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Characterization of the last step of the aerobic phenylacetic acid degradation pathway

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Phenylacetic acid (PA) degradation in bacteria involves an aerobic hybrid pathway encoded by the *paa* gene cluster. It is shown here that succinyl-CoA is one of the final products of this pathway in *Pseudomonas putida* and *Escherichia coli*. Moreover, *in vivo* and *in vitro* studies revealed that the *paaE* gene encodes the β-ketoadipyl-CoA thiolase that catalyses the last step of the PA catabolic pathway, i.e. the thiolytic cleavage of β-ketoadipyl-CoA to succinyl-CoA and acetyl-CoA. Succinyl-CoA is suggested as a common final product of aerobic hybrid pathways devoted to the catabolism of aromatic compounds.

INTRODUCTION

Aromatic compounds are widely distributed in the environment and are therefore a common carbon source for many micro-organisms (Harwood & Parales, 1996). The aerobic catabolism of aromatic compounds usually involves the oxygenolytic hydroxylation of the aromatic ring, producing central dihydroxylated aromatic intermediates (e.g. catechol, protocatechuate, gentisate, homoprotocatechuate, homogentisate and hydroxyhydroquinone). These intermediates are then cleaved by different types of ring-cleavage dioxygenases, generating aliphatic compounds that funnel into the tricarboxylic acid (TCA) cycle through a small number of central pathways, such as the well-known β-ketoadipate pathway (Fig. 1) (Harwood & Parales, 1996; Jiménez *et al.*, 2004). However, over the last few years there has been increasing evidence in several bacteria of a novel principle of aerobic aromatic catabolism that does not rely on classical hydroxylation steps, but rather on the use of substrate CoA thioesters, and which therefore resembles the conventional strategies of anaerobic catabolic pathways. As such, these novel aerobic pathways have been reported as aerobic hybrid pathways (Díaz, 2004; Gescher *et al.*, 2006; Ward & O'Connor, 2005). So far, the best-characterized aerobic hybrid pathway is that of benzoate degradation in *Azoarcus evansii*, in which all intermediates are CoA thioesters and the actual ring-cleavage reaction does not require molecular oxygen (Gescher *et al.*, 2002, 2005; Zaar *et al.*, 2004). Phenylacetic acid (PA) degradation in bacteria also involves an aerobic hybrid pathway, which was initially described in *Pseudomonas putida* U (Olivera *et al.*, 1998) and *Escherichia coli* W (Ferrández *et al.*, 1998), and which is encoded by the *paa* gene cluster [in this work we use the consensus nomenclature proposed by Luengo *et al.* (2001)]. In this pathway, PA is first activated by a phenylacetate-CoA ligase to phenylacetyl-CoA (Martínez-Blanco *et al.*, 1990), which subsequently undergoes a putative ring hydroxylation, ring opening and further β-oxidation-type degradation through a proposed pathway that involves CoA thioesters and that converges with the classical β-ketoadipate pathway at the β-ketoadipyl-CoA intermediate (Ismail *et al.*, 2003) (Fig. 1). The PA pathway is the core of the phenylacetyl-CoA catabolon, a functional unit that integrates peripheral catabolic pathways that convert several structurally related aromatic compounds, such as styrene, 2-phenylethylamine, tropic acid, and phenylacetyl esters and amides, to the common intermediate phenylacetyl-CoA (Luengo *et al.*, 2001). The PA pathway has also been described in several other Gram-negative bacteria, such as *A. evansii* (Mohamed *et al.*, 2002; Rost *et al.*, 2002), other *Pseudomonas* strains (Bartolomé-Martín *et al.*, 2004) and even Gram-positive bacteria (Navarro-Llorens *et al.*, 2005) and the genus *Thermus* (Kunishima *et al.*, 2005; Song *et al.*, 2006). Therefore, this pathway appears to be widely distributed in bacteria and is the only pathway of aerobic PA degradation reported so far in these organisms.

So far, phenylacetyl-CoA is the only intermediate of the PA pathway that has been unequivocally characterized. Although recent work strongly suggests that PA degradation involves acetyl-CoA formation (O'Leary *et al.*, 2005), there has been no experimental demonstration of whether succinyl-CoA is also a final product in the catabolism of PA (Fig. 1) (Ismail *et al.*, 2003) and of which enzyme is involved in this particular reaction. To accomplish this goal, we present here the characterization of the last step of the PA aerobic hybrid pathway.

Abbreviations: PA, phenylacetic acid; TCA cycle, tricarboxylic acid cycle.

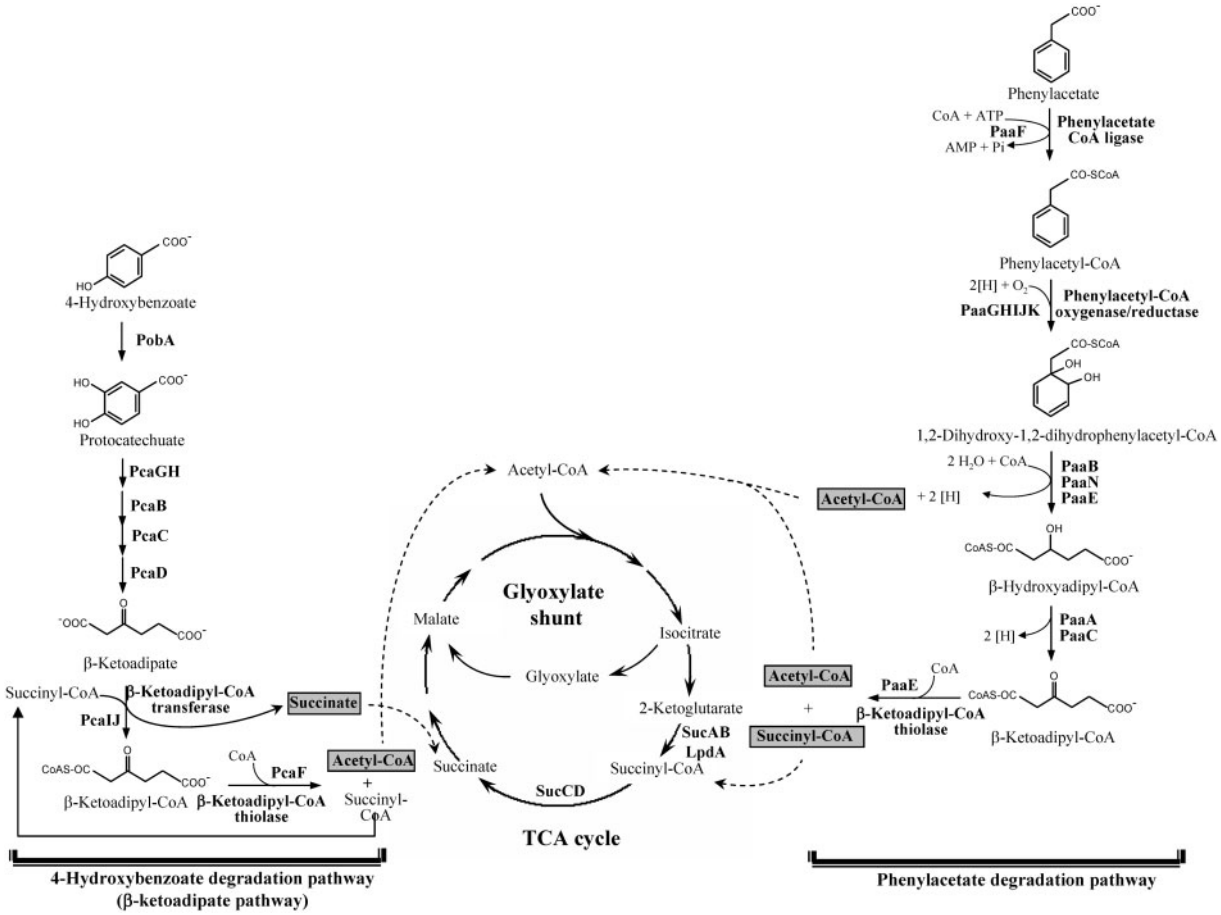

Fig. 1. Scheme of 4-hydroxybenzoate catabolism through the β-ketoadipate central pathway and proposed phenylacetate degradation pathway. The PobA monooxygenase and the Pca enzymes of the protocatechuate branch of the β-ketoadipate central pathway are indicated. The Paa enzymes and the intermediates involved in the proposed phenylacetate degradation pathway (Ismail *et al.*, 2003) are also shown [the consensus nomenclature proposed by Luengo *et al.* (2001) has been used]. Note that the amount of O_2 and [H] consumed for the metabolism of phenylacetyl-CoA is merely postulated, and could be even higher than that shown in the figure. Broken arrows show the biochemical step by which the final products (grey boxes) of the two degradation pathways enter the TCA cycle. The succinyl-CoA synthetase (SucCD) and the 2-ketoglutarate dehydrogenase complex (SucABLpdA) of the TCA cycle are indicated. The glyoxylate shunt is also shown.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this work are indicated in Table 1. *E. coli* cells were grown in Luria–Bertani (LB) medium (Sambrook & Russell, 2001) or M63 minimal medium (Miller, 1972) at 37 °C. *P. putida* cells were grown in M63 minimal medium at 30 °C. When used as carbon sources, citrate, glycerol, isoleucine, 2-ketoglutarate or succinate (0.2 %), and benzoate, 4-hydroxybenzoate or PA (5 mM) were added to the minimal medium. Where appropriate, antibiotics were added at the following concentrations: ampicillin (100 μg ml^{-1}), chloramphenicol (35 μg ml^{-1}), gentamicin (7.5 μg ml^{-1}), kanamycin (50 μg ml^{-1}), rifampicin (50 μg ml^{-1}) and tetracycline (15 μg ml^{-1}). When required, 1 mM IPTG was added to the culture medium to induce *Ptac*-driven expression.

Molecular biology techniques. Recombinant DNA techniques were carried out by published methods (Sambrook & Russell, 2001). Plasmid DNA was prepared with a High Pure plasmid isolation kit (Roche Applied Science). DNA fragments were purified with GeneClean Turbo (Q-BIOgene). Oligonucleotides were supplied by Sigma. All cloned inserts and DNA fragments were confirmed by DNA sequencing on an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Transformation of *E. coli* was carried out by using the RbCl method or by electroporation (Gene Pulser, Bio-Rad) (Sambrook & Russell, 2001). Plasmids were transferred from *E. coli* (donor strain) into *P. putida* recipient strains by triparental filter mating using *E. coli* HB101 (pRK600) as helper strain, as described previously (de Lorenzo & Timmis, 1994). Cell extracts were obtained by growing the cells in the corresponding media until they reached stationary phase. Cells were then disrupted by two consecutive passages through a French press (Aminco) operated at a pressure of 20 000 p.s.i. (138 MPa). The cell lysate was centrifuged at 13 000 ***g*** for 30 min at 4 °C, and the clear supernatant fluid was carefully decanted and used as the crude extract fraction. Proteins were analysed by SDS-PAGE, as

Table 1. Strains and plasmids used in this work

Strain/plasmid	Relevant characteristics	Reference/source
***P. putida* strains**		
KT2440	Wild-type strain; PA^+, BA^+*	Franklin *et al.* (1981)
KT2442	*P. putida* KT2440 rifampicin-resistant mutant; PA^+, BA^+	Franklin *et al.* (1981)
KT2442-150A	*P. putida* KT2442*sucD* obtained by insertion of the mini-Tn*5araC-*P_{BAD} transposon into the *sucD* gene of strain KT2442; PA^-, BA^+; Km^r	This study
KT2440d*pcaF*	*P. putida* KT2440*pcaF* obtained by disruption of the *pcaF* gene through homologous recombination; PA^+, BA^-; Km^r	This study
KT2440d*pcaF*::*paaE*	*P. putida* KT2440*pcaF* harbouring a chromosomal insertion of the $paaE_{EC}$; PA^+, BA^+; Km^r, Gm^r	This study
***E. coli* strains**		
MG1655	Wild-type *E. coli* K-12 strain	Bachmann (1987)
DH10B	F′, *mcr*A Δ(*mrr hsdRMS-mcrBC*) Φ80d*lac*ΔM15 Δ*lacX74 deoR recA1 araD*139 Δ(*ara-leu*)7697 *galU galK λ rpsL endA*1 *nupG*	Invitrogen
CC118λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA thi-1 rpsE* (Sp^r) *rpoB* (Rif^r) *argE*(Am) *recA1* λpir phage lysogen	de Lorenzo & Timmis (1994)
FB20225	MG1655*sucD* harbouring a Tn*5* transposon insertion within the *sucD* gene; Km^r	*E. coli* Genome Project; Kang *et al.* (2004)
WGAsuc26	*sucA* mutant of the wild-type *E. coli* W3110 strain	Herbert & Guest (1969)
HB101	*supE44 ara14 galK2 leuB lacY1* Δ(*gpt-proA*)62 *rps*L20 (Sm^r) *xyl-5 mtl-1 recA13* Δ(*mcrC-mrr*) *hsdS20* (r_B^- m_B^-)	Sambrook & Russell (2001)
W	Wild-type *E. coli* W strain	Davis & Mingioli (1950)
Plasmids		
pAAD	Cm^r; *ori*pSC101; low-copy-number plasmid containing a 15.5 kb DNA fragment carrying the *paa* cluster from *E. coli*	Ferrández *et al.* (1998)
pCR2.1-topo	Ap^r, Km^r; *ori*ColE1, *lacZ*; used for cloning of PCR-amplified DNA	Invitrogen
pT150A	Ap^r, Km^r; pCR2.1-topo derivative containing the *sucD* gene from *P. putida* KT2442	This study
pVLT31	Tc^r; *ori*RSF1010; broad-host-range plasmid used for cloning and expression under control of the $lacI^q$/*Ptac* system	de Lorenzo *et al.* (1993)
pV150A	Tc^r; pVLT31 derivative expressing from the *Ptac* promoter the *sucD* gene of *P. putida*	This study
pIZ1016	Gm^r; *ori*pBBR1 Mob^+; broad-host-range plasmid used for cloning and expression under control of the $lacI^q$/*Ptac* system	Moreno-Ruiz *et al.* (2003)
pIZ-paaE	Gm^r; a pIZ1016 derivative expressing the $paaE_{EC}$ gene from *Ptac*	This study
pUTmini-Tn*5*Tc	Ap^r, Tc^r; *ori*R6K RP4-Mob^+; mini-Tn*5*Tc transposon delivery plasmid	de Lorenzo *et al.* (1990)
pUTminiTn*5araC-*P_{BAD}	Ap^r, Km^r; *ori*R6K RP4-Mob^+; mini-Tn*5*Km *araC-*P_{BAD} delivery plasmid	Serina *et al.* (2004)
pUT-paaE	Ap^r, Tc^r, Gm^r; pUTmini-Tn*5*Tc derivative harbouring the Gm^r/$lacI^q$/*Ptac-paaE* 4.5 kb *Not*I-DNA cassette from plasmid pIZ-paaE	This study
pK18*mob*	Km^r; *ori*ColE1 Mob^+, *lacZα*; used for directed insertional disruption	Schäfer *et al.* (1994)
pK18F	Km^r; a pK18*mob* derivative containing an *Eco*RI/*Hin*dIII 0.8 kb internal fragment of the *pcaF* gene from *P. putida*	This study
pRK600	Cm^r; *ori*ColE1, RK2-Mob^+, RK2-Tra^+; helper plasmid for triparental matings	de Lorenzo & Timmis (1994)

*Growth on benzoate or 4-hydroxybenzoate as sole carbon sources.

described by Laemmli (1970). The protein concentrations in cell extracts were determined by the method of Bradford (1976), using BSA as the standard.

Sequence data analyses. Amino acid sequence comparison analyses were done using the TBLAST algorithm (Altschul *et al.*, 1990) at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

Random insertional mutagenesis of *P. putida* KT2442. Random insertional mutagenesis of *P. putida* KT2442 was carried out by using the mini-Tn*5araC-*P_{BAD} transposon, as described elsewhere (Serina *et al.*, 2004).

Cloning of the *sucD* gene from *P. putida*. The *sucD* gene from *P. putida* KT2442 was cloned into the pVLT31 plasmid under the control of the *Ptac* promoter, giving rise to plasmid pV150A. To construct plasmid pV150A, a 1.0 kb fragment containing the *sucD* gene of *P. putida* KT2442 was PCR-amplified by using the forward sucD-b (5′-GCGGTTTGAACATCATTGC-3′) and reverse sucDXbab (5′-GCTCTAGACGAACCCCACATACGACA-3′; an engineered *Xba*I restriction site is underlined) oligonucleotides, cloned into the pCR2.1-topo vector to produce plasmid pT150A, and then subcloned into the broad-host-range pVLT31 plasmid as an *Eco*RI–*Xba*I DNA fragment.

Cloning of the *paaE* gene from *E. coli* ($paaE_{EC}$). The $paaE_{EC}$ gene was PCR-amplified from plasmid pAAD by using oligonucleotides PaaE5′ (5′-CCGTCGACTGACCTAAGGAGGTAAATA**ATG**C-GTGAAGCCTTTATCTGTGACGGAATTC-3′; the *paaE* start codon is indicated in bold type and an engineered *Sal*I restriction site is underlined) and PaaE3′ (5′-CCAAGCTT**TCA**AACACGCTCCAGA-ATCATG-3′; the *paaE* stop codon is indicated in bold type and an engineered *Hin*dIII restriction site is underlined), and the resulting 1.2 kb DNA fragment was *Sal*I/*Hin*dIII double-digested and cloned into the *Sal*I/*Hin*dIII double-digested pIZ1016 vector. The resulting plasmid, pIZ-paaE, conferred gentamicin resistance and expressed the $paaE_{EC}$ gene under the control of the *Ptac* promoter and the LacI repressor.

Construction of the *P. putida* KT2440d*pcaF* strain. To construct a *P. putida* KT2440 mutant strain harbouring a disrupted *pcaF* gene, an internal fragment of the *pcaF* gene was PCR-amplified with primers PcaFint5′ (5′-GGGAATTCTGGATGCCGTCGGCACC-GCG-3′; an engineered *Eco*RI restriction site is underlined) and PcaFint3′ (5′-CCGAAGCTTTCACGCAGCACCGCCAGGC-3′; an engineered *Hin*dIII restriction site is underlined) and the resulting 0.8 kb DNA fragment was *Eco*RI/*Hin*dIII double-digested and cloned into the *Eco*RI/*Hin*dIII double-digested pK18*mob* vector. The resulting construct, pK18F, was transferred from *E. coli* DH10B (donor strain) to *P. putida* KT2440 (recipient strain) by triparental filter mating using *E. coli* HB101 (pRK600) as helper strain (de Lorenzo & Timmis, 1994). An exconjugant *P. putida* strain harbouring the disrupted *pcaF* gene by insertion of the suicide plasmid was isolated on kanamycin-containing M63 minimal medium supplemented with citrate as the sole carbon source for counterselection of donor cells. The mutant strain, *P. putida* KT2440d*pcaF*, was analysed by PCR to confirm the disruption of the *pcaF* gene.

Construction of the *P. putida* KT2440d*pcaF*::*paaE* strain. For the construction of the *P. putida* KT2440d*pcaF*::*paaE* strain, we first generated plasmid pUT-paaE, which carries, within a pUTmini-Tn*5*Tc vector (de Lorenzo *et al.*, 1990), the Gm^r/$lacI^q$/*Ptac-*$paaE_{EC}$ 4.5 kb *Not*I DNA cassette from plasmid pIZ-paaE. To integrate the cassette into the chromosome of *P. putida* KT2440d*pcaF*, we performed triparental filter mating, using *E. coli* CC118λpir (pUT-paaE) and *E. coli* HB101 (pRK600) as the donor and helper strains, respectively (de Lorenzo & Timmis, 1994). The *P. putida* KT2440d*pcaF*::*paaE* transconjugant was selected on M63 minimal medium agar plates supplemented with citrate, kanamycin and gentamicin. The plates were incubated at 30 °C.

HPLC analyses. HPLC conditions were as described elsewhere (Kaschabek *et al.*, 2002), with some modifications. Separations were carried out with an analytical SC column (125 × 4.6 mm; 100 RP18, 5.0 μm; LiChrospher), using as elution buffer 50 mM KH_2PO_4 (pH 5.2) and 5 % acetonitrile (v/v) at a flow rate of 1 ml min^{-1}. The column effluent was monitored by measuring A_{260}. The retention times for CoA, succinyl-CoA, β-ketoadipyl-CoA, PA and acetyl-CoA were 5.7, 7.1, 8.9, 10.3 and 13.8 min, respectively.

PA consumption experiments. PA consumption experiments were performed by monitoring through HPLC the amount of PA present in the supernatant of bacterial cultures grown in M63 minimal medium containing 3 mM PA and 0.1 % (v/v) glycerol (*E. coli* cells) or 0.1 % citrate (*P. putida* cells).

β-Ketoadipyl-CoA thiolase activity assay. The β-ketoadipyl-CoA thiolase was assayed *in vitro* spectrophotometrically by monitoring the decrease in A_{305} of the β-ketoadipyl-CoA–Mg^{2+} complex (ε_{305} 16 300 M^{-1} cm^{-1}) (Kaschabek *et al.*, 2002). One unit (U) was defined as the activity required to remove one micromole β-ketoadipyl-CoA–Mg^{2+} complex per minute and per milligram protein. To obtain β-ketoadipyl-CoA, we used crude extracts of *P. putida* KT2440d*pcaF* strain, which lacks the PcaF thiolase but harbours an active PcaIJ β-ketoadipyl-CoA transferase (Harwood & Parales, 1996; Jiménez *et al.*, 2002) (Fig. 1). To this end, *P. putida* KT2440d*pcaF* cells were grown to mid-exponential phase in 0.2 % citrate-containing minimal medium in the presence of 1 mM 4-hydroxybenzoate. The PcaIJ β-ketoadipyl-CoA transferase reaction was performed at 30 °C for 15 min using 200 mM Tris/HCl buffer, pH 8.0, 4 mM $MgCl_2$, 200 μM CoA, 200 μM succinyl-CoA, 400 μM β-ketoadipate and 150 μg crude extract from *P. putida* KT2440d*pcaF*. The $PaaE_{EC}$ thiolase was added to the above reaction assay from a crude extract (20 μg total protein) of *E. coli* DH10B (pIZ-paaE) cells that were grown to mid-exponential phase in LB medium and then induced with 1 mM IPTG for 2 h. The thiolytic reaction catalysed by $PaaE_{EC}$ was performed at 30 °C for 5 min. When using a crude extract (100 μg total protein) of *E. coli* W cells grown in PA, the PaaE-catalysed reaction was performed at 30 °C for 5 min. The thiolytic reaction catalysed by PcaF was assayed similarly but using a crude extract (100 μg total protein) of *P. putida* KT2440 cells that were grown in 4-hydroxybenzoate-containing minimal medium. The products of the thiolytic cleavage of β-ketoadipyl-CoA, i.e. succinyl-CoA and acetyl-CoA, were characterized by HPLC analysis.

RESULTS AND DISCUSSION

Succinyl-CoA is a final product of the PA catabolic pathway in *P. putida*

In the course of a functional genomic study of *P. putida* KT2442 through random insertional mutagenesis, we isolated mutant strains that were unable to grow in minimal medium containing PA as sole carbon and energy source but retained the ability to grow on other carbon sources such as succinate or citrate. By sequencing the chromosomal regions flanking the mini-Tn*5araC-*P_{BAD} insertion sites, we realized that one of the mutants, the *P. putida* KT2442-150A strain (Table 1, Fig. 2A), did not contain the transposon insertion within the *paa* gene cluster (formerly *pha* cluster) (Jiménez *et al.*, 2002, 2004) but rather on a gene (TIGR locus name

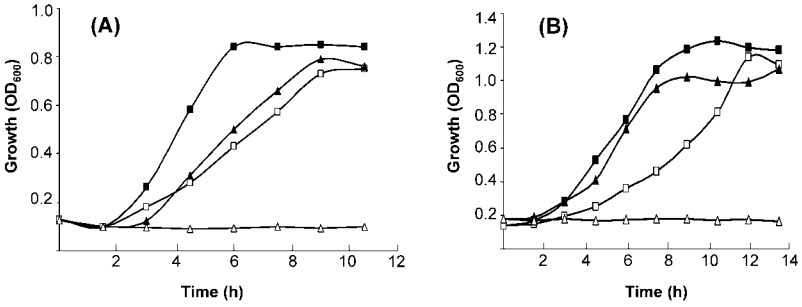

Fig. 2. Growth of wild-type and *sucD* mutant strains. (A) Wild-type *P. putida* KT2442 (squares) and *P. putida* KT2442-150A *sucD* (triangles) were cultivated in M63 medium containing either 0.2 % succinate (closed symbols) or 5 mM PA (open symbols). (B) Wild-type *E. coli* MG1655 (squares) and *E. coli* FB20225 *sucD* (triangles) strains harbouring plasmid pAAD were cultivated in M63 medium containing either 0.2 % succinate (closed symbols) or 5 mM PA (open symbols).

PP4185 of the annotated *P. putida* genome; http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gpp) whose putative product showed 97 and 88 % amino acid sequence identity with the *sucD* gene products of *P. aeruginosa* (Kapatral *et al.*, 2000) and *E. coli* (Buck *et al.*, 1986), respectively. The *sucD* gene encodes the α subunit of the succinyl-CoA synthetase (SucCD) that converts succinyl-CoA into succinate in the TCA cycle (Fig. 1). Growth of *P. putida* KT2442-150A on PA was restored when the strain harboured plasmid pV150A (Table 1), which expresses the *P. putida* wild-type *sucD* gene. Growth of *P. putida* KT2442-150A (pV150A) on PA indicated that the lack of growth of the host mutant strain was due to the absence of an active *sucD* gene rather than the putative polar effects caused by the mini-transposon insertion on flanking genes or additional mutations in the *paa* genes involved in PA catabolism in *P. putida* KT2442 (Jiménez *et al.*, 2002, 2004). Although inactivation of the *sucD* gene in *E. coli* blocks the TCA cycle at the level of succinyl-CoA, such mutants are able to grow on succinate, the next compound after succinyl-CoA in the TCA cycle (Mat-Jan *et al.*, 1989) (Fig. 1). The same behaviour was observed with the *P. putida* KT2442-150A strain, which was able to use succinate as the sole carbon source (Fig. 2A). Interestingly, whereas the wild-type strain grew on isoleucine, which is degraded via succinyl-CoA (Massey *et al.*, 1976), *P. putida* KT2442-150A was not able to use this amino acid as carbon source. Therefore, these results suggest that succinyl-CoA is a final product of the PA degradation pathway.

Succinyl-CoA is also a final product of the PA catabolic pathway in *E. coli*

Since PA degradation in *E. coli* has been shown to follow a similar pathway to that in *P. putida* (Ferrández *et al.*, 1998; Olivera *et al.*, 1998), we checked whether an *E. coli sucD* mutant strain was also unable to use PA as sole carbon source. To this end, we transformed the wild-type *E. coli* MG1655 strain and the *E. coli* FB20225*sucD* mutant strain (Table 1) with plasmid pAAD (Table 1), which contains the *paa* cluster involved in PA degradation from *E. coli* W (Ferrández *et al.*, 1998). Whereas *E. coli* MG1655 (pAAD) grew on minimal medium containing succinate or PA, the mutant strain *E. coli* FB20225 (pAAD) was able to grow on succinate but not on PA (Fig. 2B). This behaviour was similar to that observed with *P. putida* KT2442 versus *P. putida* KT2442-150A, and supports the suggestion that succinyl-CoA synthetase is required for PA degradation in *E. coli*. Since the *E. coli* FB20225 (*sucD*) mutant strain was also unable to grow on 2-ketoglutarate, the intermediate that produces succinyl-CoA in the TCA cycle by the action of the 2-ketoglutarate dehydrogenase complex (Buck *et al.*, 1986) (Fig. 1), we could not dismiss the possibility that 2-ketoglutarate was a final product in the PA catabolic pathway. To check this, we tested the growth of *E. coli* WGAsuc26 (*sucA*), a mutant strain that contains an inactive subunit of the 2-ketoglutarate dehydrogenase complex (Table 1), in PA and 2-ketoglutarate. Interestingly, whereas *E. coli* WGAsuc26 (*sucA*) containing plasmid pAAD did not grow on 2-ketoglutarate as sole carbon source, the strain grew on PA (data not shown), which indicates that 2-ketoglutarate is not produced by the aerobic catabolism of PA.

PA consumption by wild-type and *sucD* mutant strains

The experiments performed with *P. putida* and *E. coli* showed that specific blockage of the TCA cycle at the succinyl-CoA synthetase-catalysed step prevents PA mineralization, strongly suggesting the formation of succinyl-CoA as a final product in PA catabolism (Fig. 1). Interestingly, whereas wild-type cells growing in the presence of PA and citrate (*P. putida* KT2442) or glycerol (*E. coli* MG1655 harbouring plasmid pAAD) showed a complete consumption of PA after 6 h incubation, the isogenic *sucD* mutant cells showed less than 8 % PA consumption. Moreover, whereas growth of wild-type cells reached OD_{600} 1.6 and 0.8 in the presence and absence of PA, respectively, growth of the mutant cells was similar (OD_{600} 0.6) in the presence and absence of PA (data not shown). These data suggest that the accumulation of succinyl-CoA from the minor fraction of PA consumed within the mutant cells leads to a transient blockage of the whole PA degradation pathway, preventing the normal consumption of PA and its use as a carbon source. It is worth noting here that acetyl-CoA has also been shown to be a final product in PA catabolism (O'Leary *et al.*, 2005). In this sense, the proposed PA degradation pathway predicts the formation of two acetyl-CoA molecules per PA molecule (Ismail *et al.*, 2003), which might allow the growth

of *sucD* mutant cells by using the glyoxylate shunt when succinyl-CoA cannot be metabolized through the TCA cycle (Fig. 1). However, considerations of the energetics of the proposed catabolic scheme (Ismail *et al.*, 2003) appear to rule out such a possibility. Thus, the conversion of PA to the predicted dihydrodiol intermediate requires a significant consumption of ATP and reducing equivalents (Fig. 1), which might prevent a positive energetic balance if acetyl-CoA alone, and not succinyl-CoA, is finally metabolized through the glyoxylate bypass in the *sucD* mutant cells. Therefore, the *paa*-encoded pathway might be endowed with a still-unknown blockage mechanism to prevent PA consumption and avoid energetic collapse when succinyl-CoA cannot be further metabolized. Interestingly, a different metabolic strategy is found in the classical β-ketoadipate pathway, in which succinyl-CoA becomes transformed into succinate by the action of a β-ketoadipyl-CoA transferase, rather than by the activity of the SucCD succinyl-CoA synthetase of the TCA cycle (Harwood & Parales, 1996) (Fig. 1). In agreement with this, we confirmed here that the *P. putida* KT2442-150A (*sucD*) mutant was able to grow on aromatic compounds, such as benzoate and 4-hydroxybenzoate (data not shown), that are degraded via the β-ketoadipate pathway to produce succinate and acetyl-CoA as final products (Harwood & Parales, 1996) (Fig. 1).

Analysis of the β-ketoadipyl-CoA thiolase activity of the PA catabolic pathway

The formation of acetyl-CoA and succinyl-CoA as final products of the PA catabolic pathway should require a thiolase activity acting on the β-ketoadipyl-CoA intermediate proposed by Ismail *et al.* (2003) (Fig. 1). Analysis of the *paa* cluster involved in PA degradation in *E. coli* (Ferrández *et al.*, 1998) revealed the existence of the *paaE* gene (formerly named *paaJ*), whose product showed a significant amino acid sequence identity with the β-ketoadipyl-CoA thiolase (PcaF) that acts in the β-ketoadipate pathway of *P. putida* (71 %) (Harwood *et al.*, 1994) and *Acinetobacter* sp. ADP1 (66.5 %) (Kowalchuk *et al.*, 1994). Homologous *paaE* genes are also present in the *paa* clusters of *P. putida* strains (Olivera *et al.*, 1998; Jiménez *et al.*, 2002; Bartolomé-Martín *et al.*, 2004). To determine whether PaaE was the enzyme catalysing the last step in the PA degradation pathway, i.e. the thiolytic cleavage of β-ketoadipyl-CoA to succinyl-CoA and acetyl-CoA, we cloned the *paaE* gene from *E. coli*, $paaE_{EC}$, in the promiscuous and mobilizable pIZ-paaE plasmid (Table 1), as described in Methods. SDS-PAGE analysis of crude lysates from *E. coli* DH10B (pIZ-paaE) cells grown in LB medium containing gentamicin and IPTG revealed the presence of an intense band corresponding to a protein with an apparent molecular mass of 43 kDa, in good agreement with that predicted for the $paaE_{EC}$ gene product (42.2 kDa) (data not shown). To check *in vivo* whether the function of the $paaE_{EC}$ gene product was that of a β-ketoadipyl-CoA thiolase, we used plasmid pIZ-paaE to complement the lack of the PcaF β-ketoadipyl-CoA thiolase in *P. putida* KT2440d*pcaF* (Table 1), a *P. putida* KT2440*pcaF* mutant strain constructed as described in Methods. Since the *P. putida* KT2440d*pcaF* mutant strain contains a truncated β-ketoadipate pathway, it did not grow on benzoate or 4-hydroxybenzoate as sole carbon sources but, as expected, grew on PA. However, growth on benzoate and 4-hydroxybenzoate was not restored when the *P. putida* KT2440d*pcaF* strain harboured plasmid pIZ-paaE. Nevertheless, since the *P. putida* KT2440d*pcaF* (pIZ-paaE) strain grew poorly in minimal medium containing citrate plus 4-hydroxybenzoate, we suspected that overexpression of the $paaE_{EC}$ gene caused a toxic effect. Therefore, to reduce the expression level of the $paaE_{EC}$ gene, it was subcloned into a mini-transposon that allows its stable insertion as a single copy into the bacterial chromosome (see Methods), giving rise to the *P. putida* KT2440d*pcaF*::*paaE* strain (Table 1). As expected, the IPTG-induced expression of the $paaE_{EC}$ gene from the chromosome of *P. putida* KT2440d*pcaF*::*paaE* allowed growth of the strain in minimal medium containing 4-hydroxybenzoate as sole carbon source, and the growth curve was similar to that shown by a *P. putida* KT2440 wild-type strain. These data indicate that the $paaE_{EC}$ gene product was able to efficiently complement the absence of the PcaF thiolase, and therefore suggest that PaaE also functions as a β-ketoadipyl-CoA thiolase.

To confirm that the PaaE enzyme is a β-ketoadipyl-CoA thiolase, we performed *in vitro* activity assays as described in Methods. As shown in Fig. 3, addition of a crude extract containing the PcaIJ β-ketoadipyl-CoA transferase (and lacking the PcaF thiolase) to a reaction assay mixture containing CoA, succinyl-CoA and β-ketoadipate (Fig. 3B) generated a new peak in the HPLC chromatogram corresponding to a CoA derivative with a relative retention time (8.9 min) similar to that reported by Kaschabek *et al.* (2002) for β-ketoadipyl-CoA (8.4 min) (Fig. 3C). Moreover, the peak with a retention time of 8.9 min showed a characteristic absorption spectrum, with a maximum at 305 nm, which is also in agreement with the formation of a β-ketoadipyl-CoA–Mg^{2+} complex (Katagiri & Hayaishi, 1957). Interestingly, the subsequent addition to the reaction mixture of a crude extract of *E. coli* DH10B (pIZ-paaE) that overproduces the $PaaE_{EC}$ enzyme resulted in the rapid disappearance of the species absorbing at 305 nm as well as in a change in the HPLC chromatogram of CoA derivatives. Thus, addition of $PaaE_{EC}$ generated a new peak corresponding to acetyl-CoA concomitantly with a significant decrease of the β-ketoadipyl-CoA and CoA peaks and an increase of the succinyl-CoA peak (Fig. 3D). All these data are in agreement with PaaE acting as a thiolase that produces acetyl-CoA and succinyl-CoA due to thiolytic fission of β-ketoadipyl-CoA. Moreover, it should be noted that the β-ketoadipyl-CoA thiolytic cleavage due to $PaaE_{EC}$ present in crude extracts from *E. coli* W (Table 1) grown in PA [0.11 U (mg protein)$^{-1}$] was in the same range as that due to PcaF present in crude extracts from *P. putida* KT2440 grown in 4-hydroxybenzoate [0.06 U (mg protein)$^{-1}$], which is also in agreement with the data previously reported

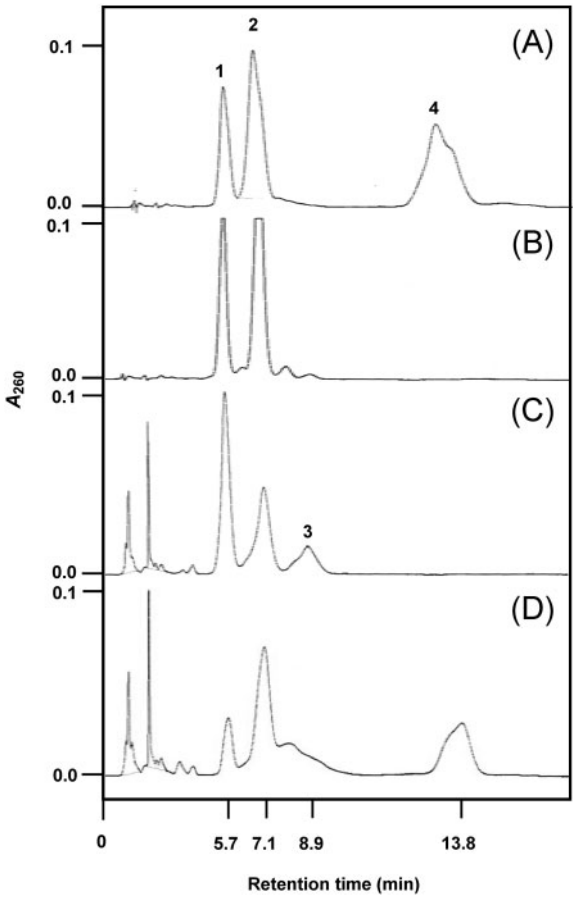

Fig. 3. HPLC chromatograms of CoA derivatives involved in the last step of aerobic PA degradation. (A) Standard compounds (200 μM): 1, CoA (peak at 5.7 min); 2, succinyl-CoA (peak at 7.1 min); 4, acetyl-CoA (peak at 13.8 min). (B) Reaction assay before adding any enzyme extract. (C) Reaction assay after addition of the PcaIJ β-ketoadipyl-CoA transferase. The reaction was performed at 30 °C for 15 min. Compound 3, β-ketoadipyl-CoA (peak at 8.9 min). (D) Addition of $PaaE_{EC}$ thiolase to the reaction assay of panel (C); the thiolytic reaction catalysed by $PaaE_{EC}$ was performed at 30 °C for 5 min.

for the PcaF thiolases from *P. putida* PRS2000 (Harwood *et al.*, 1994) and *Pseudomonas* sp. B13 (Kaschabek *et al.*, 2002).

Whereas *E. coli* has only one β-ketoadipyl-CoA thiolase ($PaaE_{EC}$), *P. putida* KT2440 has two isoenzymes, PcaF and $PaaE_{PP}$, which catalyse the thiolytic cleavage of β-ketoadipyl-CoA in two different central pathways, i.e. the classical β-ketoadipate pathway (Harwood & Parales, 1996; Jiménez *et al.*, 2002, 2004) and the PA degradation pathway, respectively (Fig. 1). According to their physiological role, the expression of the $paaE_{PP}$ and *pcaF* genes is differentially regulated in *P. putida*. Thus, whereas $paaE_{PP}$ becomes expressed in the presence of PA (García *et al.*, 2000), the *pcaF* gene is specifically induced when the *P. putida* cells grow in the presence of aromatic compounds that are degraded by the β-ketoadipate pathway, e.g. benzoate and 4-hydroxybenzoate (Harwood & Parales, 1996). It is worth noting that the $paaE_{PP}$ and *pcaF* genes from *P. putida* have a G + C content close to the mean G + C content (61 %) of the genome (Nelson *et al.*, 2002), thus suggesting that they have been present within the genome of this bacterium over a long period of evolution. However, the corresponding $PaaE_{PP}$ and PcaF enzymes share an amino acid sequence identity (68.6 %) slightly lower than that observed between the $PaaE_{PP}$ and $PaaE_{EC}$ thiolases from *P. putida* and *E. coli* (70.4 %), and significantly lower than that between PcaF and equivalent thiolases of the β-ketoadipate pathway from other *Pseudomonas* strains, such as PcaF from *Pseudomonas* sp. B13 (87.7 %) (Kaschabek *et al.*, 2002). This observation suggests that the PA and the β-ketoadipate catabolic pathways have evolved independently, and that they did not exchange common genes, such as that encoding the β-ketoadipyl-CoA thiolase, when present in the same host bacterium. Nevertheless, the gene clusters involved in PA degradation in some bacteria lack a gene encoding a β-ketoadipyl-CoA thiolase (Díaz *et al.*, 2001; Luengo *et al.*, 2001; Mohamed *et al.*, 2002; Navarro-Llorens *et al.*, 2005), which might indicate that this function can be accomplished by other ketoacyl-CoA thiolases of the cell.

In summary, this study has experimentally demonstrated that succinyl-CoA is a final product in the aerobic hybrid pathway for PA degradation and that it is produced by the PaaE thiolase acting on β-ketoadipyl-CoA. In addition, the data presented here confirm earlier work that shows that acetyl-CoA is also a final product in PA catabolism (O'Leary *et al.*, 2005). Succinyl-CoA has also been suggested to be a final product in the aerobic hybrid pathway for benzoate degradation in bacteria such as *A. evansii*, *Burkholderia xenovorans* LB400 and a *Geobacillus stearothermophilus*-like strain (Denef *et al.*, 2004; Gescher *et al.*, 2002). Therefore, within the catabolism of aromatic compounds, succinyl-CoA might be considered as a common final product that characterizes aerobic hybrid pathways.

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