2440 and the *hexR* mutant strain.

Promoter	Background	Glucose	β-gal activity
P_{edd}	Wild type	-	60 ± 3
	hexR mutant	-	120 ± 5
	Wild type	+	150 ± 5
	hexR mutant	+	200 ± 10
P_{zwf}	Wild type	-	100 ± 20
	hexR mutant	-	1260 ± 10
	Wild type	+	1210 ± 10
	hexR mutant	+	1340 ± 40
P_{hexR}	Wild type	-	220 ± 10
	hexR mutant	-	205 ± 10
	Wild type	+	295 ± 15
	hexR mutant	+	255 ± 20

b) GnuR-regulated genes. We determined the +1 of the *gnuR* and *gnuK* promoters (Figure 3). The +1 in both cases was a G located 37 and 151 bp upstream of the A of the first potential ATG. As above, in front of these genes we found potential −10/−35 hexamers similar to those sigma-70 recognizes in *Pseudomonas* (5). Overlapping them was an inverted repeat, $GTCCnTACN_3GTAnGGAC$, located between −78 and −96 in the *gnuR* promoter and +59 and +77 in the *gnuK* promoter. This motif, however, was not found in front of the other 5 genes whose level of expression increased in the absence of GnuR, suggesting they may not be under the direct control by this regulator.

c) PtxS-regulated promoters. In PtxS we determined the +1 of the *ptxS* gene (PP3380) and the operon promoter of the 2KG operon metabolism (*kguE*) (Figure 3). Both promoters had a single tsp with AT-rich −10 hexamers and relatively well conserved −35 hexamers. The potential PtxS recognition site was a 5′-TGAAACCGGTTTCA-3′ inverted repeat that overlapped the −10 region in *kguE* and covered the +1 region in *ptxS*. This motif is also present in the *ptxS* gene in *P. aeruginosa* and has been shown to be the target of PtxS in this human pathogen (35). This motif also overlaps the −10 region of the promoter of PP3384. We searched for this motif in other regulated promoters in the *ptxS* mutant background, though it was not found in the putative promoter regions of these genes.

d) GltR2 controls expression from the *gtsA* gene in response to glucose. In cells growing on glucose we mapped the +1 of *gtsA*, which corresponded to A located 96 bp upstream of the start ATG codon. The promoter exhibited relatively well conserved −10 hexamers, but lacked a defined −35 hexamer, as is typical of promoters mediated by activators that respond to chemical signals (9, 13, 34).

DISCUSSION

When *Pseudomonas putida* is exposed to glucose, the sugar—upon reaching the periplasm—can either be internalized and subsequently metabolized via glucokinase/glucose-6-phosphate dehydrogenase to 6PG or converted into 2KG in the periplasmic space via glucose dehydrogenase and gluconate dehydrogenase. Gluconate and 2KG are transported to the cytoplasm and converted into 6PG (Figure 1). The scattered distribution of the cluster of genes for each of the three pathways along the chromosome of *P. putida* suggests that each of the metabolic

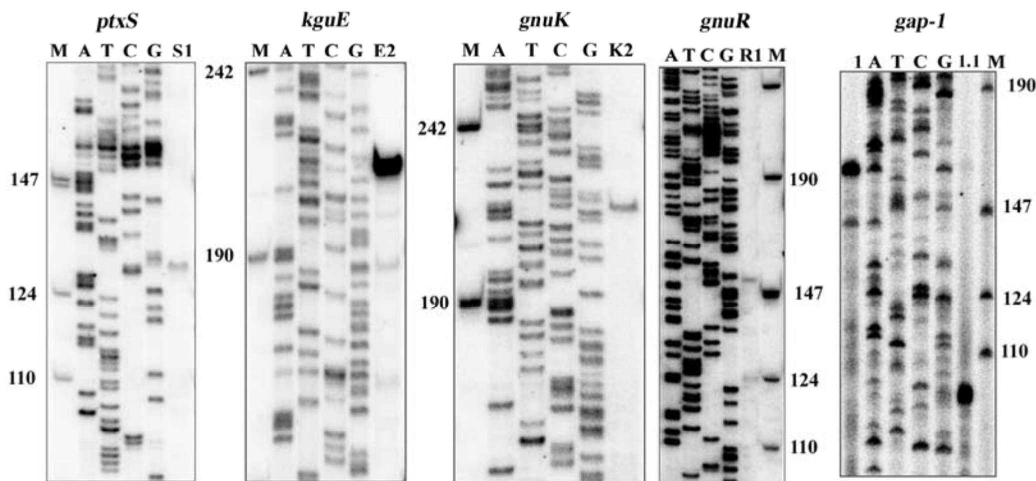


Figure 3. Transcription start point of the promoters in front of the *ptxS, kguE, gnuK, gnuR* and *gap-1* genes. Preparation of RNA from cells growing exponentially on glucose is described in Materials and Methods. Sequences of the primers used for primer extension assays are given in Supplementary Table 1. In all panels M refers to single-strand markers, and ATCG is a sequencing ladder. The primer extension product(s) for *ptxS* (S1), *kguE* (E2), *gnuK* (K2), *gnuR*(R1) and *gap1* (1 and 1.1, since two different primers were used) are shown. Other conditions are as described in Materials and Methods.

pathways has been acquired independently, and the presence of genes that encode catabolic enzymes and regulators was indicative of the potentially independent regulation of each segment. In agreement with this proposal is a series of previous studies that indicated that the glucokinase pathway was under catabolite repression control when cells were exposed to alternative C-sources such as toluene or succinate, according to a process mediated by the global Crc regulator (5, 41), whereas none of the other two pathways were under catabolic repression (5).

The set of genes for the glucokinase pathway cover a cluster consisting of PP1009 to PP1024. There are two potential transcriptional regulators within this cluster: GltR2 and HexR. The set of catabolic genes does not only include the glucokinase pathway, but also the Entner-Doudoroff *edd* and *eda* genes in a peculiar organization: the *edd* gene is co-transcribed with *glk*, and *eda* is co-transcribed with *zwf-1*. Divergently with respect to *edd* is *gap-1*, which encodes glyceraldehyde-3-phosphate dehydrogenase. The cluster also includes an operon that encodes an ABC transport system mediating the ATP-dependent transport of glucose into the periplasmic space. All of these genes except for *hexR* are inducible by glucose (4).

In *P. aeruginosa* Sage *et al.* (32) reported that the *gltR* gene encoded a product homologous to the response element of two-component systems, whose disruption caused the loss of glucose transport activity. Inactivation of the *gltR2* gene in *P. putida* had an effect on growth in the presence of glucose that resulted in a prolonged lag when cells were transferred, for example, from M9 minimal medium with citrate to glucose-containing medium as the sole C-source. This seems to be consequence of (i) the lack of induction of the *gtsABCD* genes that encode the active transport system in *P. putida*, as shown by our transcriptional fusion assays of a *gtsA::lacZ* in the *gltR* mutant background; and (ii) the lack of induction of *pgl* encoding 6-phosphogluconolactonase, an enzyme required for full operation at the highest rate of the glucokinase pathway (10, 26). It should be noted that *pgl* is under the control of HexR (Table 3) and GnuR (Table 2), which indicates that the gene can be controlled from the *zwf-1* promoter under HexR control and from its own (as yet unidentified) promoter under GltR2 control. This is somewhat surprising, but it may be related to the need to induce *pgl* and *eda* for the efficient catabolism of gluconate/2KG when these chemicals are used as the sole C-source, as is also the case in *P. fluorescens* (27), and in *E. coli* (8, 22).

Deficiency in HexR resulted in the constitutive expression of the *gap-1* gene and the *edd/glk* and *eda/zwf-1* operons. This suggests that HexR is a transcriptional repressor of these operons. This observation is in agreement with findings by Phibbs and colleagues in *P. aeruginosa* (10). In

the *hexR* mutant background, expression of the ABC glucose transport system comprising *gtsABCD* was under the control of the GltR2 protein.

Our β-galactosidase assays with a P_{edd}::*lacZ* fusion revealed that HexR exhibits a relatively broad spectrum of effectors responding not only to glucose, but also to gluconate, 2KG and fructose. Expression was equally high with all of these carbohydrates (del Castillo, T., unpublished). This is of physiological significance since the *edd*/*eda* gene products are required for *P. putida* to grow on gluconate, 2-ketogluconate and fructose. Examples of transcriptional regulators that recognize a broad range of effectors are not common in the HexR/LacI family, but some repressors of the TetR family (29) and the IclR family of regulators (21) have been reported to exhibit broad spectra. HexR also controls the expression of glyceraldehyde-3-phosphate dehydrogenase, the enzyme that acts on one of the final metabolites of the Entner-Doudoroff pathway.

The tsp of the three glucose catabolism transcriptional units regulated by HexR were mapped. This allowed us to identify the corresponding promoters, which exhibited −10/−35 hexamers by RNA polymerase with sigma-70. In *P. aeruginosa, hexR* controls the expression of *gap*-1 (24, 36) and this regulator was found to recognize an AT-rich target dyad. In *P. putida* an almost identical dyad was found covering the −10 region of the three promoters under HexR control, and we propose that in both species HexR recognizes a similar dyad motif.

In addition to the set of glucose catabolism genes, another gene (PP4488) was up-regulated in the *hexR* mutant background, suggesting that HexR might directly or indirectly regulate this gene. Sequence analysis revealed that 329 nucleotides upstream from the first ATG of the ORF encoding PP4488 was also present the 5’-CGTTTGTAN$_{14}$ACAAAAGA-3’ motif.

In *E. coli* the *edd*/*eda* genes are co-transcribed and regulated by a member of the GntR-family of repressors that responds primarily to gluconate (38). del Castillo *et al.* (4) suggested that the glucokinase pathway might contribute up to 40% of the glucose income in *P. putida*, with the 2KG loop representing the metabolism of 40% to 45% of the glucose. The set of genes involved in the initial metabolism of gluconate to 2KG forms an operon (PP3384 to PP3382), whereas the genes for 2KG transport (PP3377) and its metabolism (PP3379, PP3378 and PP3376) form another operon that is transcribed from its own promoter. The *ptxS* gene is located downstream of the *kguE* gene (Figure 2). Inactivation of the *ptxS* gene led to specific up-regulation of the promoters upstream from PP3384 and PP3379, as well as up-regulation of the *ptxS* gene (PP3380) itself. In *P. aeruginosa, ptxS* has been identified as involved in the control of the *toxA* gene and of its own synthesis (35, 40). In *P. aeruginosa* the target of PtxS is a 14-bp dyad sequence whose disruption in front of this gene resulted in its over-expression. We identified the same 14-bp dyad element (5′-TGAAACCGGTTTCA-3′) within the 35 nucleotides 5′ upstream from each of the promoter start sites, and suggest that this motif is also the target of PtxS in *P. putida*.

In *P. aeruginosa* the 2KG pathway has been described as the main route through which glucose is assimilated under aerobic conditions; however, the role of PtxS in the control of the *kguE* to *kguD* genes remains to be explored. Inspection of the sequence upstream from the first ATG of *kguE* in *P. aeruginosa* revealed that the dyad sequence was also present, which leads us to hypothesize that PtxS also controls 2KG metabolism in *P. aeruginosa*.

In *Pseudomonas putida* the direct phosphorylation of gluconate to 6PG is of minor importance in metabolic terms, which contrasts with the situation in other gram-negative (8, 22, 25, 27, 38) and gram-positive bacteria (*Corynebacterium glutamicum* [15] or *Bacillus subtilis* [43]). However, the basic mode of regulation of gluconate metabolism in *P. putida* is similar to that in other bacteria. In *P. putida* the GnuR repressor is highly homologous to the GntR repressor of *E. coli, B. subtilis* and *Corynebacterium* and controls the expression of the gluconate transporter gene and glucokinase gene. In *E. coli* and *B. subtilis,* expression of the gluconokinase is under global regulation, which is over-imposed on local specific regulation (38, 43). This level of complexity is not found in *P. putida*, probably due to the minor role of gluconokinase in the assimilation of glucose in this bacterium. The GnuR protein seems to recognize a dyad element, as is the case for the two other regulators of the glucose operons.

In summary, our results indicate that we have identified four transcriptional regulators involved in glucose catabolism. HexR controls the flux of glucose to 6PG and further down to pyruvate and glyceraldehyde-3-phosphate, due to the link between the *edd/eda* gene of the Entner-Doudoroff pathway and the *glk/zwf-1* genes, as discussed above. Concerning this pathway, it is worth noting that the positive transcriptional regulator GltR2 controls the expression of the glucose transport system that allows the internalization of glucose into the cytoplasm. PtxS controls the expression of genes that encode gluconate dehydrogenase and which convert gluconate into 2KG, and the set of genes whose products convert 2KG into 6PG. GnuR is another repressor that specifically responds to gluconate and controls its transport and phosphorylation. This intricate set of regulators guarantees the simultaneous channelling of glucose via three pathways that converge at the level of 6PG.

Acknowledgments

This study was supported by Grant SYSMO (GEN2006-27750-C5-5-E/SYS) from the EC/MEC, and by Project of Excellence from Junta de Andalucía. We thank C. Lorente and M. Fandila for secretarial assistance and K. Shashok for improving the use of English in the manuscript.

REFEREENCES

1.Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., Gaasterland, T., Glenisson, P., Holstege, F. C. P., Kim, I. F., Markowitz, V. , Matese, J. C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J., and M. Vingron. 2001. Minimum information about a microarray experiment (MIAME)—towards standards for microarray data. Nature Genet. **29:** 365-371.

2. Cuskey, S.M., J.A., Wolf, P.V. Phibbs, Jr., and R.H. Olsen. 1985. Cloning of genes specifying carbohydrate catabolism in *Pseudomonas aeruginosa* and *Pseudomonas putida*. J. Bacteriol. **162**: 865-871.

3. del Castillo, T., and J.L. Ramos. 2007. Simultaneous catabolite repression between glucose and toluene metabolism in *Pseudomonas putida* is channelled through different signalling pathways. J. Bacteriol. **189**: 6602-6610.

4. del Castillo, T., J.L. Ramos, J.J. Rodríguez-Herva, T. Fuhrer, U. Sauer, and E. Duque. 2007. Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: genomic and flux analysis. J. Bacteriol. **189**:5142-5152.

5. Domínguez-Cuevas, P., and S. Marqués. 2004. Compiling sigma-70 dependent promoters. In *Pseudomonas*: Virulence and Gene Regulation. Ed. J.L. Ramos, Vol. II. Chapter 11, pp. 319-343. Kluwer Academic/Plenum Publishers, New York.

6. Duque, E., A. J. Molina-Henares, J. de la Torre, M. A. Molina-Henares, T. del Castillo, J. Lam and J. L. Ramos. 2007b. Towards a genome-wide mutant library of *Pseudomonas putida* strains KT2440. *In* J.L. Ramos and A. Filloux eds.), *Pseudomonas*, vol. V. Chapter 8, pp. 227-251. Springer, The Netherlands.

7. Duque, E., J.J. Rodríguez-Herva, J. de la Torre, P. Domínguez-Cuevas, J. Muñoz-Rojas, and J.L Ramos. 2007a. The RpoT Regulon of *Pseudomonas putida* DOT-T1E and its role in stress endurance against solvents. J. Bacteriol. **189:** 207-219

8. Egan, S.E., R. Fliege, S. Tong, A. Shibata, R.E. Wolf, Jr., and T. Conway. 1992. Molecular characterization of the Entner-Doudoroff pathway in *Escherichia coli*: sequence analysis and localization of promoters for the *edd-eda* operon. J. Bacteriol. **174**: 4638-4646.

9. González-Pérez, M.M., S. Marqués, P. Domínguez-Cuevas, and J.L. Ramos. 2002. XylS activator and RNA polymerase binding sites at the Pm promote overlap. FEBS Lett. **519**: 117-122.

10. Hager, P.W., M.W. Calfee, and P.V. Phibbs. 2000. The *Pseudomonas aeruginosa devB/SOL* homolog, *pgl* is a member of the *hex* regulon and encodes 6-phosphogluconolactonase. J. Bacteriol. **182**: 3934-3941.

11. Higgins, C.F., S.C. Hyde, M.M. Mimmack, U. Gileadi, D.R. Gill, and M.P. Gallagher. 1990. Binding protein-dependent transport systems. J. Bioenerg. Biomembr. **22**:571-592.

12. Jiménez, J.J., B. Miñambres, J.L. García and E. Díaz. 2004. Genomic insights in the metabolism of aromatic compounds in *Pseudomonas*. In *Pseudomonas*: Biosynthesis of Macromolecules and Molecular Metabolism. Ed. J.L. Ramos. Vol. II, chapter 15, pp. 425-462. Kluwer Academic/ Plenum Publishers, New York.

13. Johnson, C.M., and R.F. Schleif. 2000. Cooperative action of the catabolite activator protein and AraC *in vitro* at the *araFGH* promoter. J. Bacteriol. **182**:1995-2000.

14. Kamilova, F., L. V. Kravchenko, A. I. Shaposhnikov, T. Azarova, N. Makarova, and B. Lugtenberg. 2006. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol. Plant Microbe Interact. **19:**250-256.

15. Letek, M., N. Valbuena, A. Ramos, E. Ordóñez, J.A. Gil, and L. Mateos. 2006. Characterization and use of catabolite-repressed promoters from gluconate genes in *Corynebacterium glutamicum*. J. Bacteriol. **188**: 409-423.

16. Llamas, M. A., J. L. Ramos, and J. J. Rodriguez-Herva. 2000. Mutations in each of the *tol* genes of *Pseudomonas putida* reveal that they are critical for maintenance of outer membrane stability. J. Bacteriol. **182:**4764-4772.

17. Llamas, M.A. J.J. Rodríguez-Herva, R.E. Hancock, W. Bitter, J. Tommassen and J.L. Ramos. 2003. Role of *Pseudomonas putida tol-oprL* gene products in uptake of solutes through the cytoplasmic membrane. J. Bacteriol. **185**:4707-4716.

18. Ma, J.-F., P.W.Hager, M.L. Howell, P.V. Phibbs, and D.J. Hassett. 1998. Cloning and characterization of the *Pseudomonas aeruginosa zwf* gene encoding glucose-6-phosphate dehydrogenase, an enzyme important in resistance to methyl viologen (paraquat). J. Bacteriol. **180**:1741-1749.

19. Marqués, S., A. Holtel, K. N. Timmis, and J. L. Ramos. 1993. Analysis of the mRNA Structure of the *Pseudomonas putida* TOL *meta*-fission pathway operon. Biochim. Biophys. Acta **1216**:227-236.

20. Miller, J. H. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

21.Molina-Henares, A.J., T. Krell, M.E. Guazzaroni, A. Segura, and J.L. Ramos. 2006. Members of the IclR family of bacterial transcriptional regulators function as activators and/or repressors. FEMS Microbiol. Rev. **20**:157-186.

22. Murray, E., and T. Conway. 2005. Multiple regulators control expression of the Entner-Doudoroff aldolase (Eda) of *Escherichia col*i. J. Bacteriol. **187**:991-1000.

23. Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A., Fouts, D. E., Gill, S. R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R. T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D. , Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K. N., Dusterhoft, A., Tümmler, B., and C. M. Fraser. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. **4:**799-808.

24. Palmer K.L., L.M. Mashburn, P.K. Singh, and M. Whiteley. 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. J. Bacteriol. **187**:5267-5277.

25. Peekhaus, N., and T. Conway. 1998. Positive and negative transcriptional regulation of the *Escherichia coli* gluconate regulon gene *gntT* by GntR and the yclic AMP (cAMP)-cAMP receptor protein complex. J. Bacteriol. **180**:1777-785.

26. Petruschka, L., K. Adolf, G. Burchhardt, J. Dernedde, J. Jurgensen, and H. Herrmann. 2002. Analysis of the zwf-pgl-eda-operon in *Pseudomonas putida* strains H and KT2440. FEMS Microbiol. Lett. **215:**89-95.

27. Quay, S.C., S.B. Friedman, and R.C. Eisenberg. 1972. Gluconate regulation of glucose catabolism in Pseudomonas fluorescens. J. Bacteriol. **112**:291-298.

28. Ramos J.L., Duque E., Ramos-González M.I. 1991. Survival in soils of an herbicide-resistant *Pseudomonas putida* bearing a recombinant TOL plasmid. Appl. Environ. Microbiol. **57**:260-266.

29. Ramos, J.L., M. Martínez-Bueno, A.J. Molina-Henares, W. Terán, K. Watanabe, X. Zhang, M.T. Gallegos, R. Brennan, R. Tobes. 2005. The TetR family of transcriptional repressors. Microbiol. Mol. Biol. Rev. **69**:326-356.

30. Rodríguez-Herva, J.J. and Ramos, J.L. 1996. Characterization of an OprL null mutant of *Pseudomonas putida*. J. Bacteriol. **178**:5836-5840.

31. Rojo, F. 1999. Repression of transcription initiation in bacteria. J. Bacteriol. **181:**2987-2991.

32. Sage, A.E., W.D. Proctor, and P.V. Phibbs, Jr. 1996. A two-component response regulator, *gltR*, is required for glucose transport activity in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **178**:6064-6066.

33. Saravolac, E.G., N.F. Taylor, R. Benz, and R.E. Hancock. 1991. Purification of glucose-inducible outer membrane protein OprB of *Pseudomonas putida* and reconstitution of glucose-specific pores. J. Bacteriol. **173**:4970-4976.

34. Schleif, R. 2000. Regulation of the L-arabinosa operon of *Escherichia coli*. Trends Genet. **16**:559-565.

35. Swanson, B.L., and A.N. Hamood. 2000. Autoregulation of the *Pseudomonas aeruginosa* protein PtxS occurs through a specific operator site within the *ptxS* upstream region. J. Bacteriol. **182**:4366-4371.

36. Temple, L., A. Sage, G.E. Christie, and P.V. Phibbs, Jr. 1994. Two genes for carbohydrate catabolism are divergently transcribed from a region of DNA containing the *hexC* locus in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **176**: 4700-4709.

37. Trias, J., E.Y. Rosenber, and H. Nikaido. 1998. Specificity of the glucose channel formed by protein D1 of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **938**:493-496.

38. Tong, S., A. Porco, T. Isturiz, and T. Conway. 1996. Cloning and molecular genetic characterization of the *Escherichia coli gntR, gntK* and *gntU genes* of GntI, the main system for gluconate metabolism. J. Bacteriol. **178**:3260-3269.

39. 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 sigma-54 Pu promoter of *Pseudomonas putida*. J. Bacteriol. **186**:8267-8275.

40. Westfall, L.W., A.M. Luna, M. San Francisco, S.P. Diggle, K.E. Worrall, P. Williams, M. Cámara, and A.N. Hamood. 2004. The *Pseudomonas aeruginosa* global regulator MvaT specifically binds to the *ptxS* upstream region and enhances *ptxS* expression. Microbiol. **150**:3797-3806.

41. Wolff, J.A., C.H. MacGregor, R.C. Eisenberg, and P.V. Phibbs. 1991. Isolation and characterization of the catabolite repression control mutants of *Pseudomonas aeruginosa* PAO. J. Bacteriol. **173**:4700-4706.

42. Yang, Y. H., S. Dudoit, P. Luu, D. M. Lin, V. Peng, J. Ngai, and T. P. Speed. 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. **30:**e15.

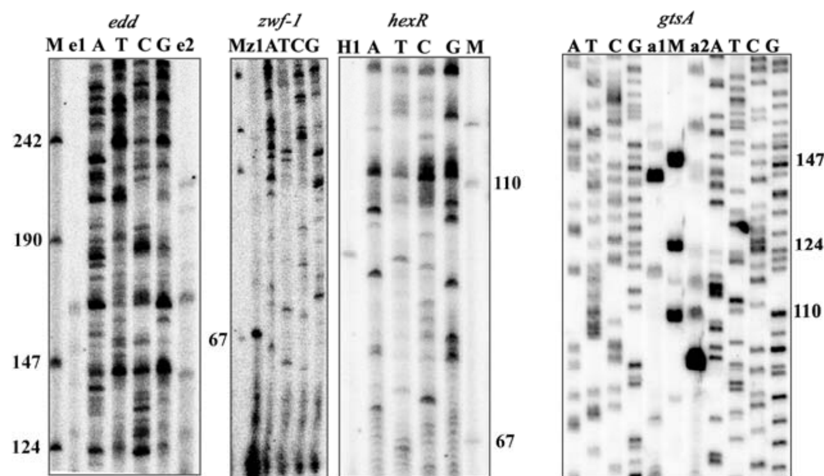
43. Yoshida, K., H. Ohmori, Y. Miwa and Y. Fujita. 1995. *Bacillus subtilis gnt* repressor mutants that diminish gluconate-binding ability. J. Bacteriol. **177**: 4813-4816.

44. Yuste, L., A.B. Hervás, 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:**166-177.

SUPPLEMENTAL MATERIAL

Supplementary Table 1. Primers used in primer extension analyses

Gen	Primer name	Sequence
ptxS	S1	5´- TAACGGGAGACGGTGGCTTT - 3´
kguE	E2	5´- GGGTGGAGTACAGGCATTCC - 3´
gnuR	R1	5´- AACGGTGCTGACCCCACGCA - 3´
gnuK	K2	5´- CGATGCAGCTTTTGCCACAA - 3´
gap-1	1	5´- AGGTCCTGGCGGTAGCCTTG - 3´
gap-1	1.1	5´- AATCCATTGATGGCGATGCG - 3´
edd	e1	5´ - GCTGGGTGACCTCAAGGATG - 3´
edd	e2	5´- AATCAGTTGCAGGTAGCGTT - 3´
zwf-1	z1	5´- GCCAAACAGGGCAAAGGTGC - 3´
hexR	H1	5´ - GATCTGTTCCAGGAGGTTTC - 3´
gtsA	a1	5´- CACTTCGACACTGCCTTTGG - 3´
gtsA	a2	5´ - AGGGGATTAACGAGGCAAAG - 3´



Supplementary Figure 1. Transcription start point of the promoters in front of the *edd, zwf-1, hexR* and *gstA* genes. Preparation of RNA from cells growing exponentially on glucose is described in Materials and Methods. Sequences of the primers used for primer extension assays are given in Supplementary Table 1. In all panels M refers to single-strand markers, and ATCG is a sequencing ladder. The primer extension product(s) for *edd* (e1 and e2, since two different primers were used), *zwf-1* (z1), *hexR*(H1) and *gstA* (a1 and a2, since two different primers were used) are shown. Other conditions are as described in Materials and Methods.

III. CAPÍTULO 3. RESUMEN.

Simultaneous catabolite repression between glucose and toluene metabolism in *Pseudomonas putida* is channeled through different signaling pathways

Teresa del Castillo & Juan L. Ramos

Pseudomonas putida KT2440 (pWW0) utiliza tolueno a través de la ruta catabólica codificada en el plámido TOL y glucosa por una serie de tres rutas periféricas de codificación cromosómica que convierten la glucosa en 6-fosfogluconato (6PG), llamadas, la ruta de la glucoquinasa, en la cual la glucosa es transformada a 6PG a través de la acción de la glucoquinasa y la glucosa-6-fosfato deshidrogenasa. Alternativamente la glucosa puede ser oxidada a gluconato y 2-cetogluconato que rinden 6PG. Nuestros resultados muestran que KT2440 cataboliza la glucosa y el tolueno simultáneamente, como revelan los análisis de flujo de glucosa utilizando ^{13}C-glucosa. La determinación de la actividad glucoquinasa y gluconoquinasa en el metabolismo de la glucosa, los ensayos de la expresión de los genes usando una fusión del promotor Pu del plásmido TOL de la ruta *upper* a *'lac*Z, y los ensayos globales de transcriptómica revelaron represión catabólica simultánea en la utilización de estas dos fuentes de carbono. El efecto del tolueno en el metabolismo de la glucosa se ejerció sobre lazo de la glucoquinasa directamente. La represión catabólica de la ruta TOL y del lazo de la glucoquinasa están mediados por dos sistemas diferentes. La expresión de Pu se reprimió principalmente vía PtsN en respuesta a altos niveles de 2-dehidro-3-deoxigluconato-6-fosfato, mientras que la represión de la ruta de la glucoquinasa se canalizó a través de Crc.

Journal Bacteriology
Vol.189, Nº. 18, pp. 6602-10, Sep 2007

JOURNAL OF BACTERIOLOGY, Sept. 2007, p. 6602–6610 Vol. 189, No. 18
0021-9193/07/$08.00+0 doi:10.1128/JB.00679-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Simultaneous Catabolite Repression between Glucose and Toluene Metabolism in *Pseudomonas putida* Is Channeled through Different Signaling Pathways▽†

Teresa del Castillo and Juan L. Ramos*

Consejo Superior de Investigaciones Científicas, Estación Experimental del Zaidín, Department of Environmental Protection, C/ Prof. Albareda, 1, E-18008 Granada, Spain

Received 30 April 2007/Accepted 2 July 2007

***Pseudomonas putida* KT2440(pWW0) can use toluene via the TOL plasmid-encoded catabolic pathways and can use glucose via a series of three peripheral chromosome-encoded routes that convert glucose into 6-phosphogluconate (6PG), namely, the glucokinase pathway, in which glucose is transformed to 6PG through the action of glucokinase and glucose-6-phosphate dehydrogenase. Alternatively, glucose can be oxidized to gluconate, which can be phosphorylated by gluconokinase to 6PG or oxidized to 2-ketogluconate, which, in turn, is converted into 6PG. Our results show that KT2440 metabolizes glucose and toluene simultaneously, as revealed by net flux analysis of [^{13}C]glucose. Determination of glucokinase and gluconokinase activities in glucose metabolism, gene expression assays using a fusion of the promoter of the Pu TOL upper pathway to *′lacZ*, and global transcriptomic assays revealed simultaneous catabolite repression in the use of these two carbon sources. The effect of toluene on glucose metabolism was directed to the glucokinase branch and did not affect gluconate metabolism. Catabolite repression of the glucokinase pathway and the TOL pathway was triggered by two different catabolite repression systems. Expression from Pu was repressed mainly via PtsN in response to high levels of 2-dehydro-3-deoxygluconate-6-phosphate, whereas repression of the glucokinase pathway was channeled through Crc.**

The *Pseudomonas putida* pWW0 TOL plasmid encodes the catabolic pathways for the mineralization of toluene and xylenes (4). Figure 1 shows the genetic organization of the catabolic operons. The chief regulator involved in the transcriptional control of TOL plasmid catabolic pathways in cells growing on aromatic hydrocarbons is XylR. This regulator drives transcription from the Pu promoter in front of the *xylUWCMABN* genes, which constitute the upper operon, for conversion of toluene and *p*- and *m*-xylenes into the corresponding benzoates, as well as from the Ps1 promoter to increase expression of the *xylS* gene (Fig. 1). This, in turn, results in the induction of the *meta* operon for the oxidation of benzoates into Krebs cycle intermediates. Transcription from Pu and Ps1 is mediated by RNA polymerase with RpoN, also known as sigma-54 (37, 39).

Expression from the Pu promoter is repressed by the presence of alternative C sources. The first indication of TOL pathway susceptibility to catabolite repression was the seminal observation by Worsey and Williams (49) that cells grown in batch cultures on a mixture of acetate and *m*-xylene contained twofold-lower levels of the upper pathway enzymes than cells grown with *m*-xylene as the sole source of carbon and energy. Definitive proof of catabolite repression was provided by Duetz et al. (12), who showed that *o*-xylene did not induce expression of the TOL catabolic pathways in continuous cultures growing either at a high rate under nonlimiting conditions (with an excess of all nutrients) or at a low rate in cultures limited in N, P, or S (all such conditions resulted in excess carbon in the medium).

In *Enterobacteriaceae* cyclic AMP (cAMP) acts as a signal molecule in catabolite repression (36). However, catabolite repression in *Pseudomonadaceae* does not involve cAMP; in fact, in *P. putida* and *Pseudomonas aeruginosa*, cAMP levels are relatively constant regardless of the growth conditions (34, 38, 41). In *Pseudomonadaceae* catabolite repression seems to integrate different signals instead, a feature that increases the complexity of the system. Up to five different potential regulators have been related to catabolite repression in *P. putida*, namely, Crc (20, 29, 38), Crp (called Vfr in *P. aeruginosa*) (43, 48), CyoB (10, 33), RelA (24, 44), and the Pts system (3, 5). The level of expression from the Pu promoter in wild-type cells growing on glucose or gluconate and toluene was one-third the level in cells growing with only toluene (2, 5, 21), and suppression of upper pathway induction in the presence of these carbon sources was proposed to operate through the *ptsN* gene product. Indeed, in a PtsN-deficient mutant background Pu expression in the presence of glucose was derepressed (5), and Aranda-Olmedo et al. (2) proposed that addition of the repressing carbon source resulted in PtsN preventing XylR binding to its target upstream activator sequences.

We recently found that in *P. putida* KT2440 6-phosphogluconate (6PG), the key metabolite of the Entner-Doudoroff pathway, was synthesized by three converging pathways; the glucose dehydrogenase (Gcd) route and the glucokinase (Glk)/glucose-6-phosphate dehydrogenase pathway accounted for almost 90% of the 6PG synthesized by the cells, whereas the remaining 10% was synthesized through direct phosphoryla-

* Corresponding author. Mailing address: EEZ-CSIC, C/ Prof. Albareda, 1, E-18008 Granada, Spain. Phone: 34 958 181600. Fax: 34 958 135740. E-mail: jlramos@eez.csic.es.
† Supplemental material for this article may be found at http://jb.asm.org/.
▽ Published ahead of print on 6 July 2007.

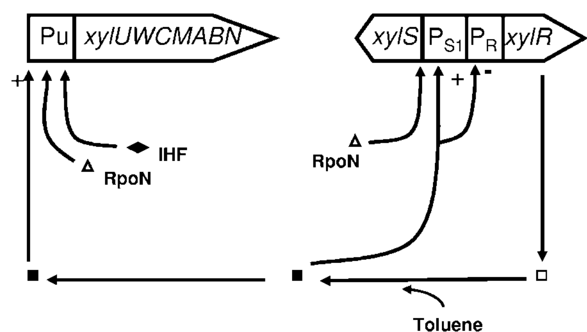


FIG. 1. TOL plasmid promoters under control of the XylR protein. The *xylR* gene, expressed from two overlapping P_R promoters, yielded an inactive XylR protein (□) which, in the presence of toluene, became active (■) and stimulated transcription (+) from Pu and Ps1 while repressing (−) its own synthesis. The alternative RpoN sigma factor participating in transcription of Pu and Ps1 is indicated. IHF has a positive role in the transcription of Pu (37).

tion of gluconate in a reaction mediated by gluconokinase (9). Velázquez et al. (46) suggested that 6PG or the products derived from its metabolism acted as signals for glucose repression of Pu. The present study was undertaken to obtain further insight into the phenomenon of glucose repression of the TOL Pu upper pathway operon promoter from the physiological and molecular points of view. We show here that in the wild-type *P. putida* KT2440 strain and isogenic mutants deficient in the operation of the Gcd and Glk pathways, toluene and glucose affected each other's metabolism in a process that counterbalanced the total amount of carbon assimilated by the cells. Our study showed that repression of toluene degradation by glucose is signaled by 2-dehydro-3-deoxygluconate-6-phosphate and that effective repression requires a functional PtsN protein, whereas toluene repression of glucose metabolism affects the Glk pathway but not the gluconate pathways. The effect of toluene on the Glk route is channeled via the Crc protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. We used *P. putida* KT2440, a derivative of *P. putida* mt-2 (1, 31) (Table 1). Isogenic *glk* (strain PSC278) and *gcd* (strain M438) mutants of this strain have been described previously (Table 1 and references therein). These strains were grown at 30°C at 200 rpm in 250-ml conical flasks with 20 ml M9 minimal medium supplemented with Fe citrate, $MgSO_4$ and trace metals (1, 13). Glucose (16 mM), toluene (6 mM), or glucose (16 mM) and toluene (6 mM) were used as carbon sources. For ^{13}C-labeling experiments we used a mixture of 20% (wt/wt) [U-^{13}C]glucose (>99% pure; Martek Biosciences Corporation, Columbia, MD) and 80% (wt/wt) natural glucose.

Analytical procedures, physiological parameters, sample preparation, and gas chromatography-mass spectrometry analysis. The glucose concentration was determined enzymatically with a commercially available kit (Roche Diagnostics). The cell dry weight, the maximum specific growth rate, the biomass yield, and the specific carbon source consumption rate were determined as described previously (9, 17).

To analyze proteinogenic amino acids, cell aliquots were harvested during the mid-exponential growth phase by centrifugation of 5 to 7 ml of culture broth at 4°C, and the pellets were hydrolyzed in 6 M HCl for 24 h at 110°C in sealed 2-ml Eppendorf tubes. Samples were desiccated, treated, and analyzed as described previously (8, 15, 17, 32).

Analysis of metabolic fluxes. For METAFoR analysis, mass spectra of four derived amino acids (glycine, serine, proline, and glutamate) were considered. These amino acids are synthesized from single metabolic intermediates, and the mass isotopomer distribution vector of these metabolites was derived from the mass isotopomer distribution vector of the amino acids. The values were used to calculate the fractional contribution of the corresponding reaction to the target metabolite pool with a set of algebraic equations implemented in the MATLAB-based program Fiat Flux, version 1.04, as described by Fischer et al. (16).

Preparation of RNA. The *P. putida* KT2440 strain was grown overnight in M9 minimal medium with glucose. Cells were then diluted until the turbidity at 660 nm (OD_{660}) was 0.05 in fresh M9 minimal medium without a carbon source, and three aliquots were removed and supplemented with glucose or glucose plus toluene. Samples were then incubated until the culture reached a turbidity at 660 nm of 0.7. Then 15-ml portions of the cultures were harvested by centrifugation at 7,000 × *g* for 5 min, and total bacterial RNA was isolated exactly as described by Marqués et al. (27). Extracts were treated with RNase-free DNase I (10 U/μl) in the presence of an RNase inhibitor cocktail (40 U/μl RNaseOUT).

***P. putida* microarrays.** The genome-wide DNA chip used in this work (printed by Progenika Biopharma) was described previously (50). It consists of an array of 5,539 oligonucleotides (50-mers) spotted in duplicate onto γ-aminosilane-treated slides and covalently linked with UV light and heat. The oligonucleotides represent 5,350 of the 5,421 predicted open reading frames annotated in the *P.*

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Reference(s)
P. putida strains		
KT2440	Wild type, prototroph; Cm^r Rif^r	1, 32
M1044[b]	*edd*:mini-Tn*5*-Km Km^r Rif^r	14
M1128[b]	*eda*:mini-Tn*5*-Km Km^r Rif^r	14
M438[b]	*gcd*:mini-Tn*5*-Km Km^r Rif^r	14
PSC278[b]	*glk*::pCHESIΩ-Km Rif^r	9
KT2440 ptsN	Km^r; *P. putida* KT2440 with a kanamycin resistance cassette interrupting the *ptsN* gene	3
KT2440 crc	Gm^r; *P. putida* KT2440 with a gentamicin resistance cassette interrupting the *crc* gene	3
KT2440 crp	Km^r; *P. putida* KT2440 with a kanamycin resistance cassette interrupting the *crp* gene	3
KT2440 cyoB	Tc^r; *P. putida* KT2440 with a tetracycline resistance cassette interrupting the *cyoB* gene	38
KT2440 relA	Km^r; *P. putida* KT2440 with a kanamycin resistance cassette interrupting the *relA* gene	44
KT2440(pWW0r PmΩ Sm)	Sm^r; *P. putida* KT2440 with a kanamycin resistance cassette interrupting the Pm gene	26
KT2440(pWW0::μ 21)	Km^r; *P. putida* KT2440 with a kanamycin resistance cassette interrupting the *xylE* gene	26
Plasmids		
pRK600	Helper plasmid; tra^+ mob^+ Cm^r	5, 19
pS10	IncP1 Sm^r *xylR*; transcriptional Pu::*lacZ*::*tet* fusion	3
pWW0	IncP9 mob^+ tra^+ $3MB^+$	49

[a] Ap^r, Cm^r, Gm^R, and Km^r, resistance to ampicillin, chloramphenicol, gentamicin, and kanamycin, respectively.
[b] Obtained from the collection of KT2440 mutants available at our institute.

TABLE 2. Growth rates and physiological parameters of *P. putida* KT2440(pWW0) and isogenic mutants of this strain growing with different carbon sources

Strain	Growth rate (h^{-1}) with:			q_{Glu} (μmol/mg [cell dry wt] h^{-1})[a]		q_{tol} (μmol/mg [cell dry wt] h^{-1})[b]	
	Glucose	Toluene	Glucose + toluene	Without toluene	With toluene	Without glucose	With glucose
KT2440(pWW0)	0.73 ± 0.03[c]	0.72 ± 0.02	0.74 ± 0.07	13.1 ± 0.8	5.7 ± 0.5	11.9 ± 0.5	6.4 ± 0.2
KT2440 gcd(pWW0)	0.42 ± 0.01	0.73 ± 0.04	0.45 ± 0.01	5.0 ± 0.5	2.4 ± 0.1	12.4 ± 0.1	11.4 ± 0.4
KT2440 glk(pWW0)	0.38 ± 0.06	0.72 ± 0.01	0.66 ± 0.01	5.1 ± 0.5	5.9 ± 0.7	11.3 ± 0.2	5.5 ± 0.3
KT2440 ptsN(pWW0)	0.67 ± 0.01	0.70 ± 0.01	0.71 ± 0.18	ND[d]	ND	ND	ND
KT2440 crc(pWW0)	0.51 ± 0.1	0.65 ± 0.1	0.65 ± 0.04	ND	ND	ND	ND

[a] Glucose consumption was determined in cells growing in the absence and in the presence of toluene.
[b] Toluene consumption was determined in cells growing in the absence and in the presence of glucose.
[c] The data are averages ± standard deviations of three to six independent assays, each done in duplicate.
[d] ND, not determined.

putida KT2440 genome. The chips also contain homogeneity controls consisting of oligonucleotides for the *rpoD* and *rpoN* genes spotted at 20 different positions, as well as duplicate negative controls at 203 predefined positions. Preparation and labeling of RNA for hybridization and data analysis were done as described previously (11, 14, 50).

β-Galactosidase assays. The pS10 plasmid was transformed into *P. putida* KT2440(pWW0) and isogenic mutants of this strain. Transformants were selected on M9 minimal medium with 5 mM 3-methylbenzoate as the sole carbon source and 10 μg/ml tetracycline. *P. putida*(pWW0, pS10) was grown on M9 minimal medium with glucose or citrate (16 mM), and when the cultures reached an OD_{660} of 0.1, they were supplemented or not supplemented with toluene in the gas phase and incubation was continued at 30°C with shaking until the OD_{660} of the cultures were 0.6 ± 0.1. The β-galactosidase activity was assayed in permeabilized whole cells using Miller's method (28). Assays were done in triplicate and were repeated at least three times.

Preparation of cell extracts and enzyme assays. Cell batches in 50 ml of minimal medium with glucose, toluene, or glucose plus toluene as the carbon source were harvested by centrifugation at 7,000 × *g* for 7 min, washed twice, and frozen at −20°C. Cells were disrupted in a French press at 120 MPa. Whole cells and debris were removed by centrifugation at 11,180 × *g* (45 min, 4°C). The clear supernatant was used as a cell extract. The protein concentrations in cell extracts were determined by the Bradford method, using bovine serum albumin as the standard. Glucokinase and gluconokinase assays were performed at 30°C at 340 nm with a Shimadzu UV-160A spectrophotometer as described previously (22, 45). Specific activities were calculated based on an NAD(P)H extinction coefficient of 6.3 mM^{-1} cm^{-1}.

RESULTS

Simultaneous utilization of glucose and toluene by *P. putida* KT2440 and isogenic mutants of this strain. *P. putida* KT2440 (Table 1) growing on M9 minimal medium with glucose, toluene, or glucose plus toluene exhibited high growth rates (Table 2). As reported previously, mutants deficient in the synthesis of Glk or Gcd were still able to grow on glucose, but the growth rates were lower (around 0.4 h^{-1}) than those of the parental strain (0.73 ± 0.03 h^{-1}) (Table 2).

The growth rates with toluene of mutants deficient in Gcd or Glk were similar to those of the parental strain (around 0.72 ± 0.03 h^{-1}) (Table 2). When the M438 (*gcd* mutant) and PSC278 (*glk* mutant) strains were grown on glucose plus toluene, we found that the growth rate of the *gcd* mutant was similar to that measured with glucose alone, whereas the *glk* mutant grew at a higher rate with glucose and toluene (0.66 ± 0.01 h^{-1}) than with glucose alone (Table 2). These results suggest that toluene influenced glucose metabolism, particularly in the *gcd* mutant, in which glucose assimilation operates via the Glk route.

When we examined the rate of glucose consumption (q_{glu}) by the parental strain and isogenic mutants of it growing in the absence and in the presence of toluene, we found that the q_{glu} for wild-type cells growing in the presence of toluene was about one-half the rate with glucose alone (Table 2). We also found that the rate of toluene assimilation (q_{tol}) in the wild-type strain in the presence of glucose dropped by about 47% compared with the rate of hydrocarbon utilization in the absence of the sugar (Table 2). This indicates that the wild-type cells were able to assimilate glucose and toluene simultaneously.

We also examined q_{glu} and q_{tol} in M438 and PSC278 mutant cells. In these mutants the level of uptake of glucose was lower than that in the parental strain, as expected from their reduced growth rates with this sugar. However, although the glucose uptake rate decreased when toluene was present in the culture medium of the *gcd* mutant (strain M438), no decrease was observed in *glk* mutant cells (strain PSC278) (Table 2). When q_{tol} in the *glk*- and *gcd*-deficient mutants was examined, we found the opposite effect, namely, that in the presence of glucose the rate of toluene consumption was not affected in the Δ*gcd* mutant, whereas it decreased by almost 50% in the *glk* mutant. Therefore, KT2440 and isogenic mutants of this strain can use glucose and toluene simultaneously in the early steps of glucose assimilation.

Results of net flux analyses support the simultaneous use of glucose and toluene by *P. putida*. To learn more about the simultaneous metabolism of glucose and toluene, we decided to carry out assays using ^{13}C-labeled glucose, as described in Materials and Methods. To this end, we grew wild-type and mutant cells in the absence and in the presence of nonlabeled toluene. We focused our attention on four amino acids: serine and glycine, made from 3-phosphoglycerate (Fig. 2), and glutamate and proline, made from the 2-ketoglutarate Krebs cycle intermediate (Fig. 2). In the absence of toluene, as expected, the percentage of labeled amino acids was about 20%, consistent with the amount of ^{13}C supplied to the cultures (Fig. 3). In the wild type in presence of toluene, the ratio of ^{13}C in glycine and serine was almost 20%, in agreement with the fact that 3-phosphoglycerate is synthesized mainly from [^{13}C]glucose. However, the percentage of ^{13}C in glutamate and proline was slightly less than 10%. This is in agreement with the dilution of ^{13}C in the tricarboxylic acid cycle because of feeding with both glucose and toluene (Fig. 3). The proportion of ^{13}C in glutamate and proline suggested that there were similar contributions by glucose and toluene to the Krebs cycle in wild-type *P. putida* KT2440 cells. These results are in agreement with the rates of glucose and toluene consumption shown in Table 2. It should be

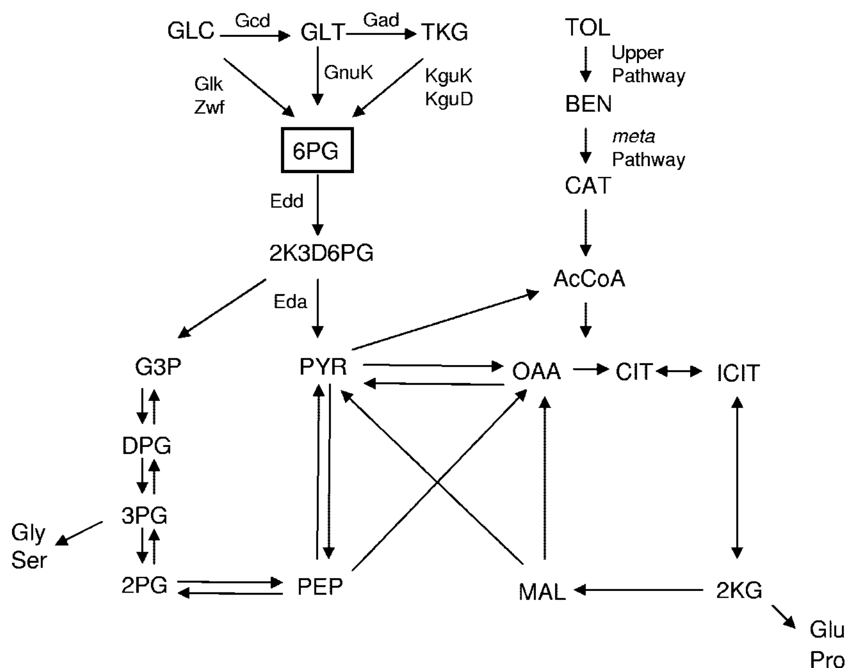


FIG. 2. Integration of glucose and toluene metabolism into central metabolism in *P. putida* KT2440. The pathways are based on experimental data reported by Worsey and Williams (49), Velázquez et al. (49), and del Castillo et al. (9). Abbreviations: GLC, glucose; GLT, gluconate; TKG, 2-ketogluconate; 6PG, 6-phosphogluconate; 2K3D6PG, 2-dehydro-3-deoxy-6-phosphogluconate; PYR, pyruvate; PEP, phosphoenolpyruvate; G3P, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; TOL toluene; BEN, benzoate; CAT, catechol; AcCoA, acetyl coenzyme A; CIT, citrate; ICIT, isocitrate; 2KG, 2-ketoglutarate; MAL, malate; OAA oxaloacetate.

noted that in *P. putida* KT2440 without the TOL plasmid, the percentage of labeled amino acids was around 20% of the total regardless of the presence of toluene, as expected.

In the KT2440 *gcd* and KT2440 *glk* mutants growing on labeled glucose in the absence of toluene, we obtained about 20% ^{13}C-labeled molecules for the four amino acids tested. This was expected since glucose was the sole C source. However, when toluene was also present, the situation was different than that described above for the wild-type strain. In this case the percentages of labeling in glycine and serine and in glutamate and proline were equivalent, as expected from their common biosynthetic origins. In the PSC278 (*glk*-deficient) mutant strain the amount of ^{13}C labeling in glycine and serine was 14%, whereas for glutamate and proline it was 8% because of dilution with unlabeled acetyl coenzyme A. In the M438 mutant strain (with the *gcd* gene knocked out) [^{13}C]glutamate and [^{13}C]proline accounted for only 1% of the total amount, whereas [^{13}C]serine and [^{13}C]glycine accounted for about 4% (Fig. 3). This was interpreted as evidence that toluene provides not only larger amounts of carbon to the Krebs cycle but also an extra flux of carbon necessary to synthesize 3-phosphoglycerate from pyruvate (Fig. 2).

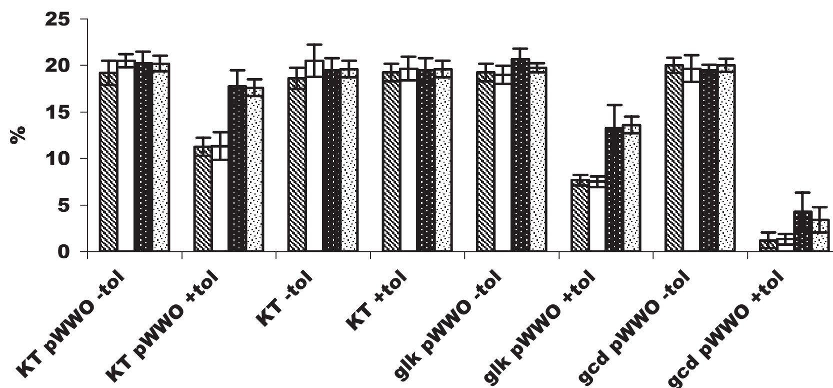


FIG. 3. ^{13}C-labeling patterns in different amino acids derived from [^{13}C]glucose. Bacterial cultures were fed with 20% (wt/wt) [^{13}C]glucose, and ^{13}C levels in serine (hatched bars), glycine (open bars), proline (stippled bars, white dots on black background), and glutamate (stippled bars, black dots on white background) were determined. The strains used are indicated as follows: KT, parental strain; glk pWWO, mutant deficient in *glk*; and gcd pWWO, mutant deficient in *gcd*. −tol indicates that toluene was absent, and +tol indicates that toluene was present. The *y* axis indicates the percentages of ^{13}C/^{12}C in the amino acids.

The results described above support the conclusions that glucose and toluene can be assimilated simultaneously and that toluene has a marked negative effect on the Glk pathway, as suggested by our finding that the flux of carbon from glucose in a *gcd* mutant was significantly limited. This effect was less substantial in the Gcd pathway. Therefore, it seems that toluene has a negative effect on the Glk pathway and a less noticeable effect on the gluconate pathways.

Global transcriptomic response of *P. putida* KT2440 cells growing with glucose and toluene. The physiological data reported above indicated that glucose affects the breakdown of toluene and that toluene affects glucose degradation too. To shed light on these effects at the transcriptional level, we decided to carry out a series of assays to compare the transcriptomes of cells growing in the exponential phase with glucose plus toluene and cells growing with glucose alone. A summary of the most relevant results for upregulated genes is shown in Table 3. Tables S1 and S2 in the supplemental material show downregulated genes and all genes identified as genes involved in glucose metabolism, respectively. The most remarkable result was that the sets of genes that formed the upper operon (*xylUWCMABN*) and the lower operon (*xylXYZLTEFGHKT*) were induced in response to toluene. The changes varied between 2.93-fold for *xylC* and 13.43-fold for *xylU* in the upper operon and between 2-fold for *xylF* and 4.68-fold for *xylZ* in the *meta* operon (Table 3). This indicates that cells induced the set of genes necessary for toluene catabolism. In addition to the TOL plasmid genes for toluene catabolism, we found that another 28 genes were induced more than twofold (Table 3). In agreement with Domínguez-Cuevas et al. (11), among the induced genes were the genes that encoded a number of stress proteins, including the IbpA chaperone (PP1982), two efflux pumps that removed excess solvent (PP3735 and PP3961), proteins involved in oxidative stress (PP3998), a number of proteins for general metabolism, and a number of hypothetical proteins. We also found two genes that encoded regulators, a sensor kinase (PP3413), and a *luxR*-like regulator (PP3717), all of which were induced.

The number of downregulated genes was small (11 genes). Four of these genes encoded proteins having unknown functions, five other genes encoded membrane proteins, including an efflux pump (PP3582) (see Table S1 in the supplemental material), and two genes encoded putative general metabolism enzymes.

We specifically analyzed the arrays in detail for glucose metabolism genes (see Table S2 in the supplemental material). Glucose metabolism involves entry of the sugar into the periplasmic space, a process that takes place through specific porins in the outer membrane (OprB porin). Glucose can then be internalized into the cytoplasm via an ABC system for subsequent conversion to glucose-6-phosphate (catalyzed by the Glk enzyme) and 6PG (catalyzed by glucose-6-phosphate dehydrogenase), or alternatively, glucose in the periplasmic space can be oxidized to gluconate and 2-ketogluconate, which are transported to the cytoplasm for conversion into 6PG (Fig. 2). We analyzed the effect of toluene on the different sets of gene clusters involved in glucose metabolism. The set of glucose metabolism genes more affected by the presence of toluene comprised the genes involved in glucose uptake via the ABC glucose transport system, made up of PP1015 through PP1018. This set of genes was repressed between 2- and 2.49-fold. The *glk* and *edd* genes, which are part of the same operon, were also repressed almost 2.0-fold. In contrast, the set of genes for the gluconate loops was affected little, if at all (see Table S2 in the supplemental material). Therefore transcrip-

TABLE 3. *P. putida* KT2440 upregulated genes in cells growing on glucose plus toluene compared to cells growing on glucose alone

Open reading frame and/or gene	Family	Fold change[a]
xylU	Probable toluene porin	13.43
xylW	Probable toluene porin	6.31
xylA	Toluene monooxygenase	6.05
xylB	Benzyl alcohol dehydrogenase	3.05
xylC	Benzaldehyde dehydrogenase	2.93
xylX	Toluate 1,2-dioxygenase	2.86
xylZ	Toluate 1,2-dioxygenase	4.68
xylL	1,2-Dihydroxy-3,5-cyclohexadiene-1–1-carboxylate	4.11
xylE	Catechol 2,3-dioxygenase	2.70
xylF	Hydrolase semialdehyde 2-hydroxymuconic	2.02
xylG	Dehydrogenase semialdehyde 2-hydroxymuconic	3.44
xylH	4-Oxalocrotonate tautomerase	3.74
xylI	4-Oxalocrotonate decarboxylase	2.78
xylK	4-Hydroxy-2-oxovalerate hydrolase	3.42
xylM	Toluene monooxygenase	3.84
xylN	Unknown function	2.61
xylQ	Acetaldehyde dehydrogenase	2.48
xylT	Ferredoxine	2.43
PP0210	Putative phycobiliprotein	2.86
PP1074 (*glpR*)	Glycerol-3-phosphate regulon repressor	2.79
PP1897	DNA internalization-related competence protein	4.36
PP2268	Phage endodeoxyribonuclease	2.19
PP2589	Aldehyde dehydrogenase family protein	2.52
PP2805	Monooxygenase flavin-binding family	2.27
PP3243	Acetyltransferase GNAT family	7.38
PP3413	Sensor histidine kinase/response regulator	2.38
PP3717	Transcriptional regulator LuxR family	2.94
PP3998	Glutathione *S*-transferase domain protein	4.05
PP4538	Putative acyl carrier protein phosphodiesterase	3.87
PP4983	Flavin-containing monamine oxidase family protein	2.23
PP5340	Acetylpolyamine aminohydrolase	2.09
pcaC	4-Carboxymuconolactone decarboxylase	2.13
pcaJ	3-Oxoadipate coenzyme A-transferase, subunit B	2.15
PP3726 (*ech*)	Enoyl coenzyme A hydratase/isomerase family protein	4.91
PP5248	Hydrolase isochorismatase family	2.46
PP5255	Hydrolase isochorismatase family	2.49
PP1982 (*ibpA*)	IbpA heat shock protein IbpA	2.00
PP3735	ABC transporter ATP-binding protein	2.00
PP3961	Putative transporter	2.00
PP1687	Hypothetical protein	2.94
PP2644	Hypothetical protein	2.97
PP3353	Conserved hypothetical protein	7.04
PP4561	Conserved hypothetical protein	2.73
PP4901	Conserved hypothetical protein	2.72
PP4981	Conserved hypothetical protein	2.02
PP4982	Conserved hypothetical protein	2.64
	Hypothetical protein pWW0 c57031-56282	2.70
	Hypothetical protein pWW0 c65777-65391	2.08
	Hypothetical protein pWW0 c94962-94570	3.65
	Hypothetical protein pWW0 c66769-66416	3.97

[a] The changes are averages of at least two assays. The *P* values were ≤ 0.05.

TABLE 4. Glucokinase activities in wild-type and mutant cells

Strain	Substrate(s)	Glucokinase activity (nmol/min/mg protein)[a]
KT2440(pWW0)	Glucose	27.3 ± 1.9
KT2440(pWW0)	Glucose + toluene	16.4 ± 1.9
KT2440(pWW0)	Toluene	1.3 ± 0.3
KT2440 pstN(pWW0)	Glucose	30.2 ± 1.4
KT2440 pstN(pWW0)	Glucose + toluene	16.2 ± 2.7
KT2440 pstN(pWW0)	Toluene	0.9 ± 0.1
KT2440 crc(pWW0)	Glucose	27.3 ± 5.4
KT2440 crc(pWW0)	Glucose + toluene	27.6 ± 1.7
KT2440 crc(pWW0)	Toluene	1.4 ± 0.2
KT2440(pWW0 Δ Pm)	Glucose	26.3 ± 0.3
KT2440(pWW0 Δ Pm)	Glucose + toluene	26.7 ± 0.4
KT2440(pWW0 Δ Pm)	Toluene	1.5 ± 0.2

[a] The data are the averages ± standard deviations of at least three independent determinations done in triplicate.

tomic analysis revealed that the catabolism of glucose through the glucokinase pathway is influenced by toluene catabolism, whereas the gluconate loop pathways are not sensitive to the presence of hydrocarbons.

Toluene affects the level of glucokinase activity in cells growing on glucose and toluene. The physiological and transcriptomic results reported above led us to measure glucokinase activity and gluconokinase activity in wild-type cells growing in the absence and in the presence of toluene. We found that in cells growing on toluene, gluconokinase and glucokinase activities were low, whereas in cells growing on glucose both enzymes were induced. This is in agreement with our previous results (9). The level of glucokinase in wild-type cells growing on glucose and toluene was almost 60% of the level in cells growing on glucose alone (Table 4). No significant effect on gluconokinase activity was observed (not shown). Therefore, the biochemical data support the pattern deduced from our transcriptomic analysis.

Identification of the glucose metabolite(s) involved in toluene repression. Wild-type *P. putida* cells, as well as cells of the *gcd* and *glk* mutants bearing the TOL plasmid, were transformed with pS10, a low-copy-number Tc^r Sm^r plasmid bearing a Pu:*lac*Z fusion and *xylR* (2). Cells were grown on M9 minimal medium with glucose, toluene, and toluene plus glucose as described in Materials and Methods. In wild-type cells, basal levels of β-galactosidase activity were found when cells were grown on glucose or citrate, and expression was about 20-fold higher in cells growing on toluene (Table 5). The β-galactosidase levels were only 30% of the maximal induced levels when they were measured in wild-type cells growing on glucose plus toluene. In the *glk* and *gcd* mutants, the β-galactosidase activity in cells growing on glucose plus toluene was lower than that in cells growing on toluene alone (Table 5). Therefore, this set of results supports previous studies that showed that glucose has a marked effect on toluene metabolism in *P. putida*.

TABLE 5. Expression of the Pu promoter fused to *lac*Z in the wild type and glucose mutants growing under different conditions[a]

Strain	β-Galactosidase activity (Miller units) with:		
	Glucose	Toluene	Glucose + toluene
KT2440(pWW0)	310 ± 20	6,620 ± 150	2,060 ± 40
KT2440 gcd(pWW0)	185 ± 15	6,035 ± 125	4,710 ± 700
KT2440 glk(pWW0)	400 ± 30	6,775 ± 100	3,870 ± 140
KT2440 ptsN(pWW0)	450 ± 20	8,100 ± 200	8,550 ± 190
KT2440 crc(pWW0)	380 ± 40	7,700 ± 500	5,680 ± 300

[a] The strains used were transformed with pS10, which carries a Pu:'*lacZ* fusion and the *xylR* gene. Assays were done in duplicate, and the data are the averages ± standard deviations of at least three independent assays.

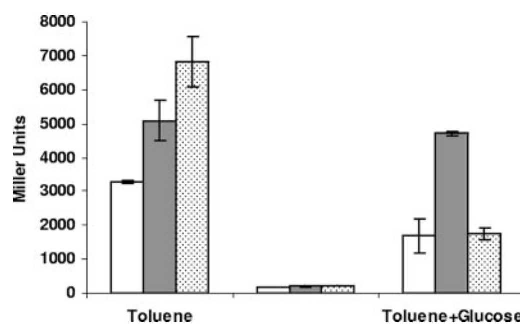


FIG. 4. Induction of Pu in parental strain *P. putida* KT2440 and *edd* and *eda* mutants of this strain. Bacterial cells were grown on citrate as the sole C source, and when cultures reached the mid-exponential phase (OD_{660}, 0.7 ± 0.1), cells were harvested, washed in M9 minimal medium without a C source, and divided into three aliquots that were supplemented with glucose, toluene, or glucose plus toluene. β-Galactosidase levels were determined 30 min later. The data are the averages and standard deviations of three independent assays. Open bars, *P. putida* KT2440(pS10); gray bars, *edd* mutant containing pS10; dotted bars, *eda* mutant.

Several research groups (9, 46, 47) have shown that *P. putida* mutants with mutations in the *edd* or *eda* genes cannot grow on glucose because the breakdown of 6PG into central metabolites is blocked (Fig. 2). To determine whether glucose or one of its metabolites was responsible for the catabolite repression described above, we decided to grow wild-type and *edd* and *eda* mutant cells on citrate and then transfer cells to minimal medium with toluene, glucose, or glucose plus toluene. After incubation for 30 min we measured β-galactosidase activity and found that it had increased from negligible levels to almost 3,000 Miller units (Fig. 4) in the parental strain and 5,000 to 7,000 Miller units in the *edd* and *eda* mutants exposed to only toluene. We also found that with glucose plus toluene the level of β-galactosidase activity in the parental strain or in the *eda* mutant was about 10 to 20% of the maximal activity measured with toluene alone, whereas in the *edd* mutant the level was close to 90% (Fig. 4). This suggests that the 2-dehydro-3-deoxygluconate-6-phosphate (Fig. 2) that accumulates in the *eda* mutant cells, not glucose or 6PG, is the true catabolite repressor signal.

To find out whether toluene or its metabolites were responsible for the effect observed on glucose assimilation, we carried out a series of assays with TOL mutants that convert toluene into benzoate (pWW0 ΔPm) or that are blocked at the level of 2,3-dioxygenase (TOL μ21) (Table 1). We found that with these TOL plasmid mutants, in which toluene does not serve as a carbon source, the glucokinase activity levels in cultures with toluene and glucose did not change compared with the levels in cells growing on glucose alone (see Table 4 for data for pWW0 ΔPm). Therefore, we concluded that utilization of the aromatic hydrocarbon as a carbon source is required for toluene to affect glucose metabolism.

Identification of master regulators involved in crossed repression of the toluene and glucose degradation genes. It was previously shown that in cells growing on glucose, the decrease in Pu expression was mediated by the PtsN protein (3, 5) and that a minor role could be ascribed to Crc (46). However, the potential regulator involved in catabolite repression of glucokinase was unknown. We decided to measure growth rates with glucose and toluene (Table 2) and to determine glucokinase activity and expression from Pu (Pu::'*lacZ*) in a series of isogenic mutants deficient in one of the global regulators involved in catabolite repression (Crc, Crp, PtsN, RelA, and CyoB). Isogenic mutant cells were transformed with pS10 (Pu::*lac*Z *xylR*), and β-galactosidase activity was determined in cells growing on glucose in the absence and in the presence of toluene. In agreement with previous studies, we found that glucose had a marked effect on Pu expression in the parental strain (Table 5). However, in the PstN-deficient background β-galactosidase levels were slightly higher in cells growing on glucose plus toluene than in cells growing on glucose alone (Table 5). The repressing effect was alleviated but not entirely eliminated in a *crc* mutant background (Table 5), and no major effects on other mutations were found (not shown).

We measured glucokinase and gluconokinase activities in all of these mutant backgrounds. As expected, we found that the activity of gluconokinase, one of the enzymes of the gluconate loop, was equally high regardless of the presence of toluene (not shown). Hence, this confirms that gluconate-metabolizing enzymes are not under catabolite repression by toluene in KT2440. Glucokinase activity was equally high in cells of the Crc mutant growing on glucose and in cells growing on glucose plus toluene (Table 4), whereas in the rest of the mutants this activity was decreased in cells growing on glucose plus toluene (see data for PtsN in Table 4). This suggests that the effect of toluene on glucokinase levels is mediated by the Crc protein.

DISCUSSION

Catabolite repression control refers to the ability of an organism to preferentially metabolize one carbon source over another when both carbon sources are present in the growth medium (3, 6, 42). Most studies of catabolite repression of the TOL plasmid catabolic pathways by carbon sources have been carried out with the nonmetabolizable toluene analogue *o*-xylene and have concentrated on the effects of sugars, organic acids, and alcohol on the transcriptional activity of the Pu promoter. The rationale behind such assays was to avoid the superimposed carbon load effect that toluene might have on its own metabolism. However, the drawback inherent in the design of such experiments was that they overlooked the potential effects of hydrocarbon metabolism on the assimilation of other C sources. Our study involved a different experimental setup for catabolite repression of the TOL plasmid Pu promoter: two assimilable carbon sources, glucose and toluene, were used simultaneously. Our results for the growth rates support the conclusion that glucose and toluene are good carbon sources and that when cells are exposed to both these carbon sources, cells counterbalance the amounts of total carbon taken up from glucose and toluene, so that cells grow at a rate similar to that observed with glucose or toluene alone. This set of results supports the earlier proposal that expression of the TOL plasmid operons is integrated into overall metabolic control in *P. putida* (37).

The observation that the total amount of carbon used by a microorganism is drawn from several carbon sources simultaneously has been reported before. *Klebsiella oxytoca* uses glycerol and sucrose simultaneously (35), and *Escherichia coli* exposed to limiting amounts of up to seven sugars can use all of them simultaneously (25). We can therefore speak of crossed catabolite repression of toluene and glucose metabolism in *P. putida*. This contrasts with the stricter catabolite repression described for other pathways. Among cases of strict catabolite repression is the preferential use of glucose over lactose in *E. coli* (23, 40, 42). This phenomenon has also been described for the metabolism of aromatic compounds, including the control of protocatechuate dioxygenase in *Pseudomonas cepacia* (52) and the control of the enzymes for the degradation of aniline in *Pseudomonas multivorans* ANl (18).

We recently showed that the early metabolism of glucose involves three convergent pathways (Fig. 2). Our microarray and enzymatic results support the conclusion that toluene catabolite repression of glucose metabolism was exerted on only one of the pathways, the glucokinase branch, rather than on the gluconate/2-ketogluconate loops. Array data indicated that OprB porin (PP1019), the ABC glucose transport system (PP1015 to PP1018), and the *glk*/*edd* operon were repressed in response to the presence of toluene, whereas the catabolic enzymes of the gluconate loops were not repressed. In agreement with these transcriptional data is the finding that the glucokinase levels in cells growing on glucose plus toluene were lower than the levels in cells grown with glucose alone (Table 4). The repressing effect of toluene on glucose metabolism was obvious in the *gcd* mutant, in which the only pathway for the assimilation of glucose is the Glk pathway. In agreement with repression of *glk* is the finding that the *gcd* mutant assimilated very small amounts of glucose in the presence of toluene (Table 2) and the finding that glucokinase activity was repressed (Table 4), which was reflected in the pattern of ^{13}C labeling of glycine, serine, glutamate, and proline (Fig. 3).

Velázquez et al. (46) proposed that either 6PG or 2-dehydro-3-deoxygluconate-6-phosphate could be the metabolic signal switching the Pu promoter pathway on or off. We ruled out the possibility that 6PG is the signal since the effect was not observed in the *edd* mutant. In contrast, we found evidence supporting the conclusion that the metabolite responsible for the effect was 2-dehydro-3-deoxygluconate-6-phosphate, since in an *eda* mutant catabolite repression of Pu by glucose was exacerbated (Fig. 4). Since the loss of *ptsN* rendered Pu unresponsive to glucose (3, 5), as monitored by '*lacZ* reporter technology with a *P. putida* strain carrying the low-copy-number plasmid pS10, we suggest that PtsN/2-dehydro-3-deoxygluconate-6-phosphate acts as the switcher in Pu inhibition. How the 2-dehydro-3-deoxygluconate-6-phosphate signal is transferred to PtsN is unknown, but Cases et al. (5) showed that PtsN inhibition required the phosphorylation of the protein at its phospho-acceptor His68 residue. Therefore, an excess of 2-dehydro-3-deoxygluconate-6-phosphate could result in a permanent PtsN-P state that could, in turn, prevent XylR from binding to its upstream activator sequences (3).

The Crc protein seems to act as the switch for the glucokinase pathway, since in a *crc*-deficient background glucokinase

was not repressed by toluene. This is in agreement with previous results for *P. aeruginosa* and *P. putida* (PpG2) which suggested that Crc controlled the expression of glucose-6-phosphate dehydrogenase, the second enzyme in the pathway that converts glucose-6-phosphate into 6PG (6, 7). Our results show that control of the glucokinase pathway in the presence of aromatic hydrocarbons also requires the active metabolism of toluene, although none of the key metabolites between toluene and catechol seemed to act as the specific signal between toluene and catechol. At present, the nature of the chemical signal is unknown, but it may be related to the energy state of the cell since Crc was proposed to launch appropriate responses based on the energy status of the cell (38).

In studies with *P. putida*, Rojo and colleagues showed that Crc may exert its effect at the posttranscriptional level, at least in the modulation of the *alk* system (30, 51). Our microarray results support a transcriptional role for Crc, but whether the effect is exerted directly by Crc or indirectly through modulation of transcription of the other regulator(s) remains unknown.

A feature that we have not overlooked is the effect of glucose on the bacterial response to toluene. Domínguez-Cuevas et al. (11) reported that exposure of *P. putida* cells to toluene resulted in the upregulation of 180 genes and the downregulation of 127 genes. In contrast, when glucose was present, only 50 genes were upregulated in response to toluene and only 11 genes were downregulated (Tables 3 and 4). In agreement with the results of Domínguez-Cuevas et al. (11) was our finding that all toluene assimilation genes were induced, but major differences were found with regard to the induction of stress genes. For instance, in cells growing on glucose plus toluene the IbpA protein was the only chaperone induced, which contrasted with the induction of 23 shock genes with toluene alone. This indicates that glucose metabolism alleviates the toxic effect of toluene.

In short, our results show that KT2440 metabolizes glucose and toluene simultaneously, as revealed by net flux analysis of [^{13}C]glucose. Simultaneous catabolite repression of the glucokinase and TOL pathways was triggered by two different catabolite repression systems; Pu was repressed mainly via PtsN in response to high levels of 2-dehydro-3-deoxygluconate-6-phosphate, whereas repression of the glucokinase pathway was channeled through Crc.

ACKNOWLEDGMENTS

This study was financed by grant BIO2006-05668 from the Spanish Ministry of Science and Education and by grant GEN2006-27750-C5-J-E from the EU SySMO Programme.

We thank Uwe Sauer and Tobias Führer for assistance with the ^{13}C assays done at the ETH in Zürich. We thank M. Mar Fandila and Carmen Lorente for secretarial assistance and K. Shashok for improving the use of 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., P. Marín, J. L. Ramos, and S. Marqués.** 2006. Role of the *pstN* gene product in catabolite repression of *Pseudomonas putida* TOL toluene degradation pathway in chemostat cultures. Appl. Environ. Microbiol. **72:**7418–7421.
3. **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:**4191–4198.
4. **Assinder, S. J., and P. A. Williams.** 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microb. Physiol. **31:**1–69.
5. **Cases, I., J. Pérez-Martín, and V. de Lorenzo.** 1999. The IIAN^{tr} (PtsN) protein of *Pseudomonas putida* mediates the C source inhibition of the σ^{54}-dependent Pu promoter of the TOL plasmid. J. Biol. Chem. **274:**15562–15568.
6. **Collier, D. N., P. W. Hager, and P. V. Phibbs, Jr.** 1996. Catabolite repression control in the pseudomonads. Res. Microbiol. **147:**551–561.
7. **Collier, D. N., C. Spencer, 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.
8. **Dauner, M., and U. Sauer.** 2000. GC-MS analysis of amino acids rapidly provides rich information for isotopomer balancing. Biotechnol. Prog. **16:** 642–649.
9. **del Castillo, T., J. L. Ramos, J. J. Rodríguez-Herva, T. Führer, U. Sauer, and E. Duque.** 2007. Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: genomic and flux analysis. J. Bacteriol. **189:**5142–5152.
10. **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.
11. **Domínguez-Cuevas, P., J. E. González-Pastor, S. Marqués, J. L. Ramos, and V. de Lorenzo.** 2006. Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. J. Biol. Chem. **281:**11981–11991.
12. **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:**601–606.
13. **Duque, E., A. Haïdour, F. Godoy, and J. L. Ramos.** 1993. Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. J. Bacteriol. **175:**2278–2283.
14. **Duque, E., A. J. Molina-Henares, J. de la Torre, M. A. Molina-Henares, T. del Castillo, J. Lam, and J. L. Ramos.** 2007. Towards a genome-wide mutant library of *Pseudomonas putida* strains KT2440. *In* J. L. Ramos (ed.), *Pseudomonas*, vol. V, in press Kluwer. London, United Kingdom.
15. **Fischer, E., and U. Sauer.** 2003. Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS. Eur. J. Biochem. **270:** 880–891.
16. **Fischer, E., N. Zamboni, and U. Sauer.** 2004. High-throughput metabolic flux analysis based on gas chromatography-mass spectrometry derived ^{13}C constraints. Anal. Biochem. **325:**308–316.
17. **Führer, T., E. Fischer, and U. Sauer.** 2005. Experimental identification and quantification of glucose metabolism in seven bacterial species. J. Bacteriol. **187:**1581–1590.
18. **Helm, V., and H. Reber.** 1979. Investigation on the regulation of aniline utilization in *Pseudomonas multivorans* strain AN1. Eur. J. Appl. Microbiol. Biotechnol. **7:**191–199.
19. **Herrero, M., and V. de Lorenzo.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
20. **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.
21. **Holtel, A., S. Marqués, I. Mohler, 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.
22. **Hunt, J. C., and P. V. Phibbs.** 1983. Regulation of alternate peripheral pathways of glucose catabolism during aerobic and anaerobic growth of *Pseudomonas aeruginosa*. J. Bacteriol. **154:**793–802.
23. **Inada, T., K. Kimata, and H. Aiba.** 1996. Mechanism responsible for glucose-lactose diauxie in *Escherichia coli*: challenge to the cAMP model. Genes Cells **1:**293–301.
24. **Jishage, M., K. Kvint, V. Shingler, and T. Nystrom.** 2002. Regulation of sigma factor competition by the alarmone ppGpp. Genes Dev. **16:**1260–1270.
25. **Lendenmann, U., M. Snozzi, and T. Egli.** 1996. Kinetics of the simultaneous utilization of sugar mixtures by *Escherichia coli* in continuous culture. Appl. Environ. Microbiol. **62:**1493–1499.
26. **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.
27. **Marqués, S., J. L. Ramos, and K. N. Timmis.** 1993. Analysis of the mRNA structure of the *Pseudomonas putida* TOL *meta*-fission pathway operon around the transcriptional initiation point, the *xylTE* and the *xylFJ* regions. Biochim. Biophys. Acta **1216:**227–236.

28. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
29. **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.
30. **Moreno, R., A. Ruíz-Manzano, L. Yuste, and F. Rojo.** 2007. The *Pseudomonas putida* Crc global regulator is an RNA binding protein that inhibits translation of the AlkS transcriptional regulator. Mol. Microbiol. **64:**665–676.
31. **Nakazawa, T.** 2002. Travels of a *Pseudomonas*, from Japan around the world. Environ. Microbiol. **4:**782–786.
32. **Nanchen, A., T. Führer, and U. Sauer.** 2007. Determination of metabolic flux ratios from ^{13}C-experiments and gas chromatography-mass spectrometry data: protocol and principle. Methods Mol. Biol. **358:**177–198.
33. **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.
34. **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.
35. **Piñar, G., K. Kovárová, T. Egli, and J. L. Ramos.** 1998. Influence of carbon source on nitrate removal by nitrate-tolerant *Klebsiella oxytoca* CECT 4460 in batch and chemostat cultures. Appl. Environ. Microbiol. **64:**2970–2976.
36. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543–594.
37. **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.
38. **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.
39. **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, vol. II, Kluwer Academic Publishers, New York, NY.
40. **Santillán, M., and M. C. MacKey.** 2004. Influence of catabolite repression and inducer exclusion on the bistable behavior of the *lac* operon. Biophys. J. **86:**1282–1292.
41. **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.
42. **Stulke, J., and W. Hillen.** 1999. Carbon catabolite repression in bacteria. Curr. Opin. Microbiol. **2:**195–201.
43. **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.
44. **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.
45. **Temple, L., A. Sage, G. E. Christie, and P. Phibbs, Jr.** 1994. Two genes for carbohydrate catabolism are divergently transcribed from a region of DNA containing the *hexC* locus in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **176:**4700–4709.
46. **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.
47. **Vicente, M., and J. L. Cánovas.** 1973. Glucolysis in *Pseudomonas putida*: physiological role of alternative routes from the analysis of defective mutants. J. Bacteriol. **116:**908–914.
48. **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.
49. **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.
50. **Yuste, L., A. B. Hervás, 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:**165–177.
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.
52. **Zylstra, G. J., R. Olsen, and D. P. Ballou.** 1989. Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase. J. Bacteriol. **171:**5907–5917.

Suppl. Table 1. *Pseudomonas putida* downregulated genes in cells growing on glucose plus toluene compared to cells grown on glucose alone.

ORF	Gene	Function	Fold change
PP0076		Putative glycine betaine-binding protein	-2.05
PP1904	*murB*	UDP-N-acetylenolpyruvoylglucosamine reductase	-2.01
PP5379	*copB*	Copper resistance protein B	-2.25
			-2.02
PP1206	*acrD*	OprD-porin D	-1.92
PP2968		Putative membrane protein	-2.05
PP3330		Putative outer membrane ferric siderophore receptor	-2.36
PP3582		RND efflux transporter outer membrane protein	-2.23
PP3245		Hypothetical protein	-2.18
PP3480		Hypothetical protein	-2.62
PP3642		Hypothetical protein	-2.09
PP3963		Conserved domain protein	-2.27

Suppl. Table 2. Fold-change of genes encoding proteins for glucose metabolism in cells growing on glucose plus toluene and glucose alone.

ORF	Gene	Family	Fold change
PP1015		Sugar ABC transporter periplasmic sugar-binding protein	-2.00
PP1017		Sugar ABC transporter permease protein	-2.41
PP1018		Sugar ABC transporter ATP-binding subunit	-2.49
PP1010	*edd*	6-phosphogluconate dehydratase	-1.90
PP1011	*glk*	Glucokinase	-2.09
PP1012	*gltR-2*	DNA-binding response regulator GltR	-1.26
PP1019	*oprB-1*	Porin B	-1.42
PP1445	*oprB-2*	Porin B	1.08
PP1022	*zwf-1*	Glucose-6-phosphate 1-dehydrogenase	-1.07
PP1023	*pgl*	6-phosphogluconolactonase	-1.45
PP1024	*eda*	2-dehydro-3-deoxyphosphogluconate aldolase	-1.4
PP1808	*pgi-1*	Glucose-6-phosphate isomerase	-1.12
PP3376	*kguD*	2-ketogluconate-6-phosphate reductase	-1.71
PP3377		Putative 2-ketogluconate transporter	-1.33
PP3378	*kguK*	2-ketogluconate kinase	-1.2
PP3379	*kguE*	Putative epimerase kguE	-1.16
PP3380	*ptxS*	Transcriptional regulator PtxS	1.04
PP3383		Gluconate dehydrogenase	1.05
PP3415		Transcriptional regulator LacI family	-1.04
PP3416	*gnuK*	Gluconokinase	-1.25
PP3417	*gntP*	Gluconate transporter	-1.16
PP4701	*pgi-2*	Glucose-6-phosphate isomerase	-1.21
PP4960	*fda*	Fructose-1,6-bisphosphate aldolase	-1.04
PP5040	*fbp*	Frutose 1,6-bisphosphatase	-1.14

DISCUSIÓN GENERAL

Los primeros estudios del metabolismo de carbohidratos realizados en bacterias del género *Pseudomonas* se remontan a los años 70, y ya entonces se determinó que tenía lugar fundamentalmente a través de la ruta de Entner-Doudoroff debido a la falta del gen que codifica la 6-fosfofructoquinasa, que impide la utilización de la ruta de Embden-Meyerhof. Si comparamos el número de carbohidratos que utilizan la mayoría de *Pseudomonas* con el de otros géneros, éste es muy reducido (glucosa, gluconato, 2-cetogluconato, fructosa, manitol y sorbitol). El metabolismo de todos estos carbohidratos tienen un intermediario común, el 6-fosfogluconato que a través de la ruta Entner-Doudoroff y por acción de las enzimas Edd y Eda rinde gliceraldehído-3-fosfato y piruvato que son canalizadas hasta intermediarios del ciclo de Krebs.

Blevins *et al*. (1975) demostraron que mutantes en el gen *edd* de *P. aeuriginosa* PAO1 eran incapaces de utilizar manitol, glucosa, gluconato o 2-cetogluconato. Vicente y Cánovas (1973a) aislaron mutantes en el gen *edd* y *eda* en *P. putida* ATCC 12633, los cuales no utilizaban glucosa, gluconato, y 2-cetogluconato como fuente de carbono. Posteriormente, Velázquez *et al.* (2004) confirmaron que mutantes en *eda* de *P. putida* MAD2 eran incapaces de utilizar glucosa y fructosa como única fuente de carbono y además nosotros comprobamos que mutantes en *edd* y *eda* de *P. putida* KT2440 tampoco consumían manitol ni sorbitol (datos no publicados). Estos estudios confirman que las bacterias del género *Pseudomonas* utilizan únicamente la ruta de Entner-Doudoroff en el catabolismo de carbohidratos.

1. Estudio del metabolismo de la glucosa en *Pseudomonas putida*

Se había descrito que el metabolismo de la glucosa hasta 6-fosfogluconato en bacterias del género *Pseudomonas* podía seguir dos vías excluyentes; la oxidativa, que tiene lugar en el periplasma y transforma la glucosa en gluconato, el cual es transportado al citoplasma y fosforilado a 6-fosfogluconato (Midgley & Dawes, 1973; Narrod & Wood, 1956; Roberts *et al*., 1973) o es oxidado en el mismo periplasma a 2-cetogluconato (Matsushita & Ameyama, 1982; Matsushita *et al.,* 1982), el cual es transportado al interior de la célula (Lynch & Franklin, 1978; Torrontegui *et al.,* 1976;

Whiting *et al.,* 1976a; Whiting *et al.,* 1976b), fosforilado y reducido hasta 6-fosfogluconato (Frampton & Wood, 1961; Mukadda *et al.,* 1973; Narrod & Wood, 1956; Roberts *et al*., 1973). La otra vía se conoce como fosforilativa y ocurre en el citoplasma por acción de la glucoquinasa y de la glucosa-6-fosfato deshidrogenasa dando lugar al 6-fosfogluconato (Coffee & Hu, 1970; Coffee & Hu, 1972; Eisenberg *et al.*, 1974; Guymon & Eagon, 1974; Hunt y Phibbs Jr, 1983; Lessie y Neidhardt, 1967; Lynch & Franklin, 1978; Midgley & Dawes, 1973; Tiwari & Campbell, 1969; Whiting *et al.,* 1976b; Wood & Schwerdt, 1953). Los estudios bioquímicos iniciales del metabolismo de la glucosa hasta 6-fosfogluconato en *P. putida* ATCC 12633 sugerían que tenía lugar a través de la vía oxidativa, siendo el lazo del 2-cetogluconato la vía principal y la fosforilación directa del gluconato la vía minoritaria (Vicente & Cánovas 1973a).

El análisis de la secuenciación del genoma de *Pseudomonas putida* KT2440 reveló que la información codificada sobre el metabolismo de la glucosa no coincidía de pleno con los estudios bioquímicos previos (Velázquez *et al.,* 2004), ya que se identificaron mediante análisis de BLAST todos los genes que codificarían los enzimas de las tres rutas. Para arrojar luz sobre el posible papel de la ruta fosforilativa, decidimos aislar y construir mutantes de *P. putida* KT2440 en cada una de las rutas potenciales, para lo que se generaron mutantes concretamente en los genes: *glk, gcd, gnuK* y *kguD*, con ello pretendíamos vislumbrar el uso de más de una ruta periférica en el metabolismo inicial de la glucosa. Nuestros resultados sugieren que *P. putida* KT2440 utiliza simultáneamente todas las rutas periféricas que conducen a la síntesis del 6-fosfogluconato, y la inactivación de cualquiera de ellas tiene un efecto en la tasa de crecimiento (μ_{max}). Así los mutantes en el lazo de la vía de la glucoquinasa y en el lazo del 2-cetogluconato crecían mucho más lento que la cepa silvestre, indicando que cuantitativamente eran más importantes que la fosforilación directa del gluconato. La presencia de estas rutas convergentes garantiza a la cepa la utilización de la fuente de carbono en su nicho natural, la rizosfera de plantas, en los que la glucosa es uno de los productos más abundantes exudados por las raíces (Kamilova *et al.,* 2006).

Las enzimas del metabolismo de la glucosa, de la vía del gluconato y de la glucosa-6-fosfato son inducibles según análisis de *microarray,* fusiones transcripcionales y determinaciones enzimáticas. La glucosa induce a la glucoquinasa y el gluconato a la gluconoquinasa. Ambas actividades presentaron un nivel basal en células cultivadas en citrato que aumentó unas 10 veces en células cultivadas en glucosa. Estos resultados concuerdan con el aumento de expresión de *glk* y *gnuK* en los *microarrays*. Las actividades enzimáticas y los datos de transcripción revelaron la inducción simultánea tanto de las enzimas de la fosforilación directa de la glucosa, como de los lazos del gluconato y 2-cetogluconato, lo que nos llevó a proponer que *P. putida* KT2440 utiliza a la vez la vía oxidativa y fosforilativa para metabolizar la glucosa.

Se realizaron análisis de flujo basados en ^{13}C para establecer como funcionaba y que respuesta tenían las diferentes entradas de glucosa. El análisis de los intermediarios metabólicos revelaron que en el ciclo de los ácidos tricarboxílicos (TCA) en la cepa silvestre la fracción mayor del metabolismo consiste en el *bypass* del piruvato a oxalacetato, representando una menor fracción el que tiene lugar a través de la malato deshidrogenasa. Esta observación coincide con lo descrito en otras *Pseudomonas*, pero contrasta con los flujos en *Escherichia coli* y *Bacillus subtilis* (Fuhrer *et al.,* 2005). Esto indica que en *P. putida* el exceso de carbono se canaliza a través de la vía del piruvato. Los análisis de flujo revelaron que la operación del ciclo de TCA y el *bypass* del piruvato funciona de la misma manera en los mutantes *gcd* y *glk*, a pesar de que la cantidad total de carbono consumida en los mutantes fuese más baja, debido probablemente a que la tasa de crecimiento fue también más baja en los mutantes. El análisis de flujo confirmó que *Pseudomonas putida* utiliza la ruta de Entner-Doudoroff para metabolizar la glucosa y que la ruta de las pentosas fosfato no contribuye al metabolismo de éste azúcar como se sabía de estudios previos (Aparicio *et al.,* 1971).

Para entender el patrón de expresión de los genes de las rutas convergentes se analizó primero la localización física de los genes de las distintas rutas del metabolismo inicial de la glucosa, luego se construyeron mutantes mediante mutagénesis dirigida y se estudió el patrón global de expresión utilizando *arrays* y

fusiones transcripcionales a `*lacZ*. Los genes de las rutas iniciales de la glucosa se disponen formando operones, dándose combinaciones de genes que codifican las enzimas catabólicas, un conjunto de porinas y transportadores específicos, de manera que se garantiza la entrada y el metabolismo del correspondiente metabolito. Así, OprB1 sería la porina que permite la entrada de la glucosa al periplasma para después ser transportada al interior de la célula por un sistema de transporte tipo ABC (PP1015-PP1018). Se pueden distinguir otros cinco operones de genes del metabolismo de la glucosa. Uno de los operones está formado por los genes que codifican la porina OprB-2 y la glucosa deshidrogenasa (Gcd), que no se transcriben juntos y el gen que codifica la enzima Gcd no está sujeto a represión catabólica. El análisis de secuencias nos llevó descubrir que en *P. putida* KT2440 los genes de las enzimas de la ruta Entner-Doudoroff están genéticamente ligados a los genes de la vía fosforilativa, así el gen *glk* de la ruta de la glucoquinasa forma parte del operón con el gen *edd,* y que el gen *zwf-1* forma parte de otro operón con el gen *eda*. Esta organización física en el genoma *edd/glk* y *zwf-1/eda* explica que la ruta fosforilativa tiene que ser operativa en *P. putida* porque el producto de ambos genes *edd* y *eda* son necesarios para el metabolismo del 6-fosfogluconato hasta los intermediarios del ciclo de Krebs. El gen *hexR* codifica el regulador HexR que controla la expresión del regulón *hex* formado por los dos operones *edd/glk* y *zwf-1/eda* y el gen *gap-1*. En el metabolismo del gluconato, hemos puesto de manifiesto el papel de GnuR que se transcribe divergentemente a la glucoquinasa (GnuK). El gen *gnuK* se localiza adyacente al gen del transportador de gluconato (GntP) y una proteína hipotética, estos dos últimos se transcriben formando un único tránscrito. El gen del regulador transcripcional PtxS se transcribe conjuntamente con los genes necesarios para metabolizar el 2-cetogluconato a 6-fosfogluconato (*kguE, kguK, kguT* y *kguD*). Corriente debajo del gen *ptxS* se ha localizado una transposasa (ISPpu9) seguida de los genes que codifican las dos subunidades de la gluconato deshidrogenasa (Gad).

2. Estudio del metabolismo de la glucosa en bacterias del género *Pseudomonas*

Cuando recopilamos la información que existía sobre el metabolismo de la glucosa previa a la secuenciación en las diferentes especies del género *Pseudomonas* observamos que se describía que las bacterias de la especie *P. aeruginosa* PAO1

metabolizaban la glucosa hasta 6-fosfogluconato a través de la vía oxidativa en condiciones de aerobiosis y de la vía fosforilativa en anaerobiosis (Hunt *et al.,* 1981; Hunt *et al.,* 1983), mientras que *P. fluorescens* A3.12 utilizaría únicamente la vía oxidativa (Narrod & Wood, 1955), igual que se pensaba en *P. putida* (Vicente & Cánovas, 1973a). Hasta el momento, en *P. syringae* y *P. entomophila* no se ha descrito que vía utilizan.

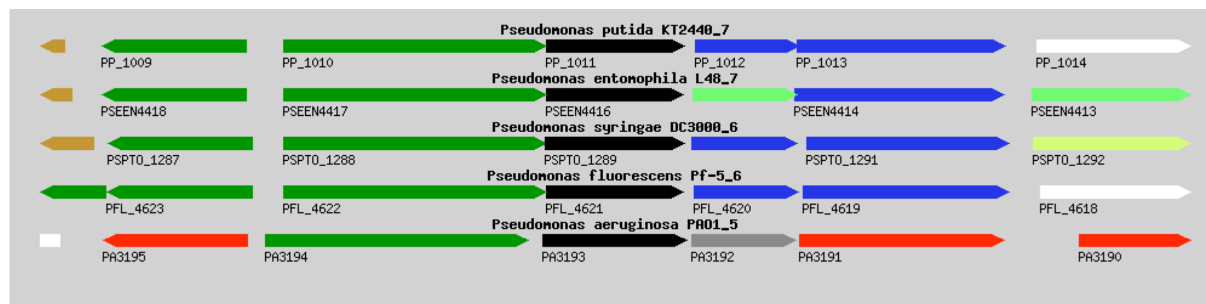
Para vislumbrar la posible utilización de la vía fosforilativa en las diferentes especies del género *Pseudomonas,* se decidió analizar si la organización de los genes del metabolismo de la glucosa de *P. putida* KT2440 se conservaba en los genomas de cepas de diferentes especies de *Pseudomonas,* a saber: *P. aeruginosa* PAO1 (Stover *et al.,* 2000), *P. entomophila* L48 (Vodovar *et al.*, 2006), *P. syringae* DC3000 (Buell *et al.,* 2003) y *P. fluorescens* Pf-5 (Paulsen *et al.,* 2005).

El regulón *hex* y su entorno genético

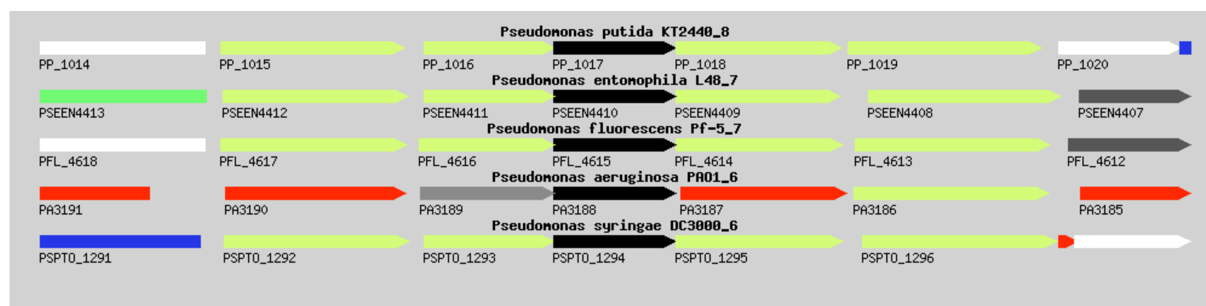
Los genes que codifican las enzimas de la ruta de Entner-Doudoroff responsables del metabolismo de la glucosa a piruvato y gliceraldehído-3-fosfato forman en *P. putida* parte del regulón *hex*, que se encuentran bajo el control del represor HexR en *P. putida* KT2440 según demostraron nuestros ensayos de *microarray* y fusiones transcripcionales. El regulón *hex* está formado por los genes *zwf, pgl, eda*, *edd*, *glk, gltR2* y *gap*. Estos genes se disponen en dos operones, uno formado por *edd/glk/gltR2,* y otro, constituido por *zwf/pgl/eda.* Corriente arriba de *edd/glk/gltR2* y divergentemente se transcribe el gen *gap-1* (Figura 1A). La misma organización se repite en las otras cuatro especies de *Pseudomonas*, concluyéndose que todas deben utilizar la vía fosforilativa ya que la expresión de los genes *edd* y *eda* es *sine qua non* para la operación de la ruta Entner-Doudoroff. Es curioso que en 3´con respecto al operón *edd/glk/gltR2* se localiza un gen que codifica una histidín quinasa sensora (PP1013) junto con el gen que codifica una proteína hipotética (PP1014). Acontinuación se localiza el sistema de transporte de la glucosa tipo ABC *(gtsABCD)* y el gen que codifica la porina OprB1, quedando por tanto todos los genes del metabolismo de la glucosa agrupados. La única diferencia encontrada en los genomas de las bacterias de *P. aeruginosa* PAO1 y *P. syringae* DC3000 es que no

está presente el gen que codifica la proteína hipotética (PP1014) de *P. putida* KT2440 (Figura 1A).

A



B



C

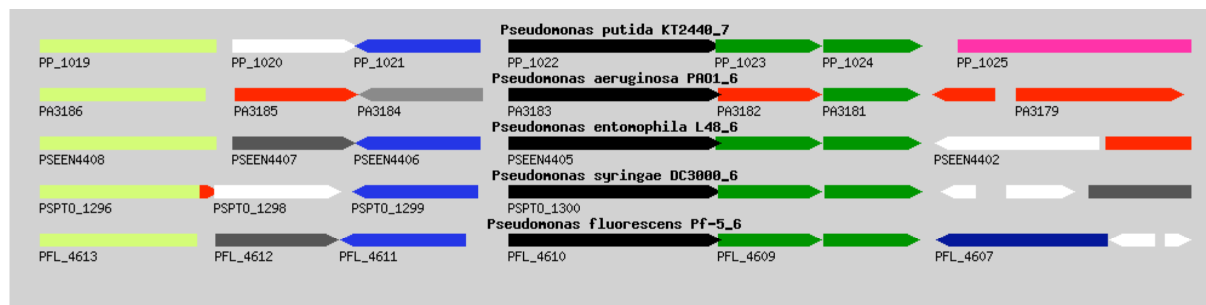


Figura 1. Organización de los genes del regulón *hex* y región adyacente. La figura 1A engloba a los genes *gap*-1 (PP1009), al operón *edd-glk-gltR* (PP1010-PP1011-PP1012), la histidín quinasa sensora (PP1013) y la proteína hipotética (PP1014) de KT2440. En la figura 1B se representan los genes del sistema de transporte de glucosa tipo ABC (PP1015-1018) y la porina OprB1 (PP1019) de KT2440. En la figura 1C se representan la proteína hipotética (PP1020), el regulador transcripcional HexR (PP1021) y el operón *zwf-pgl-eda* (PP1022-PP1023-PP1024) de KT2440 y en las demás especies de *Pseudomonas*. Las figuras son imágenes obtenidas de www.tigr.org

El genoma de KT2440 contiene cuatro genes implicados en el sistema de transporte de la glucosa de tipo ABC (PP1015 - PP1018) (Figura 1B), que permite a este azúcar ser transportado a través de la membrana interna y llegar al citoplasma. En las bacterias de la especie *P. aeruginosa* ATCC 15692 se le llamó sistema GBP (Glucose Binding Protein) (Stinson *et al.*, 1977) y se describió que un mutante en *P.*

aeruginosa PAO1 en este sistema perdía la capacidad de transportar glucosa, así como la respuesta quimiotáctica a este azúcar (Sage *et al.,* 1996). Este sistema se encuentra muy conservado entre las especies de *Pseudomonas* (Anexo 1). En 3´ con respecto a los genes del sistema de transporte, encontramos el gen de la porina OprB1 (PP1019) y una proteína hipotética conservada (PP1020), seguidos del regulador transcripcional (*hexR*) (Figura 1C).

Según la anotación del genoma de *P. putida* KT2440 y *P. entomophila* L48, el gen que codifica la glucosa-6-fosfato deshidrogenasa presenta tres alelos, mientras que en *P. aeruginosa* PAO1, *P. syringae* DC3000 y *P. fluorescens* Pf-5 sólo aparecen dos alelos (Anexo 2). El alelo *zwf-1* forma parte del regulón *hex* en todas las especies de *Pseudomonas* (Figura 1A), mientras que *zwf-2* (PP4042) y *zwf-3* (PP5351) están localizados en zonas diferentes en el genoma de KT2440 y podrían no estar implicados en el metabolismo de la glucosa, ya que en nuestros estudios de *microarrays* ni *zwf-2* ni *zwf-3* se indujeron en presencia de glucosa.

El operón del metabolismo del gluconato

El operón del metabolismo del gluconato no se había descrito en *Pseudomonas*, en cambio en *Bacillus subtilis* gracias al trabajo de Fujita *et al.* (1986) se sabía que estaba compuesto por *gntR, gntK, gntP* y *gntZ,* que codifican un regulador transcripcional negativo, gluconato quinasa, permeasa del gluconato y un gen de función no conocida, respectivamente. Fujita y Fujita (1987), describieron que GntR es un represor transcripcional del operón *gnt* y un mutante en este gen hacía que el operón se expresase de forma constitutiva.

Más adelante se estudió el metabolismo del gluconato en *E. coli* y se comprobó que estaba formado por dos loci; GntI y GntII. GntI está integrado por los genes *gntR, gntK* y *gntU*, que codifican una proteína reguladora, gluconato quinasa y el transportador de gluconato, respectivamente (Tong *et al.,* 1996). El loci GntII está compuesto por dos genes; *gntS* y *gntV* (Klemm *et al.* 1996). La expresión de *gntR* es constitutiva y responsable de la inducción de los genes *edd* y *eda* de la ruta de Entner-Doudoroff, mientras que *gntKU* son inducibles por gluconato y glucosa. Tong *et al.*

(1996) propusieron un modelo de inducción por gluconato, donde el gluconato se unía a GntR desplazándolo del sitio del operador que impedía la transcripción de *gntKU* por interferir en la zona promotora.

En *Pseudomonas* se conocía que las especies de *P. aeruginosa*, *P. fluorescens* y *P. putida* utilizaban el gluconato como fuente de carbono, el cual era transportado al interior de la célula y fosforilado por la gluconoquinasa dando lugar a 6-fosfogluconato que se metaboliza a través de la ruta de Entner-Doudoroff (Midgley & Dawes, 1973; Narrod & Wood, 1956; Roberts *et al*., 1973). Nosotros hemos descrito la organización del operón del metabolismo del gluconato implicado también en el metabolismo de la glucosa en KT2440 compuesto por el regulador transcripcional de la familia LacI (*gnuR*) (PP3415) que se transcribe divergentemente a los genes que codifican la glucoquinasa (*gnuK*) (PP3416), el transportador de gluconato (*gntP*) (PP3417) y una proteína hipotética (PP3418), estos dos últimos genes se transcriben formando un único tránscrito en *P. putida* KT2440 (Figura 2). La organización génica de KT2440 se repite en *P. entomophila* L48, siendo la similitud a nivel de proteína muy alto (80 - 86 %), mientras que en las otras especies de *Pseudomonas* no encontramos la proteína hipotética y la similitud entre proteínas es algo menor (79 – 67 %) (Anexo 3 y Figura 2).

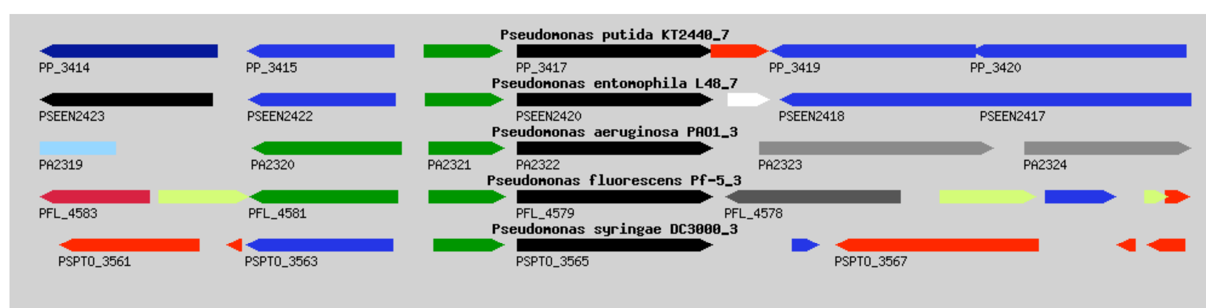


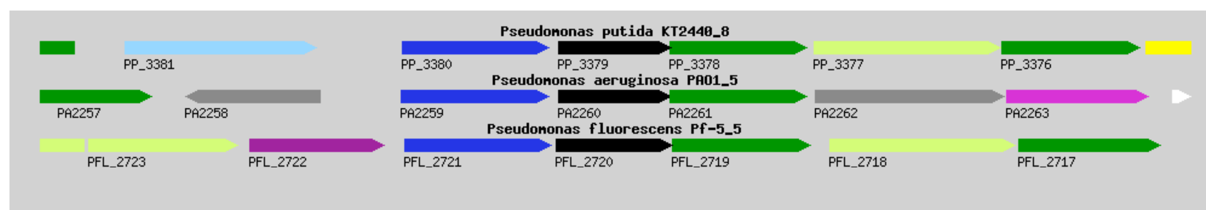
Figura 2. Representación de los genes que codifican operón del gluconato. El regulador transcripcional GnuR (PP3415), la gluconoquinasa (GnuK) (PP3416), el transportador de gluconato (GntP) (PP3417) y la proteína hipotética (PP3418) en KT2440 y en las demás especies de *Pseudomonas.* La figura es imagen obtenida de www.tigr.org

El operón del metabolismo del 2-cetogluconato

El operón que codifica los genes necesarios para el metabolismo del 2-cetogluconato en *P. aeruginosa* PAO1 fue descrito por Swanson *et al.* (2000a). Está formado por el gen *ptx*S, junto a los genes que codifican la 2-cetogluconato epimerasa (*kguE*), 2-cetogluconato quinasa (*kguK*), el transportador de 2-cetogluconato (*kguT*) y 2-cetogluconato-6-fosfato reductasa (*kguD*). Nosotros hemos encontrado que este operón en *P. putida* KT2440 y *P. fluorescens* Pf-5 conserva la misma organización (Figura 3A) y una alta similitud (86 – 63 %) (Anexo 4), pero estos genes no están presentes en los genomas de *P. entomophila* L48 y *P. syringae* DC3000, por tanto estas dos especies deben haber perdido la capacidad de metabolizar el 2-cetogluconato.

En el genoma de KT2440, corriente debajo del gen *ptxS* se localizó una transposasa (ISPpu9) (Ramos-González *et al.,* 2006) (PP3381) seguida de dos genes que codifican las subunidades de la gluconato deshidrogenasa (Gad) (PP3382-PP3383) y una proteína hipotética (PP3384) que también se regula por PtxS (Figura 3B). La transposasa tiene una similitud del 96 % con los genes PP1133, PP1260, PP3381, PP3586, PP4603 y PP4791, y esta transposasa no se encuentra en las otras *Pseudomonas* (Ramos-González *et al.,* 2006). Las bacterias del género *P. aeruginosa* PAO1 tienen los genes que codifican la proteína hipotética y las dos unidades de la gluconato deshidrogenasa (Gad) corriente abajo del gen *kguD*, mientras que en *P. fluorescens* Pf-5 se encuentran en otra localización diferente del genoma (Figura 3B). En los genomas de *P. entomophila* L48 y *P. syringae* DC3000 tampoco se encuentran los genes que codifican la gluconato deshidrogenasa.

A



B

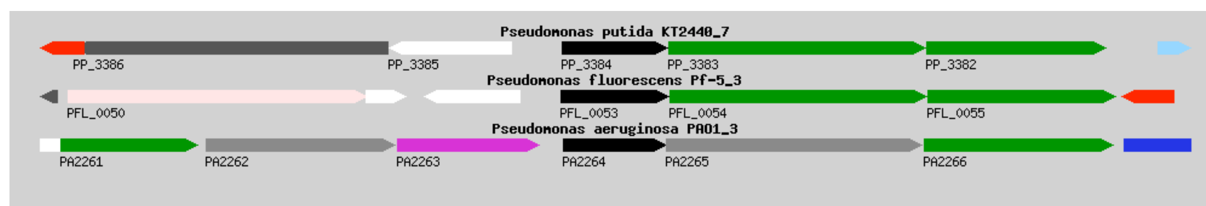


Figura 3. Representación de los genes del metabolismo del 2-cetogluconato. En la figura 3A se representan los genes que codifican la transposasa ISPpu9 (PP3381), el regulador transcripcional PtxS (PP3380), la 2-cetogluconato epimerasa (KguE) (PP3379), la 2-cetogluconato quinasa (KguK) (PP3378), el transportador de 2-cetogluconato (KguT) (PP3377), la 2-cetogluconato-6-fosfato reductasa (KguD) (PP3376) en KT2440 y la organización del mismo operón en P. *fluorescens Pf-5* y *P. aeruginosa* PAO1. En la figura 3B se representan al gen que codifica la proteína hipotética (PP3384) junto al gen de la gluconato deshidrogenasa (Gad) (PP3383) y el gen de la gluconato deshidrogenasa citocromo c (PP3382) de KT2440 y en las especies de P. *fluorescens Pf-5* y *P. aeruginosa* PAO1. Las figuras son imágenes obtenidas de www.tigr.org

3. Reguladores del metabolismo de la glucosa en bacterias del género *Pseudomonas*

El regulador GltR

El gen que codifica *gltR* es homólogo a los reguladores de respuesta a dos componentes y está implicado en la expresión del sistema de transporte activo en *Pseudomonas aeruginosa* PAO1 (Sage *et al.,* 1996). Corriente abajo del gen *gltR* se encuentra un gen que codifica una sensor histidín quinasa típica de la familia de reguladores OmpR, pero si el correspondiente producto génico participa o no en el metabolismo de la glucosa no se conoce todavía. Una mutación del gen *gltR2* dio lugar a la pérdida de transporte de glucosa en *P. aeruginosa* PAO1, donde este regulador controla la expresión de la porina OprD, que es específica de glucosa y al gen *gltB* que está implicado en el transporte activo de glucosa (Sage *et al.,* 1996). Esta misma organización se encuentra en las demás especies de *Pseudomonas*. Nuestros estudios con fusiones transcripcionales del promotor del gen *gtsA* a *´lacZ* en KT2440

mostraron que GltR2 controla la expresión del sistema de transporte de glucosa (*gtsABCD*). También se determinó el punto de inicio de la transcripción del gen *gtsA* que se encuentra a 96 pb del ATG del gen.

El regulador GnuR

Nuestros resultados de transcriptómica sugieren que la proteína GnuR controla la transcripción de GnuK y al transportador de gluconato. Se determinó el punto de inicio de la transcripción del gen *gnuR* que se encuentra a 37 pb del ATG del gen y en el caso de *gnuK* a 151 pb de su ATG. Curiosamente, la secuencia -10 de *gnuK* solapa en 5 nucleótidos con la secuencia -10 de *gnuR*. Además encontramos una secuencia palindrómica (5´-GTCCATACTGCGTAAGGAC-3´) situada entre +59 y +77 del promotor *gnuK* a la que el regulador GnuR podría unirse para controlar la transcripción del gen *gnuK*.

El regulador PtxS

En *P. aeruginosa*, el gen *ptxS* que codifica un represor transcripcional de la familia GalR-LacI, interviene induciendo la síntesis de exotoxina A, junto con la proteína PtxR, la cual pertenece a la familia LysR de activadores transcripcionales. Swanson *et al.* (1999), describieron que en *P. aeruginosa* PAO1, el gen *ptxS* se transcribía divergentemente al gen *ptxR*. Aunque el mecanismo de regulación de la expresión de ambos genes no se conoce (Swanson *et al.,* 2000a). El gen *ptxS* forma parte del operón de utilización del 2-cetogluconato en *P. aeruginosa* PAO1 junto a *kguEKTD* (Swanson *et al.,* 2000a). Utilizando la técnica de análisis de la huella dactilar (footprinting), Swanson *et al.* (2000b) determinaron que PtxS se unía a una región palindrómica corriente arriba del gen *ptxS*, dando lugar a su autorregulación (OP1). Posteriormente estos autores también demostraron que la proteína PtxS, se unía a otro operador (OP2) corriente abajo del gen *ptxS* controlando así al resto de genes del operón. La secuencia palindrómica es: 5´- TGAAACCGGTTTCA- 3´. El 2-cetogluconato inhibe la unión de PtxS a OP2 más eficientemente que PtxS a OP1 en *P. aeruginosa* PAO1.

Nuestros estudios *in silico* en KT2440 revelaron que existe la misma secuencia palindrómica en la región promotora de *ptxS* y del operón *kgu,* así como en la región promotora del gen que codifica la proteína hipotética (PP3384) que forma operón con los dos genes adyacentes que codifican la gluconato deshidrogenasa. Por tanto, proponemos que el regulador PtxS se une a estos operadores y controla a los dos operones y su propia síntesis. Cuando determinamos el punto de iniciación de la transcripción de *ptxS* y *kguE* observamos que la secuencia palindrómica solapaba con la +1 en el caso de *ptxS* y con la -10 en el caso de *kguE*.

El regulador HexR

La proteína HexR en *P. aeruginosa* controla la expresión de todas las enzimas del regulón *hex* por unión a secuencias específicas en un conjunto de promotores. La inactivación del gen *hexR* por inserción de un casete de resistencia a gentamicina en el cromosoma, dio lugar a la expresión constitutiva del regulón *hex* (Hager *et al.* 2000). Nuestros resultados de *microarray* y las fusiones transcripcionales del promotor del gen *zwf* a *lacZ* mostraron que el regulador HexR controla la expresión de los genes *gap-1, edd, glk, gltR2, zwf-1, pgl, eda*. Cuando determinamos el punto de inicio de la transcripción para los genes *gap-1, edd, zwf-1* y *hexR*, observamos que el motivo de reconocimiento del represor (5´-GntTtTaN$_{12}$TAAAA-C -3´) solapaba con los sitios de unión de la ARN polimerasa en los promotores de *gap-1, edd, zwf-1*, lo cual explica como el regulador HexR controla la transcripción de los genes mencionados.

En resumen, se puede decir que en la cepa bajo estudio en este trabajo de Tesis Doctoral, *Pseudomonas putida* KT2440, utiliza tres rutas periféricas para metabolizar la glucosa hasta 6-fosfogluconato, las cuales se encuentran organizadas en clusters en el genoma de la cepa. La expresión concertada de las rutas implica un juego de represores y activadores que responden a glucosa y que permiten la expresión simultánea de las rutas. Hemos establecido que la ruta fosforilativa se encuentra bajo el control catabólico cuando la cepa crece simultáneamente con glucosa y tolueno. La información de este estudio se ha ampliado mediante análisis *in silico* a otras especies del género *Pseudomonas,* cuyos genomas estaban secuenciados. El estudio reveló que el regulón *hex*, su entorno genético y el operón del metabolismo del gluconato de *P.*

putida se conservan en *P. entomophila, P. aeruginosa, P. syringae* y *P. fluorescens*, mientras que el operón del metabolismo del 2-cetogluconato sólo está presente en *P. aeruginosa* y *P. fluorescens*. Proponemos que *P. aeruginosa* y *P. fluorescens* guarden un comportamiento similar a *P. putida* respecto a la utilización de las tres rutas periféricas, mientras que las demás especies utilizarían sólo la vía fosforilativa y la vía del gluconato. El análisis sistemático de mutantes en cepas de esas especies debe arrojar luz experimental sobre esta propuesta.

ANEXOS

En los anexos 1 a 4 se presenta la identidad y la similitud que tienen las proteínas de las especies *P. entomophila* L48, *P. fluorescens* Pf-5, *P. syringae* DC3000 y *P. aeruginosa* PAO1 frente a la proteína correspondiente en *P. putida* KT2440.

Anexo 1. Regulón *hex* y su entorno genético

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT 2440	**Gap-1**	PP1009			1002
P. entomophila L48		PSEEN4418	90,7	94,3	1002
P. fluorescens Pf-5		PFL4623	85,0	98,2	1005
P. syringae DC3000		PSPTO1287	83,4	88,6	1002
P. aeruginosa PAO1		PA3195	73,2	83,4	1005

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Edd**	PP1010			1827
P. entomophila L48		PSEEN4417	91,1	95,4	1827
P. fluorescens Pf-5		PFL4622	84,2	89,8	1827
P. syringae DC3000		PSPTO1288	85,7	92,4	1827
P. aeruginosa PAO1		PA3194	76,0	86,6	1827

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Glk**	PP1011			960
P. entomophila L48		PSEEN4416	85,2	88,7	957
P. fluorescens Pf-5		PFL4621	66,8	76,9	957
P. syringae DC3000		PSPTO1284	60,8	73,1	966
P. aeruginosa PAO1		PA3193	52,4	64,4	996

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**GltR2**	PP1012			726
P. entomophila L48		PSEEN4415	92,9	97,1	723
P. fluorescens Pf-5		PFL4620	83,1	89,9	732
P. syringae DC3000		PSPTO1283	83,5	87,8	738
P. aeruginosa PAO1		PA3192	79,7	85,7	729

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP1013			1455
P. entomophila L48	PSEEN4414	71,3	74,8	1455
P. fluorescens Pf-5	PFL4619	66,5	73,2	1428
P. syringae DC3000	PSPTO1291	63,9	72,3	1407
P. aeruginosa PAO1	PA3191	61,5	71,3	1422

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP1014			1251
P. entomophila L48	PSEEN4413	60,7	70,1	1218
P. fluorescens Pf-5	PFL 4618	58,5	68,4	1260

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP1015			1287
P. entomophila L48	PSEEN4412	89,9	91,9	1287
P. fluorescens Pf-5	PFL 4617	77,3	83,4	1293
P. syringae DC3000	PSPTO1292	84,5	88,9	1287
P. aeruginosa PAO1	PA3190	62,3	72,4	1263

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP1016			909
P. entomophila L48	PSEEN4411	86,7	88,7	909
P. fluorescens Pf-5	PFL 4616	78,1	83,4	945
P. syringae DC3000	PSPTO1293	80,7	86,0	909
P. aeruginosa PAO1	PA3189	75,7	82,4	933

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP1017			846
P. entomophila L48	PSEEN4410	77,6	79,4	846
P. fluorescens Pf-5	PFL 4615	71,5	77,9	846
P. syringae DC3000	PSPTO1294	74,0	79,7	846
P. aeruginosa PAO1	PA3188	69,3	76,6	846

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP1018			1155
P. entomophila L48	PSEEN4409	91,4	95,1	1149
P. fluorescens Pf-5	PFL 4614	85,0	89,4	1161
P. syringae DC3000	PSPTO1295	84,8	91,6	1161
P. aeruginosa PAO1	PA3187	80,8	88,5	1161

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**OprB1**	PP1019			1344
P. entomophila L48		PSEEN4408	93,7	95,1	1344
P. fluorescens Pf-5		PFL 4613	70,1	82,2	1350
P. syringae DC3000		PSPTO1296	78,7	87,7	1362
P. aeruginosa PAO1		PA3186	80,7	88,4	1365

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP1020			855
P. entomophila L48	PSEEN4407	96,1	98,2	855
P. fluorescens Pf-5	PFL 4612	72,1	82,9	855
P. syringae DC3000	PSPTO1298	72,1	82,5	876
P. aeruginosa PAO1	PA3185	49,3	65,5	852

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**HexR**	PP1021			873
P. entomophila L48		PSEEN4406	93,8	94,1	873
P. fluorescens Pf-5		PFL 4611	86,1	90,9	870
P. syringae DC3000		PSPTO 1299	87,4	91,3	867
P. aeruginosa PAO1		PA3184	78,9	87,7	870

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Zwf-1**	PP1022			1470
P. entomophila L48		PSEEN4405	88,8	91,8	1470
P. fluorescens Pf-5		PFL 4610	79,8	86,7	1470
P. syringae DC3000		PSPTO 1300	78,7	85,5	1470
P. aeruginosa PAO1		PA3183	72,5	81,6	1470

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Pgl**	PP1023			729
P. entomophila L48		PSEEN4404	74,0	77,3	729
P. fluorescens Pf-5		PFL 4609	59,9	68,4	714
P. syringae DC3000		PSPTO 1301	58,6	70,5	714
P. aeruginosa PAO1		PA3182	59,2	68,5	717

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Eda**	PP1024			681
P. entomophila L48		PSEEN4403	96,9	98,2	675
P. fluorescens Pf-5		PFL 4608	76,9	87,5	666
P. syringae DC3000		PSPTO 1302	71,4	86,2	675
P. aeruginosa PAO1		PA3181	64,1	79,4	663

Anexo 2. Glucosa-6-fosfato deshidrogenasa: Zwf-2 y Zwf-3.

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Zwf-2**	PP4042			1506
P. entomophila L48		PSEEN1940	92,2	94,4	1497
P. fluorescens Pf-5		PFL 3143	75,0	82,8	1518
P. syringae DC3000		PSPTO3121	75,2	83,4	1533

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Zwf-3**	PP5351			1458
P. entomophila L48		PSEEN5500	89,9	94,2	1458
P. aeruginosa PAO1		PA5439	70,9	81,8	1467

Anexo 3. Operón del metabolismo del gluconato

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**GnuR**	PP3415			1020
P. entomophila L48		PSEEN2422	75,3	80,5	1020
P. fluorescens Pf-5		PFL4581	55,8	67,7	1032
P. syringae DC3000		PSPTO3563	60,5	71,5	1017
P. aeruginosa PAO1		PA2320	57,6	70,4	1032

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**GnuK**	PP3416			540
P. entomophila L48		PSEEN2421	78,2	84,4	540
P. fluorescens Pf-5		PFL4580	62,3	72,6	534
P. syringae DC3000		PSPTO3564	60,8	67,7	489
P. aeruginosa PAO1		PA2321	63,5	72,9	522

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**GntP**	PP3417			1353
P. entomophila L48		PSEEN2420	82,1	84,7	1353
P. fluorescens Pf-5		PFL4579	69,0	77,8	1353
P. syringae DC3000		PSPTO3565	72,8	79,9	1353
P. aeruginosa PAO1		PA2322	68,6	77,5	1353

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP4041			1881
P. entomophila L48	PSEEN1941	84,8	91,3	1797
P. syringae DC3000	PSPTO3119	65,7	78,2	1818

Anexo 4. Operón del metabolismo del 2-cetogluconato

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**KguD**	PP3376			963
P. fluorescens Pf-5		PFL2717	63,3	75,2	987
P. aeruginosa PAO1		PA2263	66,6	75,4	987

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**KguT**	PP3377			1293
P. fluorescens Pf-5		PFL2718	77,4	84,7	1290
P. aeruginosa PAO1		PA2262	81,2	86,9	1308

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**KguK**	PP3378			951
P. fluorescens Pf-5		PFL2719	68,4	76,5	963
P. aeruginosa PAO1		PA2261	68,7	75,9	951

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**KguE**	PP3379			780
P. fluorescens Pf-5		PFL2720	55,4	65,0	810
P. aeruginosa PAO1		PA2260	54,5	63,5	783

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**PtxS**	PP3380			1020
P. fluorescens Pf-5		PFL2717	68,5	78,3	1017
P. aeruginosa PAO1		PA2263	73,8	84,3	1023

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Gad cytc**	PP3382			1254
P. fluorescens Pf-5		PFL0055	82,0	89,7	1305
P. aeruginosa PAO1		PA2266	76,4	86,9	1320

Especie		Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	**Gad**	PP3383			1787
P. fluorescens Pf-5		PFL2717	84,7	90,9	1785
P. aeruginosa PAO1		PA2263	72,7	82,8	1776

Especie	Localización	% Identidad	% Similitud	Longitud del gen (pb)
KT2440	PP3384			741
P. fluorescens Pf-5	PFL0053	60,2	68,9	756
P. aeruginosa PAO1	PA2264	51,0	62,9	717

CONCLUSIONES

El conjunto de resultados obtenidos a lo largo de esta Tesis Doctoral nos ha permitido establecer las siguientes conclusiones:

1. El catabolismo de la glucosa en la cepa *Pseudomonas putida* KT2440 tiene lugar a través de tres rutas (glucoquinasa, gluconato quinasa y el lazo del 2-cetogluconato), que convergen a nivel del 6-fosfogluconato y que funcionan simultáneamente. El lazo del 2-cetogluconato y la ruta de la glucoquinasa son mayoritarias con respecto a la fosforilación directa del gluconato.

2. Las enzimas de las tres rutas periféricas de asimilación de glucosa son inducibles por éste azúcar, tal y como revelan los análisis de *microarrays* a nivel transcriptómico global, así como la determinación de las correspondientes actividades enzimáticas.

3. Cuando la glucosa es la única fuente de carbono el análisis de flujo demostró que el malato del ciclo de Krebs se canaliza a través de piruvato a oxalacetato.

4. Se ha identificado que el activador transcripcional GltR2 controla la expresión del gen de una de las porinas (OprB-1) de entrada de glucosa al periplasma y la expresión del sistema de transporte de la glucosa al citoplasma, denominado GtsABCD.

5. El regulador HexR controla los genes de la vía fosforilativa *glk/zwf-1* y de la ruta de Entner-Doudoroff *edd/eda* que se encuentran formando dos operones (*glk/edd* y *zwf/plg/eda)*, así como la expresión del gen *gap-1* que codifica la gliceraldehído-3-fosfato deshidrogenasa.

6. Se ha identificado un represor implicado en el metabolismo del 2-cetogluconato, PtxS, que controla la expresión de los genes que codifican las enzimas responsables de la oxidación del gluconato a 2-cetogluconato, su transporte y metabolismo hasta 6-fosfogluconato. Este regulador también controla un conjunto de genes no relacionados con el metabolismo de la glucosa.

7. Se ha identificado un represor implicado en el metabolismo del gluconato, GnuR, que responde específicamente a éste y controla su transporte y fosforilación.

8. El conjunto de reguladores HexR, GltR2, PtxS y GnuR garantizan la canalización simultánea de la glucosa a través de tres vías que convergen a nivel del 6PG.

9. *P. putida* KT2440 portando el plásmido pWW0 metaboliza glucosa y tolueno simultáneamente como revela nuestros estudios fisiológicos y el análisis de flujo de la glucosa marcada con ^{13}C.

10. Los estudios de transcriptómica y las medidas de actividades enzimáticas realizadas en *P. putida* KT2440 (pWW0) en respuesta a glucosa y tolueno demostraron que el tolueno reducía la expresión de los genes de la vía fosforilativa, mientras que la expresión de los genes de la ruta oxidativa no se afectaba.

11. La represión catabólica simultánea de la glucoquinasa y la ruta TOL se disparan por dos sistemas diferentes de represión catabólica: Pu es reprimido vía PtsN en respuesta a los altos niveles de 2-ceto-3-deoxi-6-fosfogluconato, mientras la represión de la ruta glucoquinasa se canaliza a través de la proteína Crc.

BIBLIOGRAFÍA

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.

Anderson, R. L., and W. A. Wood. 1969. Carbohydrate metabolism in microorganisms. Annu Rev Microbiol **23:**539-78.

Aparicio, M. L., M. Ruiz-Amil, M. Vicente, and J. L. Canovas. 1971. The role of phosphoglycerate kinase in the metabolism of *Pseudomonas putida*. FEBS Lett **14:**326-328.

Aranda-Olmedo, I., R. Tobes, M. Manzanera, J. L. Ramos, and S. Marques. 2002. Species-specific repetitive extragenic palindromic (REP) sequences in *Pseudomonas putida*. Nucleic Acids Res **30:**1826-33.

Assinder, S. J., and P. A. Williams. 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv Microb Physiol **31:**1-69.

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.

Bagdasarian, M., and K. N. Timmis. 1982. Host: vector systems for gene cloning in *Pseudomonas*. Curr Top Microbiol Immunol **96:**47-67.

Banerjee, P. C., R. I. Vanags, A. M. Chakrabarty, and P. K. Maitra. 1983. Alginic acid synthesis in *Pseudomonas aeruginosa* mutants defective in carbohydrate metabolism. J Bacteriol **155:**238-45.

Basu, A., and P. S. Phale. 2006. Inducible uptake and metabolism of glucose by the

phosphorylative pathway in *Pseudomonas putida* CSV86. FEMS Microbiol Lett **259:**311-6.

Bergey, D. H., G. M. Garrity, N. R. Krieg, and J. T. Staley. 2005. Bergey´s Manual of Systematic Bacteriology., p. 15-19. *In* D. J. Brenner, N. R. Krieg, and J. T. Staley (ed.), vol. 2. Springer, New York.

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.

Blevins, W. T., T. W. Feary, and P. V. Phibbs, Jr. 1975. 6-Phosphogluconate dehydratase deficiency in pleiotropic carbohydrate-negative mutant strains of *Pseudomonas aeruginosa*. J Bacteriol **121:**942-9.

Botsford, J. L. 1981. Cyclic nucleotides in procaryotes. Microbiol Mol Biol Rev **45:**620-642.

Braun, V. 1995. Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. FEMS Microbiol Rev **16:**295-307.

Buell, C. R., V. Joardar, M. Lindeberg, J. Selengut, I. T. Paulsen, M. L. Gwinn, R. J. Dodson, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, S. Daugherty, L. Brinkac, M. J. Beanan, D. H. Haft, W. C. Nelson, T. Davidsen, N. Zafar, L. Zhou, J. Liu, Q. Yuan, H. Khouri, N. Fedorova, B. Tran, D. Russell, K. Berry, T. Utterback, S. E. Van Aken, T. V. Feldblyum, M. D'Ascenzo, W. L. Deng, A. R. Ramos, J. R. Alfano, S. Cartinhour, A. K. Chatterjee, T. P. Delaney, S. G. Lazarowitz, G. B. Martin, D. J. Schneider, X. Tang, C. L. Bender, O. White, C. M. Fraser, and A. Collmer. 2003. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato

DC3000. Proc Natl Acad Sci U S A **100:**10181-6.

Cases, I., J. Perez-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.

Coffee, C. J., and A. S. Hu. 1970. The purification and partial characterization of gluconokinase from a *Pseudomonad*. J Biol Chem **245:**3703-7.

Coffee, C. J., and A. S. Hu. 1972. The kinetic characterization of gluconokinase from a *Pseudomonad*. Arch Biochem Biophys **149:**549-59.

Cuskey, S. M., J. A. Wolff, P. V. Phibbs, Jr., and R. H. Olsen. 1985. Cloning of genes specifying carbohydrate catabolism in *Pseudomonas aeruginosa* and *Pseudomonas putida*. J Bacteriol **162:**865-71.

De Ley, J. 1960. Comparative carbohydrate metabolism and localization of enzymes in *Pseudomonas* and related microorganisms. J Appl Bacteriol **23:**400-41.

Dejonghe, W., N. Boon, D. Seghers, E. M. Top, and W. Verstraete. 2001. Bioaugmentation of soils by increasing microbial richness: missing links. Environ Microbiol **3:**649-57.

Delgado, A., E. Duque, and J. L. Ramos. 1992. Behavior in agricultural soils of a recombinant *Pseudomonas* bacterium that simultaneously degrades alkyl- and haloaromatics. Microb Releases **1:**23-8.

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.

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.

Dunn, N. W., and I. C. Gunsalus. 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J Bacteriol **114:**974-9.

Eisenberg, R. C., S. J. Butters, S. C. Quay, and S. B. Friedman. 1974. Glucose uptake and phosphorylation in *Pseudomonas fluorescens*. J Bacteriol **120:**147-53.

Entner, N., and M. Doudoroff. 1952. Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*. J Biol Chem **196:**853-62.

Erb, R. W., C. A. Eichner, I. Wagner-Dobler, and K. N. Timmis. 1997. Bioprotection of microbial communities from toxic phenol mixtures by a genetically designed *pseudomonad*. Nat Biotechnol **15:**378-82.

Espinosa-Urgel, M., R. Kolter, and J. L. Ramos. 2002. Root colonization by *Pseudomonas putida*: love at first sight. Microbiology **148:**341-3.

Esteve-Nuñez, A., G. Lucchesi, B. Philipp, B. Schink, and J. L. Ramos. 2000. Respiration of 2,4,6-trinitrotoluene by *Pseudomonas* sp. strain JLR11. J Bacteriol **182:**1352-5.

Frampton, E. W., and W. A. Wood. 1961. Carbohydrate oxidation by *Pseudomonas fluorescens* VI. Conversion of 2-keto-6-phosphogluconate to pyruvate. J Biol Chem **236:**2571-7.

Franklin, F. C., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis. 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 U S A **78:**7458-62.

Franklin, F. C., P. R. Lehrbach, R. Lurz, B. Rueckert, M. Bagdasarian, and K. N. Timmis. 1983. Localization and functional analysis of transposon mutations in regulatory genes of the TOL catabolic pathway. J Bacteriol **154:**676-85.

Fuhrer, T., Fischer, E., Sauer, U. 2005. Experimental identification and quantification of glucose metabolism in seven bacterial species. Journal of Bacteriology **187:**1581-1590.

Fujita, Y., T. Fujita, Y. Miwa, J. Nihashi, and Y. Aratani. 1986. Organization and transcription of the gluconate operon, *gnt*, of *Bacillus subtilis*. J Biol Chem **261:**13744-53.

Fujita, Y., and T. Fujita. 1987. The gluconate operon *gnt* of *Bacillus subtilis* encodes its own transcriptional negative regulator. Proc Natl Acad Sci U S A **84:**4524-8.

Galán, B., E. Díaz, and J. L. García. 2000. Enhancing desulphurization by engineering a flavin reductase-encoding gene cassette in recombinant biocatalysts. Environ Microbiol **2:**687-94.

Gallegos, M. T., C. Michan, and J. L. Ramos. 1993. The XylS/AraC family of regulators. Nucleic Acids Res **21:**807-10.

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.

Gallegos, M. T., R. Schleif, A. Bairoch, K. Hofmann, and J. L. Ramos. 1997.

Arac/XylS family of transcriptional regulators. Microbiol Mol Biol Rev **61:**393-410.

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.

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.

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.

Guymon, L. F., and R. G. Eagon. 1974. Transport of glucose, gluconate, and methyl alpha-D-glucoside by *Pseudomonas aeruginosa*. J Bacteriol **117:**1261-9.

Haas, D., and B. W. Holloway. 1978. Chromosome mobilization by the R plasmid R68.45: a tool in *Pseudomonas* genetics. Mol Gen Genet **158:**229-37.

Hager, P. W., M. W. Calfee, and P. V. Phibbs. 2000. The *Pseudomonas aeruginosa* *devB*/SOL homolog, *pgl*, is a member of the *hex* regulon and encodes 6-phosphogluconolactonase. J Bacteriol **182:**3934-41.

Harayama, S., P. R. Lehrbach, and K. N. Timmis. 1984. Transposon mutagenesis analysis of *meta*-cleavage pathway operon genes of the TOL plasmid of *Pseudomonas putida* mt-2. J Bacteriol **160:**251-5.

Harayama, S., M. Rekik, K. L. Ngai, and L. N. Ornston. 1989. Physically

associated enzymes produce and metabolize 2-hydroxy-2,4-dienoate, a chemically unstable intermediate formed in catechol metabolism via meta cleavage in *Pseudomonas putida*. J Bacteriol **171:**6251-8.

Harayama, S., and M. Rekik. 1990. The *meta* cleavage operon of TOL degradative plasmid pWW0 comprises 13 genes. Mol Gen Genet **221:**113-20.

Haywood, G. W., A. J. Anderson, D. F. Ewing, and E. A. Dawes. 1990. Accumulation of a Polyhydroxyalkanoate Containing Primarily 3-Hydroxydecanoate from Simple Carbohydrate Substrates by *Pseudomonas* sp. Strain NCIMB 40135. Appl Environ Microbiol **56:**3354-3359.

Higgins, C. F., S. C. Hyde, M. M. Mimmack, U. Gileadi, D. R. Gill, and M. P. Gallagher. 1990. Binding protein-dependent transport systems. J Bioenerg Biomembr **22:**571-92.

Hochster, R. M., and H. Katznelson. 1958. On the mechanism of glucose-beta-phosphate oxidation in cell-free extracts of *Xanthomonas phaseoli* (XP8). Can J Biochem Physiol **36:**669-89.

Hunt, J. C., and P. V. Phibbs, Jr. 1981. Failure of *Pseudomonas aeruginosa* to form membrane-associated glucose dehydrogenase activity during anaerobic growth with nitrate. Biochem Biophys Res Commun **102:**1393-9.

Hunt, J. C., and P. V. Phibbs, Jr. 1983. Regulation of alternate peripheral pathways of glucose catabolism during aerobic and anaerobic growth of *Pseudomonas aeruginosa*. J Bacteriol **154:**793-802.

Hylemon, P. B., and P. V. Phibbs, Jr. 1972. Independent regulation of hexose catabolizing enzymes and glucose transport activity in *Pseudomonas aeruginosa*. Biochem Biophys Res Commun **48:**1041-8.

Inouye, S., A. Nakazawa, and T. Nakazawa. 1984. Nucleotide sequence of the promoter region of the *xylDEGF* operon on TOL plasmid of *Pseudomonas putida*. Gene **29:**323-30.

Inouye, S., A. Nakazawa, and T. Nakazawa. 1987a. Overproduction of the *xylS* gene product and activation of the *xylDLEGF* operon on the TOL plasmid. J Bacteriol **169:**3587-92.

Inouye, S., A. Nakazawa, and T. Nakazawa. 1987b. Expression of the regulatory gene *xylS* on the TOL plasmid is positively controlled by the *xylR* gene product. Proc Natl Acad Sci U S A **84:**5182-6.

Jamir, Y., T. Xiaoyan, and J. R. Alfano. 2004. The genome of *Pseudomonas syringae* tomato DC3000 and functional genomic studies to better understand plant pathogenesis, p. 113-138. *In* J. L. Ramos (ed.), *Pseudomonas*, vol. I. Kluwer Academic/Plenum Publishers, New York.

Jiménez, J. I., Miñambres, B., García, J.L., Díaz, E. 2002. Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. Enviromental Microbiology **4:**824-841.

Kamilova, F., L. V. Kravchenko, A. I. Shaposhnikov, T. Azarova, N. Makarova, and B. Lugtenberg. 2006. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol Plant Microbe Interact **19:**250-6.

Kellerhals, M. B., B. Kessler, and B. Witholt. 1999. Closed-loop control of bacterial high-cell-density fed-batch cultures: production of mcl-PHAs by *Pseudomonas putida* KT2442 under single-substrate and cofeeding conditions. Biotechnol Bioeng **65:**306-15.

Kessler, B., S. Marques, T. Kohler, 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.

Klemm, P., S. Tong, H. Nielsen, and T. Conway. 1996. The *gntP* gene of *Escherichia coli* involved in gluconate uptake. J Bacteriol **178:**61-7.

Kraak, M. N., T. H. Smits, B. Kessler, and B. Witholt. 1997. Polymerase C1 levels and poly(R-3-hydroxyalkanoate) synthesis in wild-type and recombinant *Pseudomonas* strains. J Bacteriol **179:**4985-91.

Kunz, D. A., and P. J. Chapman. 1981. Catabolism of pseudocumene and 3-ethyltoluene by *Pseudomonas putida* (arvilla) mt-2: evidence for new functions of the TOL (pWWO) plasmid. J Bacteriol **146:**179-91.

Lee, D. G., J. M. Urbach, G. Wu, N. T. Liberati, R. L. Feinbaum, S. Miyata, L. T. Diggins, J. He, M. Saucier, E. Deziel, L. Friedman, L. Li, G. Grills, K. Montgomery, R. Kucherlapati, L. G. Rahme, and F. M. Ausubel. 2006. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol **7:**R90.

Lessie, T., and F. C. Neidhardt. 1967. Adenosine triphosphate-linked control of *Pseudomonas aeruginosa* glucose-6-phosphate dehydrogenase. J Bacteriol **93:**1337-45.

Lessie, T. G., and P. V. Phibbs, Jr. 1984. Alternative pathways of carbohydrate utilization in *pseudomonads*. Annu Rev Microbiol **38:**359-88.

Lessmann, D., K. L. Schimz, and G. Kurz. 1975. D-glucose-6-phosphate

dehydrogenase (Entner-Doudoroff enzyme) from *Pseudomonas fluorescens*. Purification, properties and regulation. Eur J Biochem **59:**545-59.

Lockwood, L. B., B. Tabenkin, and G. E. Ward. 1941. The production of gluconic acid and 2-Keto-gluconic acid from glucose by species of *Pseudomonas* and *Phytomonas*. J Bacteriol **42:**51-61.

Lynch, W. H., and M. Franklin. 1978. Effect of temperature on the uptake of glucose, gluconate, and 2-ketogluconate by *Pseudomonas fluorescens*. Can J Microbiol **24:**56-62.

Marqués, S., and J. L. Ramos. 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Mol Microbiol **9:**923-9.

Marqués, S., J. L. Ramos, and K. N. Timmis. 1993. Analysis of the mRNA structure of the *Pseudomonas putida* TOL *meta* fission pathway operon around the transcription initiation point, the *xylTE* and the *xylFJ* regions. Biochim Biophys Acta **1216:**227-36.

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.

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.

Matsushita, K., and M. Ameyama. 1982. D-Glucose dehydrogenase from *Pseudomonas fluorescens,* membrane-bound. Methods Enzymol **89 Pt D:**149-54.

Matsushita, K., E. Shinagawa, and M. Ameyama. 1982. D-Gluconate dehydrogenase from bacteria, 2-keto-D-gluconate-yielding, membrane-bound. Methods Enzymol **89 Pt D:**187-93.

Maurer, P., D. Lessmann, and G. Kurz. 1982. D-Glucose-6-phosphate dehydrogenases from *Pseudomonas fluorescens*. Methods Enzymol **89 Pt D:**261-70.

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.

Midgley, M., and E. A. Dawes. 1973. The regulation of transport of glucose and methyl alpha-glucoside in *Pseudomonas aeruginosa*. Biochem J **132:**141-54.

Morett, E., and L. Segovia. 1993. The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J Bacteriol **175:**6067-74.

Mukkada, A. J., G. L. Long, and A. H. Romano. 1973. The uptake of 2-deoxy-D-glucose by *Pseudomonas aeruginosa* and its regulation. Biochem J **132:**155-62.

Nandadasa, H. G., M. Andreesen, and H. G. Schlegel. 1974. The utilization of 2-ketogluconate by *Hydrogenomonas eutropha* H 16. Arch Microbiol **99:**15-23.

Narrod, S. A., and W. A. Wood. 1956. Carbohydrate oxidation by *Pseudomonas fluorescens*. V. Evidence for gluconokinase and 2-ketogluconokinase. J Biol Chem **220:**45-55.

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.

Ng, F. M.W., and E. A. Dawes. 1973. Chemostat studies on the regulation of glucose metabolism in *Pseudomonas aeruginosa* by citrate. Biochem J **132:**129-140.

Nikaido, H. 1992. Porins and specific channels of bacterial outer membranes. Mol Microbiol **6:**435-42.

O'Brien, R., D. T. Chuang, B. L. Taylor, and M. F. Utter. 1977. Novel enzymic machinery for the metabolism of oxalacetate, phosphoenolpyruvate, and pyruvate in *Pseudomonas citronellolis*. J Biol Chem **252:**1257-63.

Olivera, E. R., D. Carnicero, B. Garcia, B. Minambres, M. A. Moreno, L. Canedo, C. C. Dirusso, G. Naharro, and J. M. Luengo. 2001. Two different pathways are involved in the beta-oxidation of n-alkanoic and n-phenylalkanoic acids in *Pseudomonas putida* U: genetic studies and biotechnological applications. Mol Microbiol **39:**863-74.

Palleroni, N. J., R. W. Ballard, E. Ralston, and M. Doudoroff. 1972. Deoxyribonucleic acid homologies among some *Pseudomonas species*. J Bacteriol **110:**1-11.

Palleroni, N. J. 1993-1994. *Pseudomonas* classification. A new case history in the taxonomy of gram-negative bacteria. Antonie Van Leeuwenhoek **63:**231-51.

Palleroni, N. J. 2003. Prokaryote taxonomy of the 20th century and the impact of studies on the genus *Pseudomonas*: a personal view. Microbiology **149:**1-7.

Palumbo, S. A., and L. D. Witter. 1969. The influence of temperature on the pathways of glucose catabolism in *Pseudomonas fluorescens*. Can J Microbiol **15:**995-1000.

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.

Peréz-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 U S A **92:**9392-6.

Petruschka, L., K. Adolf, G. Burchhardt, J. Dernedde, J. Jurgensen, and H. Herrmann. 2002. Analysis of the *zwf-pgl-eda*-operon in *Pseudomonas putida* strains H and KT2440. FEMS Microbiol Lett **215:**89-95.

Phibbs, P. V., Jr., and R. G. Eagon. 1970. Transport and phosphorylation of glucose, fructose, and mannitol by *Pseudomonas aeruginosa*. Arch Biochem Biophys **138:**470-82.

Phibbs, P. V. J., S. M. McCowen, T. W. Feary, and W. T. Blevins. 1978. Mannitol and fructose catabolic pathways of *Pseudomonas aeruginosa* carbohydrate-negative mutants and pleiotropic effects of certain enzyme deficiencies. J Bacteriol **133:**717-28.

Quay, S. C., S. B. Friedman, and R. C. Eisenberg. 1972. Gluconate regulation of glucose catabolism in *Pseudomonas fluorescens*. J Bacteriol **112:**291-8.

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 U S A **83:**8467-71.

Ramos, J. L., N. Mermod, and K. N. Timmis. 1987a. Regulatory circuits controlling transcription of TOL plasmid operon encoding *meta*-cleavage pathway for degradation of alkylbenzoates by *Pseudomonas.* Mol Microbiol **1:**293-300.

Ramos, J. L., A. Wasserfallen, K. Rose, and K. N. Timmis. 1987b. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science **235:**593-6.

Ramos, J. L., F. Rojo, L. Zhou, and K. N. Timmis. 1990. A family of positive regulators related to the *Pseudomonas putida* TOL plasmid XylS and the *Escherichia coli* AraC activators. Nucleic Acids Res **18:**2149-52.

Ramos, J. L., E. Diaz, D. Dowling, V. de Lorenzo, S. Molin, F. O'Gara, C. Ramos, and K. N. Timmis. 1994. The behavior of bacteria designed for biodegradation. Biotechnology (N Y) **12:**1349-56.

Ramos, J. L., S. Marques, 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.

Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-González, A. Rojas, W. Teran, and A. Segura. 2002. Mechanisms of solvent tolerance in gram-

negative bacteria. Annu Rev Microbiol **56:**743-68.

Ramos-González, M. I., E. Duque, and J. L. Ramos. 1991. Conjugational transfer of recombinant DNA in cultures and in soils: host range of *Pseudomonas putida* TOL plasmids. Appl Environ Microbiol **57:**3020-7.

Ramos-González, M. I., M. J. Campos, J. L. Ramos, and M. Espinosa-Urgel. 2006. Characterization of the *Pseudomonas putida* mobile genetic element ISPpu10: an occupant of repetitive extragenic palindromic sequences. J Bacteriol **188:**37-44.

Raps, S., and R. D. Demoss. 1962. Glycolytic enzymes in *Zymomonas mobilis*. J Bacteriol **84:**115-8.

Regenhardt, D., H. Heuer, S. Heim, D. U. Fernandez, C. Strompl, E. R. Moore, and K. N. Timmis. 2002. Pedigree and taxonomic credentials of *Pseudomonas putida* strain KT2440. Environ Microbiol **4:**912-5.

Rheinwald, J. G., A. M. Chakrabarty, and I. C. Gunsalus. 1973. A transmissible plasmid controlling camphor oxidation in *Pseudomonas putida*. Proc Natl Acad Sci U S A **70:**885-9.

Roberts, B. K., M. Midgley, and E. A. Dawes. 1973. The metabolism of 2-oxogluconate by *Pseudomonas aeruginosa*. J Gen Microbiol **78:**319-29.

Rojas, A., E. Duque, A. Schmid, A. Hurtado, J. L. Ramos, and A. Segura. 2004. Biotransformation in double-phase systems: physiological responses of *Pseudomonas putida* DOT-T1E to a double phase made of aliphatic alcohols and biosynthesis of substituted catechols. Appl Environ Microbiol **70:**3637-43.

Rojo, F., D. H. Pieper, K. H. Engesser, H. J. Knackmuss, and K. N. Timmis. 1987. Assemblage of ortho cleavage route for simultaneous degradation of chloro- and

methylaromatics. Science **238:**1395-8.

Sage, A. E., W. D. Proctor, and P. V. Phibbs, Jr. 1996. A two-component response regulator, *gltR*, is required for glucose transport activity in *Pseudomonas aeruginosa* PAO1. J Bacteriol **178:**6064-6.

Saravolac, E. G., N. F. Taylor, R. Benz, and R. E. Hancock. 1991. Purification of glucose-inducible outer membrane protein OprB of *Pseudomonas putida* and reconstitution of glucose-specific pores. J Bacteriol **173:**4970-6.

Sawyer, M. H., P. Baumann, L. Baumann, S. M. Berman, J. L. Canovas, and R. H. Berman. 1977. Pathways of D-fructose catabolism in species of *Pseudomonas*. Arch Microbiol **112:**49-55.

Schindler, J., and H. G. Schlegel. 1969. [Regulation of the glucose-6-phosphate dehydrogenase of different bacterial species by ATP]. Arch Mikrobiol **66:**69-78.

Schleissner, C., A. Reglero, and J. M. Luengo. 1997. Catabolism of D-glucose by *Pseudomonas putida* U occurs via extracellular transformation into D-gluconic acid and induction of a specific gluconate transport system. Microbiology **143 (Pt 5):**1595-603.

Schmid, A., J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, and B. Witholt. 2001. Industrial biocatalysis today and tomorrow. Nature **409:**258-68.

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.

Shingler, V., and H. Pavel. 1995. Direct regulation of the ATPase activity of the

transcriptional activator DmpR by aromatic compounds. Mol Microbiol **17:**505-13.

Shuster, C. W., and M. Doudoroff. 1967. Purification of 2-keto-3-deoxy-6-phosphohexonate aldolases of *Pseudomonas saccharophila*. Arch Mikrobiol **59:**279-86.

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.

Spangler, W. J., and C. M. Gilmour. 1966. Biochemistry of nitrate respiration in *Pseudomonas stutzeri*. I. Aerobic and nitrate respiration routes of carbohydrate catabolism. J Bacteriol **91:**245-50.

Stainer, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic *Pseudomonads*: a taxonomic study. J Gen Microbiol **43:**159-271.

Stern, I. J., C. H. Wang, and C. M. Gilmour. 1960. Comparative catabolism of carbohydrates in *Pseudomonas* species. J Bacteriol **79:**601-11.

Stinson, M. W., M. A. Cohen, and J. M. Merrick. 1977. Purification and properties of the periplasmic glucose-binding protein of *Pseudomonas aeruginosa*. J Bacteriol **131:**672-81.

Stokes, F. N., and J. J. Campbell. 1951. The oxidation of glucose and gluconic acid by dried cells of *Pseudomonas aeruginosa*. Arch Biochem **30:**121-5.

**Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S.

N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959-64.

Swanson, B. L., J. A. Colmer, and A. N. Hamood. 1999. The *Pseudomonas aeruginosa* exotoxin A regulatory gene, *ptxS*: evidence for negative autoregulation. J Bacteriol **181:**4890-5.

Swanson, B. L., P. Hager, P. Phibbs, Jr., U. Ochsner, M. L. Vasil, and A. N. Hamood. 2000a. Characterization of the 2-ketogluconate utilization operon in *Pseudomonas aeruginosa* PAO1. Mol Microbiol **37:**561-73.

Swanson, B. L., and A. N. Hamood. 2000b. Autoregulation of the *Pseudomonas aeruginos*a protein PtxS occurs through a specific operator site within the *ptxS* upstream region. J Bacteriol **182:**4366-71.

Tan, Y., M. Xu, X. Tan, X. Wang, Y. Saikawa, T. Nagahama, X. Sun, M. Lenz, and R. M. Hoffman. 1997. Overexpression and large-scale production of recombinant L-methionine-alpha-deamino-gamma-mercaptomethane-lyase for novel anticancer therapy. Protein Expr Purif **9:**233-45.

Temple, L., S. M. Cuskey, R. E. Perkins, R. C. Bass, N. M. Morales, G. E. Christie, R. H. Olsen, and P. V. Phibbs, Jr. 1990. Analysis of cloned structural and regulatory genes for carbohydrate utilization in *Pseudomonas aeruginosa* PAO. J Bacteriol **172:**6396-402.

Timmis, K. N., R. J. Steffan, and R. Unterman. 1994. Designing microorganisms for the treatment of toxic wastes. Annu Rev Microbiol **48:**525-57.

Tiwari, N. P., and J. J. Campbell. 1969. Enzymatic control of the metabolic activity

of *Pseudomonas aeruginosa* grown in glucose or succinate media. Biochim Biophys Acta **192:**395-401.

Tong, S., A. Porco, T. Isturiz, and T. Conway. 1996. Cloning and molecular genetic characterization of the *Escherichia coli gntR, gntK*, and *gntU* genes of GntI, the main system for gluconate metabolism. J Bacteriol **178:**3260-9.

Torrontegui, D., R. Díaz, and J. L. Cánovas. 1976. The uptake of 2-ketogluconate by *Pseudomonas putida*. Arch Microbiol **110:**43-8.

Trias, J., E. Y. Rosenberg, and H. Nikaido. 1988. Specificity of the glucose channel formed by protein D1 of *Pseudomonas aeruginosa*. Biochim Biophys Acta **938:**493-6.

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.

Velázquez, F., K. Pfluger, I. Cases, L. I. De Eugenio, and V. de Lorenzo. 2007. The phosphotransferase system formed by PtsP, PtsO, and PtsN proteins controls production of polyhydroxyalkanoates in *Pseudomonas putida*. J Bacteriol **189:**4529-33.

Vicente, M., and J. L. Cánovas. 1973a. Glucolysis in *Pseudomonas putida:* physiological role of alternative routes from the analysis of defective mutants. J Bacteriol **116:**908-14.

Vicente, M., and J. L. Cánovas. 1973b. Regulation of the glucolytic enzymes in *Pseudomonas putida*. Arch Mikrobiol **93:**53-64.

Vicente, M. 1975. The uptake of fructose by *Pseudomonas putida*. Arch Microbiol

102:163-6.

Vodovar, N., D. Vallenet, S. Cruveiller, Z. Rouy, V. Barbe, C. Acosta, L. Cattolico, C. Jubin, A. Lajus, B. Segurens, B. Vacherie, P. Wincker, J. Weissenbach, B. Lemaitre, C. Medigue, and F. Boccard. 2006. Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. Nat Biotechnol **24:**673-9.

Walsh, U. F., J. P. Morrissey, and F. O'Gara. 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. Curr Opin Biotechnol **12:**289-95.

Weinel, C., K. E. Nelson, and B. Tummler. 2002. Global features of the *Pseudomonas putida* KT2440 genome sequence. Environ Microbiol **4:**809-18.

Whitfield, C. D., L. M. Hupe, C. Nugent, K. E. Urbani, and H. J. Whitfield. 1982. Increased hexose transport in Chinese hamster ovary cells resistant to 3-O-methyl-D-glucose. J Biol Chem **257:**4902-6.

Whiting, P. H., M. Midgley, and E. A. Dawes. 1976a. The regulation of transport of glucose, gluconate and 2-oxogluconate and of glucose catabolism in *Pseudomonas aeruginosa*. Biochem J **154:**659-68.

Whiting, P. H., M. Midgley, and E. A. Dawes. 1976b. The role of glucose limitation in the regulation of the transport of glucose, gluconate and 2-oxogluconate, and of glucose metabolism in *Pseudomonas aeruginosa*. J Gen Microbiol **92:**304-10.

Willard, T., T. Blevins, W. Ferry, and P. Jr. 1975. 6-Phosphogluconate dehydratase deficiency in pleiotropic carbohydrate-negative mutant strain of *Pseudomonas aeruginosa.* J Bacteriol **121:**942-9.

Williams, P. A., and K. Murray. 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (arvilla) mt-2: evidence for the existence of a TOL plasmid. J Bacteriol **120:**416-23.

Williams, P. A., L. M. Shaw, C. W. Pitt, and M. Vrecl. 1997. XylUW, two genes at the start of the upper pathway operon of TOL plasmid pWW0, appear to play no essential part in determining its catabolic phenotype. Microbiology **143 (Pt 1):**101-7.

Wood, W. A., and R. F. Schwerdt. 1953. Carbohydrate oxidation by *Pseudomonas fluorescens*. I. The mechanism of glucose and gluconate oxidation. J Biol Chem **201:**501-511.

Wood, W. A., and R. F. Schwerdt. 1954. Carbohydrate oxidation by *Pseudomonas fluorescens*. II. Mechanism of hexose phosphate oxidation. J Biol Chem **206:**625-35.

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.

Wubbolts, M. G., and K. N. Timmis. 1990. Biotransformation of substituted benzoates to the corresponding cis-diols by an engineered strain of *Pseudomonas oleovorans* producing the TOL plasmid-specified enzyme toluate-1,2-dioxygenase. Appl Environ Microbiol **56:**569-71.

Zeyer, J., P. R. Lehrbach, and K. N. Timmis. 1985. Use of cloned genes of *Pseudomonas* TOL plasmid to effect biotransformation of benzoates to cis-dihydrodiols and catechols by *Escherichia coli* cells. Appl Environ Microbiol **50:**1409-13.