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

Juan Nogales,1 Raffaella Macchi,2 Federico Franchi,2 Dagania Barzaghi,2 Cristina Fernández,1 José L. García,1 Giovanni Bertoni2 and Eduardo Díaz1

1Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas-CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain
2Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, 20133 Milan, Italy

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, protocatechuic, gentisic, homoprotocatechuic, homogentisic 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-ketoacidipate 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 hybrid pathways have been described 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 Azotobacter 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-ketoacidipate pathway at the β-ketoacidipyle-CoA intermediate (Ismail et al., 2003) (Fig. 1). The PA pathway is the core of the phenylacetetyl-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 phenylacetetyl-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, phenylacetetyl-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.
**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 20000 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

---

**Fig. 1.** Scheme of 4-hydroxybenzoate catabolism through the β-ketoacid pathway and proposed phenylacetate degradation pathway. The PobA monooxygenase and the Pca enzymes of the protocatechuate branch of the β-ketoacid 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 O₂ 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.
Table 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. putida strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KT2440</td>
<td>Wild-type strain; PA⁺, BA⁺⁺</td>
<td>Franklin et al. (1981)</td>
</tr>
<tr>
<td>KT2442</td>
<td>P. putida KT2440 rifampicin-resistant mutant; PA⁺, BA⁺</td>
<td>Franklin et al. (1981)</td>
</tr>
<tr>
<td>KT2442-150A</td>
<td>P. putida KT2442sucD obtained by insertion of the mini-Tn5araC-PBAD transposon into the sucD gene of strain KT2442; PA⁺, BA⁺⁺; Km⁺</td>
<td>This study</td>
</tr>
<tr>
<td>KT2440pcaF</td>
<td>P. putida KT2440pcaF obtained by disruption of the pcaF gene through homologous recombination; PA⁺, BA⁺⁺; Km⁺</td>
<td>This study</td>
</tr>
<tr>
<td>KT2440pcaF::paaE</td>
<td>P. putida KT2440pcaF harbouring a chromosomal insertion of the paaEEC; PA⁺, BA⁺⁺; Km⁺, Gm⁺</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>Wild-type E. coli K-12 strain</td>
<td>Bachmann (1987)</td>
</tr>
<tr>
<td>DH10B</td>
<td>F−, mcrA Δ(mrr hsdRMS-mcrBC) F80lacAM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK thi-1 rpsL endA1 F80 lac-D galX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK thi-1 rpsL endA1 F80 lac-D</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CC118pir</td>
<td>Δ(ara-leu) araD ΔlacX74 galE galK phoA thi-1 rpsE (Sp') rpoB (Rif') argE(Am) recA1 Δpir phage lysogen</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
</tr>
<tr>
<td>FB20225</td>
<td>MG1655sucD harbouring a Tn5 transposon insertion within the sucD gene; Km⁺</td>
<td>E. coli Genome Project; Kang et al. (2004)</td>
</tr>
<tr>
<td>WGAsuc26</td>
<td>sucA mutant of the wild-type E. coli W3110 strain</td>
<td>Herbert &amp; Guest (1969)</td>
</tr>
<tr>
<td>HB101</td>
<td>supE44 ara14 galK2 leuB lacY1 Δ(gpt-proA)62 rpsL20 (Sm⁺) xyl-5 mtl-1 recA13 Δ(mcrC-mrr) hsdS20 (r2 B m2 B)</td>
<td>Sambrook &amp; Russell (2001)</td>
</tr>
<tr>
<td>W</td>
<td>Wild-type E. coli W strain</td>
<td>Davis &amp; Mingioli (1950)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAAD</td>
<td>Cm⁺; oriSC101; low-copy-number plasmid containing a 15.5 kb DNA fragment carrying the paa cluster from E. coli</td>
<td>Ferrández et al. (1998)</td>
</tr>
<tr>
<td>pCR2.1-topo</td>
<td>Ap', Km'; oriColE1, lacZ; used for cloning of PCR-amplified DNA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pT150A</td>
<td>Ap', Km'; pCR2.1-topo derivative containing the sucD gene from P. putida KT2442</td>
<td>This study</td>
</tr>
<tr>
<td>pVLT31</td>
<td>Tc'; oriRSF1010; broad-host-range plasmid used for cloning and expression under control of the lacIq/Ptac system</td>
<td>de Lorenzo et al. (1993)</td>
</tr>
<tr>
<td>pV150A</td>
<td>Tc'; pVLT31 derivative expressing from the Ptac promoter the sucD gene of P. putida</td>
<td>This study</td>
</tr>
<tr>
<td>pIZ1016</td>
<td>Gm⁺; oriPBBR1 Mob⁺; broad-host-range plasmid used for cloning and expression under control of the lacIq/Ptac system</td>
<td>Moreno-Ruiz et al. (2003)</td>
</tr>
<tr>
<td>pIZ-paaE</td>
<td>Gm⁺; a pIZ1016 derivative expressing the paaEEC gene from Ptac</td>
<td>This study</td>
</tr>
<tr>
<td>pUTmini-Tn5Tc</td>
<td>Ap', Tc'; oriR6K RP4-Mob⁺; mini-Tn5Tc transposon delivery plasmid</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
<tr>
<td>pUTminiTn5araC-PBAD</td>
<td>Ap', Km'; oriR6K RP4-Mob⁺; mini-Tn5Km araC-PBAD delivery plasmid</td>
<td>Serina et al. (2004)</td>
</tr>
<tr>
<td>pUT-paaE</td>
<td>Ap', Tc', Gm⁺; pUTmini-Tn5Tc derivative harbouring the Gm⁺/lacIq/Ptac-paaE 4.5 kb NotI-DNA cassette from plasmid pIZ-paaE</td>
<td>This study</td>
</tr>
<tr>
<td>pK18mob</td>
<td>Km⁺; oriColE1 Mob⁺⁺, lacZs; used for directed insertional disruption</td>
<td>Schafer et al. (1994)</td>
</tr>
<tr>
<td>pK18F</td>
<td>Km⁺; a pK18mob derivative containing an EcoRI/HindIII 0.8 kb internal fragment of the pcaF gene from P. putida</td>
<td>This study</td>
</tr>
<tr>
<td>pRK600</td>
<td>Cm⁺; oriColE1, RK2-Mob⁺⁺, RK2-Tra⁺⁺; helper plasmid for triparental matings</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
</tr>
</tbody>
</table>

*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-Tn5araC-PBAD 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 pVLT3 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’-GGGTTTGAACATCCATGGC-3’) and reverse sucDxa-b (5’-GGTCTAAGAGACCACATACGACA-3’); an engineered XbaI restriction site is underlined) oligonucleotides, cloned into the pCR2.1-topo vector to produce plasmid pT150A, and then sub-cloned into the broad-host-range plasmid pVLT31 plasmid as an EcoRI–XbaI DNA fragment.

**Cloning of the paaE gene from* E. coli (paaE)C*.* The paaEEC gene was PCR-amplified from plasmid pAAD by using oligonucleotides PaaE5’ (5’-GGTCTAGATCTAATAGGTTAAATATGC-3’) and PaaE3’ (5’-GGAGAGGTTATCTGAGGAAATTCC-3’); the paaE start codon is indicated in bold type and an engineered SalI restriction site is underlined) oligonucleotides, cloned into the pCR2.1-topo vector to produce plasmid pT150A, and then sub-cloned into the broad-host-range plasmid pVLT31 plasmid as an EcoRI–XbaI DNA fragment.

**Construciton of the* P. putida* KT2440dpcAf 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’-GGGTTTGAACATCCATGGC-3’) and PcaFint3’ (5’-GGGTTTGAACATCCATGGC-3’), an engineered HindIII restriction site is underlined) oligonucleotides, cloned into the pCR2.1-topo vector to produce plasmid pT150A, and then sub-cloned into the broad-host-range plasmid pVLT31 plasmid as an EcoRI–XbaI DNA fragment.

**RESULTS AND DISCUSSION**

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

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-Tn5araC-PBAD 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 KT2440dpcAf::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 on a analytical SC column (125 × 4.6 mm; 100 RP18, 5.0 μm; LiChrospher), using an elution buffer 50 mM KH2PO4 (pH 5.2) and 5% acetonitrile (v/v) at a flow rate of 1 ml min⁻¹. The column effluent was monitored by measuring A530. The retention times for CoA, succinyl-CoA, β-ketoacipyl-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-Ketoacyl-CoA thiolase activity assay.** The β-ketoacyl-CoA thiolase was assayed in vitro spectrophotometrically by monitoring the decrease in A346 of the β-ketoacyl-CoA–Mg2⁺ complex (βₜₐ₅₃ 16 300 M⁻¹ cm⁻¹) (Kaschabek et al., 2002). One unit (U) was defined as the activity required to remove one micromole β-ketoacyl-CoA–Mg2⁺ complex per minute and per milligram protein. To obtain β-ketoacyl-CoA, we used crude extracts of *P. putida* KT2440dpcAf strain, which lacks the PcaF thiolase but harbours an active PaaIE β-ketoacyl-CoA thiolase (Harwood & Parales, 1996; Jiménez et al., 2002) (Fig. 1). To this end, *P. putida* KT2440dpcAf cells were grown to mid-exponential phase in 0.2% citrate-containing minimal medium in the presence of 1 mM 4-hydroxybenzoate. The PcaII β-ketoacyl-CoA thiolase reaction was performed at 30°C for 15 min using 200 mM Tris/HCl buffer, pH 8.0, 4 mM MgCl₂, 200 μM CoA, 200 μM succinyl-CoA, 400 μM β-ketoacidipate and 150 μg crude extract from *P. putida* KT2440dpcAf. The PaaEEC thiolase was added to the above reaction assay from a crude extract (20 μg total protein) of *E. coli* DH10B (pLZ-paaE) cells that were grown to mid-exponential phase in LB medium and then induced with 1 mM IPTG for 2 h. The thiolitiic reaction catalysed by PaaEEC was performed at 30°C for 5 min. When using a crude extract (100 μg total protein) of *E. coli* cells grown in PA, the PaaE-catalysed reaction was performed at 30°C for 5 min. The thiolactic 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 thiolitic cleavage of β-ketoacidipyl-CoA, i.e. succinyl-CoA and acetyl-CoA, were characterized by HPLC analysis.

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

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-Tn5araC-PBAD 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 KT2440dpcAf::paaE transconjugant was selected on M63 minimal medium agar plates supplemented with citrate, kanamycin and gentamicin. The plates were incubated at 30°C.
PP4185 of the annotated *P. putida* genome; [http://www.tigr.org/igr-scripts/CMR2/GenomePage3.spl?database=ggp](http://www.tigr.org/igr-scripts/CMR2/GenomePage3.spl?database=ggp) 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 ϵ subunit of the succinyl-CoA synthetase (SuccCD) 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 able 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 pPAAD (Table 1), which contains the *paa* cluster involved in PA degradation from *E. coli* W (Ferrández et al., 1998). Whereas *E. coli* MG1655 (pPAAD) grew on minimal medium containing succinate or PA, the mutant strain *E. coli* FB20225 (pPAAD) 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 pPAAD 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 pPAAD) 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 OD₆₀₀ 1.6 and 0.8 in the presence and absence of PA, respectively, growth of the mutant cells was similar (OD₆₀₀ 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 succinyll-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 dihydriodiol 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 succinyll-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 succinyll-CoA cannot be further metabolized. Interestingly, a different metabolic strategy is found in the classical β-ketoadipate pathway, in which succinyll-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 succinyll-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 paaI), 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, paaEKC, in the promiscuous and mobilizable plIZ-paaE plasmid (Table 1), as described in Methods. SDS-PAGE analysis of crude lysates from E. coli DH10B (plIZ-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 paaEKC gene product (42.2 kDa) (data not shown). To check in vivo whether the function of the paaEKC gene product was that of a β-ketoadipyl-CoA thiolase, we used plasmid plIZ-paaE to complement the lack of the PcaF β-ketoadipyl-CoA thiolase in P. putida KT2440dpcAF (Table 1), a P. putida KT2440pcaF mutant strain constructed as described in Methods. Since the P. putida KT2440dpcAF 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 KT2440dpcAF strain harboured plasmid plIZ-paaE. Nevertheless, since the P. putida KT2440dpcAF (pIZ-paaE) strain grew poorly in minimal medium containing citrate plus 4-hydroxybenzoate, we suspected that overexpression of the paaEKC gene caused a toxic effect. Therefore, to reduce the expression level of the paaEKC 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 KT2440dpcAF::paaE strain (Table 1). As expected, the IPTG-induced expression of the paaEKC gene from the chromosome of P. putida KT2440dpcAF::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 paaEKC 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–Mg2+ complex (Katagiri & Hayaishi, 1957). Interestingly, the subsequent addition to the reaction mixture of a crude extract of E. coli DH10B (plIZ-paaE) that overproduces the PaaEKC 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 PaaEKC 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 thiolase cleavage due to PaaEKC 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.
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 $\beta$-ketoadipyl-CoA thiolase (PaaE$_{EC}$), P. putida KT2440 has two isoenzymes, PcaF and PaaE$_{PP}$, which catalyse the thiolytic cleavage of $\beta$-ketoadipyl-CoA in two different central pathways, i.e. the classical $\beta$-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 $\beta$-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 $\beta$-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 $\beta$-ketoadipate catabolic pathways have evolved independently, and that they did not exchange common genes, such as that encoding the $\beta$-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 $\beta$-ketoadipyl-CoA thiolase (Diaz 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 $\beta$-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. evansi, Burkholderia xenovarans 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.

ACKNOWLEDGEMENTS

We thank M. K. B. Berlyn (Yale University) for providing strain E. coli WGAsuc26. We gratefully acknowledge the technical assistance of I. Alonso. This work was supported by EU contract QLK3-CT2000-00170, and by grants GEN2001-4698-C05-02 and BIO2003-05309-C04-02 from the Comisión Interministerial de Ciencia y Tecnología, and FIRB2001-RBAU01KHM2 from the Ministerio dell’Istruzione, dell’Università e della Ricerca, Rome. J. N. and C. F. were the recipients of an I3P predoctoral fellowship from the Consejo Superior de Investigaciones Científicas (CSIC) and a Formación de Personal Investigador (FPI) predoctoral fellowship from the Ministerio de Educación y Ciencia (MEC), respectively.

REFERENCES

Aerobic benzoyl-CoA (CoA) catabolic pathway in


Edited by: M. A. Kertesz