A Novel Pathway of Aerobic Benzoate Catabolism in the Bacteria Azoarcus evansii and Bacillus stearothermophilus*

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The aerobic catabolism of benzoate was studied in the Gram-negative proteobacterium Azoarcus evansii and in the Gram-positive bacterium Bacillus stearothermophilus. In contrast to earlier proposals, benzoate was not converted into hydroxybenzoate or gentisate. Rather, benzoyl-CoA was a product of benzoate catabolism in both microbial species under aerobic conditions in vivo. Benzoyl-CoA was converted into various CoA thioesters by cell extracts of both species in oxygen- and NADPH-dependent reactions. Using [ring-13C6]benzoyl-CoA as substrate, cis-3,4-[2,3,4,5,6-13C5]dehydrodipicolinate-CoA, trans-2,3-[2,3,4,5,6-13C5]dehydrodipicolinate-CoA, the 3,6-lactone of 3-[2,3,4,5,6-13C5]2-hydroxydipicolinate-CoA, and 3-[2,3,4,5,6-13C5]2-hydroxydipicolinate-CoA were identified as products by NMR spectroscopy. A protein mixture of A. evansii transformed [ring-13C6]benzoyl-CoA in an NADPH- and oxygen-dependent reaction into 6-[2,3,4,5,6-13C5]2-hydroxy-3-hexenoyl-CoA. The data suggest a novel aerobic pathway of benzoate catabolism via CoA intermediates leading to β-ketoacyl-CoA, an intermediate of the known β-ketoacyl path.

The aerobic metabolism of benzoate (compound 1) (Fig. 1) in bacteria has been studied in considerable detail (for a recent review, see Ref. 1). Catechol (1,2-dihydroxybenzene, compound 3) and protocatechuate (3,4-dihydroxybenzene, compound 2) were identified as early intermediates. Both compounds serve as substrates for dioxygenases that cleave the aromatic ring between the hydroxyl groups, leading to 3-ketoacyl (compound 4), which is converted into succinyl-CoA (compound 6) and acetyl-CoA (compound 7) via compound 5 (ortho-cleavage pathway) (Fig. 1).

However, some observations could not be explained by the established mechanisms. Thus, cell extracts of some Bacillus spp. grown on benzoate (compound 1) or 3-hydroxybenzoate (compound 9) utilized gentisate (compound 11), but not catechol (compound 3) or protocatechuate (compound 2) (Fig. 2). In an attempt to explain these findings, hydroxylation of benzoate (compound 1) affording 3-hydroxybenzoate (compound 9) (Fig. 2) and gentisate (compound 11) was proposed (2–4). However, direct evidence for the suggested intermediates and reactions has not been obtained up to now.

More recently, it was shown that the Gram-positive bacterium Bacillus stearothermophilus (5) and the facultative denitrifying Gram-negative bacterium Azoarcus evansii (9) are able to utilize benzoate (compound 1), 3-hydroxybenzoate (compound 9), or gentisate (compound 11) as the sole source of carbon and energy (5, 8, 9). 2- and 4-hydroxybenzoate, protocatechuate, catechol, and 2,3-dihydroxybenzoate do not support growth (5, 8). In conjunction with the presence of an aerobic inducible benzoate-CoA ligase (AMP-forming) and gentisate 1,2-dioxygenase, the degradation of benzoate was proposed to proceed via benzyol-CoA (compound 8) and either 2- or 3-hydroxybenzoyl-CoA (compound 10) as intermediates (5, 8). Further hydroxylation of either compound hypothetically could yield gentisyl-CoA, which might undergo thioester hydrolysis to gentisate (compound 11) (Fig. 2) (5, 8). However, the enzyme reactions catalyzing the proposed pathway to gentisate remained elusive.

This report describes the conversion of [13C6]benzoyl-CoA by cell extracts of A. evansii and B. stearothermophilus under aerobic conditions. Intermediates identified by NMR spectroscopy point to a gentisate-independent and hitherto unknown mechanism of aerobic benzoate degradation.

EXPERIMENTAL PROCEDURES

Materials—[ring-13C6]Benzoyl-CoA was obtained from Merck Sharp & Dohme (Montreal, Canada). Gentisyl-CoA was purchased from MSD Isotopes (Montreal, Canada). NADPH (98% purity) was from Maciso Biotech (Uppsala, Sweden), and [ring-13C6]benzoyl-CoA was prepared by a slight modification of a published procedure (15). A reaction mixture containing 5 mM of [ring-13C6]benzoyl-CoA, 5 mM of N-hydroxysuccinimide, and 5 mM of dicyclohexylcarbodiimide in 30 ml of dioxane was filtered, and the

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solvent was evaporated under reduced pressure. An aliquot of the succinimidyl ester (400 μmol) of the residue was dissolved in 1 ml of dioxane. Aliquots of 100 μl were added to 20 ml of 0.1 mM sodium bicarbonate (pH 8) containing 200 μmol of coenzyme A. The reaction mixture was stirred at room temperature. Aliquots were retrieved at intervals, and thioester formation was monitored by the nitroprusside method (16). After 1 h, the mixture was acidified to pH 3.5 by addition of 4 ml of 2 N formic acid and was then extracted with diethyl ether (3 × 50 ml). The aqueous phase was lyophilized. The residue was dissolved in 5 ml of 20 mM ammonium formate (pH 3.5) containing 2% (v/v) methanol and finally with 10 ml of a linear gradient of 11–40% (v/v) acetonitrile, and the supernatant was evaporated under reduced pressure at 45 °C. The dry residue was dissolved in 50 μl of water and applied to an HPLC column of Grom-Sil 120 ODS-4 HE (5 μm, 120 × 4 mm; Grom, Herrenberg, Germany) that had been equilibrated with 50 mM potassium phosphate (pH 4.5) (solvent 2) containing 2% (v/v) acetonitrile. The column was developed at a flow rate of 1 ml/min with 15 ml of solvent 2 containing 2% (v/v) acetonitrile, then with 25 ml of 50 mM potassium phosphate (pH 6.7) (solvent 3) containing 11% (v/v) acetonitrile, and finally with 10 ml of a linear gradient of 11–40% (v/v) acetonitrile in solvent 3. The effluent was monitored with a radioactivity detector (Raytest solid scintillator cell) and a UV diode array detector (Waters, Eschborn, Germany). Retention times were as follows: unknown polar products, 2 min; gentisate, 6 min; 3-hydroxybenzoate, 18 min; benzoyl-CoA, 25 min; benzoyl-CoA, 32 min; and hydrolytic degradation product of benzoyl-CoA, 45 min. In an attempt to inhibit the aerobic benzoate catabolism, 5 mM trifluoroacetate or 5 mM 2-, 3-, or 4-fluorobenzoate was added to the assay and incubated for 20 min at 37 °C prior to addition of 1 μM [14C]benzoate (0.11 GBq/mmol); 3-hydroxybenzoate and gentisate were not added. The subsequent steps were performed as described above.

In Vitro Assays with Cell Extracts of A. evansii—Assay mixtures (0.25 ml, pH 6.3) containing 0.6 mM NADPH, 0.2 mM [ring-14C]benzoyl-CoA (66.6 MBq/mmol), and 25 μl of cell extracts of benzoate- or 3-hydroxybenzoate-grown A. evansii (52 mg of protein/ml) (20) were stirred at 22 °C. Aliquots (50 μl) were retrieved at intervals. The enzyme reaction was stopped by adding 200 μl of ethanol (−80 °C). Denatured protein was removed by centrifugation. The supernatant was evaporated under reduced pressure at 45 °C, and the residue was dissolved in 100 μl of water. Aliquots of 30 μl were applied to a column of LiChrospher RP18 RF19 (5 μm, 125 × 4 mm; Merck, Darmstadt, Germany) that had been equilibrated with solvent 3 containing 2% (v/v) acetonitrile. The column was eluted at a flow rate of 1 ml/min with 10 ml of solvent 3 containing 2% (v/v) acetonitrile and then with 25 ml of a linear gradient of 2–10% (v/v) acetonitrile in solvent 3. Residual benzoyl-CoA was eluted with 10 ml of a linear gradient of 10–40% (v/v) acetonitrile in solvent 3. The effluent was monitored with the radioactivity detector and the UV diode array detector. Retention times were as follows: polar products, 1.5 min; benzoyl-CoA, 6 min; product 1, 17 min; product 2, 23 min; product 3, 28 min; benzoate, 41 min; and hydrolytic degradation product of benzoyl-CoA, 42.5 min.

Control assays were performed as follows. (i) NADPH was replaced by NADH (0.6 mM); (ii) FAD (0.2 mM) was added; (iii) NADPH was omitted; (iv) oxygen was removed by flushing the assay with nitrogen; and (v) [ring-14C]benzoyl-CoA was replaced by [ring-14C]benzoyl-CoA (0.2 mM). Large-scale experiments (180 ml) were performed with 0.2 mM [ring-14C]benzoyl-CoA (99.9% 13C) in the presence of [14C]benzoyl-CoA (6.7 MBq/mmol) as tracer and 0.6 mM NADPH as described above. After 5 min, the reaction was stopped by addition of 0.72 liters of ethanol (−80 °C). Denatured protein was removed by centrifugation. Aliquots of 3.5 ml were applied to a column of Grom-Sil 120 ODS-4 HE (5 μm, 250 × 20 mm) that had been equilibrated with solvent 3 containing 2% (v/v) acetonitrile. The column was eluted at a flow rate of 8 ml/min with...
30 ml of solvent 3 containing 2% (v/v) acetonitrile, then with 75 ml of a linear gradient of 0–10% (v/v) acetonitrile in solvent 3, and finally with 30 ml of a linear gradient of 10–40% (v/v) acetonitrile in solvent 3. Retention times were as follows: polar products, 1.5 min; benzoate, 6 min; and product 6, 32.5 min. The fractions were desalted as described above.

NMR Spectroscopy—The lyophilized samples were dissolved in 0.5 ml of D$_2$O (pH 6) (uncorrected glass electrode reading). $^1$H and $^{13}$C NMR spectra were measured at 20 °C using a four-channel Bruker DRX 500 spectrometer. One-dimensional experiments and two-dimensional HMOC, HMOC-TOCSY, and INADEQUATE experiments were performed according to standard Bruker software (XWINNMR). The duration of the $^1$H spin lock was 60 ms in the HMOC-TOCSY experiment. Chemical shift predictions were performed with SpecInfo (Chemical Concepts, Darmstadt).

RESULTS

Experiments with Suspensions of Whole Cells of A. evansi—Suspensions of whole cells of benzoate-grown A. evansi were incubated with [14C]benzoate (0.27 mM) at room temperature. The mixture contained 3-hydroxybenzoate (1 mM) and gentisate (1 mM) to trap simultaneously formed [14C]-labeled 3-hydroxybenzoate or gentisate. At intervals, aliquots of the incubation mixture were retrieved, extracted, and analyzed by HPLC as described under “Experimental Procedures.” 3-Hydroxybenzoate and [14C]benzoate were consumed simultaneously. The analysis of gentisate consumption was not possible since gentisate is coeluted with other ultraviolet-light-absorbing compounds. After 15 s, a radiolabeled intermediate (5% of the initially added radioactivity) coeluting with an authentic sample of benzoyle-CoA was detected. After 10 min, the putative benzoyle-CoA intermediate disappeared, and polar products appeared, which did not bind to the column. None of the radiolabeled intermediates coeluted with 3-hydroxybenzoate, gentisate, or 3-hydroxybenzoyl-CoA, and unlabelled 3-hydroxybenzoate was still present (data not shown). These results indicate that 3-hydroxybenzoate, 3-hydroxybenzoyl-CoA, and gentisate do not serve as intermediates in the aerobic catabolism of benzoate in A. evansi.

In an attempt to accumulate unknown intermediates, whole cells of A. evansi were incubated with [14C]benzoate (0.27 mM)
and fluoroacetate or fluorobenzoate specimens (5 mM) in the absence of 3-hydroxybenzoate and gentisate. HPLC analysis revealed that radiolabeled 3-hydroxybenzoate, 3-hydroxybenzoyl-CoA, and gentisate did not accumulate. However, the rates of product formation were significantly decreased. In summary, experiments with whole cells of A. evansi showed that [14C]benzoate was not converted into 3-hydroxybenzoate or gentisate. Rather, benzoyl-CoA was formed as an intermediate.

Experiments with Cell Extracts of A. evansi—Extracts of benzoate-grown A. evansi cells were incubated with [14C]benzoyl-CoA or [14C]benzoate in the presence of NADPH (0.6 mM) and oxygen (air saturation). Aliquots were retrieved at intervals and analyzed by HPLC optimized for the separation of CoA thioesters. Product formation was not observed with [14C]benzoate as substrate. In contrast, several peaks reflecting 14C-labeled products were detected when [14C]benzoyl-CoA was used as substrate (Fig. 3A). More specifically, a minor fraction (~5%) of [14C]benzoyl-CoA was found to be hydrolyzed to labeled benzoate and coenzyme A probably due to nonspecific thioesterases present in the crude cell extracts. The major part of benzoyl-CoA was converted into products (products 1–3) (Fig. 3A and Table I) that eluted from the reversed-phase column at retention times typical for CoA adducts. The product pattern shown in Fig. 3A was detected over a pH range of 6–8. The products were collected, lyophilized, and analyzed by HPLC as described above. The chromatography of product 1 reflected the original compound (retention time, 17 min; 70% of radioactivity) and a new compound with a retention time of 28 min (30% of radioactivity). Obviously, product 1 was partially converted into a novel compound during isolation. As shown below, NMR analysis of the fraction containing product 1 confirmed the presence of two compounds. Rechromatography of product 2 afforded one peak at a retention time of the original peak. Rechromatography of product 3 showed a major peak (75% of radioactivity) at the retention time of product 2 (25 min). NMR analysis confirmed that product 3 was converted into product 2 during isolation (see below).

Fig. 4A shows the kinetics of substrate consumption and product formation in more detail. After 5 min, 80% of the initially added benzoyl-CoA was consumed. This process was accompanied by the formation of the major product 1 (maximum after 5 min; peak intensity reflected 40% of the initially added radiolabeled benzoyl-CoA) and the formation of the minor products 2 and 3. Products 1 and 3 were subsequently consumed in the course of the reaction, whereas product 2 seemed not to be converted any further. The amount of polar products increased steadily from the beginning on up to 40% of the initially added radioactivity. The total radioactivity decreased slightly after 7 min presumably due to the formation of labeled volatile 14CO2.

Addition of FAD (0.2 mM) inhibited the catabolism of benzo-
10 signals with one-bond $^{13}$C-$^{13}$C coupling constants of 50–30 Hz (Table II). Additionally, some signals showed long-range couplings with coupling constants of 2–5 Hz. On the basis of the chemical shift patterns, the relative fractions of compounds 22 and 21 were determined from one-dimensional $^{13}$C NMR spectra. Atoms coupled to the respective index atoms are in parentheses.

Two of the five intense $^{13}$C NMR signals of product 2 (Fig. 6) and product 6 (Table II) had chemical shift values that were consistent with alkene motifs. One doublet signal of product 2 was found at a chemical shift value of 181.5 ppm, typical for a carboxylic acid moiety; and two signals were detected at 35.1 and 28.3 ppm, reflecting two carbon atoms with $sp^3$ hybridization. The doublet signal of product 6 was found at 60.6 ppm, suggesting a $^{13}$CH$_2$OH moiety. The observed chemical shift pattern, as well as the carbon-carbon connectivities obtained from INADEQUATE experiments (Fig. 7 and Table II), establishes product 6 as 6-[2,3,4,5,6-$^{13}$C$_5$]hydroxy-3-hexenoyl-CoA (compound 21). In line with many model compounds in spectral data bases (e.g., Specinfo), compound 22 was tentatively assigned as the 3,6-lactone of $[\text{ring-}$-$^{13}$C$_6]\text{benzoyl-CoA}$. From the $^{13}$C NMR signal integrals, the relative fractions of compounds 22 and 21 were estimated as 2:1 in the NMR sample.

Experiments with B. stearothermophilus — The conversion of $[\text{ring-}$-$^{13}$C$_6]\text{benzoyl-CoA}$ and $[^{14}$C]$\text{benzoyl-CoA}$ by cell extracts of B. stearothermophilus was studied as described for A. evansi, but in the presence of 1 mM iodoacetamide. It was found that iodoacetamide increased the accumulation of early prod-

### Table II

| Position | Chemical shift$^a$ | Coupling constants (J$_{cc}$)$^d$ | Correlation experiments |
|----------|-------------------|-------------------------------|-------------------------|
|          | $^{13}$C$^b$ | $^1$H | H$_2$ | HMOC-TOCSY | INADEQUATE |
| ppm      | Hz          | Hz    | Hz    | Hz     | Hz           |
| 3,6-Lactone of 3-[2,3,4,5,6-$^{13}$C$_5$]hydroxyadipyl-CoA (product 1, compound 22) | | | | | |
| 2 | 47.88 (d) | 2.89 | 38.9 (3) | 2, 3 | 3 |
| 3 | 78.14 (m) | 4.82 | 38.9 (2), 31.8 (4), 4.8 (6) | 3, 2 | 2, 4 |
| 4 | 26.37 (t) | 2.27, 1.83 | 32.4 (3, 5) | 4, 4', 5, 3 | 3, 5 |
| 5 | 28.14 (dd) | 2.45 | 48.2 (6), 32.5 (4) | 5, 4', 4 | 4, 6 |
| 6 | 181.12 (d) | 5.18 (5) | 50.9 (6), 42.9 (4), 3.5 | 4, 6 |
| 3-[2,3,4,5,6-$^{13}$C$_5$]Hydroxyadipyl-CoA (product 1, compound 21) | | | | | |
| 2 | 50.32 (d) | 2.61 | 37.3 (2), 1.8 (5) | 2, 3 |
| 3 | 67.46 (td) | 3.90 | 37.2 (2, 4), 3.5 (6) | 3, 2 |
| 4 | 30.97 (t) | 1.63, 1.54 | 36.7 (3, 5) | 4, 4', 5 | 3, 5 |
| 5 | 29.80 (dd) | 2.28 | 52.7 (6), 35.4 (4), 1.8 (2) | 5, 4', 4 | 4, 6 |
| 6 | 177.92 (dd) | 53.6 (5), 3.8 (3) | 5 |
| trans-2-[2,3,4,5,6-$^{13}$C$_5$]Dehydroadipyl-CoA (product 2, compound 20) | | | | | |
| 2 | 127.85 (dd) | 6.10 (d, 15 Hz) | 69.0 (3), 3.4 (5) | 2, 3, 4, 5 (w)$^b$ |
| 3 | 146.62 (dd) | 6.84 (dt) | 41.1 (4), 69.0 (2) | 3, 2, 4, 5 | 2, 4 |
| 4 | 28.28 (dd) | 2.33 | 33.7 (5), 42.1 (3) | 4, 3, 5 | 2, 3, 5 |
| 5 | 35.99 (dd) | 2.22 | 34.1 (4), 51.5 (6) | 5, 4, 3, 5 (w) | 4, 6 |
| 6 | 181.46 (dd) | 51.4 (5) | 5 |
| cis-3-[2,3,4,5,6-$^{13}$C$_5$]Dehydroadipyl-CoA (product 4, compound 18) | | | | | |
| 2 | 41.41 (d) | ND | 42.5 (3), 3.1 | 4 |
| 3 | 128.81 (dd) | 5.22 (q, 7 Hz) | 72.1 (3), 43.5 (2) | 3, 2, 4, 5 |
| 4 | 121.86 (dd) | 5.43 (q, 7 Hz) | 72.1 (3), 42.5 (5), 4.0 | 5 |
| 5 | 35.70 (dd) | 2.22 | 50.9 (6), 42.9 (4), 3.5 | 4, 6, 5 |
| 6 | 183.07 (d) | 50.9 (5) | 5 |
| 6-[2,3,4,5,6-$^{13}$C$_5$]Hydroxy-3-hexenoyl-CoA (product 6, compound 19) | | | | | |
| 2 | 41.65 (dd) | 3.15 | 42.3 (3), 3.6 | 2, 3, 4 (w) |
| 3 | 131.08 (dd) | 5.45 | 42.3 (3), 71.3 (4) | 3, 4, 2, 5 |
| 4 | 122.35 (dd) | 5.36 | 42.3 (5), 71.1 (3), 3.6 | 4, 3 |
| 5 | 29.50 (dd) | 2.08 | 36.6 (6), 41.6 (4), 3.4 | 5, 6, 4 |
| 6 | 60.57 (d) | 3.40 | 37.1 (5), 2.3 | 6, 5, 4 |

$^a$ Referenced to external 3-trimethylsilylpropane 1-sulfonate.

$^b$ Signal multiplicities in parentheses (t, pseudo-triplet; d, doublet; dd, double doublet; ddd, triple doublet; q, pseudo-quartet; m, multiplet).

$^c$ Determined from one-dimensional $^{13}$C NMR spectra. Atoms coupled to the respective index atoms are in parentheses.

$^d$ w, weak signal intensity.
products, most likely by inhibiting a late step in the overall transformation. HPLC (Fig. 3B) and NMR analysis identified the same products 1–3 as described above. Moreover, two additional products (products 4 and 5) (Fig. 3B) could be detected. Fig. 4B shows the kinetics of substrate consumption and product formation in more detail. Product 4 was the earliest of the detected products and was consumed in the course of the reaction. Products 2 and 3 were detected later. More polar products (non-CoA thioesters) accumulated steadily.

The 13C NMR spectrum of the fraction containing products 2 and 4 displayed five major signals that were identical to the NMR signature of trans-2,3-[2,3,4,5,6-13C5]dehydroadipyl-CoA (product 2, compound 20). Five novel signals were attributed to product 4 (Table II). The 13C NMR chemical shifts again indicated a dehydroadipyl derivative. In contrast to compound 20, both signals of the alkene moiety displayed simultaneous coupling to two adjacent 13C atoms. The carbon connectivity was further analyzed by INADEQUATE experiments, establishing a 13CH2–13CH–13CH2 motif (Table II). Thus, product 4 was assigned as 3,4-[2,3,4,5,6-13C5]dehydroadipyl-CoA (compound 18). A cis-configuration is suggested from the analysis of the 1H NMR signature of H-3 and H-4 (7-Hz couplings) (Table II). The amount of product 5 was too low for NMR analysis.

**DISCUSSION**

*In vivo* experiments indicated that aerobic benzoate catabolism in *A. evansii* proceeds via benzoyl-CoA (compound 8) and possibly other CoA intermediates, but not via 3-hydroxybenzoate (compound 9), 3-hydroxybenzoyl-CoA (compound 10), or gentisate (compound 11). β-[2,3,4,5,6-13C5]Hydroxyadipyl-CoA (compound 21), the 3,6-lactone of β-[2,3,4,5,6-13C5]hydroxyadipyl-CoA (compound 22), and trans-2,3-[2,3,4,5,6-13C5] dehydroadipyl-CoA (compound 20) were identified by two-dimensional NMR spectroscopy in aerobic reaction mixtures containing [ring-13C6]benzoyl-CoA (compound 8), NADPH, and crude cell extracts of *A. evansii*. Using a protein fraction of *A. evansii*, [ring-13C6]benzoyl-CoA was converted into 6-[2,3,4,5,6-13C5]hydroxy-3-hexenoyl-CoA (compound 19). With cell extracts of *B. stearothermophilus*, compounds 20–22 and cis-3,4-[2,3,4,5,6-13C5]dehydroadipyl-CoA (compound 18) were detected. Structure assignments were facilitated by the use of the multiply 13C-labeled substrate conducive to enhanced sensitivity and selectivity of INADEQUATE, HMQC, and HMQC-TOCSY experiments with minimal amounts of samples (~200 μg of CoA adducts).

The identified compounds cannot be explained by conventional pathways (Fig. 1) or by previously suggested mechanisms via gentisate (compound 11) (Fig. 2), but point at a gentisate-independent novel pathway operative in the microbial species under study. A hypothetical pathway of aerobic benzoate metabolism in *A. evansii* and *B. stearothermophilus* integrating the identified intermediates is shown in Fig. 8.
A. evansii and B. stearothermophilus appear to metabolize benzoate (compound 1) via coenzyme A thioesters. The first step of the pathway is the formation of benzoyl-CoA (compound 8) from benzoate (compound 1) and coenzyme A by a benzoate-CoA ligase.

The formation of CoA esters of 13C5-labeled adipate analogs (C6 compounds) from [ring-13C6]benzoyl-CoA (C7 compound) requires enzymes that hydroxylate and cleave the aromatic ring and that decarboxylate putative intermediates. A protein mixture obtained from crude extracts of A. evansii after two chromatographic steps converted benzoyl-CoA in an NADPH- and oxygen-dependent reaction into 6-[2,3,4,5,6-13C5]hydroxy-3-hexenoyl-CoA (compound 19).

Obviously, compound 19 or a compound closely related to compound 19 is an early intermediate of the new aerobic benzoate pathway. The compound was detected only with the protein mixture, but not with crude cell extracts. Probably, compound 19 could be oxidized to compounds 18 and 20 in assays containing crude cell extracts and a large excess of NADP+ (Fig. 8).

The conversion of benzoyl-CoA into 6-hydroxy-3-hexenoyl-CoA (compound 19) is a multistep reaction involving oxygenation, ring cleavage, and decarboxylation. A hypothetical mechanism of benzoyl-CoA (compound 8) conversion into compound 19 is shown in Fig. 8. A 2,3-dioxygenation of benzoyl-CoA (compound 8) could yield the 2,3-dihydrodiol compound 13. Isomerization of compound 13 could afford compound 16, and the keto form of compound 16 could be converted into compound 15 or 17 by non-oxygenolytical cleavage of the 2,3- or 1,2-bond catalyzed by a hydrolase. Decarboxylation of compound 15 or 17 could then yield compound 19.

A more conventional 2,3-dioxygenation of benzoyl-CoA (compound 8) followed by oxygenolytical intradiol ring cleavage would result in compounds 18 via compounds 12 and 14 (Fig. 8). However, the reduction of compound 18 to compound 19 is hardly conceivable under the assay conditions. Therefore, a pathway via compound 16 appears more probable.

The postulated benzoyl-CoA oxygenase is currently under study. Remarkably, an iron-sulfur flavoprotein (BoxA protein) catalyzing the FAD- and benzoyl-CoA-dependent oxidation of NADPH (H2O2-forming) has been purified from A. evansii. The boxA gene shows similarity to reductase domains of various hydroxylases. Moreover, boxA is adjacent to boxB in a hypothesized operon that is induced by benzoate. boxB has similarity to domains of putative multicomponent oxygenases. We suggest that a BoxA-BoxB complex is involved in benzoyl-CoA oxygenation in A. evansii. However, up to now, we were not able to reconstitute the native BoxA-BoxB complex.

Hydration of 2,3-dehydroxylipid-CoA (compound 20) could afford β-hydroxyadipyl-CoA (compound 21), which can be interconverted into the 3,6-lactone of β-hydroxyadipyl-CoA (compound 22). Alternatively, 2,3-dehydroxylipid-CoA (compound 20) could react to the lactone compound 22, which could be opened hydrolytically into the 3-hydroxy compound 21. Oxida-
tion could then yield β-ketoacyl-CoA (compound 5), a known intermediate of the ortho-cleavage pathway. β-Ketoacyl-CoA can be further elaborated thiolynthionically into succinyl-CoA and acetyl-CoA (Fig. 1). The corresponding thiolase gene was found next to the boxA and boxB genes in A. evansiisi.3

The kinetics of product formation (Fig. 4) is in line with the proposed mechanism. Product 4 (compound 22) specifies as an early intermediate. The formation of compound 22 can be easily explained via product 6 (compound 19), which was identified only in assays with partially purified protein. Product 1 (compounds 21 and 22) and product 2 (compound 20) show kinetics of later products, with an uncertain order of formation.

It must be emphasized that the proposed reaction sequence remains speculative at the present level of experimental evidence. Moreover, it remains to be established whether the novel pathway is operative in other Bacillus strains and in the halophilic archaeabacterium Haloferax sp., which have been claimed to metabolize benzoate via gentisate (2–4, 22).

We can only speculate about the selective advantage of the proposed pathway. (i) One advantage may be that more complex aromatic substrates such as phenyl propionate and cinamat can be converted by β-oxidation to benzyol-CoA rather than benzoate. Recently, it was found that phenyl derivatives containing an odd or even number of carbon atoms will be catabolized through β-oxidation to either benzyol-CoA (odd) or phenylacetyl-CoA (even), which could be further catabolized to the intermediates of general metabolism (23). (ii) Another advantage for facultative anaerobic bacteria could be that benzyol-CoA, which is a characteristic intermediate of the anaerobic aromatic metabolism, may be immediately used as substrate of the aerobic metabolism when oxygen becomes available. This would allow a rapid shift from the anoxic to oxic mode of growth and vice versa. (iii) Also, thioester formation allows an efficient trapping of aromatic acids, very much the same as in the case of alphabetic fatty acids, e.g. in Escherichia coli. Fatty acid-CoA ligase is found in the soluble fraction after cell disruption, but is thought to act as a membrane-associated protein in whole cells, trapping incoming fatty acids (24). The energy-rich thioester bond is retained in the course of the pathway; therefore, the energy initially spent is useful not only for the active transport, but at the same time, for the activation of the acid. One may therefore speculate that benzoate-CoA ligase could behave similarly to fatty acid-CoA ligase. It was found that Pseudomonas putida U lost the ability to take up phenyl acetate when the gene coding for phenylacetyl-CoA ligase, the first enzyme of a new pathway of catabolism of phenylacetic acid, was mutated (23).

It is remarkable that besides benzoate, also 2-aminobenzoate (19, 25) and phenyl acetate (26–28) are aerobically metabolized in A. evansiisi (19, 26) and possibly in some other bacteria via coenzyme A thioesters (10–13). An inducible CoA ligase was mutated (23). A halophilic archaebacterium, a novel pathway is operative in other bacteria via an inducible CoA ligase was mutated (23).

esterified carboxyl group may facilitate enzymatic steps that otherwise would not be possible with free acids. One example is the hydroxylation and subsequent reduction of 2-aminobenzoyl-CoA by the flavoenzyme 2-aminobenzoyl-CoA monoxygenase/nitrate reductase (31–33). Another example is the hydrolytic chloride elimination in 4-chlorobenzoyl-CoA (34, 35). It will be interesting to see which kind of initial reactions benzyol-CoA and phenylacetyl-CoA will undergo and what kind of role the coenzyme A carboxyl thioester group plays in these unprecedented reactions.

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