JOURNAL OF BACTERIOLOGY, June 1992, p. 3621–3628
0021-9193/92/113621-08$02.00/0

Vol. 174, No. 11

Anaerobic Degradation of 2-Aminobenzoic Acid (Anthranilic Acid) via Benzoyl-Coenzyme A (CoA) and Cyclohex-1-Enecarboxyl-CoA in a Denitrifying Bacterium

CHRISTA LOCHMEYER, JÜRGEN KOCH, AND GEORG FUCHS*

Angewandte Mikrobiologie, University of Ulm, P.O. Box 4066, W-7900 Ulm, Germany

Received 26 December 1991/Accepted 2 March 1992

The enzymes catalyzing the initial reactions in the anaerobic degradation of 2-aminobenzoic acid (anthranilic acid) were studied with a denitrifying *Pseudomonas* sp. anaerobically grown with 2-aminobenzoate and nitrate as the sole carbon and energy sources. Cells grown on 2-aminobenzoate are simultaneously adapted to growth with benzoate, whereas cells grown on benzoate degrade 2-aminobenzoate several times less efficiently than benzoate. Evidence for a new reductive pathway of aromatic metabolism and for four enzymes catalyzing the initial steps is presented. The organism contains 2-aminobenzoate-coenzyme A ligase (2-aminobenzoate-CoA ligase), which forms 2-aminobenzoyl-CoA. 2-Aminobenzoyl-CoA is then reductively deaminated to benzoyl-CoA by an oxygen-sensitive enzyme, 2-aminobenzoyl-CoA reductase (deaminating), which requires a low potential reductant [Ti(III)]. The specific activity is 15 nmol of 2-aminobenzoyl-CoA reduced min^{-1} mg^{-1} of protein at an optimal pH of 7. The two enzymes are induced by the substrate under anaerobic conditions only. Benzoyl-CoA is further converted in vitro by reduction with Ti(III) to six products; the same products are formed when benzoyl-CoA or 2-aminobenzoyl-CoA is incubated under reducing conditions. Two of them were identified preliminarily. One product is cyclohex-1-enecarboxyl-CoA, the other is *trans*-2-hydroxycyclohexanecarboxyl-CoA. The complex transformation of benzoyl-CoA is ascribed to at least two enzymes, benzoyl-CoA reductase (aromatic ring reducing) and cyclohex-1-enecarboxyl-CoA hydratase. The reduction of benzoyl-CoA to alicyclic compounds is catalyzed by extracts from cells grown anaerobically on either 2-aminobenzoate or benzoate at almost the same rate (10 to 15 nmol min^{-1} mg^{-1} of protein). In contrast, extracts from cells grown anaerobically on acetate or grown aerobically on benzoate or 2-aminobenzoate are inactive. This suggests a sequential induction of the enzymes.

Anthranilic acid (2-aminobenzoic acid) is an intermediate in the synthesis and degradation of tryptophane (4, 5, 9, 36, 41); it has been shown or postulated to be an intermediate in the degradation of other compounds containing an indole moiety, both aerobically (16, 19, 24, 26, 27) and anaerobically (6, 31, 40). The aerobic metabolism proceeds via catechol (4, 25) or gentisic acid (11) and requires molecular oxygen. The pathway of anaerobic degradation of 2-aminobenzoate is unknown. Indirect evidence with a methanogenic enrichment culture suggested that benzoate is an intermediate under these conditions (39).

There are numerous examples demonstrating that the anaerobic metabolism of aromatic acids in general is initiated by ATP-dependent coenzyme A (CoA) thioester formation (12, 20–23, 30, 32). We have recently purified two CoA ligase isoenzymes for 2-aminobenzoate activation from denitrifying *Pseudomonas* strain KB 740, which grows on this substrate aerobically as well as anaerobically under denitrifying conditions (3). Surprisingly, one of the enzymes is required for a novel aerobic degradation pathway, which is atypical in many respects (1, 29). It is induced aerobically by 2-aminobenzoate and is encoded by a small plasmid. The other isoenzyme is induced anaerobically by 2-aminobenzoate and is encoded by the chromosome (2a). The anaerobic isoenzyme, therefore, is thought to catalyze the first step in anaerobic 2-aminobenzoate metabolism.

The question of which are the initial steps in anaerobic 2-aminobenzoate metabolism was addressed with a related denitrifying *Pseudomonas* strain, K 172. This strain was chosen since it is especially interesting in that it also readily oxidizes toluene and phenol anaerobically to CO_2 (2, 37); in addition, the anaerobic reduction of benzoyl-CoA to alicyclic compounds has been documented in this strain (25a).

It is shown that the anaerobic metabolism of 2-aminobenzoate proceeds via activation to 2-aminobenzoyl-CoA, reductive deamination to benzoyl-CoA, and ring reduction of benzoyl-CoA to yield alicyclic acyl-CoA compounds.

MATERIALS AND METHODS

Materials. Chemicals were obtained from Aldrich-Chemie (Steinheim, Germany), Fluka (Neu-Ulm, Germany), Heraeus (Karlsruhe, Germany), Merck (Darmstadt, Germany), and Serva (Heidelberg, Germany). Biochemicals were obtained from Boehringer GmbH (Mannheim, Germany), [U-^{14}C]2-aminobenzoic acid was from Sigma (Deisenhofen, Germany; specific radioactivity, 421.8 MBq $mmol^{-1}$), thin-layer chromatographic (TLC) plates were from Merck, scintillation cocktail Rotiszint 2200 was from Roth (Karlsruhe, Germany), and gases were from Linde (Höllriegelskreuth, Germany). *Pseudomonas* strain K 172 was isolated in our laboratory (37). *Pseudomonas* strain KB 740 was a kind gift from K. Braun (8).

Growth of bacteria. *Pseudomonas* strain K 172 was grown at 28°C under anaerobic conditions in mineral salts medium with 5 mM aromatic acid (benzoate or 2-aminobenzoate) or 10 mM acetate and 20 mM nitrate as the sole sources of energy and cell carbon, respectively. Under aerobic conditions, the same medium was used but without nitrate (37).

* Corresponding author.

Growth was monitored photometrically as optical density at 578 nm (1-cm light path). Cultures (10 liters) were harvested by continuous centrifugation with a Westfalia separator at an optical density of 0.8, yielding 8 g (wet weight) of cells. The cells were stored in liquid nitrogen or at −70°C. *Pseudomonas* strain KB 740 was grown anaerobically with 2-aminobenzoate and nitrate at 28°C as described previously (8).

Preparation of cell extracts. The cell extract was prepared under anaerobic conditions. Cells (1 g) were suspended in 1 or 1.5 ml of 3-morpholinopropanesulfonic acid (MOPS)-KOH buffer, pH 7.2, containing 4 mM $MgCl_2$, 2 mM dithioerythritol (DTE), and 0.2 mg of DNase I. In some experiments, MOPS buffer was replaced by the buffer used in the enzyme assay. The cells were disintegrated at 130 MPa in a cooled French pressure cell gassed with N_2 and then subjected to anaerobic centrifugation at 17,000 × *g* (4°C, 20 min). The supernatant was used as the cell extract. An extract of *Pseudomonas* KB 740 cells was prepared as described previously (38). The amount of protein was determined by the Bradford method (7).

Simultaneous adaptation experiment. The simultaneous adaptation experiment was carried out under anaerobic conditions. *Pseudomonas* strain K 172 was grown under denitrifying conditions in 1-liter infusion bottles (Müller and Krempel AG, Bülach, Switzerland) sealed with latex rubber septa (Maag Technic AG, Dübendorf, Switzerland) in the mineral salts medium described above; 5 mM benzoic acid or 2-aminobenzoic acid served as the sole carbon source. Cells were harvested at the end of the exponential growth phase (ΔA_{578}, ≤0.8) by centrifugation at 8,000 × *g* (10 min, 12°C) and were washed with mineral salts medium without growth substrates. The cell pellet was suspended in mineral salts medium which contained 10 mM nitrate but no carbon source. The final cell density of the cell suspension corresponded to a ΔA_{578} of 10 (corresponding to approximately 4 mg of cell dry matter per ml). Aliquots (10 ml) of each suspension were dispensed anaerobically into Hungate tubes (Bellco Glass Inc.), which were closed with rubber stoppers. The experiment was started by adding growth substrates after the temperature was adapted to 30°C. Each cell suspension was tested for the degradation of benzoic acid and 2-aminobenzoic acid. After different incubation periods, 0.5-ml samples were withdrawn, cooled on ice, and rapidly centrifuged at 10,000 × *g* (4°C). The repeated sampling may result in oxygen contamination at the later time points, resulting in a decrease of the substrate consumption rate. The pellet was discarded and the UV absorption spectrum of the supernatant was recorded between 200 and 350 nm. For a determination of benzoic acid (λ_{max} = 273 nm; ε = 0.97 mM^{-1} cm^{-1}) (13), the solution was acidified to pH 2; for quantitation of 2-aminobenzoic acid (λ_{max} = 310 nm; ε = 2.98 mM^{-1} cm^{-1}) (15), the neutral supernatant was used.

PAGE. The protein pattern of cell extracts of *Pseudomonas* strain K 172 grown on benzoic acid or 2-aminobenzoic acid was analyzed (7 μg of protein per lane) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE (12.5% polyacrylamide gels) was performed by the method of Laemmli (28). Molecular mass standards were purchased from Pharmacia (Freiburg, Germany). Gels were stained with silver (33).

Purification of 2-aminobenzoate-CoA ligase. 2-Aminobenzoate-CoA ligase was purified from *Pseudomonas* KB 740 cells grown anaerobically on 2-aminobenzoate and nitrate (3). The purified enzyme had a specific activity of 130 nkat mg^{-1} of protein.

Synthesis of 2-aminobenzoyl-CoA. 2-Aminobenzoyl-CoA was enzymatically synthesized and purified as described previously (10).

In vitro assays of 2-aminobenzoyl-CoA reduction. The enzymatic reduction of 2-aminobenzoyl-CoA was studied in vitro under strictly anaerobic, reducing conditions at 30°C. Assays were performed in 0.5-ml Durham tubes or 0.5-ml glass cuvettes closed with rubber stoppers and gassed with N_2. All additions and samplings were done with gas-tight Hamilton microliter syringes.

Spectrophotometric assay. The assay mixture (0.5 ml, total volume) contained 150 mM MOPS–KOH buffer (pH 7.2), 0.2 mM 2-aminobenzoate, 4 mM $MgCl_2$, 2 mM ATP, 2 mM DTE, 1 mM CoA, 3 mM titanium(III) citrate, 50 μl of cell extract (2 mg of protein). The enzymatic formation and reduction of 2-aminobenzoyl-CoA was monitored spectrophotometrically at 365 nm (ε = 5.5 mM^{-1} cm^{-1}). First, the enzymatic formation of 2-aminobenzoyl-CoA by endogenous 2-aminobenzoate-CoA ligase was measured, and therefore the test was started by the addition of ATP; this reaction was accompanied by an absorption increase. After the nearly complete formation of 2-aminobenzoyl-CoA, the reduction of 2-aminobenzoyl-CoA was started by adding titanium(III) citrate; this reaction was accompanied by an absorption decrease.

Radioisotope assay with [U-^{14}C]2-aminobenzoate. The assay mixture (0.35 ml, total volume) consisted of 150 mM MOPS–KOH buffer, pH 7.2, with 4 mM $MgCl_2$ and 2 mM DTE; 50 μl of cell extract (8,000 × *g*), 1.3 mg of protein; a [U-^{14}C]2-aminobenzoyl-CoA generating system consisting of 0.2 mM [U-^{14}C]2-aminobenzoate (37 kBq), 1.7 mM ATP, 1.1 mM CoA, and 0.4 nkat of purified 2-aminobenzoate-CoA ligase from *Pseudomonas* KB 740, 3.4 μg of protein; an ATP regenerating system consisting of 3.3 mM phosphoenolpyruvic acid, 100 nkat of pyruvate kinase (20 μg of protein), and 12 nkat of myokinase (2 μg of protein); 3.3 mM titanium(III) citrate was the reducing component. The anaerobic assay mixture was prepared at 4°C and preincubated for 30 min at 30°C in order to form 2-aminobenzoyl-CoA, and the reaction was started by adding cell extract of *Pseudomonas* strain K 172. After different incubation periods, samples were withdrawn. For the analysis of CoA thioesters, samples (50 μl) were acidified with 20% H_2SO_4 (7 μl) and centrifuged (20 min, 10,000 × *g*, 4°C). For the analysis of thioester-bound acids, samples (50 μl) were first treated with 5 μl of 4 N KOH, pH 12 (20 min, 80°C), acidified with 2 μl of 50% H_2SO_4 to pH 2, and centrifuged (10,000 × *g*, 20 min, 4°C). The released ^{14}C-labeled organic acids in the supernatant were analyzed. The formation of [U-^{14}C]2-aminobenzoyl-CoA in the assays was monitored. At the end of the experiment, an aliquot (50 μl) of the assay mixture was acidified (7 μl of 20% H_2SO_4) and centrifuged (10,000 × *g*, 20 min, 4°C). The supernatant was analyzed by TLC and autoradiography, and the amount of ^{14}C in the 2-aminobenzoate area was determined. This amount of radioactivity corresponded to residual 2-aminobenzoate, which was not in the CoA thioester form.

Chromatographic separation, detection, preliminary identification, and quantitation of reaction products. [U-^{14}C]2-aminobenzoate and labeled products derived thereof were analyzed by TLC on aluminum plates (20 by 20 cm) with a 0.2-mm silica gel (Kieselgel 60 F254; Merck). The following two solvents were used: (i) system A (benzene-dioxan-acetic acid, 8:1:1, vol/vol/vol) and (ii) system B (isopropylether-butanol, 75:25, vol/vol). Radioactive areas on TLC plates were located by autoradiography using X-ray film (Kodak X-OMAT, XAR-351; Sigma); radioactive spots were scraped

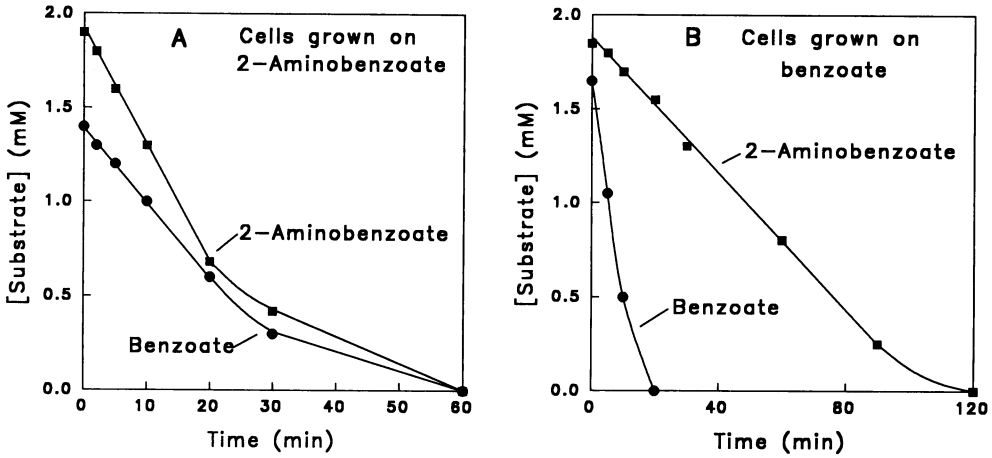

FIG. 1. 2-Aminobenzoate and benzoate degradation by suspensions of *Pseudomonas* K 172. Substrate consumption by cells anaerobically grown with 2-aminobenzoate (A) or benzoate (B) and nitrate is shown. ■, 2-aminobenzoate; ●, benzoate. The decrease in substrate consumption in panel A may be caused by oxygen introduced by repeated sampling. For details, see Materials and Methods.

off, and the TLC material was extracted directly in a 4-ml scintillation cocktail. ^{14}C labeling was determined by liquid scintillation counting by using an external standard. Organic standard acids were detected by UV light at 254 nm or after spraying (35) with 1% (wt/vol) vanillin in concentrated H_2SO_4 or 5% (wt/vol) potassium dichromate in concentrated H_2SO_4.

RESULTS

Adaptation of whole cells to anaerobic 2-aminobenzoate degradation. *Pseudomonas* strain K 172 degrades 2-aminobenzoate, benzoate, and other aromatic acids completely to CO_2, both under aerobic and anaerobic, denitrifying conditions (37). These compounds also serve as the sole source for cell carbon. The generation times for anaerobic growth were 6.5 h with benzoate and 10 h with 2-aminobenzoate. The simultaneous adaptation of cells grown on 2-aminobenzoate for the metabolism of benzoate was investigated with dense suspensions of whole cells. Similarly, the utilization of 2-aminobenzoate by cells grown on benzoate was studied. Short lag phases in substrate utilization can be detected only by using this method. All cells were precultivated several times on either 2-aminobenzoate or benzoate. The rate of conversion of these aromatic acids to nonaromatic products by preadapted, suspended cells corresponded to the substrate consumption rate of growing cultures and amounted to 1 to 2 mM substrate consumed per 30 min by a cell suspension with a ΔA_{578} of 10.

Cells grown on 2-aminobenzoate immediately degraded benzoate and 2-aminobenzoate with almost identical initial rates (Fig. 1A). Cells grown with 2-aminobenzoate, therefore, were simultaneously adapted to metabolize benzoate without a lag phase. Cells grown on benzoate degraded benzoate severalfold faster than 2-aminobenzoate. 2-Aminobenzoate was used slowly without a noticeable lag phase, and the rate of 2-aminobenzoate consumption did not increase within 90 min of incubation (Fig. 1B). Although the substrate consumption rate varied by almost a factor of 2 depending on the batch of cells used, the relative difference in the 2-aminobenzoate and benzoate consumption rates in one batch of cells was reproducible. Cells grown anaerobically with benzoate, therefore, appear to have some basal capability to metabolize 2-aminobenzoate as well. Yet, the full induction appears to take more than 90 min.

Formation of 2-aminobenzoyl-CoA and reductive transformation of 2-aminobenzoyl-CoA. We have recently purified a 2-aminobenzoate-CoA ligase from *Pseudomonas* strain KB 740 grown anaerobically with nitrate and 2-aminobenzoate. This enzyme was anaerobically induced by 2-aminobenzoate (3). Therefore, we expected that in the related *Pseudomonas* strain K 172, 2-aminobenzoate is activated to 2-aminobenzoyl-CoA, which may then become reductively deaminated to the central intermediate benzoyl-CoA.

Extracts of cells grown anaerobically on 2-aminobenzoate catalyzed the Mg^{2+}-ATP- and CoA-dependent activation of 2-aminobenzoate and the reductive conversion of 2-aminobenzoyl-CoA to several products (see below). The formation of 2-aminobenzoyl-CoA from 2-aminobenzoate, CoA, and Mg^{2+}-ATP by endogenous 2-aminobenzoate-CoA ligase, which was monitored in a spectrophotometric assay, was nearly stoichiometric after a short incubation and was 90% of the expected amount; the stoichiometry for the substrate 2-aminobenzoate is shown in Fig. 2.

When, after nearly complete formation of 2-aminobenzoyl-CoA, the reducing agent titanium(III) citrate was added to the assay, an absorption decrease at 365 nm due to 2-aminobenzoyl-CoA reduction was observed (Fig. 2). The reaction proceeded linearly over time until the residual concentration of 2-aminobenzoyl-CoA was about 20 μM. This suggests that the apparent K_m for 2-aminobenzoyl-CoA is ≤20 μM. The specific activity of the 2-aminobenzoyl-CoA reductase was 15 nmol min^{-1} mg^{-1} of protein. The reaction was dependent on 2-aminobenzoyl-CoA, ATP, the amount of protein added, and the concentration of titanium(III) citrate used.

Enzyme assays. Two independent assays for 2-aminobenzoyl-CoA reduction were developed, a spectrophotometric assay and a radioisotope assay (see Materials and Methods). The convenient spectrophotometric assay was carried out as outlined above, with 3 mM titanium(III) citrate as the reductant. The radioisotope assay allowed us to also examine the nature of the products of enzymatic [U-^{14}C]2-aminobenzoyl-CoA reduction. The radioactive acids, which were obtained upon alkaline treatment of the assay mixture, were separated by TLC in two solvent systems.

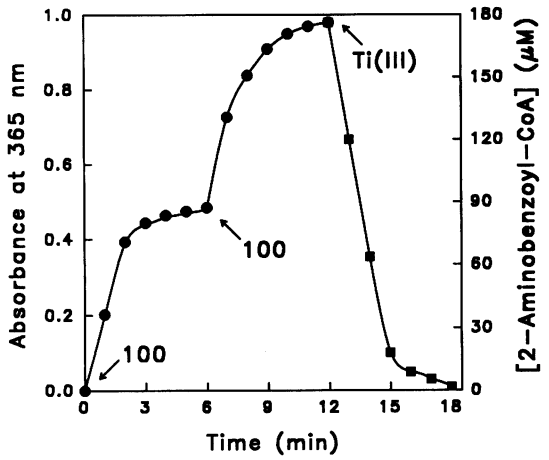

FIG. 2. Stoichiometry of 2-aminobenzoate-CoA ligase and 2-aminobenzoyl-CoA reductase reactions catalyzed by cell extracts. Reactions were monitored with the spectrophotometric assay. 2-Aminobenzoate was added twice, each at 100 μM. Then Ti(III) citrate (3 mM) was added, resulting in the complete consumption of 2-aminobenzoyl-CoA. The 0.5-ml assay contained 2 mg of protein. ●, 2-aminobenzoyl-CoA formation; ■, 2-aminobenzoyl-CoA reduction.

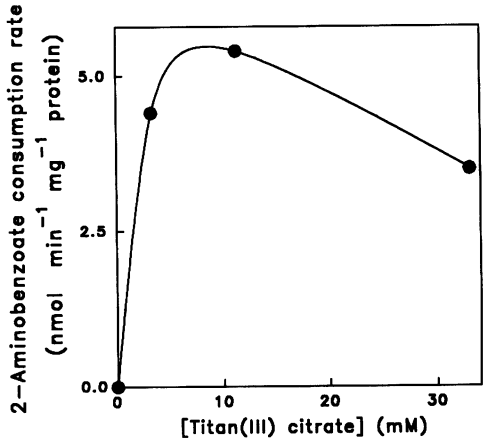

FIG. 3. [U-^{14}C]2-aminobenzoyl-CoA reduction by cell extracts with different concentrations of Ti(III) citrate as the reductant. The radioisotope assay used is described in Materials and Methods. Cells were anaerobically grown on 2-aminobenzoate and nitrate.

Requirements of 2-aminobenzoyl-CoA reductase (deaminating). The dependence of the overall reaction on the individual test components was tested by using the radioisotope assay (Table 1). The transformation of 2-aminobenzoate was dependent on the extract, Mg^{2+}-ATP, CoA, and Ti(III) and was inhibited by oxygen. The requirement for CoA is probably indirect since it was necessary for 2-aminobenzoyl-CoA formation. The effect of ATP is described below. Although ATP alone was sufficient, an ATP-regenerating system was used when the assay conditions were modified to avoid ATP limitation due to ATPases. For similar reasons, DTE and purified 2-aminobenzoate-CoA ligase from *Pseudomonas* strain KB 740 were included, although they did not stimulate the assay under standard conditions. DTE stabilizes the oxygen-sensitive enzyme, and the endogenous 2-aminobenzoate-CoA ligase in the extract may be limiting when cells which were grown on substrates other than 2-aminobenzoate are tested. Furthermore, extracts contained an unspecific thioesterase activity which may exhaust the substrate.

TABLE 1. Requirements of in vitro reduction of 2-aminobenzoyl-CoA to benzoyl-CoA catalyzed by 2-aminobenzoyl-CoA reductase (deaminating) from *Pseudomonas* K 172

Assay	Sp act (%)[a]	% 2-Aminobenzoate at 30 min[b]
Complete	100	1
− Ti(III)	9	2
− ATP	<1	100
− ATP-regenerating system, + ATP	70	2
− CoA	4	71
− Exogenous 2-aminobenzoate-CoA ligase	99	2
− Extract	<1	1
Complete, aerobic	10	1

[a] In the complete assay, the specific activity of 2-aminobenzoyl-CoA reductase was 8 nmol min^{-1} mg^{-1} of cell protein (100%). Values represent percentages of that for the complete assay.

[b] To test whether 2-aminobenzoate was converted to 2-aminobenzoyl-CoA or products, the radioisotope assay as described in Materials and Methods, was used to determine the amount of 2-aminobenzoate after 30 min of incubation. Values refer to free 2-aminobenzoate in the assay.

The reaction proceeded linearly over time from 0 to 10 min, and the rate was linearly dependent on the amount of protein added in the range of 0 to 2 mg of protein per 0.35-ml assay mixture. The dependence of the reaction on titanium (III) citrate was strict, and the optimal concentration was around 10 mM (Fig. 3). The pH optimum of 2-aminobenzoyl-CoA reduction determined by using MOPS-KOH and TRIS-HCl was around pH 7.

In the spectrophotometric assay, the dependence of the enzyme on synthetically formed 2-aminobenzoyl-CoA and titanium(III) citrate was shown. 2-Aminobenzoate was not used as the substrate. The reaction rate increased in a similar manner with the titanium(III) citrate concentration, and the optimal concentration was about 3 mM. CoA was not required if the substrate 2-aminobenzoyl-CoA was added, but the reaction was dependent on Mg^{2+}-ATP.

Products of 2-aminobenzoyl-CoA metabolism. The radioactive products formed from [U-^{14}C]2-aminobenzoyl-CoA were analyzed. Seven radioactive products, designated 1 through 6 and B (Fig. 4), were found. The product pattern was reproducible and was independent of the pH of the assay mixture and the batch of cells. Three types of enzymatic 2-aminobenzoyl-CoA reduction can be envisaged as follows. (i) 2-Aminobenzoyl-CoA could be reduced to the aldehyde, and the reaction product should be 2-aminobenzaldehyde. (ii) The aromatic ring could be directly reduced, resulting in unknown products. (iii) The amino substituent could be reductively removed, whereby benzoyl-CoA and products obtained by further metabolism of benzoyl-CoA are expected.

The labeled products were still CoA thioesters, because the free acids were released only after alkaline hydrolysis of the thioester bond (Fig. 4, lanes 11; note that CoA thioesters do not migrate in this system). The reduction of 2-aminobenzoyl-CoA to the corresponding aldehyde (reduction type i) was therefore excluded. Product 3 could be an exception to this rule, since it was present in the CoA-free form already after the acidic termination of the assay. Three of the reaction products were preliminarily identified by cochromatography by using TLC. Product B cochromatographed

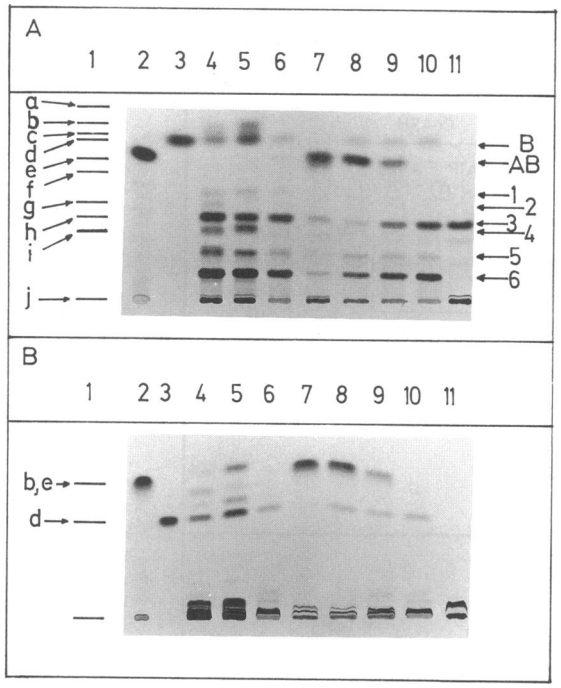

FIG. 4. Separation by TLC of the products of the reductive conversion of [U-^{14}C]2-aminobenzoyl-CoA to [^{14}C]benzoyl-CoA and six additional major products. Unless otherwise indicated, the thioesters were hydrolyzed by alkaline treatment before chromatography. (A) Separation by TLC system A; (B) separation by TLC system B. Lane 1, schematic representation of the R_f values of the following reference compounds: cyclohexanecarboxylic acid (a), cyclohex-1-enecarboxylic acid (b), 2-oxocyclohexanecarboxylic acid (c), benzoic acid (d), 2-aminobenzoic acid (e), salicylic acid (f), pimelic acid (g), *cis*-2-hydroxycyclohexanecarboxylic acid (h), *trans*-2-hydroxycyclohexanecarboxylic acid (i), and CoA thioesters (j). Lane 2, [U-^{14}C]2-aminobenzoate; lane 3, [U-^{14}C]benzoate; lane 4, assay mixture containing [U-^{14}C]benzoyl-CoA, 10 min incubation with extract from cells grown on 2-aminobenzoate; lane 5, same as lane 4 but extracts from cells grown on benzoate; lane 6, assay mixture containing [U-^{14}C]2-aminobenzoyl-CoA, 10 min incubation with extract from cells grown on 2-aminobenzoate; lanes 7 to 10, same as lane 6 but incubation for 15 s, and 2.5, 5, and 30 min, respectively; lane 11, same as lane 10 but before alkaline treatment. Numbers 1 to 6 to the right refer to the six products found by this method. B, benzoate; AB, 2-aminobenzoate. For descriptions of the TLC systems and the radioisotope assay, see Materials and Methods.

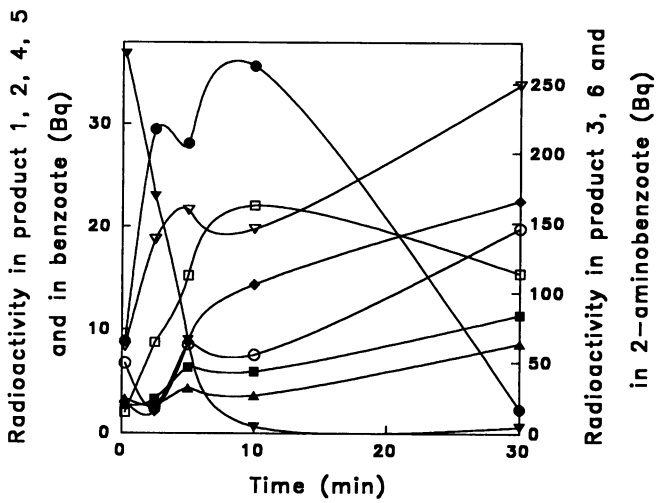

FIG. 5. Kinetics of [U-^{14}C]2-aminobenzoate consumption and formation of benzoate and six additional products. Benzoate and most of the other products were present as CoA thioesters. The amount of ^{14}C in the products refers to the 5-μl assay mixture treated with alkali. The first sample was withdrawn after 15 s of incubation. ▼, 2-aminobenzoate; ●, benzoate; ■, product 1; ▲, product 2; ◆, product 3; ○, product 4; ▽, product 5; □, product 6. For a description of the radioisotope assay, see Materials and Methods.

with benzoate. This suggests that the aromatic ring of 2-aminobenzoyl-CoA was not directly reduced, excluding reduction type ii. It is concluded that benzoyl-CoA is the immediate product of 2-aminobenzoyl-CoA transformation (reduction type iii).

The reductive 2-aminobenzoyl-CoA conversion, therefore, was catalyzed by a deaminating 2-aminobenzoyl-CoA reductase. Since benzoyl-CoA is completely oxidized to CO_2, cell extracts might be able to metabolize benzoyl-CoA further by reduction of the aromatic ring. This reaction may result in the formation of the other labeled products obtained during [U-^{14}C]2-aminobenzoate transformation.

In order to test this possibility, the product pattern was studied with [U-^{14}C]benzoate instead of [U-^{14}C]2-aminobenzoate. [U-^{14}C]benzoyl-CoA is formed by purified 2-aminobenzoate-CoA ligase of *Pseudomonas* strain KB 740, which in addition to 2-aminobenzoate also activates benzoate and fluorobenzoate (3). The same product pattern was observed with [U-^{14}C]benzoate, which was unvaried when cells anaerobically grown on benzoate or 2-aminobenzoate were examined. However, benzoate-grown cells were unable to metabolize [U-^{14}C]2-aminobenzoate (see below). This shows clearly that all other labeled products of enzymatic [U-^{14}C]2-aminobenzoyl-CoA reduction were formed secondarily from [U-^{14}C]benzoyl-CoA. This requires a separate reductase system which reductively attacks the aromatic ring of benzoyl-CoA.

The products of reductive [U-^{14}C]benzoyl-CoA conversion were studied after alkaline treatment by cochromatography with different TLC and HPLC systems with authentic standards. The product migrating faster than benzoate (Fig. 4A, lanes 4 and 5) was identified as cyclohex-1-enecarboxylic acid. Product 4 was preliminarily identified as *trans*-2-hydroxycyclohexanecarboxylic acid (25a). The other products were not identical with any of the following compounds: 2-hydroxybenzoic acid, 2-oxocyclohexanecarboxylic acid, *cis*-2-hydroxycyclohexanecarboxylic acid, cyclohexanecarboxylic acid, or pimelic acid.

The kinetics of the formation of labeled products from [U-^{14}C]2-aminobenzoyl-CoA are shown in Fig. 5. Benzoate and products 3, 5, and 6 obtained by benzoyl-CoA reduction seemed to be early labeled products, whereas products 1, 2, and 4 appeared to be formed later. The main products were 3 and 6 (each 70 μM, maximal concentration). The other products were formed in smaller amounts (benzoate and product 5, 16 μM; product 4, 10 μM; products 1 and 2, 4 μM). All products plus residual 2-aminobenzoate accounted for 80 to 90% of the labeling of all samples.

Expression of 2-aminobenzoate-CoA ligase (AMP forming) and 2-aminobenzoyl-CoA reductase (deaminating) activities. The induction of the initial two enzyme activities responsible for anaerobic 2-aminobenzoate metabolism, 2-aminobenzoate-CoA ligase (AMP forming), and 2-aminobenzoyl-CoA

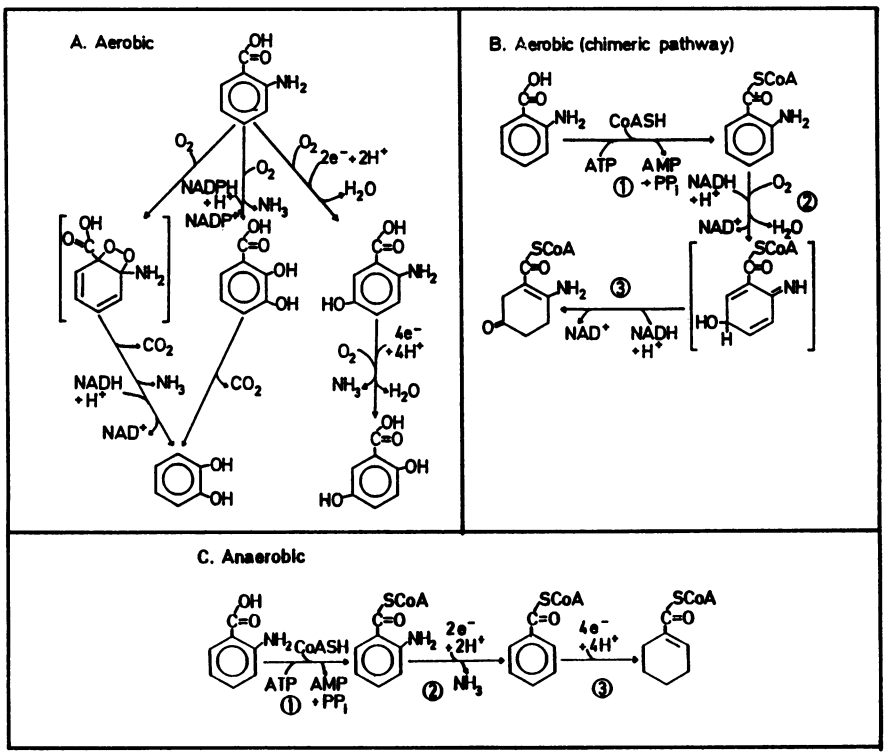

FIG. 6. Initial reactions in different pathways of 2-aminobenzoate breakdown. (A) Aerobic degradation via catechol and gentisic acid. (B) Aerobic breakdown in a related *Pseudomonas* strain KB 740 via CoA thioesters. ①, 2-aminobenzoate-CoA ligase; ②, 2-aminobenzoyl-CoA monooxygenase; ③, 2-aminobenzoyl-CoA reductase. (C) Pathway of anaerobic 2-aminobenzoate degradation in *Pseudomonas* strain K 172 as proposed from the results of this study. ①, 2-aminobenzoate-CoA ligase (AMP forming); ②, 2-aminobenzoyl-CoA reductase (deaminating); ③, benzoyl-CoA reductase (aromatic ring reducing).

reductase (deaminating) was studied by comparing cells grown anaerobically on 2-aminobenzoate, benzoate, or acetate or those grown aerobically on 2-aminobenzoate. A regulation of this pathway was observed in the experiments with whole cells by using the simultaneous adaptation method.

2-Aminobenzoyl-CoA reduction was catalyzed only by extracts of cells anaerobically grown on 2-aminobenzoate. No activity after aerobic growth was observed, and almost no activity was found with cells grown anaerobically on benzoate (<5%) or acetate (<2%). The specific 2-aminobenzoate-CoA ligase activity was four times higher (25 nmol min^{-1} mg^{-1} of protein) in cells grown anaerobically on 2-aminobenzoate compared with cells grown on benzoate (6 to 7 nmol min^{-1} mg^{-1} of protein). These findings suggest that the syntheses of the first and second enzymes of the pathway are controlled in a similar manner.

Comparison of the protein pattern of cells grown anaerobically on 2-aminobenzoate and benzoate. The regulatory pattern of enzyme activities was interpreted as the result of differential induction of the enzymes of the anaerobic 2-aminobenzoate degradation pathway. In order to get an insight into how complex the enzyme system is which converts 2-aminobenzoate to benzoyl-CoA, the protein pattern of extracts from cells grown on 2-aminobenzoate and benzoate was compared by SDS-PAGE. Cells grown on 2-aminobenzoate showed four additional protein bands (80, 61, 59, and 42 kDa) which were almost lacking in benzoate-grown cells. In benzoate-grown cells, two proteins (69 and 36 kDa) seemed to be present in significantly higher amounts compared with results with 2-aminobenzoate-grown cells.

DISCUSSION

We have provided evidence for the anaerobic degradation of 2-aminobenzoate via activation to 2-aminobenzoyl-CoA and reductive deamination to benzoyl-CoA, the central aromatic intermediate (Fig. 6C) (17, 18). A new enzyme, which catalyzes the reductive removal of the *ortho* amino group of the activated aromatic acid, was found; this enzyme is referred to as 2-aminobenzoyl-CoA reductase (deaminating). The physiological electron donor is not known; the low-potential reductant Ti(III) is active in vitro. The specific activity (15 nmol min^{-1} mg^{-1} of protein) is close to the estimated 2-aminobenzoate degradation rate of cells (30 to 40 nmol min^{-1} mg^{-1} of protein) growing with a generation time of 10 h.

Both the oxygen-sensitive 2-aminobenzoyl-CoA reductase and the oxygen-insensitive 2-aminobenzoate–CoA ligase were induced by 2-aminobenzoate under denitrifying conditions; both enzyme activities were not detectable or were much lower in cells grown under different conditions. The residual 2-aminobenzoate-CoA ligase activity in benzoate-grown cells is probably due to benzoate-CoA ligase, which also activates 2-aminobenzoate (28% relative activity) (3). The data suggest that 2-aminobenzoate or 2-aminobenzoyl-CoA acts as an inducer and that the expression of the two enzymes is under oxygen control. Since benzoyl-CoA is formed directly in the anaerobic degradation of 2-aminobenzoate and other aromatic acids, the benzoyl-CoA reducing enzyme should be induced by benzoyl-CoA. This would imply a sequential induction. The comparison of the protein pattern of cells grown on benzoate and 2-aminobenzoate

revealed four additional protein bands in 2-aminobenzoate-grown cells. The aromatic acid-CoA ligases purified so far are composed of one or two identical subunits with a M_r of about 60,000 (3). Therefore, it is expected that one of the proteins corresponds to the CoA ligase, and the 2-aminobenzoyl-CoA reductase may consist of several subunits.

2-Aminobenzoyl-CoA reductase catalyzes an interesting, novel type of reaction, the reductive removal of a substituent from the aromatic nucleus. It requires relatively high concentrations of a strong reductant [Ti(III)] and possibly ATP. The ATP effect needs to be studied in detail with substrate amounts of 2-aminobenzoyl-CoA and requires purification of the enzyme. In the anaerobic metabolism of aniline and 4-aminobenzoate, similarly 4-aminobenzoyl-CoA is reductively deaminated to benzoyl-CoA (34). The reductive removal of other substituents of the aromatic ring has been discussed elsewhere (14, 21, 38).

The further metabolism of benzoyl-CoA appears to be common for such diverse aromatic substrates as benzoate, phenol, 4-hydroxybenzoate, toluene, benzyl alcohol, benzaldehyde, *p*-cresol, 4-hydroxybenzylalcohol, 4-hydroxybenzaldehyde, phenylacetate, and 4-hydroxyphenylacetate (2, 12, 25a, 32a, 38). The labeled products formed from [U-^{14}C] 2-aminobenzoate and [U-^{14}C]benzoate were the same. One of the products was cyclohex-1-enecarboxyl-CoA. Its formation requires the four-electron reduction of the benzene nucleus, either in a single step or in sequential two-electron reduction steps. The formation of the second identified product, *trans*-2-hydroxycyclohexanecarboxyl-CoA, is easily explained by *trans* addition of water to the cyclohex-1-ene double bond. Whereas these CoA thioesters were hydrolyzed only by alkali, one of the products was already present in the CoA-free form after the acidic stop of the reaction. This suggests that this product may be more sensitive to hydrolysis than the other activated acids. Work is in progress to identify the products of benzoyl-CoA reduction and the sites and the stereochemistry of the reductive attack at the aromatic nucleus.

So far, three quite different types of microbial 2-aminobenzoate metabolism have been reported. The initial steps of the anaerobic metabolism in denitrifying bacteria, as studied in this work, are summarized in Fig. 6C. Three classical aerobic pathways, which require dioxygenases for ring cleavage, lead either to catechol or gentisic acid as intermediates (4, 11, 25) (Fig. 6A). Recently, a chimeric pathway of aerobic 2-aminobenzoate metabolism, which combines characteristics of both the aerobic and the anaerobic degradation pathways, was found (1, 29). It proceeds via CoA thioesters as in anaerobic degradation, but the aromatic ring reduction is coupled with simultaneous O_2-dependent hydroxylation of the benzene ring in the *para* position to the amino group (Fig. 6B).

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

1. **Altenschmidt, U., C. Eckerskorn, and G. Fuchs.** 1990. Evidence that enzymes of a novel aerobic 2-aminobenzoate metabolism in denitrifying *Pseudomonas* are coded on a small plasmid. Eur. J. Biochem. **194:**647–653.
2. **Altenschmidt, U., and G. Fuchs.** 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central intermediate. Arch. Microbiol. **156:**152–158.
2a. **Altenschmidt, U., and G. Fuchs.** Eur. J. Biochem., in press.
3. **Altenschmidt, U., B. Oswald, and G. Fuchs.** 1991. Purification and characterization of benzoate-coenzyme A ligase and 2-aminobenzoate-coenzyme A ligases from a denitrifying *Pseudomonas* sp. J. Bacteriol. **173:**5494–5501.
4. **Anderson, J. J., and S. Dagley.** 1981. Catabolism of tryptophane, anthranilate, and 2,3-dihydroxybenzoate in *Trichosporon cutaneum*. J. Bacteriol. **146:**291–297.
5. **Balba, M. T., and W. C. Evans.** 1980. Methanogenic fermentation of the naturally occurring aromatic amino acids by a microbial consortium. Biochem. Soc. Trans. **8:**625–627.
6. **Berry, D. F., E. L. Madsen, and J.-M. Bollag.** 1987. Conversion of indole to oxindole under methanogenic conditions. Appl. Environ. Microbiol. **53:**180–182.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Braun, K., and D. T. Gibson.** 1984. Anaerobic degradation of 2-aminobenzoate (anthranilic acid) by denitrifying bacteria. Appl. Environ. Microbiol. **48:**102–107.
9. **Brock, T. D., and M. T. Madigan.** 1988. Biology of microorganisms, 5th ed., p. 362–363. Prentice-Hall, Inc., Englewood Cliffs, N.J.
10. **Buder, R., K. Ziegler, G. Fuchs, B. Langkau, and S. Ghisla.** 1989. 2-Aminobenzoyl-CoA monooxygenase/reductase, a novel type of flavoenzyme. Studies on the stoichiometry and the course of the reaction. Eur. J. Biochem. **185:**637–643.
11. **Cain, R. B.** 1968. Anthranilic acid metabolism by microorganisms. Formation of 5-hydroxyanthranilate as an intermediate in anthranilate metabolism by *Nocardia opaca*. Antonie van Leeuwenhoek **34:**417–432.
12. **Dangel, W., R. Brackmann, A. Lack, M. Mohamed, J. Koch, B. Oswald, B. Seyfried, A. Tschech, and G. Fuchs.** 1991. Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA. Arch. Microbiol. **155:**256–262.
13. **Dawson, R. X., D. C. Elliott, W. H. Elliott, and M. J. Jones.** 1986. Data for biochemical research, 3rd ed., p. 36–37. Clarendon Press, Oxford.
14. **Dolfing, J., and J. M. Tiedje.** 1987. Growth yield increase linked to reductive dechlorination in a defined 3-chlorobenzoate degrading methanogenic coculture. Arch. Microbiol. **149:**102–105.
15. **Doy, C. H., and F. Gibson.** 1959. 1-(o-Carboxyphenylamino)-1-deoxyribulose. A compound formed by mutant strains of *Aerobacter aerogenes* and *Escherichia coli* blocked in the biosynthesis of tryptophane. Biochem. J. **72:**585–589.
16. **Egebo, L. A., S. V. S. Nielsen, and B. U. Jochimsen.** 1991. Oxygen-dependent catabolism of indole-3-acetic acid in *Bradyrhizobium japonicum*. J. Bacteriol. **173:**4897–4901.
17. **Evans, W. C.** 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature (London) **270:**17–22.
18. **Evans, W. C., and G. Fuchs.** 1988. Anaerobic degradation of aromatic compounds. Annu. Rev. Microbiol. **42:**289–317.
19. **Fujioka, M., and H. Wada.** 1968. The bacterial oxidation of indole. Biochim. Biophys. Acta **158:**70–78.
20. **Geissler, J. F., C. S. Harwood, and J. Gibson.** 1988. Purification and properties of benzoate-coenzyme A ligase, a *Rhodopseudomonas palustris* enzyme involved in the anaerobic degradation of benzoate. J. Bacteriol. **170:**1709–1714.
21. **Glöckler, R., A. Tschech, and G. Fuchs.** 1989. Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA in a denitrifying, phenol-degrading *Pseudomonas* species. FEBS Lett. **251:**237–240.
22. **Harwood, C. S., and J. Gibson.** 1986. Uptake of benzoate by *Rhodopseudomonas palustris* grown anaerobically in light. J. Bacteriol. **165:**504–509.
23. **Hutber, G. N., and D. W. Ribbons.** 1983. Involvement of coenzyme A esters in the metabolism of benzoate and cyclohexanecarboxylate by *Rhodopseudomonas palustris*. J. Gen. Microbiol. **129:**2413–2420.
24. **Kamath, A. V., and C. S. Vaidyanathan.** 1990. New pathway for the biodegradation of indole in *Aspergillus niger*. Appl. Envi-

ron. Microbiol. **56**:275–280.
25. **Kobayashi, S., S. Kuno, and N. Itada.** 1964. O^{18} studies on anthranilate hydroxylase—a novel mechanism of double hydroxylation. Biochim. Biophys. Res. Commun. **16**:556–561.
25a. **Koch, J., and G. Fuchs.** 1992. Enzymatic reduction of benzoyl-CoA to alicyclic compounds, a key reaction in anaerobic aromatic metabolism. Eur. J. Biochem. **205**:195–202.
26. **Kunapuli, S. P., and C. S. Vaidyanathan.** 1983. Purification and characterization of a new indole oxygenase from the leaves of *Tecoma stans* L. Plant Physiol. **71**:19–23.
27. **Kunapuli, S. P., and C. S. Vaidyanathan.** 1984. Indolic compounds in the leaves of *Tecoma stans*. Phytochemistry **23**:1826–1827.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
29. **Langkau, B., S. Ghisla, R. Buder, K. Ziegler, and G. Fuchs.** 1990. 2-Aminobenzoyl-CoA monooxygenase/reductase, a novel type of flavoenzyme. Identification of the reaction products. Eur. J. Biochem. **191**:365–371.
30. **Löffler, F., and R. Müller.** 1991. Identification of 4-chlorobenzoyl-coenzyme A as intermediate in the dehalogenation catalysed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3. FEBS Lett. **290**:224–226.
31. **Madsen, E. L., and J.-M. Bollag.** 1989. Pathway of indole metabolism by a denitrifying microbial community. Arch. Microbiol. **151**:71–76.
32. **Merkel, S. M., A. E. Eberhard, J. Gibson, and C. S. Harwood.** 1989. Involvement of coenzyme A thioesters in anaerobic metabolism of 4-hydroxybenzoate by *Rhodopseudomonas palustris*. J. Bacteriol. **171**:1–7.
32a. **Mohamed, M., B. Seyfried, A. Tschech, and G. Fuchs.** Unpublished results.
33. **Morrissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
34. **Schnell, S., and B. Schink.** 1991. Anaerobic aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*. Arch. Microbiol. **155**:183–190.
35. **Stahl, E.** 1967. Geräte und allgemeine Techniken der DC, p. 79–81. *In* E. Stahl (ed.), Dünnschichtchromatographie, 2th ed. Springer-Verlag KG, Berlin.
36. **Tatum, E. L., and D. M. Bonner.** 1943. Synthesis of tryptophane from indole and serine by *Neurospora*. J. Biol. Chem. **151**:349.
37. **Tschech, A., and G. Fuchs.** 1987. Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying *pseudomonads*. Arch. Microbiol. **148**:213–217.
38. **Tschech, A., and G. Fuchs.** 1989. Anaerobic degradation of phenol via carboxylation to 4-hydroxybenzoate: in vitro study of isotope exchange between $^{14}CO_2$ and 4-hydroxybenzoate. Arch. Microbiol. **152**:594–599.
39. **Tschech, A., and B. Schink.** 1988. Methanogenic degradation of anthranilate (2-aminobenzoate). Syst. Appl. Microbiol. **11**:9–12.
40. **Wang, Y.-T., M. T. Suidan, and J. T. Pfeffer.** 1984. Anaerobic biodegradation of indole to methane. Appl. Environ. Microbiol. **48**:1058–1060.
41. **Yanofsky, C.** 1956. The enzymatic conversion of anthranilic acid to indole. J. Biol. Chem. **223**:171–184.