Bacterial phenylalanine and phenylacetate catabolic pathway revealed

R. Teufel[a], V. Mascaraque[b,1], W. Ismail[a,1,2], M. Voss[a], J. Perera[b], W. Eisenreich[c], W. Haehnel[d], and G. Fuchs[a,3]

Departments of [a]Mikrobiologie and [d]Biochemie der Pflanzen, Fakultät für Biologie, Albert-Ludwigs-Universität Freiburg, D-79104 Freiburg, Germany; [b]Departamento de Bioquímica y Biología Molecular, I, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain; and [c]Center of Isotopologue Profiling, Lehrstuhl für Biochemie, Technische Universität München, D-85748 Munich, Germany

Edited by Caroline S. Harwood, University of Washington, Seattle, WA, and approved June 24, 2010 (received for review April 20, 2010)

Aromatic compounds constitute the second most abundant class of organic substrates and environmental pollutants, a substantial part of which (e.g., phenylalanine or styrene) is metabolized by bacteria via phenylacetate. Surprisingly, the bacterial catabolism of phenylalanine and phenylacetate remained an unsolved problem. Although a phenylacetate metabolic gene cluster had been identified, the underlying biochemistry remained largely unknown. Here we elucidate the catabolic pathway functioning in 16% of all bacteria whose genome has been sequenced, including *Escherichia coli* and *Pseudomonas putida*. This strategy is exceptional in several aspects. Intermediates are processed as CoA thioesters, and the aromatic ring of phenylacetyl-CoA becomes activated to a ring 1,2-epoxide by a distinct multicomponent oxygenase. The reactive nonaromatic epoxide is isomerized to a seven-member O-heterocyclic enol ether, an oxepin. This isomerization is followed by hydrolytic ring cleavage and β-oxidation steps, leading to acetyl-CoA and succinyl-CoA. This widespread paradigm differs significantly from the established chemistry of aerobic aromatic catabolism, thus widening our view of how organisms exploit such inert substrates. It provides insight into the natural remediation of man-made environmental contaminants such as styrene. Furthermore, this pathway occurs in various pathogens, where its reactive early intermediates may contribute to virulence.

enoyl-CoA hydratase | epoxide | oxepin | oxygenase | phenylacetic acid

The biggest challenge for organisms using aromatic compounds as growth substrates is to overcome the stabilizing resonance energy of the aromatic ring system. This aromatic structure makes the substrates unreactive toward oxidation or reduction and thus requires elaborate degradation strategies. How microorganisms cope with this problem depends primarily on the availability of oxygen (1). Aerobic pathways use oxygen both for hydroxylation and for cleavage of the ring (2, 3). In contrast, under anaerobic conditions the common strategy consists of activation by CoA-thioester formation, shortening of the side chain, and energy-driven ring reduction, which also applies to phenylacetate catabolism (ref. 4 and literature cited therein).

The aerobic strategy is illustrated by the metabolism of phenylacetate and phenylacetyl-CoA, which are derived from a variety of substrates such as phenylalanine, lignin-related phenylpropane units, 2-phenylethylamine, phenylalkanoic acids with an even number of carbon atoms, or even environmental contaminants such as styrene and ethylbenzene (5–7). Rarely, phenylalanine is hydroxylated to tyrosine, which can be converted into 4-hydroxyphenylpyruvate, followed by hydroxylation to homogentisate (2,5-dihydroxyphenylacetate) as the central intermediate. The aromatic ring of homogentisate then is split by a ring-cleaving homogentisate dioxygenase, and finally fumarate and acetoacetate are produced (8). In most cases, however, phenylalanine is converted into phenylacetate. A conventional aerobic route for phenylacetate, which also leads to homogentisate, occurs in fungi (9). In bacteria, generally, neither phenylalanine hydroxylation to tyrosine nor phenylacetate hydroxylation to homogentisate occurs, leaving unsolved the question of how most bacteria use phenylalanine or phenylacetate.

Although first reports of aerobic phenylacetate-metabolizing bacteria appeared more than 50 y ago (10), the pathway itself remained enigmatic. The induction of a phenylacetate-CoA ligase in *Pseudomonas putida* under aerobic conditions was reported 2 decades ago (11). That report suggested an unconventional strategy for an oxygen-dependent metabolism using CoA-thioesters as intermediates (12). The ligase gene was found to be clustered together with 13 other phenylacetic acid (*paa*) genes in three transcriptional units, which were suspected to be involved in phenylacetate assimilation (13). Moreover, many of these genes were mandatory for growth on this substrate (14). A similar gene organization was reported in *E. coli* (15) (Fig. 1*A*) and in several *Pseudomonas* species (13, 16, 17); the high sequence similarity allows the prediction of a similar structure and function of the homologous proteins in these organisms. It is the only known bacterial aerobic degradation pathway for phenylacetate and is the most common for phenylalanine.

Many attempts to identify pathway intermediates other than phenylacetyl-CoA led only to rough outlines (14, 18–20). We set out to elucidate the pathway for phenylacetate degradation by producing purified enzymes coded by the *E. coli* K12 and *Pseudomonas* sp. strain Y2 *paa* gene clusters. This widespread catabolic pathway differs fundamentally from known aromatic catabolic pathways and involves the use of CoA thioesters throughout, epoxide formation, isomerization of the epoxide to an oxepin, and hydrolytic ring cleavage.

Results

The use of ^{13}C- and ^{14}C-labeled phenylacetate (Fig. 1*B*, compound I) was key to investigating this long-standing problem. These compounds were chemically transformed or enzymatically converted to phenylacetyl-CoA (Fig. 1*B*, compound II) using phenylacetate-CoA ligase (PaaK, the first enzyme of the pathway). Products were detected by UV and radioactivity detection and identified by ^{13}C-NMR spectroscopy, assisted by mass spectrometry.

Epoxidation of the Aromatic Ring. Phenylacetyl-CoA is the substrate of a presumed multicomponent oxygenase, PaaABCDE. This oxygenase is a key enzyme of the pathway, proposed to be responsible for the introduction of oxygen into the aromatic ring of phenylacetyl-CoA (6, 14, 21). First we cloned the five genes encoding this enzyme. The genes for all subunits were expressed

Author contributions: R.T., J.P., and G.F. designed research; R.T., V.M., W.I., M.V., W.E., and W.H. performed research; R.T., W.E., and W.H. analyzed data; and R.T. and G.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]V.M. and W.I. contributed equally to this work.

[2]Present address: Biotechnology Program, College of Graduate Studies, Arabian Gulf University, Al Manamah, Kingdom of Bahrain.

[3]To whom correspondence should be addressed. E-mail: georg.fuchs@biologie.uni-freiburg.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1005399107/-/DCSupplemental.

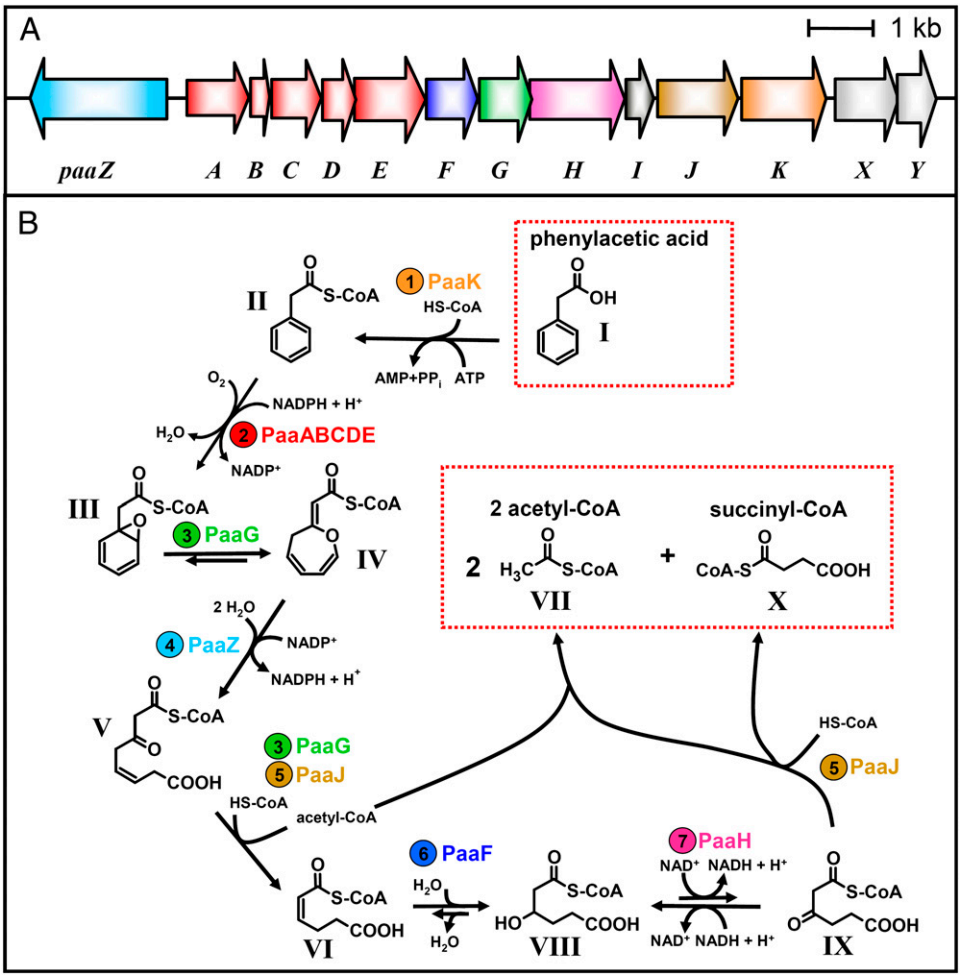

Fig. 1. Aerobic phenylacetate catabolic pathway. (*A*) Catabolic gene cluster for phenylacetate degradation in *E. coli* K12. (*B*) Reactions and intermediates of the pathway as studied in *E. coli* K12 and *Pseudomonas* sp. strain Y2. Proposed enzyme names (Table S1): 1: phenylacetate-CoA ligase (AMP forming); 2: ring 1,2-phenylacetyl-CoA epoxidase (NADPH); 3: ring 1,2-epoxyphenylacetyl-CoA isomerase (oxepin-CoA forming), postulated 3,4-dehydroadipyl-CoA isomerase. 4: oxepin-CoA hydrolase/ 3-oxo-5,6-dehydrosuberyl-CoA semialdehyde dehydrogenase ($NADP^+$); 5: 3-oxoadipyl-CoA/ 3-oxo-5,6-dehydrosuberyl-CoA thiolase; 6: 2,3-dehydroadipyl-CoA hydratase; 7: 3-hydroxyadipyl-CoA dehydrogenase (NAD^+) (probably (*S*)-3-specific). Compounds: I, phenylacetate; II, phenylacetyl-CoA; III, ring 1,2-epoxyphenylacetyl-CoA; IV, 2-oxepin-2(3H)-ylideneacetyl-CoA; V, 3-oxo-5,6-dehydrosuberyl-CoA, VI, 2,3-dehydroadipyl-CoA; VII, acetyl-CoA; VIII, 3-hydroxyadipyl-CoA; IX, 3-oxoadipyl-CoA; X, succinyl-CoA.

simultaneously with PaaA as a maltose-binding protein (mbp)-tagged protein. Purification carried out through amylose resin chromatography. Subunits PaaB, PaaC, and PaaE copurified with PaaA, whereas PaaD, a suspected subunit of this complex, eluted separately. The purified PaaABCE complex catalyzed a phenylacetyl-CoA–induced and strictly oxygen-dependent NADPH consumption in a spectrophotometric assay with ≈1 μmol min^{-1} mg^{-1} protein, implying phenylacetyl-CoA oxygenase/reductase activity. The product derived from phenylacetyl-CoA was highly unstable and was purified using reverse phase (RP)-HPLC (Fig. 2*A* and *B*). Mass spectrometry showed the incorporation of a single oxygen atom, suggesting the complex acts as a monooxygenase (Table 1). To identify PaaABC(D)E unambiguously as an oxygenase, we applied ^{18}O-labeling studies and showed the incorporation of a single ^{18}O atom from $^{18}O_2$ (Table 1). In contrast, no labeled oxygen was introduced to the ring when $H_2{}^{18}O$ was used in the assay, clearly demonstrating the monooxygenase activity of PaaABC(D)E. ^{13}C-NMR analysis substantiated ring 1,2-epoxyphenylacetyl-CoA (Fig. 1*B*, compound III) as the product (Table 2). Further proof was provided by derivatization of the epoxide (Table 1). PaaABC(D)E thus functions as a ring 1,2-phenylacetyl-CoA epoxidase. Interestingly, the ability of PaaABC(D)E to oxygenate its substrate was largely lost within 5 min in an enzymatic assay. The multicomponent oxygenase thus appears to have been inactivated by its reactive epoxide product. To test a possible impact of PaaD on phenylacetyl-CoA oxygenation, we added purified mbp-tagged PaaD separately to the activity tests. The addition of the preparation did not affect the specific activity of PaaABCE significantly.

C–C Bond Cleavage Through Isomerization. The *paaG* and *paaZ* knockout mutants of *E. coli* released 2-hydroxyphenylacetate when cultivated on phenylacetate (14), probably because of the decomposition of ring 1,2-epoxyphenylacetyl-CoA. Therefore these genes probably are involved in early steps of the pathway. We purified his-tagged PaaG and found that it catalyzed a reversible conversion of the epoxide to a new product (Fig. 2*C*) that exhibited the same mass as the epoxide (Table 1). ^{13}C-NMR spectroscopy identified this isomer as an unusual unsaturated, oxygen-containing, seven-member heterocyclic enol ether, namely 2-oxepin-2(3H)-ylideneacetyl-CoA (oxepin-CoA) (Fig. 1*B*, compound IV and Table 2). PaaG therefore acted as a ring 1,2-epoxyphenylacetyl-CoA isomerase (oxepin-CoA–forming).

Table 1. Mass spectrometry data of intermediates of the phenylacetic acid catabolic pathway

Enzyme	Product	MH^+ (monoisotopic) Measured	Calculated
PaaABC(D)E	Ring 1,2-epoxyphenylacetyl-CoA (III)	902.158	902.159
	^{18}O-labeled ring 1,2-epoxyphenylacetyl-CoA (III)	904.164	904.164
	N,N-diethyldithiocarbamate derivative of (III)	1,051.192	1,051.193
PaaG	Oxepin-CoA (IV)	902.160	902.159
PaaZ	3-Oxo-5,6-dehydrosuberyl-CoA (V)	936.164	936.165
PaaJ	Acetyl-CoA (VII)	810.133	810.133
PaaJ/PaaG	2,3-Dehydroadipyl-CoA (VI)	894.154	894.154
PaaF	3-Hydroxyadipyl-CoA (VIII)	912.165	912.165
PaaJ	Succinyl-CoA (X)	868.139	868.139

Roman numerals indicate the compounds in Fig. 1*B*.

Hydrolytic Ring Cleavage of the C–O Heterocycle. PaaZ is the most likely candidate for the ring-cleavage reaction. It is a fusion protein consisting of a C-terminal MaoC-like domain and an N-terminal aldehyde dehydrogenase domain. MaoC-like Hotdog-fold proteins act as *R*-specific hydratases (22). Addition of purified mbp-tagged PaaZ to enzymatically produced epoxide and oxepin (in the presence of PaaG) led to a complete $NADP^+$-dependent conversion of epoxide and oxepin into 3-oxo-5,6-dehydrosuberyl-CoA (Fig. 1*B*, compound V, and Fig. 2*D*), as shown by mass spectrometry (Table 1) and ^{13}C-NMR spectroscopy (Table 2). PaaZ thus functioned as an oxepin-CoA hydrolase/ 3-oxo-5,6-dehydrosuberyl-CoA semialdehyde dehydrogenase catalyzing the two-step conversion of the oxepin-CoA via the open-chain aldehyde intermediate to 3-oxo-5,6-dehydrosuberyl-CoA (Fig. 1*B*). This oxepin-CoA ring cleavage was affected by PaaZ with a specific activity of ≈20 μmol min^{-1} mg^{-1} protein.

β-Oxidation of the Ring-Opened Intermediates. PaaJ, a β-ketothiolase, probably transforms the resulting β-keto C_8 intermediate with CoA to the C_6 intermediate dehydroadipyl-CoA (Fig. 1*B*, compound VI) and acetyl-CoA (Fig. 1*B*, compound VII) (14), besides catalyzing the last step of the pathway, in which 3-oxoadipyl-CoA similarly is cleaved to acetyl-CoA and succinyl-CoA. This last function has been shown previously (19). To test if the C_8 compound also is a substrate for the β-ketothiolase, we added purified PaaJ to an enzymatic assay containing PaaABC(D)E, PaaG, and PaaZ. A complete conversion of 3-oxo-5,6-dehydrosuberyl-CoA into 2,3-dehydroadipyl-CoA and acetyl-CoA (Fig. 1*B*) with accompanying CoA consumption was demonstrated by RP-HPLC (Fig. 2*E*). The identity of the products was confirmed by mass spectrometry (Table 1) and ^{13}C-NMR spectroscopy (Table 2).

PaaF is an enoyl-CoA hydratase. Purified PaaF, when added to the assay (Fig. 2*E*), catalyzed the reversible conversion of enzymatically produced 2,3-dehydroadipyl-CoA into 3-hydroxyadipyl-CoA (Fig. 1*B*, compound VIII and Fig. 2*F*), evidenced by mass spectrometry (Table 1). This analysis identified PaaF as a 2,3-dehydroadipyl-CoA hydratase (Fig. 1*B*), in accordance with previous suggestions (14).

PaaH is a member of the alcohol dehydrogenase family. To clarify the last steps of the aerobic phenylacetate pathway, we added purified mbp-tagged PaaH to enzymatically produced 3-hydroxyadipyl-CoA. We observed the formation of NADH, succinyl-CoA, and acetyl-CoA in the presence of CoA, NAD^+, and PaaJ (Fig. 2*G*). PaaH therefore functioned as an NAD^+-dependent [probably (*S*)-3–specific] 3-hydroxyadipyl-CoA dehydrogenase forming 3-oxoadipyl-CoA (Fig. 1*B*, compound IX). PaaJ finally cleaved 3-oxoadipyl-CoA into the central metabolites succinyl-CoA (Fig. 1*B*, compound X) and acetyl-CoA (Fig. 1*B*, compound VII), completing the pathway.

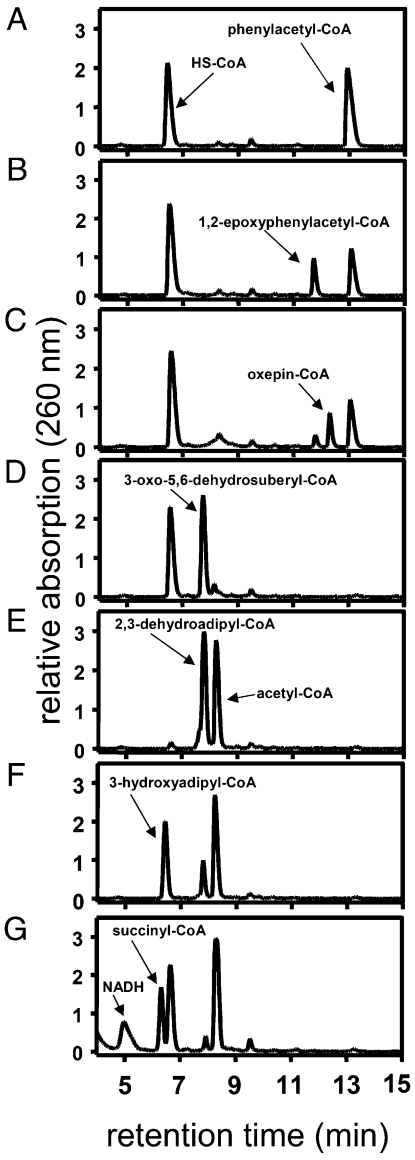

Fig. 2. Conversion of phenylacetyl-CoA to succinyl-CoA and acetyl-CoA at 30 °C by heterologously produced enzymes of the phenylacetate pathway. The initial reaction mixture (0.25 mL) contained 50 mM Tris-HCl (pH 8.0), 0.5 mM CoA, 1 mM NADPH, 1 mM $NADP^+$, and 0.8 mg of PaaABC(D)E. The reaction was started by addition of 0.5 mM phenylacetyl-CoA. The products were separated by RP-HPLC and detected at 260 nm. (*A*) Control without PaaABC(D)E. (*B*) Three minutes after substrate addition (ring 1,2-epoxyphenylacetyl-CoA). (*C*) Three minutes after addition of 10 μg PaaG (oxepin-CoA). Because of an inactivation of the enzymes (likely through nonconverted epoxides), a second reaction mixture (0.4 mL) was used for the following samples. From the start of the reaction it contained PaaABC(D)E, as above, and 10 μg of PaaG and 30 μg of PaaZ. (*D*) After 2 min (3-oxo-5,6-dehydrosuberyl-CoA). (*E*) Four minutes after addition of 7 μg PaaJ (2,3-dehydroadipyl-CoA and acetyl-CoA, consumption of CoA). (*F*) Three minutes of incubation with 2 μg of PaaF (3-hydroxyadipyl-CoA). (*G*) Three minutes of incubation with 16 μg of PaaH, 0.5 mM CoA, and 1 mM NAD^+ (succinyl-CoA and acetyl-CoA). The PaaH product 3-oxoadipyl-CoA is directly converted by PaaJ and therefore is not shown.

Discussion

Unorthodox Aerobic Aromatic Degradation Strategy. This paradigm for aerobic aromatic catabolism differs significantly from estab-

Table 2. ^{13}C-NMR data of products derived from [U-^{13}C] phenylacetyl-CoA

Compound position	Chemical shift [ppm] δ^{13}C Observed	Predicted	Coupling constants [Hz] J_{cc}
[U-^{13}C]ring 1,2-epoxy-phenylacetyl-CoA (III)			
1	195.3 (d)	198	46.1 (**2**)
2	38.4 (t, broad)	48	45 (1, 3)
3	48.7 (m, broad)	59	
4	48.7 (m, broad)	59	
5	114–128 (m)	131	
6	114–128 (m)	128	
7	114–128 (m)	125	
8	114–128 (m)	135	
[U-^{13}C]oxepin-CoA (IV)			
1	187.5 (dd)	187	66.5 (**2**), 4.0 (**9**)
2	102.2 (ddd)	103	80.8 (3), 66.5 (**1**), 6.0
3	162.8 (dd)	172	80.8 (2), 41.5 (9)
4	34.3 (t, broad)	37	41 (**8**, **3**)
5	127.9 (dd)	125	67.2 (7), 37.4 (9)
6	125.8 (dd)	129	67.2 (**8**), 52.4 (6)
7	109.5 (dd)	106	74.8 (**5**), 52.4 (7)
8	142.9 (d)	141	74.8 (**6**)
[U-^{13}C]3-oxo-5,6-dehydrosuberyl-CoA (V)			
1	195.6 (d)	196	46.7 (**2**)
2	55.3 (broad)	55	n.d.
3	205.4 (t)	206	38.1 (**4**, 2)
4	41.0 (broad)	43	n.d.
5	122.2 (dd)	125	71.8 (**6**), 43.2 (**3**)
6	128.2 (dd)	125	71.8 (**5**), 43.2 (**7**)
7	35.3 (dd)	38	51.5 (**8**), 42.8 (**6**)
8	179.7(d)	176	51.5 (**7**)
[U-^{13}C]2,3-dehydro- adipyl-CoA (VI)			
1	189.3 (dd)	187	61.5 (**2**), 6.3
2	127.9 (ddd)	132	69.8 (**3**), 61.5 (**1**), 3.3
3	146.1 (dd)	151	69.4 (**2**), 41.3 (**4**)
4	28.1 (ddd)	28	41.1 (**3**), 34.3 (**5**), 6.5
5	34.4 (dd)	35	52.6 (**6**), 34.1 (**4**)
6	178.2 (dd)	177	52.2 (**5**), 3.0
[U-^{13}C]acetyl-CoA (VII)			
1	195.5 (d)	196	47.2 (**2**)
2	29.1 (d)	27	47.2 (**1**)

The roman numerals indicate the compounds in Fig. 1*B*. Coupling constants caused by $^{13}C^{13}C$ coupling (Jcc) to the indexed atom are indicated in parentheses. Numbers in bold type indicate coupling pairs observed in the INADEQUATE spectrum. The signals are designated as follows: d, doublet; dd, double-doublet; ddd, double-doublet-doublet; m, multiplet; n.d., not determined; t, triplet. For numbering of C-atoms see Fig. 3.

lished knowledge. The first enzyme of the pathway, phenylacetate-CoA ligase, converted phenylacetate into phenylacetyl-CoA (Fig. 1*B*). All further intermediates likewise were processed as CoA-thioesters, a feature typical of anaerobic rather than aerobic aromatic metabolism. The identification of ring 1,2-epoxide as the second pathway intermediate is contrary to the formerly assumed dioxygenation of phenylacetyl-CoA to a dihydrodiol by a dioxygenase/reductase (14), a process that would have resembled conventional aerobic aromatic catabolism. Previous *in silico* studies designated PaaABC(D)E as the first member of a group of the family of bacterial di-iron multicomponent oxygenases acting on CoA-esters (21). This enzyme family consists exclusively of monooxygenases [e.g., methane monooxygenase (23)], which also may form epoxides. The subunits PaaAC(D) probably act as the core oxygenase complex, because similar multicomponent oxygenases have been reported (24). PaaB probably acts as an effector protein, whereas PaaE likely functions as an iron-sulfur oxidoreductase transferring electrons from NADPH to the active center of the oxygenase complex (24).

PaaD has been suggested to be part of the oxygenase complex (21), possibly acting as a stabilizing element (24). Alternatively, PaaD might not be a part of the catalytic complex, which may consist of two subunits instead of three, as known to occur in other members of the family (24). Sequence comparisons with the National Center for Biotechnology Information (NCBI) database revealed the similarity of PaaD to proteins of the SUF operon. Proteins encoded by this operon are involved in the assembly of iron-sulfur clusters (25). The *paaD*-related gene is adjacent to the cysteine desulfurase gene *sufS*. A role in the assembly of iron-sulfur clusters is likely, although the actual function is unknown. PaaD therefore may take part in the formation of the predicted iron-sulfur cluster of the reductase subunit PaaE. Further studies are required to resolve this question.

The ring 1,2-epoxyphenylacetyl-CoA isomerase PaaG is a member of the versatile crotonase superfamily that catalyzes very different metabolic reactions (26). It shares greatest similarity with Δ^3,Δ^2-enoyl-CoA isomerases, containing a single conserved aspartic acid in the active center acting as a proton relay (27). We therefore suggest the following mechanism for the isomerization of the ring 1,2-epoxide to the oxepin (Fig. 1*B*, Fig. 3*A*): PaaG mediates the formation and stabilization of the enolate form by abstracting a proton from the side chain at C2 of ring 1,2-epoxyphenylacetyl-CoA. Addition of the abstracted proton to C8 (becoming C4, as shown in Fig. 3*A*) of the ring leads to a rearrangement of the double bonds and results in a C–C cleavage of the two epoxy–C–O bonds, yielding the oxepin. Spontaneous equilibrium between benzene-epoxide and the respective oxepin was observed more than 40 y ago (28). In this case, a similar but enzyme-mediated epoxide–oxepin equilibrium is involved in a biochemical pathway. The electron-withdrawing character of the CoA thioester facilitates the abstraction of a proton from C2 of the side chain and stabilizes the resulting double bond of the side chain of the oxepin compound.

Ring opening probably is catalyzed by the MaoC domain of PaaZ, because oxepins are susceptible to hydrolysis. The MaoC domain introduces water at the double bond of the side chain. We propose that this attack may be facilitated by the mesomerism of the oxepin compound resulting in a positively charged ether O atom and an enolate anion of the CoA-thioester (Fig. 3*B*). This structure enables the addition of a proton to C2 and of a hydroxyl group to C3, respectively. The proton of the attached hydroxyl group dissociates, thus forming a keto group with concomitant ring cleavage between C3 and the ether O atom. The resulting ring-opened C_8 alcohol rearranges through keto-enol tautomerism to the aldehyde, yielding 3-oxo-5,6-dehydrosuberyl-CoA semialdehyde (Fig. 3*B*). The aldehyde group is oxidized further to the respective carboxylic acid by the aldehyde dehydrogenase domain under formation of NADPH (Fig. 1*B*). Again, the hydrolytic rather than oxygenolytic cleavage is uncommon for aerobic aromatic catabolism and is achieved through an unprecedented reaction.

The next β-oxidation steps leading to acetyl-CoA and succinyl-CoA were catalyzed by β-ketothiolase PaaJ, enoyl-CoA hydratase PaaF, and alcohol dehydrogenase PaaH (Fig. 1*B*). It should be stressed that the expected product of a thiolytic cleavage of 3-oxo-5,6-dehydrosuberyl-CoA by PaaJ ought to be 3,4-dehydroadipyl-CoA, rather than the observed 2,3-dehydro compound. This 3,4–2,3 isomerization reaction probably was catalyzed by enoyl-CoA isomerase PaaG, which was present in the assay. However, PaaJ itself also may carry out this reaction, because intrinsic isomerase activity has been described for rat liver thiolase (29). Note also that the cis configuration of 2,3-dehydroadipyl-CoA (Fig. 1*B*, compound VI) is hypothetical, because its stereochemistry has not yet been determined. PaaF, like PaaG, is a member of the crotonase superfamily, but sequence comparison reveals a signif-

icant difference between these two members of this superfamily. Whereas the isomerase PaaG shows a conserved aspartic acid, PaaF lacks this aspartic acid but harbors two strictly conserved glutamic acids, which have been shown to be essential in the incorporation or elimination of water in rat enoyl-CoA hydratase (26) (Fig. S1).

In total, the formation of two molecules of acetyl-CoA and one molecule of succinyl-CoA from phenylacetate follows the equation: 1 phenylacetic acid +1 O_2 + 2 H_2O + 3 CoA + 1 ATP + 1 $NAD^+ \rightarrow$ 2 acetyl-CoA + 1 succinyl-CoA + 1 AMP + 1 PP_i + 1 NADH + 1 H^+.

Functions of Other Gene Products of the *Paa* Cluster. PaaI is predicted to be a thioesterase, and its gene is part of the conserved *paa* gene cluster. It has been shown that PaaI acts on hydroxylated phenylacetyl-CoA substrates but poorly on aliphatic CoA-esters; i.e., it acts preferentially on early rather than late intermediates of the pathway (30). Natural substrates thus could be ring 1,2-epoxyphenylacetyl-CoA, the subsequent oxepin-CoA [PaaABC(D)E and PaaG products], or the breakdown product 2-hydroxyphenylacetyl-CoA. Because of the instability of the epoxide, such a salvage reaction could be vital for the bacteria to avoid CoA depletion resuling from the formation of the dead-end product 2-hydroxyphenylacetyl-CoA. PaaX has been shown to act as a transcriptional repressor in *E. coli*, with phenylacetyl-CoA as a specific inducer that prevents PaaX from binding its target sequences (31). PaaY is a protein of unknown function resembling acetyltransferases. Because it is located within the same transcriptional unit as PaaX, a role in regulation seems likely, possibly by inactivating PaaK, the phenylacetate-CoA ligase, through acetylation. A similar mechanism recently has been shown for anaerobic benzoate-CoA ligase in *Rhodopseudomonas palustris* (32). PaaY therefore may down-regulate phenylacetate degradation on the enzymatic level, allowing rapid metabolic adjustment in response to increased acetyl-CoA levels.

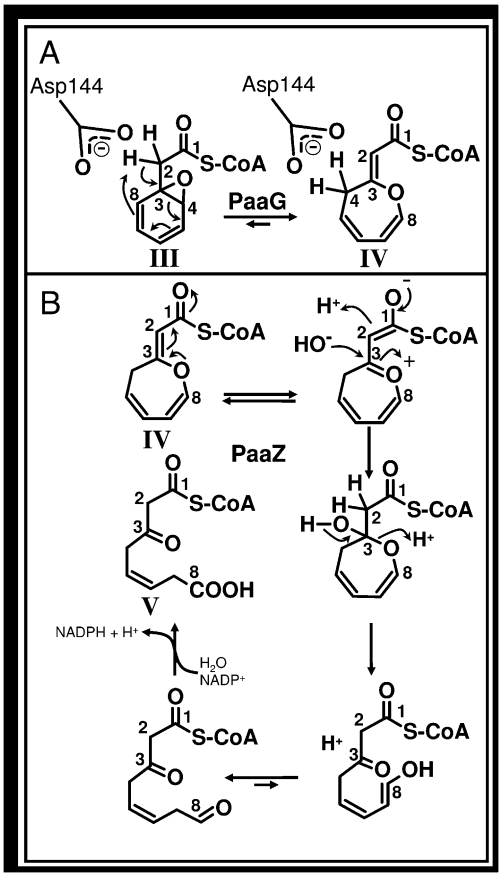

Fig. 3. (*A*) Proposed PaaG-catalyzed ring expansion (oxepin-CoA–forming) through isomerization. Note that the numbering of ring C atoms changes through oxepin-CoA formation. (*B*) Proposed hydrolytic ring cleavage of oxepin-CoA and subsequent aldehyde oxidation by fusion protein PaaZ. The roman numerals indicate the compounds in Fig. 1*B*.

CoA-Thioesters in Aerobic Aromatic Catabolism. Why are the intermediates of this pathway processed as CoA-thioesters? Anaerobic catabolism profits from the electron-withdrawing character of thioesters, which facilitates ATP-dependent or other energy-driven reduction of the aromatic system. In aerobic catabolism, oxygen is used to dearomatize and cleave the ring. Use of CoA-esters in an aerobic aromatic degradation pathway therefore may appear futile at first glance. However, the PaaG-mediated oxepin formation is facilitated by the CoA-ester, as discussed above. Furthermore, CoA-bound intermediates are retained within the cell and can be recognized rapidly and bound through CoA-binding motives of the processing enzymes. This rapid recognition and binding could be particularly important for the highly reactive intermediate ring 1,2-epoxyphenylacetyl-CoA, because free epoxides would harm the cell.

Virulence Through Reactive Pathway Intermediates. Recently, connections between virulence and gene products of the phenylacetate catabolism have been shown in *Burkholderia cenocepacia* and have been proposed in *Mycobacterium abscessus (33–35)*; both organisms cause severe and often fatal lung infections in cystic fibrosis or immunocompromised patients. The *paaE* knockout mutants of *B. cenocepacia* were unable to survive in a rat pulmonary infection model (34), and knockout of *paaA* or *paaE* genes led to an attenuated phenotype in a *Caenorhabditis elegans* model. In contrast, knockout of *paaZ* or *paaF* increased virulence slightly (35). Therefore, accumulation of early products of phenylacetate degradation probably has toxic effects on the host. Ring-1,2-epoxide and its phenolic breakdown product 2-hydroxyphenylacetate are obvious candidates for causing such damage. It is an intriguing question whether secretion of such substances contributes to host cell death, which would supply feed stock for the pathogen. Arguments for the toxicity of these compounds come from the very similar chemistry of benzene metabolism in humans. Cytochromes P450 are known to metabolize benzene to benzene-epoxide that equilibrates spontaneously with the oxepin. Further ring opening yields reactive aldehydes (36). Those compounds and also the spontaneous phenolic breakdown product of benzene-oxide are known to be reactive toward proteins and DNA and are potentially carcinogenic (37). Even more intriguing is the demonstration that catabolic gastrointestinal activities of gut bacteria leading to the formation of phenols are causal factors for leukemia (38). Therefore phenylacetate catabolism of *E. coli* and other (micro)aerobic gut bacteria may contribute to forms of de novo leukemia.

Relevance and Distribution of the Reported Strategy. Is this pathway ephemeral or is it of major importance? It is the only proven pathway for aerobic phenylacetate degradation in bacteria, a central key intermediate of aromatic catabolism; moreover, phenylalanine generally is metabolized via phenylacetate. Conserved genes of the phenylacetate pathway have been identified thus far in 16% of the sequenced bacterial species, strongly indicating its relevance (Fig. S2). These species include many members of Proteobacteria, such as the model organisms *E. coli* or *P. putida* (13, 15), pathogens such as *Bordetella pertussis, Shigella dysenteriae*, or opportunistic pathogens such as *B. cenocepacia*. Gram-positive organisms such as *Rhodococcus sp.* or members of the *Deinococcus/Thermus* group also make use of this route (6, 7) (Fig. S2). This pathway evolved in the bacterial domain, because the

only Archaea harboring key genes of the pathway are some members of the Halobacteria, which may have acquired a multitude of bacterial genes (39).

Is this strategy restricted to the metabolism of phenylacetate? We suppose that a similar type of aerobic benzoate metabolism also involves CoA thioesters, ring epoxy, and possibly oxepin intermediates as well as a hydrolytic ring cleavage. This benzoate pathway occurs in many bacteria, showing again the wide usage of the strategy (40). Therefore, this paradigm for aromatic degradation probably is widespread and closes a substantial gap in our understanding of how microorganisms exploit inert substrates and how metabolism may trigger virulence in certain pathogens.

Materials and Methods

Recombinant enzymes from *Escherichia coli* (PaaZ, PaaH) or *Pseudomonas* sp. strain Y2 (PaaABC(D)E, PaaG, PaaJ, PaaF) were tagged with His_6, His_{10}, or mbp and purified accordingly via Ni^{2+}-affinity or amylose resin chromatography.

Labeled [U-^{14}C, U-^{13}C]phenylacetate was derived from L-[U-^{14}C]phenylalanine or L-[U-^{13}C,^{15}N]phenylalanine, and labeled or unlabeled phenylacetyl-CoA was synthesized chemically from the activated precursor phenylacetyl succinimide or enzymatically using phenylacetate-CoA ligase.

Enzymatic activity of PaaABC(D)E was determined at 30 °C by following NADPH consumption in a photometric assay at 365 nm after the addition of phenylacetyl-CoA. In addition, activities and product formation of PaaABC(D)E, PaaG, PaaZ, PaaJ, PaaF, and PaaH were tested by RP-HPLC with optical detection between 200–400 nm. A C18-E column was used to separate CoA-esters using an acetonitrile gradient in ammonium acetate buffer. The initial reaction mixture (0.25 mL) contained 50 mM Tris-HCl (pH 8.0), 0.5 mM CoA, 1 mM NADPH, 1 mM $NADP^+$, and 0.8 mg PaaABC(D)E. The reaction was started by the addition of 0.5 mM phenylacetyl-CoA. When the effect of PaaD on phenylacetyl-CoA transformation was tested, the assay included in addition 0.1 mg of mbp-tagged PaaD. Further enzymes were added step by step.

For mass spectrometry, fractions of product peaks from RP-HPLC runs were injected into a Fourier transform ion cyclotron resonance mass spectrometer. ^{13}C-labeled intermediates were acquired through enzymatic conversions of [U-^{13}C]-phenylacetyl-CoA by PaaABC(D)E, PaaG, PaaZ, and PaaJ. Labeled intermediates were prepared by RP-HPLC and subsequently analyzed by ^{13}C-NMR using a Bruker DRX 500 spectrometer.

The BLASTP searches were performed via the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) with the amino acid sequences of PaaA and PaaC as queries against assembled bacterial genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). A BLASTP search was performed with PaaA and PaaC against 640 completely sequenced bacterial genomes.

The exact procedures of cloning and heterologous expression of genes from *E. coli* K12 and *Pseudomonas* sp. strain Y2, enzyme purification, enzyme measurements, syntheses, identification of compounds, and all other methods are described in *SI Materials and Methods*. For primers and plasmids see Table S2.

ACKNOWLEDGMENTS. We thank Magdy Mohamed and Gertjan Heijman for their contributions during the initial stages of this work and Bettina Knapp for mass spectrometry measurements. We also thank Christine Schwarz, Nasser Gad'on, and Christa Ebenau-Jehle for technical assistance and Ivan Berg and Sophie Sp. Phillips for critical reading. This work was supported by Deutsche Forschungsgemeinschaft (G. F., W.E., and W.H.) and Spanish Ministry of Education and Science Project BFU2006-15214-C03-02.

1. Fuchs G (2008) Anaerobic metabolism of aromatic compounds. *Ann N Y Acad Sci* 1125: 82–99.
2. Wang YZ, Lipscomb JD (1997) Cloning, overexpression, and mutagenesis of the gene for homoprotocatechuate 2,3-dioxygenase from *Brevibacterium fuscum*. *Protein Expr Purif* 10:1–9.
3. Gibson DT, Parales RE (2000) Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr Opin Biotechnol* 11:236–243.
4. Boll M, Fuchs G (1995) Benzoyl-coenzyme A reductase (dearomatizing), a key enzyme of anaerobic aromatic metabolism. ATP dependence of the reaction, purification and some properties of the enzyme from *Thauera aromatica* strain K172. *Eur J Biochem* 234:921–933.
5. Luengo JM, García JL, Olivera ER (2001) The phenylacetyl-CoA catabolon: A complex catabolic unit with broad biotechnological applications. *Mol Microbiol* 39:1434–1442.
6. Erb TJ, Ismail W, Fuchs G (2008) Phenylacetate metabolism in thermophiles: Characterization of phenylacetate-CoA ligase, the initial enzyme of the hybrid pathway in *Thermus thermophilus*. *Curr Microbiol* 57:27–32.
7. Navarro-Llorens JM, et al. (2005) Phenylacetate catabolism in *Rhodococcus* sp. strain RHA1: A central pathway for degradation of aromatic compounds. *J Bacteriol* 187: 4497–4504.
8. Arias-Barrau E, et al. (2004) The homogentisate pathway: A central catabolic pathway involved in the degradation of L-phenylalanine, L-tyrosine, and 3-hydroxyphenylacetate in *Pseudomonas putida*. *J Bacteriol* 186:5062–5077.
9. Rodríguez-Sáiz M, et al. (2001) Reduced function of a phenylacetate-oxidizing cytochrome p450 caused strong genetic improvement in early phylogeny of penicillin-producing strains. *J Bacteriol* 183:5465–5471.
10. Kunita N (1955) Bacterial oxidation of phenylacetic acid. I. The pathway through homoprotocatechuic acid. *Med J Osaka Univ* 6:697–702.
11. Martínez-Blanco H, Reglero A, Rodriguez-Aparicio LB, Luengo JM (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.
12. Vitovski S (1993) Phenylacetate-coenzyme A ligase is induced during growth on phenylacetic acid in different bacteria of several genera. *FEMS Microbiol Lett* 108:1–5.
13. Olivera ER, et al. (1998) Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: The phenylacetyl-CoA catabolon. *Proc Natl Acad Sci USA* 95:6419–6424.
14. Ismail W, et al. (2003) Functional genomics by NMR spectroscopy. Phenylacetate catabolism in *Escherichia coli*. *Eur J Biochem* 270:3047–3054.
15. Ferrández A, et al. (1998) Catabolism of phenylacetic acid in *Escherichia coli*. Characterization of a new aerobic hybrid pathway. *J Biol Chem* 273:25974–25986.
16. Di Gennaro P, et al. (2007) Styrene lower catabolic pathway in *Pseudomonas fluorescens* ST: Identification and characterization of genes for phenylacetic acid degradation. *Arch Microbiol* 188:117–125.
17. Bartolomé-Martín D, et al. (2004) Characterization of a second functional gene cluster for the catabolism of phenylacetic acid in *Pseudomonas* sp. strain Y2. *Gene* 341: 167–179.
18. Rost R, Haas S, Hammer E, Herrmann H, Burchhardt G (2002) Molecular analysis of aerobic phenylacetate degradation in *Azoarcus evansii*. *Mol Genet Genomics* 267: 656–663.
19. Nogales J, et al. (2007) Characterization of the last step of the aerobic phenylacetic acid degradation pathway. *Microbiology* 153:357–365.
20. Mohamed Mel-S, Ismail W, Heider J, Fuchs G (2002) Aerobic metabolism of phenylacetic acids in *Azoarcus evansii*. *Arch Microbiol* 178:180–192.
21. Fernández C, Ferrández A, Miñambres B, Díaz E, García JL (2006) Genetic characterization of the phenylacetyl-coenzyme A oxygenase from the aerobic phenylacetic acid degradation pathway of *Escherichia coli*. *Appl Environ Microbiol* 72:7422–7426.
22. Park SJ, Lee SY (2003) Identification and characterization of a new enoyl coenzyme A hydratase involved in biosynthesis of medium-chain-length polyhydroxyalkanoates in recombinant *Escherichia coli*. *J Bacteriol* 185:5391–5397.
23. Murray LJ, Lippard SJ (2007) Substrate trafficking and dioxygen activation in bacterial multicomponent monooxygenases. *Acc Chem Res* 40:466–474.
24. Notomista E, Lahm A, Di Donato A, Tramontano A (2003) Evolution of bacterial and archaeal multicomponent monooxygenases. *J Mol Evol* 56:435–445.
25. Takahashi Y, Tokumoto U (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. *J Biol Chem* 277:28380–28383.
26. Holden HM, Benning MM, Haller T, Gerlt JA (2001) The crotonase superfamily: Divergently related enzymes that catalyze different reactions involving acyl coenzyme a thioesters. *Acc Chem Res* 34:145–157.
27. Kichise T, Hisano T, Takeda K, Miki K (2009) Crystal structure of phenylacetic acid degradation protein PaaG from *Thermus thermophilus* HB8. *Proteins* 76:779–786.
28. Vogel E, Günther H (1967) Benzene oxide-oxepin valence tautomerism. *Angew Chem Int Ed Engl* 6:385–401.
29. Wu L, et al. (2007) Inactivation of thiolase by 2-alkynoyl-CoA via its intrinsic isomerase activity. *Org Lett* 9:3877–3880.
30. Song F, et al. (2006) Structure, function, and mechanism of the phenylacetate pathway hot dog-fold thioesterase PaaI. *J Biol Chem* 281:11028–11038.
31. Ferrández A, García JL, Díaz E (2000) Transcriptional regulation of the divergent paa catabolic operons for phenylacetic acid degradation in *Escherichia coli*. *J Biol Chem* 275:12214–12222.
32. Crosby HA, Heiniger EK, Harwood CS, Escalante-Semerena JC (2010) Reversible N epsilon-lysine acetylation regulates the activity of acyl-CoA synthetases involved in anaerobic benzoate catabolism in *Rhodopseudomonas palustris*. *Mol Microbiol* 76:874–888.
33. Ripoll F, et al. (2009) Non mycobacterial virulence genes in the genome of the emerging pathogen *Mycobacterium abscessus*. *PLoS ONE* 4:e5660.
34. Hunt TA, Kooi C, Sokol PA, Valvano MA (2004) Identification of *Burkholderia cenocepacia* genes required for bacterial survival in vivo. *Infect Immun* 72:4010–4022.
35. Law RJ, et al. (2008) A functional phenylacetic acid catabolic pathway is required for full pathogenicity of *Burkholderia cenocepacia* in the *Caenorhabditis elegans* host model. *J Bacteriol* 190:7209–7218.
36. Monks TJ, Butterworth M, Lau SS (2010) The fate of benzene-oxide. *Chem Biol Interact*, 184:201–206 10.1016/j.cbi.2009.12.025.
37. Golding BT, Watson WP (1999) Possible mechanisms of carcinogenesis after exposure to benzene. *IARC Sci Publ* 150:75–88.
38. McDonald TA, Holland NT, Skibola C, Duramad P, Smith MT (2001) Hypothesis: Phenol and hydroquinone derived mainly from diet and gastrointestinal flora activity are causal factors in leukemia. *Leukemia* 15:10–20.
39. Kennedy SP, Ng WV, Salzberg SL, Hood L, DasSarma S (2001) Understanding the adaptation of *Halobacterium* species NRC-1 to its extreme environment through computational analysis of its genome sequence. *Genome Res* 11:1641–1650.
40. Rather LJ, Knapp B, Haehnel W, Fuchs G (2010) Coenzyme A-dependent aerobic metabolism of benzoate via epoxide formation. *J Biol Chem* 285:20615–20624.