Intracellular 2-keto-3-deoxy-6-phosphogluconate is the signal for carbon catabolite repression of phenylacetic acid metabolism in *Pseudomonas putida* KT2440

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The growth pattern of *Pseudomonas putida* KT2440 in the presence of glucose and phenylacetic acid (PAA), where the sugar is used in preference to the aromatic compound, suggests that there is carbon catabolite repression (CCR) of PAA metabolism by glucose or gluconate. Furthermore, CCR is regulated at the transcriptional level. However, this CCR phenomenon does not occur in PAA-amended minimal medium containing fructose, pyruvate or succinate. We previously identified 2-keto-3-deoxy-6-phosphogluconate (KDPG) as an inducer of glucose metabolism, and this has led to this investigation into the role of KDPG as a signal compound for CCR. Two mutant strains, the *edd* mutant (non-KDPG producer) and the *eda* mutant (KDPG overproducer), grew in the presence of PAA but not in the presence of glucose. The *edd* mutant utilized PAA even in the presence of glucose, indicating that CCR had been abolished. This observation has additional support from the finding that there is high phenylacetyl-CoA ligase activity in the *edd* mutant, even in the presence of glucose + PAA, but not in wild-type cells under the same conditions. Unlike the *edd* mutant, the *eda* mutant did not grow in the presence of glucose + PAA. Interestingly, there was no uptake and/or metabolism of PAA in the *eda* mutant cells under the same conditions. Targeted disruption of *PaaX*, a repressor of the PAA operon, had no effect on CCR of PAA metabolism in the presence of glucose, suggesting that there is another transcriptional repression system associated with the KDPG signal. This is the first study to demonstrate that KDPG is the true CCR signal of PAA metabolism in *P. putida* KT2440.

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INTRODUCTION

In *Pseudomonas putida*, the phenylacetic acid (PAA) catabolic gene clusters consist of 15 genes that are organized into five contiguous operons (del Peso-Santos *et al.*, 2008; Di Gennaro *et al.*, 2007; Jiménez *et al.*, 2002; Luengo *et al.*, 2001; Olivera *et al.*, 1998). The PAA pathway (Fig. 1) is a major route of a complex functional unit that catalyses the transformation of structurally related compounds such as styrene, tropic acid, ethylbenzene and 2-phenylethylalanine into phenylacetyl-CoA (PA-CoA), a common intermediate (Luengo *et al.*, 2001). It has been reported that PA-CoA, and not PAA, is the true inducer of the PAA catabolic pathway and that the *PaaX* repressor controls PAA-catabolic gene expression (Ferrández *et al.*, 2000; García *et al.*, 2000). Binding of *PaaX* to its cognate

DNA binding sites is abolished when the inducer, PA-CoA, interacts with the *PaaX* protein (Bartolomé-Martín *et al.*, 2004; del Peso-Santos *et al.*, 2006; Ferrández *et al.*, 2000; García *et al.*, 2000). Interestingly, along with the StyR/StyS system, *PaaX* also regulates the styrene degradation pathway by binding to the promoter region of the styrene upper pathway in *Pseudomonas* sp. strain Y2 (del Peso-Santos *et al.*, 2006). *PaaX* is also involved in the transcriptional regulation of the *pac* gene, which encodes penicillin G acylase in *Escherichia coli* W (Galán *et al.*, 2004; Kim *et al.*, 2004). *PaaX* is involved in the common central pathway of several aromatic biodegradation reactions (Luengo *et al.*, 2001).

In glucose-amended minimal medium or Luria–Bertani (LB) medium, *paaA* expression in *P. putida* is repressed even in the presence of PAA (Kim *et al.*, 2007; Luengo *et al.*, 2001). This carbon catabolite repression (CCR) mechanism of PAA metabolism in *P. putida* is not yet fully understood. An RNA-binding global regulator in *P. putida*, Crc (Moreno & Rojo, 2008), appears to be involved in CCR

Abbreviations: CCR, carbon catabolite repression; ED pathway; Entner, Doudoroff pathway; KDPG, 2-keto-3-deoxy-6-phosphogluconate; PAA, phenylacetic acid; PA-CoA, phenylacetyl-CoA; 6PG, 6-phosphogluconate.

Fig. 1. The ED and PAA metabolic pathways in *P. putida*. The *paa* genes and the intermediates involved in the proposed PA degradation pathway (Olivera *et al.*, 1998) are shown [the consensus nomenclature proposed by (Luengo *et al.*, 2001) was used]. Metabolites from glucose metabolism may inhibit PAA degradation. *zwf-1*, Glucose-6-phosphate dehydrogenase; *edd*, 6PG dehydratase; *eda*, KDPG aldolase; *paaF*, pentylacetyl-CoA ligase; *paaGHIJK*, ring-hydroxylating complex; *paaN*, ring-opening enzyme; *paaAB*, enoyl-CoA hydratase 1, 2; *paaC*, 3-OH-acyl-CoA dehydrogenase; *paaD*, putative thioesterase; *paaE*, ketothiolase.

of the degradation pathways for several aromatic compounds, such as benzoate and 4-hydroxybenzoate, but not PAA (Morales *et al.*, 2004). In *E. coli*, the cAMP receptor protein Crp is involved in CCR of PAA degradation (Ferrández *et al.*, 2000; Kimata *et al.*, 1997; Notley-McRobb *et al.*, 1997). However, the level of cAMP in *P. putida* remains constant regardless of the carbon source and cAMP is unlikely to be involved in CCR in this species (Basu *et al.*, 2007; Siegel *et al.*, 1977). CCR of PAA metabolism appears to be unaffected by organic acids, such as pyruvate or succinate (Kim *et al.*, 2007), suggesting that CCR is generated by glucose intermediates (Fig. 1).

Glucose catabolically represses the toluene degradation system (del Castillo & Ramos, 2007; Holtel *et al.*, 1994; Velázquez *et al.*, 2004). CCR of toluene metabolism by glucose is mediated by 2-keto-3-deoxy-6-phosphogluconate (KDPG), an intermediate in the Entner–Doudoroff (ED) pathway of glucose metabolism in *P. putida* (del Castillo & Ramos, 2007; Velázquez *et al.*, 2004). Interestingly, the regulation of glucose metabolism is controlled by the HexR repressor (Hager *et al.*, 2000; Petruschka *et al.*, 2002; Temple *et al.*, 1994). We have shown that HexR binding to its cognate operator sites is inhibited by KDPG (Kim *et al.*, 2008). It seems likely that KDPG is an important signal generated during glucose metabolism that may control the preferential utilization of glucose over other aromatic carbon sources in *P. putida*. Our hypothesis is that KDPG may be the intracellular CCR signal of PAA metabolism in *P. putida* KT2440, and our

data indicate that KDPG is indeed required for CCR of PAA metabolism in this strain.

METHODS

Bacterial strains, culture conditions and DNA manipulation.

Bacteria (Table 1) were grown at 37 °C (*E. coli*) or 30 °C (*P. putida*) in LB medium (Sambrook *et al.*, 1989) or mineral salts medium (M9) (Sambrook *et al.*, 1989) containing different carbon sources (succinate, glucose, gluconate, fructose and pyruvate), each at a concentration of 10 mM. Plasmid isolation, gel electrophoresis, transformation and PCR were performed using standard procedures (Ausubel *et al.*, 1999). *P. putida* KT2440R was selected from spontaneous mutations of *P. putida* KT2440 (laboratory stock) on LB medium containing rifampicin (200 µg ml⁻¹) after prolonged incubation and sequential transfers. Tetracycline (15 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) were added to bacterial cultures when necessary. The *edd* and *eda* mutants were purchased from Bio-Iliberis R&D (Spain).

Cloning procedures and mutant construction.

The broad-host-range promoter-probe vector pRK415gfp (Yin *et al.*, 2003) was used to construct the reporter plasmid pRK_{*paaA*::gfp}. Briefly, a 534 bp fragment from the *paaA* promoter was amplified using the primers kt *paaA* pro1 (5'-CGCGAATTCCAGCGGGCCAAATGTAGCA-3') and kt *paaA* pro2 (5'-CGCGGATCCCGGGCCGTTGCAGGGTA ATGAGC-3'). The amplicon was inserted into the *EcoRI*/*Bam*HI cloning site of the pRK415gfp vector to generate pRK_{*paaA*::gfp}. The constructed plasmid was introduced into *E. coli* Top10 by electroporation. Then, the pRK_{*paaA*::gfp} plasmid was introduced by triparental conjugation into *P. putida* KT2440R to create *P. putida* KT2440R(pRK_{*paaA*::gfp}). Conjugation was performed by filter mating using *E. coli* Top10(pRK_{*paaA*::gfp}), *E. coli* HB101(pRK2013) and *P. putida*

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics*	Reference(s)
<i>P. putida</i> KT2440		
KT2440	Wild-type	Lab stock
KT2440R	Wild-type; Rf ^R	Lab stock
KT2440R (pRK _{paaA::gfp})	Strain KT2440 harbouring pRK _{paaA::gfp} ; Tc ^R Rf ^R	This study
ΔPaaX	Derivation of strain KT2440, insertion of pVIK-PaaX; Rf ^R Km ^R	This study
ΔPaaX (pRK _{paaA::gfp})	Strain ΔPaaX harbouring pRK _{paaA::gfp} ; Rf ^R Km ^R Tc ^R	This study
ΔEdd	<i>edd</i> : mini-Tn5-Km; Rf ^R Km ^R	Duque <i>et al.</i> (2007)
ΔEdd (pRK _{paaA::gfp})	Strain ΔEdd harbouring pRK _{paaA::gfp} ; Rf ^R Km ^R Tc ^R	This study
ΔEda	<i>eda</i> : mini-Tn5-Km; Rf ^R Km ^R	Duque <i>et al.</i> (2007)
<i>E. coli</i>		
Top10	<i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>), <i>φ80lacZ</i> ΔM15	Promega
HB101	Strain harbouring pRK2013	Fredrickson <i>et al.</i> (1988)
S17-1λpir	Tp ^R Sm ^R <i>recA thi pro hsdM</i> ⁺ RP4-2-Tc::Mu::Km Tn7; <i>hsdR</i> mutant	Simon <i>et al.</i> (1983)
Plasmids		
pRK415gfp	Broad-host-range vector; Tc ^R Mob+	Yin <i>et al.</i> (2003)
pRK _{paaA::gfp}	pRK415gfp carrying the KT2440 <i>paaA</i> full promoter region	This study
pVIK112	R6KoriV, suicide vector, LacZ translation fusion vector; Km ^R	Kalogeraki & Winans (1997)
pVIK-PaaX	Partial region of <i>paaX</i> gene cloned into pVIK112	This study
pRK2013	<i>tra</i> ⁺ Km ^R derivative of RK2 containing ColE1 replicon	Fredrickson <i>et al.</i> (1988)

*Km^R, Rf^R, Sm^R, Tc^R, Tp^R, Resistance to kanamycin, rifampicin, streptomycin, tetracycline or trimethoprim, respectively.

KT2440R as donor, helper and recipient, respectively (Park *et al.*, 2003). Using the *kt paaX* KO1 (5'-CGCGAATTCAGAAAAGGTGGG-CGCAGCGAGTTA-3') and *kt paaX* KO2 (5'-CGCGAATTCGTCAGCAGCTCGTCGGGTAGTT-3') primers, we deleted the amplified 538 bp fragment of the *paaX* internal region by cloning it into the *EcoRI* cloning site of the pVIK112 vector (Kalogeraki & Winans, 1997) to generate pVIK-*paaX*. The constructed plasmid was introduced into *E. coli* S17-1 λpir by electroporation. Conjugation was performed by filter mating with *E. coli* S17-1 λpir(pVIK-*paaX*) and *P. putida* KT2440R (Lee *et al.*, 2006) as the donor and recipient, respectively. The transconjugant was selected on LB medium containing kanamycin and rifampicin at 30 °C. PCR with the *paaX* KO1/MCS-R primer pairs was performed to verify the transconjugant. The MCS-R primer was designed based on the pVIK112 plasmid sequence (Park *et al.*, 2003). *P. putida* KT2440R(Δ*paaX*/pRK_{paaA::gfp}) was constructed as described above (Table 1).

Quantification of GFP fluorescence. Overnight cultures of the reporter strains in LB medium were collected using a microcentrifuge (~15 800 g) and washed twice with M9 medium without a carbon source. The strains (1:250) were regrown to the exponential phase (OD₆₀₀, ~0.2±0.1) in each of the carbon sources. PAA (5 mM) was added during the early exponential phase. After a 3 h incubation, the samples were collected using a microcentrifuge (~15 800 g) and washed twice with PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). The OD₆₀₀ and GFP fluorescence intensity of the resuspended cultures were quantified using a microtitre plate reader (Victor3; Bio-Rad). The reporter strain expresses a stable GFP variant that absorbs light at 488 nm. One fluorescence unit was defined as [(fluorescence intensity of cells–fluorescence intensity of PBS buffer)]/OD₆₀₀ of cells.

Northern blot analysis. Total RNA was isolated from 5 or 10 ml cells from each growth phase using an RNeasy kit (Qiagen), according to the manufacturer's instructions. Total RNA (5 µg) concentration was determined by measuring the absorbance at 260 nm. The fractionated RNA was transferred to a nylon membrane (Schleicher

& Schuell) using a Turboblotter (Schleicher & Schuell), and the amount of *paaA* mRNA was determined by hybridizing the membrane with an [α -³²P]-labelled probe specific for each gene (Takara Bio). The *paaA*-Pp1 (5'-CCGCCAGCAGGGTGAAG-3') and *paaA*-Pp2 (5'-GGCCAGCCGGAATCAA-3') primers were used as the specific probe. The band intensity of Northern blot data was measured using a densitometry instrument (800×1600 d.p.i.; Umax UTA 2100XL).

Determination of glucose, gluconate and PAA concentrations.

To analyse residual substrates, the supernatants in each growth phase were collected using a microcentrifuge (15 800 g for 3 min). Residual glucose concentration was measured by the glucose oxidase enzymic method (Keston, 1956). Residual gluconate concentration was measured using a gluconic acid assay kit (Megazyme), according to the manufacturer's instructions. Residual PAA concentration was analysed by HPLC (Ferrández *et al.*, 1998; Martínez-Blanco *et al.*, 1990). We used a Cosmosil 5C₁₈-AR-II column (4.6×250 mm) with a Younglin model SP930D pump at room temperature. The mobile phase was 50% methanol/H₂O at a flow rate of 1.0 ml min⁻¹. Elution peaks corresponding to the retention time of an authentic PAA standard (2.6 min) were monitored at 220 nm.

PA-CoA ligase assay. PA-CoA ligase was assayed by measuring the rate of generation of phenylacetylhydroxamate in the presence of ATP, CoA, PAA and neutral hydroxylamine (Martínez-Blanco *et al.*, 1990). The assay mixture contained 12.5 µl 0.2 M MgCl₂, 50 µl 0.1 M ATP, 15 µl 20 mM CoA, 30 µl 0.2 M PAA and 50 µl hydroxylamine solution (1 ml 5 M hydroxylamine hydrochloride, 250 µl distilled water and 1.25 ml 4 M KOH). In standard tests PAA, ATP, MgCl₂ or CoA were omitted. Reactions were started by adding 100 µl enzyme solution and the incubations were carried out for 1 h at 30 °C. Reactions were stopped by adding 450 µl ferric chloride reagent (0.37 M ferric chloride, 20 mM trichloroacetic acid and 0.66 M hydrochloric acid) and immediately incubated on ice for 30 min. The reactions were centrifuged for 2 min and monitored using an Optizen 2120 UV/Vis spectrophotometer (Mecasys) at 540 nm. The extinction

coefficient of phenylacetylhydroxamate under these conditions was $0.9 \text{ mM}^{-1} \text{ cm}^{-1}$. Protein concentration was measured by the method of Bradford (Ausubel *et al.*, 1999)

RESULTS AND DISCUSSION

PAA metabolism is repressed by the presence of glucose and gluconate

Consistent with other observations (Morales *et al.*, 2004; Schleissner *et al.*, 1994), we have previously shown that the PAA catabolic genes (*paaA*, *paaG* and *paaL*) are repressed in glucose + PAA-amended minimal medium (Kim *et al.*, 2007). We measured the *paaA* expression level using a GFP-based reporter strain, *P. putida* KT2440R(pRKP_{*paaA*::gfp}). The results indicated that the *paaA* promoter activity in PAA-amended minimal medium was repressed by the presence of glucose or gluconate, whereas fructose, pyruvate or succinate did not affect *paaA*-promoter expression, suggesting that there is CCR of PAA metabolism by glucose or gluconate (Fig. 2). These results suggested that an intermediate of these carbon sources may be a key signal for CCR. A similar CCR by glucose has been reported in the metabolism of toluene, which indicated that KDPG is the signal for toluene CCR (del Castillo & Ramos, 2007; Velázquez *et al.*, 2004). Previously, we have shown that HexR, a repressor of glucose metabolism, binds to the *zwf-1* promoter region, and that HexR binding is inhibited by KDPG. Taken together, we hypothesized that KDPG may be a crucial signal for CCR of PAA metabolism by glucose.

Fig. 2. Quantification of GFP expression in wild-type reporter strains grown in the presence of various carbon sources with or without PAA. Measurement of GFP was performed as described in Methods. The following carbon sources and concentrations were used: glucose, 10 mM; gluconate, 10 mM; fructose, 10 mM; pyruvate, 10 mM; succinate, 10 mM; PAA, 5 mM.

KDPG is the signal for the PAA catabolite repression

edd (encoding a 6-phosphogluconate dehydratase) or *eda* (encoding a KDPG aldolase) gene-deleted strains were used to test our hypothesis. The *edd* mutant does not make KDPG, whereas the *eda* mutant accumulates KDPG (Fig. 1). Because the degradation of 6-phosphogluconate (6PG) or KDPG into central metabolites is blocked in the ED pathway, these mutants did not grow on glucose or gluconate (del Castillo *et al.*, 2007; Velázquez *et al.*, 2004; Table 2). However, the strains grew on other substrates or in LB medium (Table 2). Interestingly, the growth patterns of the mutant strains indicated that the *eda* mutant (KDPG overproducer) did not grow on minimal medium supplemented with glucose + PAA, whereas the wild-type and *edd* mutant strains did grow under such conditions; however, the *eda* mutant grew in minimal medium containing only PAA (Fig. 3a, Table 2). These growth patterns of wild-type and mutants were also observed under gluconate-amended conditions (Table 2). *eda* mutant cells also grew in the presence of glucose plus other aromatic carbon sources, including benzoate or 4-hydroxybenzoate, indicating that their utilization is not under CCR by glucose (Morales *et al.*, 2004; Nichols & Harwood, 1995; Putrins *et al.*, 2007).

Previously, we have shown that the expression of *paaL*, which encodes a PAA permease, is repressed by glucose in PAA-amended minimal medium (Kim *et al.*, 2007). Therefore, we can rule out the possibility that the KDPG accumulated during glucose metabolism directly inhibits the PAA transport system in *P. putida* KT2440. KDPG may be involved in a repression system in which the PAA metabolic genes, including the genes involved in the transport of PAA, are not expressed. When the *eda* mutant cells were grown to exponential phase in LB medium and transferred to a minimal medium containing glucose + PAA, the glucose was completely metabolized after 16 h incubation, whereas the concentration of PAA remained the same (Fig. 3b). The uptake and/or the metabolism of

Table 2. Growth of *P. putida* KT2440 and the *edd* and *eda* mutant strains in minimal medium with various substrates

Growth was measured in medium containing 5 mM substrate after 72 h. Initial OD₆₀₀ ~0.03–0.05. A final OD₆₀₀ of <0.1 was scored as no growth (–); a final OD₆₀₀ of >0.28 was scored as wild-type growth (+). The experiment was repeated five times. Depending on the substrate, the measured OD₆₀₀ values varied from ~0.28 to 1.5. Glu, Glucose; Glc, gluconate. Wild-type growth was recorded for all three strains with PAA, glucose + benzoate, benzoate, glucose + 4-hydroxybenzoate, and 4-hydroxybenzoate.

Substrate type	Glu	Glu + PAA	Glc	Glc + PAA
WT	+	+	+	+
ΔEDD	–	+	–	+
ΔEDA	–	–	–	–

Fig. 3. (a) Growth of the *eda* mutant strain in minimal medium with PAA (●) or glucose+PAA (○). (b) Residual PAA (●) or glucose (○) when the *eda* mutant strain was grown in minimal medium with glucose (5 mM)+PAA (5 mM). The cells were cultured in LB medium to exponential phase (OD₆₀₀ ~0.6) and then harvested, washed and aliquoted into minimal medium with glucose or glucose+PAA. (c) Viability test of the *eda* mutant strain using serial dilution. Cells cultured in medium with glucose+PAA were serially diluted and spotted onto LB agar.

PAA appeared to be inhibited in the *eda* mutant in the presence of glucose (Fig. 3b). There was no decrease in viability of the *eda* mutant cells when cells cultured in glucose plus PAA medium were serially diluted and spotted onto LB agar (Fig. 3c).

PAA catabolite repression is abolished in the *edd* mutant

We observed a triauxic growth pattern in the wild-type cells when they were grown in the presence of glucose+PAA (Fig. 4a). The reason for the second lag period is not clear but may reflect the accumulation of PAA metabolites. The residual PAA concentration was not reduced while glucose remained in the culture medium (Fig. 4a). The consumption of PAA was initiated once the glucose was consumed, which demonstrated glucose as a repressor of PAA metabolism. Northern blot analysis revealed that *paaA* was strongly induced after the complete exhaustion of glucose (Fig. 4a). The growth rate of the *edd* mutant strain was diminished in minimal medium with glucose+PAA probably due to a blockage of glucose metabolism through the ED pathway (Fig. 4b). This observation is consistent with other reports in which the

accumulation of phosphorylated intermediates of glucose metabolism was a suspected cause of slow growth under these conditions (Sawyer *et al.*, 1977; Velázquez *et al.*, 2004; Vicente & Canovas, 1973). Despite the poor growth of the *edd* mutant in the presence of glucose+PAA, the *edd* mutant simultaneously consumed glucose and PAA (Fig. 4b). Coincidentally, *paaA* expression was highly induced in the early exponential phase under the same conditions (Fig. 4b). Our finding has also shown that this CCR of PAA occurs in the presence of gluconate and is abolished in the *edd* mutant (Fig. 4c, d). Interestingly, in the presence of gluconate+PAA, the final OD₆₀₀ of the *edd* mutant strain reached ~1 (Fig. 4d); with glucose+PAA it reached up to ~2 (Fig. 4b). We speculated that this discrepancy is due to the activity of glucose-6-phosphate dehydrogenase and availability of its precursor substrate (glucose). The *edd* mutant could obtain byproducts such as NADPH produced by glucose-6-phosphate dehydrogenase activity under conditions where glucose+PAA are present, but not in the presence of gluconate+PAA. This phenomenon might explain why all glucoses are not converted to gluconate, and glucose uptake via the direct glucose uptake route may be preferred in *P. putida* KT2440. Furthermore, the GFP reporter assay of *edd* mutant *paaA* promoter

Fig. 4. Wild-type (a, c) and *edd* mutant (b, d) growth ($\bullet$) and analysis of residual glucose (5 mM; $\diamond$), gluconate (5 mM; $\triangle$) and PAA (5 mM; $\blacklozenge$) when cells were cultured in minimal medium with glucose + PAA or gluconate + PAA. The box represents the Northern blot analysis showing that *paaA* expression is induced when glucose and PAA are consumed. The level of *paaA* expression is shown as fold induction level with the control (background of Northern blot analysis). (e) Quantification of GFP expression in wild-type (black bars) and *edd* mutant (white bars) reporter strains grown in minimal medium containing PAA or glucose + PAA or gluconate + PAA.

activity in the presence of glucose + PAA or gluconate + PAA indicated that KDPG is involved in CCR of PAA under glucose- or gluconate-amended conditions (Fig. 4e). Our studies with these mutants demonstrated that the CCR of PAA metabolism by glucose or gluconate is mediated by KDPG, not by 6PG, in *P. putida*.

To confirm the results presented above, we also measured the activity of the first enzyme of PAA biodegradation, PA-CoA ligase, with glucose + PAA (Martínez-Blanco *et al.*, 1990). In the presence of PAA as sole carbon source, PA-CoA ligase activity remained very high during exponential growth phase (OD₆₀₀ = 0.3–0.7) in both wild-type and the *edd* mutant (Fig. 5). However, no PA-CoA ligase activity was observed in the presence of PAA + glucose in wild-type cells (Fig. 5a). In contrast, the specific activity of PA-CoA ligase remained very high during exponential phase of the *edd* mutant

(OD₆₀₀ = 0.5–0.7) even in the presence of glucose + PAA (Fig. 5b). From this, it is apparent that PAA and glucose are simultaneously consumed in the *edd* mutant.

The PaaX protein is not responsible for CCR of PAA

We inserted the *paaA::gfp* reporter into a strain in which PaaX is inactivated by Campbell-type homologous recombination to verify whether a repressor of a PAA-catabolic genes, PaaX, is involved in this CCR event. Targeted disruption of PaaX showed CCR of PAA metabolism in the presence of glucose and gluconate, but not fructose, pyruvate or succinate (Fig. 6). The absence of the PaaX repressor may have allowed constitutively high expression of *paaA* in the absence of PAA, leading to the

Fig. 5. PA-CoA ligase activities of the wild-type (a) and the *edd* mutant (b) in minimal medium with PAA (grey bars) or glucose + PAA (black bars). One unit of enzyme activity is defined as the catalytic activity leading to the formation of 1 nmol phenylacetylhydroxamate min⁻¹.

transcriptional initiation of *paaA* by RNA polymerase (Fig. 6). The data suggested that PaaX is not involved in CCR of PAA metabolism by glucose and that another repression mechanism might be preventing RNA polymerase from initiating transcription when repressive carbon sources are present. Because KDPG and blockage of RNA polymerase are required for CCR of PAA metabolism, it is plausible to suggest that there is a link between the KDPG signal and another repression system.

PAA catabolite repression occurred in the presence of either glucose or gluconate, but not with fructose, pyruvate or succinate (Fig. 2). These repressive carbon sources are metabolized via the ED pathway in *P. putida*. It was recently reported that *P. putida* KT2440 can simultaneously use all three peripheral pathways (glucokinase pathway, 2-ketogluconate loop, gluconokinase pathway) in glucose uptake (del Castillo *et al.*, 2007). Despite the preference for the glucose uptake pathway, both 6PG and KDPG are key metabolites in the ED pathway of *Pseudomonas* strains (Kim *et al.*, 2008).

Fig. 6. Quantification of GFP expression in the *paaX* mutant reporter strain *P. putida* KT2440R(Δ PaaX/pRKP_{*paaA::gfp*}) grown in the presence of various carbon sources. The following carbon sources and concentrations were used: glucose 10 mM; gluconate, 10 mM; fructose, 10 mM; pyruvate, 10 mM; succinate, 10 mM; PAA, 5 mM.

Although PAA catabolic genes are intact in the *eda* mutant, the most intriguing finding of this study was that the *eda* deletion mutant did not grow on minimal medium in the presence of glucose + PAA (Table 2, Fig. 3a). We have provided the first evidence that KDPG is the signal for CCR of PAA metabolism by glucose, and that CCR occurs at the transcriptional level. Interestingly, we found that *zwf-1*, which encodes glucose-6-phosphate dehydrogenase, was highly induced by glucose and gluconate, whereas *zwf-1* promoter activity was not induced by fructose, pyruvate or succinate, even though the metabolic pathway of fructose produces 6PG and KDPG (Kim *et al.*, 2008). Our finding might be due to the fact that *P. putida* possesses another fructose metabolic pathway which does not produce KDPG (Velázquez *et al.*, 2004). This would explain why fructose does not induce CCR of PAA catabolic genes; perhaps fructose generates KDPG levels that are not high enough to trigger catabolite repression.

It has been shown that KDPG inhibits the binding of HexR to its cognate promoter regions and thus enables expression genes involved in the ED pathway (Kim *et al.*, 2008). KDPG is involved in the regulation of both *paaA*, the first gene of PAA metabolism, and *zwf-1*, the first gene of glucose metabolism. These results strongly suggest that the generation of KDPG in the ED pathway may signal CCR of PAA metabolism. Additional molecular studies of the role of KDPG in CCR of PAA metabolism of *P. putida* KT2440 are warranted.

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REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struthi, K. (1999). *Current Protocols in Molecular Biology*. New York: Wiley.
- Bartolomé-Martín, D., Martínez-García, E., Mascaraque, V., Rubio, J., Perera, J. & Alonso, S. (2004). Characterization of a second functional gene cluster for the catabolism of phenylacetic acid in *Pseudomonas* sp. strain Y2. *Gene* **341**, 167–179.
- Basu, A., Shrivastava, R., Basu, B., Apte, S. K. & Phale, P. S. (2007). Modulation of glucose transport causes preference utilization of aromatic compounds in *Pseudomonas putida* CSV86. *J Bacteriol* **189**, 7556–7562.
- del Castillo, T. & Ramos, J. L. (2007). Simultaneous catabolic repression between glucose and toluene metabolism in *Pseudomonas putida* is channeled through different signaling pathways. *J Bacteriol* **189**, 6602–6610.
- del Castillo, T., Ramos, J. L., Rodríguez-Herva, J. J., Fuhrer, T., Sauer, U. & Duque, E. (2007). Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: genomic and flux analysis. *J Bacteriol* **189**, 5142–5152.
- del Peso-Santos, T., Bartolomé-Martín, D., Fernández, C., Alonso, S., García, J. L., Díaz, E., Shingler, V. & Perera, J. (2006). Coregulation by phenylacetyl-Coenzyme A-responsive PaaX integrates control of the upper and lower pathways for catabolism of styrene by *Pseudomonas* sp. strain Y2. *J Bacteriol* **188**, 4812–4821.
- del Peso-Santos, T., Shingler, V. & Perera, J. (2008). The styrene-responsive StyS/StyR regulation system controls expression of an auxiliary phenylacetyl-coenzyme A ligase: implications for rapid metabolic coupling of the styrene upper- and lower-degradative pathways. *Mol Microbiol* **69**, 317–330.
- Di Gennaro, P., Ferrara, S., Ronco, I., Galli, E., Sello, G., Papacchini, M. & Bestetti, G. (2007). Styrene lower catabolic pathway in *Pseudomonas fluorescens* ST: identification and characterization of genes for phenylacetic acid degradation. *Arch Microbiol* **188**, 117–125.
- Duque, E., Molina-Henares, A. J., de la Torre, J., Molina-Henares, M. A., del Castillo, T., Lam, J. & Ramos, J. L. (2007). Towards a genome-wide mutant library of *Pseudomonas putida* strains KT2440. In *Pseudomonas*, vol. V, chapter 8, pp. 227–251. Edited by J. L. Ramos & A. Filoux. The Netherlands: Springer.
- Ferrández, A., Miñambres, B., García, B., Olivera, E. R., Luengo, J. M., García, J. L. & Díaz, E. (1998). Catabolism of phenylacetic acid in *Escherichia coli*. *J Biol Chem* **273**, 25974–25986.
- Ferrández, A., García, J. L. & Díaz, E. (2000). Transcriptional regulation of the divergent *paa* catabolic operons for phenylacetic acid degradation in *Escherichia coli*. *J Biol Chem* **275**, 12214–12222.
- Fredrickson, J. K., Bezdicek, D. F., Brockman, F. J. & Li, S. W. (1988). Enumeration of Tn5 mutant bacteria in soil by using a most-probable number-DNA hybridization procedure and antibiotic resistance. *Appl Environ Microbiol* **54**, 446–453.
- Galán, B., García, J. L. & Prieto, M. A. (2004). The PaaX repressor, a link between penicillin G acylase and the phenylacetyl-coenzyme A catabolon of *Escherichia coli* W. *J Bacteriol* **186**, 2215–2220.
- García, B., Olivera, E. R., Miñambres, B., Carnicero, D., Muñoz, C., Naharro, G. & Luengo, J. M. (2000). Phenylacetyl-Coenzyme A is the true inducer of the phenylacetic acid catabolism pathway in *Pseudomonas putida* U. *Appl Environ Microbiol* **66**, 4575–4578.
- Hager, P. W., Calfee, M. W. & Phibbs, P. V. (2000). The *Pseudomonas aeruginosa* devB/SOL homolog, *pgl*, is a member of the *hex* regulon and encodes 6-phosphogluconolactonase. *J Bacteriol* **182**, 3934–3941.
- Holtel, A., Marques, S., Mohler, I., Jakubzik, U. & Timmis, K. N. (1994). Carbon source-dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. *J Bacteriol* **176**, 1773–1776.
- Jiménez, J. I., Miñambres, B., García, J. L. & Díaz, E. (2002). Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ Microbiol* **4**, 824–841.
- Kalogeraki, V. S. & Winans, S. C. (1997). Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to genes of diverse bacteria. *Gene* **188**, 69–75.
- Keston, A. S. (1956). Specific colorimetric enzymatic analytical reagents for glucose. In *Abstracts of the 129th Meeting of the American Chemical Society*, Dallas, TX, pp. 310–314.
- Kim, H. S., Kang, T. S., Hyun, J. S. & Kang, H. S. (2004). Regulation of penicillin G acylase gene expression in *Escherichia coli* by repressor PaaX and the cAMP–cAMP receptor protein complex. *J Biol Chem* **279**, 33253–33262.
- Kim, J., Jeon, C. O. & Park, W. (2007). A green fluorescent protein-based whole-cell bioreporter for the detection of phenylacetic acid. *J Microbiol Biotechnol* **17**, 1727–1732.
- Kim, J., Jeon, C. O. & Park, W. (2008). Dual regulation of *zwf-1* by both 2-keto-3-deoxy-6-phosphogluconate and oxidative stress in *Pseudomonas putida*. *Microbiology* **154**, 3905–3916.
- Kimata, K., Takahashi, H., Inada, T., Postma, P. & Aiba, H. (1997). cAMP receptor protein–cAMP plays a crucial role in glucose–lactose diauxie by activating the major glucose transporter gene in *Escherichia coli*. *Proc Natl Acad Sci U S A* **94**, 12914–12919.
- Lee, Y., Ahn, E., Park, S., Madsen, E. L., Jeon, C. O. & Park, W. (2006). Construction of a reporter strain *Pseudomonas putida* for the detection of oxidative stress caused by environmental pollutants. *J Microbiol Biotechnol* **16**, 386–390.
- Luengo, J. M., García, J. L. & Olivera, E. R. (2001). The phenylacetyl-CoA catabolon: a complex catabolic unit with broad biotechnological application. *Mol Microbiol* **39**, 1434–1442.
- Martínez-Blanco, H., Reglero, A., Rodríguez-Aparicio, L. B. & Luengo, J. M. (1990). Purification and biochemical characterization of phenylacetyl-CoA ligase from *Pseudomonas putida*. A specific enzyme for the catabolism of phenylacetic acid. *J Biol Chem* **265**, 7084–7090.
- Morales, G., Linares, J. F., Beloso, A., Albar, J. P., Martínez, J. L. & Rojo, F. (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.
- Moreno, R. & Rojo, F. (2008). The target for the *Pseudomonas putida* *Crc* global regulator in the benzoate degradation pathway is the BenR transcriptional regulator. *J Bacteriol* **190**, 1539–1545.
- Nichols, N. N. & Harwood, C. (1995). Repression of 4-hydroxybenzoate transport and degradation by benzoate: a new layer of regulatory control in the *Pseudomonas putida* β -ketoadipate pathway. *J Bacteriol* **177**, 7033–7040.
- Notley-McRobb, L., Death, A. & Ferenci, T. (1997). The relationship between external glucose concentration and cAMP levels inside *Escherichia coli*: implications for models of phosphotransferase-mediated regulation of adenylate cyclase. *Microbiology* **143**, 1909–1918.
- Olivera, E. R., Miñambres, B., García, B., Muñoz, C., Moreno, M. A., Ferrández, A., Díaz, E., García, J. L. & Luengo, J. M. (1998). Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: the phenylacetyl-CoA catabolon. *Proc Natl Acad Sci U S A* **95**, 6419–6424.
- Park, W., Jeon, C. O., Hohnstock-Ashe, A. M., Wnas, S. C., Zylstra, G. J. & Madsen, E. L. (2003). Identification and characterization of the conjugal transfer region of the pCgl plasmid from naphthalene-degrading *Pseudomonas putida* Cg1. *Appl Environ Microbiol* **69**, 3263–3271.

- Petruschka, L., Adolf, K., Burchhardt, G., Dervedde, J., Jürgensen, J. & Herrmann, H. (2002).** Analysis of the *zwf-pgl-eda*-operon in *Pseudomonas putida* strains H and KT2440. *FEMS Microbiol Lett* **215**, 89–95.
- Putriš, M., Tover, A., Tegova, R., Saks, Ü. & Kivisaar, M. (2007).** Study of factors which negatively affect expression of the phenol degradation operon *pheBA* in *Pseudomonas putida*. *Microbiology* **153**, 1860–1871.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sawyer, M. H., Baumann, L., Berman, S. M., Canovas, J. L. & Berman, R. H. (1977).** Pathways of D-fructose catabolism in species of *Pseudomonas*. *Arch Microbiol* **112**, 49–55.
- Schleissner, C., Olivera, E. R., Fernandez-Valverde, M. & Luengo, J. M. (1994).** Aerobic catabolism of phenylacetic acid in *Pseudomonas putida* U: biochemical characterization of a specific phenylacetic acid transport system and formal demonstration that phenylacetyl-coenzyme A is a catabolic intermediate. *J Bacteriol* **176**, 7667–7676.
- Siegel, L. S., Hylemon, P. B. & Phibbs, P. V. (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.
- Simon, R., Priefer, U. & Puehler, A. (1983).** A broad host range mobilization system in Gram-negative bacteria. *Biotechnology (N Y)* **1**, 784–791.
- Temple, L., Sage, A., Christie, G. E. & Phibbs, P. V. (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.
- Velázquez, F., di Bartolo, I. & de Lorenzo, V. (2004).** Genetic evidence that catabolites of the Entner–Doudoroff pathway signal C sources repression of the σ^{54} *Pu* promoter of *Pseudomonas putida*. *J Bacteriol* **186**, 8267–8275.
- Vicente, M. & Canovas, J. L. (1973).** Regulation of the glucolytic enzymes in *Pseudomonas putida*. *Arch Mikrobiol* **93**, 53–64.
- Yin, S., Fuanthong, M., Laratta, W. P. & Shapleigh, J. P. (2003).** Use of a green fluorescent protein-based reporter fusion for detection of nitric oxide produced by denitrifiers. *Appl Environ Microbiol* **69**, 3938–3944.

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