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Anaerobic Degradation of Catechol by *Desulfobacterium* sp. Strain Cat2 Proceeds via Carboxylation to Protocatechuate

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Under anoxic conditions, most methoxylated mononuclear aromatic compounds are degraded by bacteria, with catechol being formed as an important intermediate. On the basis of our experiments with the sulfate-reducing bacterium *Desulfobacterium* sp. strain Cat2, we describe for the first time the enzymatic activities involved in the complete anaerobic oxidation of catechol and protocatechuate. Results obtained from experiments with dense cell suspensions of strain Cat2 demonstrated that all enzymes necessary for protocatechuate and benzoate degradation were induced during growth with catechol. In addition, anaerobic oxidation of catechol was found to be a CO_2-dependent process. Phenol was not degraded in suspensions of cells grown with catechol. In cell extracts of *Desulfobacterium* sp. strain Cat2, protocatechuyl-coenzyme A (CoA) was formed from catechol, bicarbonate, and uncombined CoA. This oxygen-sensitive reaction requires high concentrations of both bicarbonate and protein, and only very low levels of enzyme were detected. In a second oxygen-sensitive step, protocatechuyl-CoA was reduced to 3-hydroxybenzoyl-CoA by reductive elimination of the *p*-hydroxyl group. Further dehydroxylation to benzoyl-CoA was not detectable. Key reactions described for anaerobic degradation of benzoate were catalyzed by cell extracts of strain Cat2, too.

Aerobic biodegradation of lignin by fungi and bacteria produces several different aromatic compounds carrying methylated hydroxyl groups. Since the ligninolytic enzymes of these organisms are located extracellularly, diffusion of methoxy aromatics into anoxic habitats is possible, and as a consequence, anaerobic degradation can take place (8, 11).

An important criterion for degradability of lignin derivatives is their molecular mass. Until now, investigations of anaerobic degradation of oligomeric products were contradictory, but there were some indications of low levels of degradation activity in lake sediments when the relative molecular weight was lower than 600 (8). In contrast, there is no doubt about anaerobic transformation and degradation of monomeric derivatives. In 1979, Healy and Young (16) demonstrated the fermentive degradation of methoxylated aromatic acids by undefined methanogenic mixed cultures, and soon after, the respective hydroxyl derivatives were identified as products of this process (1). Most lignin monomers tested were transformed to catechol, which therefore is an important intermediate in anaerobic degradation of aromatic compounds released from wood (18).

Although anaerobic degradation of catechol was known for a long time (16), no physiological or biochemical investigations were carried out. In an early hypothesis, phenol was favored as the first intermediate of the pathway, but until now, no data confirming this hypothesis have been published (2). Recently, pure cultures growing anaerobically with catechol and sulfate were isolated (20, 24, 26), but no evidence of reductive dehydroxylation to phenol was found. In contrast, some indications of carboxylation as an initial reaction were obtained in investigations with a newly isolated *Desulfotomaculum* strain (20).

In the present study, we report results of experiments with cell suspensions and cell extracts of *Desulfobacterium* sp. strain Cat2 which allow us to propose a new pathway for anaerobic catechol degradation.

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MATERIALS AND METHODS

Growth conditions. *Desulfobacterium* sp. strain Cat2, DSM 8540, was grown in saltwater-mineral medium as described previously (24). Aromatic substrates were stored anoxically as neutralized stock solutions in sterile infusion bottles under nitrogen gas and were added to cultures with syringes. Cultivation of strain Cat2 was performed in 1,200-ml infusion bottles sealed with natural rubber septa, with 2 mM aromatic substrate and 20 mM Na_2SO_4 under an N_2-CO_2 (90:10) atmosphere at 28°C in the dark.

Experiments with dense cell suspensions. Cell suspensions were prepared as previously described (14). Cell density was adjusted to an optical density at 578 nm of 15, and experiments were performed anoxically under N_2 in 5-ml, rubber-sealed Hungate tubes. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM dithioerythritol, 1 to 2 mM substrate, 5 mM $MgCl_2$, 20 mM Na_2SO_4, and 0.2 M NaCl. Samples were taken with gas-tight glass syringes and diluted in H_3PO_4 to a 0.1 M final concentration.

The induction of degradative capacities was tested in the presence and absence of chloramphenicol (30 $\mu g \cdot mg$ of protein^{-1}). The dependence of substrate degradation on CO_2 was tested in 50 mM potassium phosphate buffer (pH 7.0) supplemented with 2 mM dithioerythritol, 5 mM $MgCl_2$, and 0.2 M NaCl by the addition or omission of 50 mM $NaHCO_3$.

Enzyme assays. Cell extracts were prepared anoxically by French pressure cell treatment of freshly harvested cells at 138 MPa of pressure. Cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 4°C. Cell extracts were active for 6 to 8 h if kept on ice. No or only low enzyme activity was detected in extracts prepared from pellets stored in liquid nitrogen.

Enzyme assays were carried out under anoxic conditions at 25°C either in tests discontinuously analyzed by high-pressure liquid chromatography (HPLC) or in continuous photometric assays. All test vessels were evacuated and gassed with N_2

through gas-tight rubber septa. Samples for HPLC analysis were taken with syringes. All assays were carried out with at least three independently prepared cell extracts. Each single measurement was checked by the addition of two different amounts of cell extract in two parallels.

Carboxylation of catechol was assayed discontinuously with 30 to 850 mM $NaHCO_3$ with 100 mM Tricine-KOH buffer (pH 9.0 or 9.2). The assay mixture contained 1 to 5 mM catechol, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 to 10 mM ATP, and 0.5 to 4.2 mg of protein per ml. To combine the carboxylation reaction with a subsequent coenzyme A (CoA)-ligase reaction, uncombined CoA (CoASH) was added in some test series. Cofactors such as biotin, thiamine PP_i, pyridoxal phosphate, pyridoxamine hydrochloride, pyridoxal hydrochloride, or vitamin B_6 (50 to 100 μM each) were tested in this assay system as well.

In addition, carboxylation of catechol and phenol was tested after the exchange of ^{14}C of [^{14}C]carbonate into the carboxyl group of protocatechuate and 4-hydroxybenzoate in a discontinuous assay as described previously (28). The assays were performed in 100 mM Tris-HCl buffer (pH 9.2).

Reduction of catechol was assayed after phenol formation by the addition of dithionite-reduced benzyl or methyl viologen, titanium(III) citrate, NAD(P)H, or formate (1 to 5 mM each) (14). Alternatively, the headspace of the test vial was flushed with hydrogen.

Reductive dehydroxylation of aromatic CoA esters, glutaryl-CoA dehydrogenase (EC 1.3.99.7), and glutaconyl-CoA decarboxylase (EC 4.1.1.70) was assayed as described recently (14).

Protocatechuate decarboxylase was measured, and a decrease of protocatechuate at 288 nm (ε_{288} = 3.65 $mM^{-1} \cdot cm^{-1}$) was recorded. The assay mixture contained 100 mM potassium phosphate buffer (pH 6.0), 5 mM $MgCl_2$, and 0.3 mM protocatechuate. Inhibition of the decarboxylating enzyme was performed by the addition of avidin (0.05 to 0.2 mg of avidin per mg of cell protein).

Carbon monoxide dehydrogenase (EC 1.2.99.2) was measured, and a benzyl viologen reduction upon addition of carbon monoxide at 578 nm (10) was recorded. Formate dehydrogenase (EC 1.2.1.2) was assayed analogously.

Crotonase (EC 4.2.1.17), 3-hydroxybutyryl–CoA dehydrogenase (EC 1.1.1.157), and β-ketothiolase (EC 2.3.1.16) were measured by standard methods (3).

PP_i phosphohydrolase (EC 3.6.1.1) was tested by measuring P_i formation from PP_i catalyzed by the cell extract. The assay mixture contained 100 mM Tris-HCl buffer (pH 8.2), 2 mM dithioerythritol, 1 mM $MgCl_2$, and 5 mM Na_4 PP_i.

Chemical determinations and chemicals. Aromatic compounds, CoASH, CoA esters, ATP, ADP, and AMP were analyzed and quantified by HPLC (6, 14). Identification of CoA esters was performed as described previously (14).

P_i was quantified as described by Chen et al. (7).

Protein was determined as described by Bradford (5), with bovine serum albumin as the standard.

Aromatic CoA esters were synthesized from CoASH and the corresponding anhydride (22). In addition, protocatechuyl-CoA was prepared enzymatically in a CoA-ligase reaction with cell extract of a newly isolated denitrifying bacterium (strain AR1) grown anaerobically with protocatechuate (13). The reaction was stopped by centrifugation at 3,000 × *g* in a Centriprep concentrator (Amico, Witten, Germany) for 60 min at 4°C. The filtrate contained up to 0.15 mM protocatechuyl-CoA, which was purified by HPLC.

All other chemicals used were of analytical grade and were obtained from Boehringer, Mannheim, Germany; Fluka, Neu-Ulm, Germany; Merck, Darmstadt, Germany; Serva, Heidelberg, Germany; and Sigma, Deisenhofen, Germany.

RESULTS

Induction experiments with dense cell suspensions of strain Cat2. Anaerobic degradation of catechol, protocatechuate, 4-hydroxybenzoate, 3-hydroxybenzoate, benzoate, and phenol was tested with cell suspensions of strain Cat2 grown with catechol and sulfate. In suspension experiments with strain Cat2, catechol was degraded at a rate of 11 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$, whereas in analogous assays with phenol substrate, degradation started with a lag phase of 2 h only at a low rate (2 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$). Protocatechuate was mainly decarboxylated to catechol in the assay mixture. The concentration of total aromatic compounds decreased during the incubation experiment at a significant rate (3 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$). Benzoate and 4-hydroxybenzoate were degraded within 5 h, but no decrease in substrate concentration was observed with 3-hydroxybenzoate.

Dependence of catechol oxidation on CO_2. The rate of catechol degradation was significantly enhanced upon addition of bicarbonate (10 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$). In bicarbonate-free assays, the oxidation started slowly after a lag phase of 1 h. The rate of 4-hydroxybenzoate degradation was independent of bicarbonate (16 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ in both assays).

Enzyme activities. In assays with cell extracts of catechol-grown cells, a catechol-reducing activity forming phenol could not be detected. Besides dithionite-reduced viologen derivatives, NAD(P)H and titanium(III) citrate were used as electron donors (1 to 5 mM each).

A catechol-carboxylating enzyme activity was not found in either direct assays or assays monitoring ^{14}C exchange with the carboxyl group of protocatechuate. In contrast, protocatechuate decarboxylase was detected at high activity in cell extracts (Fig. 1a). The specific activity was 6.2 to 6.3 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ if cells were grown with catechol and 4.2 to 4.8 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ after growth with protocatechuate. No activity was detected if strain Cat2 was cultivated with 3-hydroxybenzoate or benzoate. The decarboxylating enzyme had an apparent optimum pH of 6.0 (Fig. 1b). No activity was measured at pH values below 4 and above 9. Therefore, further enzyme assays with protocatechuate were performed under these pH conditions. The specific activity of the protocatechuate decarboxylase was not influenced by addition of avidin or EDTA (5 mM).

Under alkaline conditions (pH 9.2), protocatechuate and 4-hydroxybenzoate were activated to their CoA esters in an ATP- and Mg^{2+}-dependent reaction by extracts of catechol-grown cells of strain Cat2. The specific activity was determined to be 22 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ for 4-hydroxybenzoate and 15 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ for protocatechuate. 3-Hydroxybenzoate was not activated.

The complete CoA-ligase assay was applied to carboxylation experiments, and catechol was used as a substrate again. If $NaHCO_3$ was added at high concentrations (0.2 to 0.5 M), a slow decrease in catechol, CoASH, and ATP was detected, while AMP and a product coeluting with protocatechuyl-CoA were produced. The specific activity of catechol consumption and protocatechuyl-CoA formation was 1 to 3 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$. No such activity or production of 4-hydroxybenzoyl–CoA was found if phenol was used as substrate. The addition of avidin did not influence this enzyme activity, but exposure of cell extract to air for 5 min caused total loss of activity. The catechol-carboxylating activity was characterized by low stability even during storage on ice or in liquid nitrogen. Therefore, reproducible results were achieved with at least nine independently prepared cell extracts.

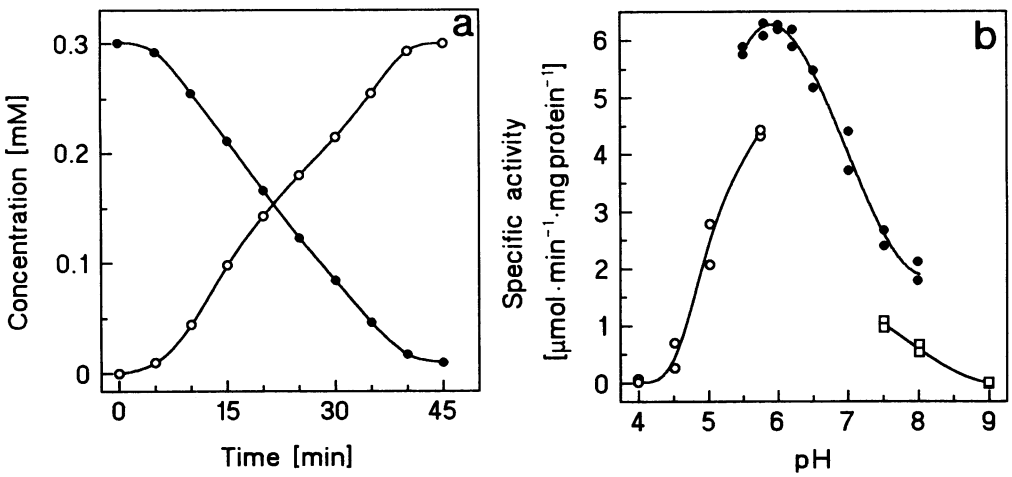

FIG. 1. (a) Decarboxylation of protocatechuate in cell extracts prepared from catechol-grown cells of strain Cat2. Symbols: ●, protocatechuate; ○, catechol. (b) Influence of pH on protocatechuate decarboxylase activity. Symbols: ○, 30 mM acetate buffer; ●, 50 mM potassium phosphate buffer; □, 50 mM Tris-HCl buffer.

Reductive dehydroxylation of aromatic CoA esters was assayed in a combined test with CoA-ligase or by addition of enzymatically synthesized substrate (0.2 to 0.3 mM) in the presence of an electron donor (1 to 5 mM). Cell extracts prepared from catechol-grown cells catalyzed reductive elimination of the *p*-hydroxyl group of protocatechuyl-CoA or of 4-hydroxybenzoyl–CoA. A typical time course of a combined test is shown in Fig. 2. Benzyl viologen and methyl viologen proved suitable as electron donors (Table 1). A decrease in protocatechuyl-CoA concentration started immediately upon addition of dithionite, and there was a stoichiometric increase in the 3-hydroxybenzoyl–CoA concentration. The highest specific activity was 12 nmol · min^{-1} · mg of $protein^{-1}$ with dithionite-reduced methyl viologen for both substrates, protocatechuyl-CoA and 4-hydroxybenzoyl–CoA. Protocatechuyl-CoA reductase activity was sensitive to oxygen: treatment with a gentle airstream irreversibly inactivated the enzyme within 3 to 4 min.

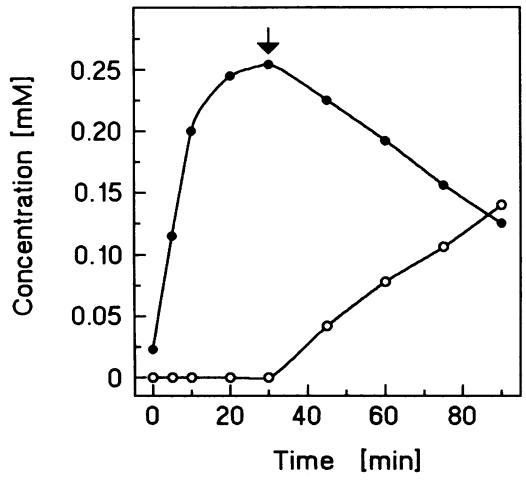

FIG. 2. Activation of protocatechuate and reductive dehydroxylation of protocatechuyl-CoA to 3-hydroxybenzoyl–CoA in cell extracts of strain Cat2 grown with catechol. Methyl viologen was used as the electron donor. Symbols: ●, protocatechuyl-CoA; ○, 3-hydroxybenzoyl–CoA; ↓, addition of sodium dithionite.

Cell extracts of catechol-grown cells catalyzed key reactions necessary for complete catechol oxidation. In addition to glutaryl-CoA dehydrogenase activity, enzymes of β-oxidation of fatty acids and of the carbon monoxide pathway were detected (Table 2).

DISCUSSION

In early investigations of anaerobic catechol degradation by undefined mixed cultures, phenol was suggested to be an intermediate produced by reductive dehydroxylation (2). This hypothesis was maintained until the first pure cultures of catechol-degrading, sulfate-reducing bacteria, e.g., *Desulfobacterium catecholicum*, were isolated (20, 24, 26). Growth tests with these strains showed that catechol degradation was not always linked to the ability to grow with phenol. In contrast, a different combination of aromatic growth substrates was found to be common to all known catechol-degrading bacteria: protocatechuate, 3-hydroxybenzoate, 4-hydroxybenzoate, and benzoate.

Protocatechuate as intermediate in anaerobic catechol metabolism. In dense suspensions of catechol-grown cells of strain Cat2, no phenol degradation was detected even if

TABLE 1. Specific activities of protocatechuyl-CoA reductase and 4-hydroxybenzoyl–CoA reductase detected in cell extracts of strain Cat2 grown with catechol and sulfate

Electron donor	Sp act (nmol · min^{-1} · mg of $protein^{-1}$)[a]	
	Protocatechuyl-CoA	4-Hydroxybenzoyl–CoA
Methyl viologen[b]	10–12	9–12
Benzyl viologen[b]	6–7	8
Titanium(III) citrate	<0.1	<0.1
NAD(P)H	<0.1	<0.1

[a] Averages determined from duplicate experiments are given. For discontinuously measured enzyme activities, the rate was calculated from six to eight sampling points.

[b] Concentrations (2 mM each) were reduced by the addition of sodium dithionite.

TABLE 2. Enzyme activities in cell extracts of strain Cat2 grown with catechol and sulfate

Enzyme	Sp act (nmol · min^{-1} · mg protein^{-1})[a]
Glutaryl-CoA dehydrogenase	68–73
Crotonase	6,230–6,300
3-Hydroxybutyryl–CoA dehydrogenase	345–370
β-Ketothiolase	582–595
CO dehydrogenase[b]	4,460–4,890
Formate dehydrogenase[b]	1,470–1,550
PP_i phosphohydrolase	110–122

[a] Averages determined from duplicate experiments are given. For discontinuously measured enzyme activities, the rate was calculated from six to eight sampling points.

[b] With 10 mM benzyl viologen as the electron acceptor.

protein biosynthesis was not inhibited by the addition of chloramphenicol. Thus, enzymes necessary for phenol breakdown were not present, indicating that this aromatic compound was probably not involved in catechol degradation.

Anaerobic degradation of other nonacidic aromatic compounds was found to be CO_2 dependent (14, 21, 25). Also, the rate of catechol degradation by strain Cat2 was significantly accelerated by the addition of bicarbonate, indicating participation of a carboxylating reaction in the pathway. This CO_2-consuming step might be the carboxylation of catechol, which is chemically favored in an *o* or *p* position to a hydroxyl group, forming 2,3-dihydroxybenzoate or protocatechuate. 2,3-Dihydroxybenzoate was not used as a growth substrate by strain Cat2. Therefore, formation of protocatechuate as an initial reaction seems to be more likely.

Decarboxylation of protocatechuate detected in cell suspensions and cell extracts of catechol-grown strain Cat2 is a contraproductive process because the reversed reaction probably initiates the anaerobic breakdown of catechol. To date, we have no physiological explanation for this observation, but protocatechuate decarboxylation was also described for a catechol-oxidizing *Desulfotomaculum* strain, indicating that this reaction might be widespread among bacteria degrading catechol anaerobically (20). The protein catalyzing this decarboxylation had some properties in common with other enzymes decarboxylating hydroxybenzoate derivatives (12, 19); the apparent optimum pH was 6.0, and decarboxylation was a biotin-independent reaction, since addition of avidin was without effect.

To prevent protocatechuate decarboxylation during further experiments, alkaline assay conditions were chosen. In such assays, catechol was carboxylated and immediately esterified with CoASH in a reaction which requires high bicarbonate concentrations, ATP, Mg^{2+}, Mn^{2+}, and CoASH. Since the equilibrium constant for the initial catechol carboxylation was calculated to be 7.5×10^{-5}, detection of a weak catechol carboxylase activity could be possible only through the removal of protocatechuate by esterification with CoASH and immediate hydrolysis of PP_i ($\Delta G^{0'} = -21.9$ kJ · mol^{-1}) (27), a product of the following CoA-ligase reaction. The overall reaction can be summarized as follows: catechol + CO_2 + ATP + CoASH + H_2O→protocatechuyl-CoA + AMP + 2 P_i ($\Delta G^{0'} \approx -4.4$ kJ · mol of catechol^{-1}) ($\Delta G^{0'}$ was calculated by comparison with esterification of acetate to acetyl-CoA [27]). In a manner analogous to that of anaerobic phenol degradation, catechol was first carboxylated in the *p* position to a hydroxyl function before further modification of the aromatic ring took place. Like the phenol carboxylase of a *Pseudomonas* sp. (21), the catechol-carboxylating activity was extremely sensitive to oxidation. In contrast, an isotope exchange with [^{14}C]carbonate was not catalyzed by cell extracts of strain Cat2. Since we used alkaline conditions for this assay to prevent protocatechuate decarboxylation and since the optimum pH for phenol carboxylase was around 6, the purified enzyme might catalyze such an exchange as well.

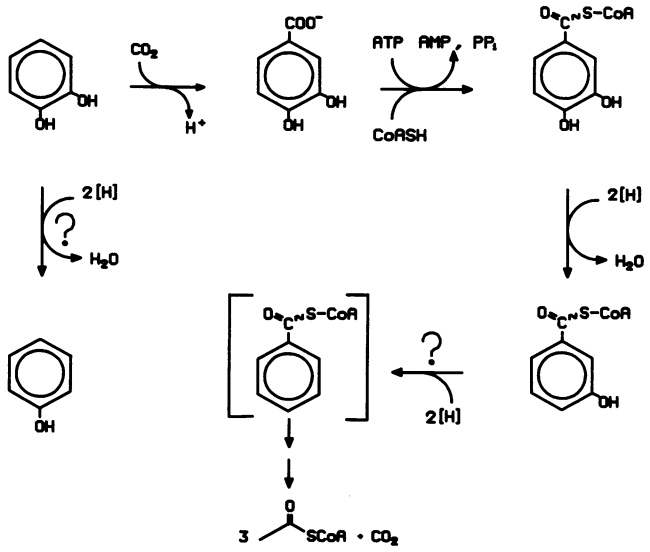

FIG. 3. Initial reactions of anaerobic catechol degradation by *Desulfobacterium* sp. strain Cat2, as proposed from the results of this study.

For phenol degradation, it appears likely that a phosphorylated intermediate is involved. Unfortunately, a similar experiment could not yet be performed with strain Cat2 since catechyl phosphate is not commercially available.

Further degradation of protocatechuate involves reductive dehydroxylation. Under anoxic conditions, phenolic compounds unsuitable for easy transformation into resorcinol or phloroglucinol are generally degraded via benzoyl-CoA (23). Reductive elimination of hydroxyl or amino functions from the aromatic ring requires activation to the CoA derivative (23). This was also true for anaerobic degradation of protocatechuate. CoA-ligase activity catalyzed by cell extracts of strain Cat2 grown with catechol was measured with protocatechuate, 4-hydroxybenzoate, and benzoate but not with 3-hydroxybenzoate as substrate. This might be an explanation for the lack of 3-hydroxybenzoate degradation observed in suspension experiments with catechol-grown cells.

In the 1960s, it was demonstrated in experiments with rat intestinal flora that aromatic acid derivatives are dehydroxylated, predominantly at the *p* position (9). For example, protocatechuate was converted to 3-hydroxybenzoate as the main product. This is exactly what we found in enzyme assays with *Desulfobacterium* sp. strain Cat2. With dithionite-reduced methyl viologen as the electron donor, protocatechuyl-CoA was reduced to 3-hydroxybenzoyl–CoA in cell extracts. Even 4-hydroxybenzoyl–CoA could be dehydroxylated, but not 3-hydroxybenzoyl–CoA, which shows clearly that the dehydroxylating activity is restricted to *p* eliminations. The recently purified 4-hydroxybenzoyl–CoA reductase enzyme of *Pseudomonas* sp. strain K172 also reacts specifically with the 4-hydroxy derivative and not with 3-hydroxybenzoyl–CoA (4). After growth with 3-hydroxybenzoate, cell extracts of this *Pseudomonas* strain catalyze the elimination of the *m*-hydroxyl group of

3-hydroxybenzoyl–CoA, with reduced benzyl viologen as the electron donor. This kind of reaction was detected neither in our assays with *Desulfobacterium* sp. strain Cat2 nor with a newly isolated, protocatechuate-degrading, denitrifying bacterium (13). Therefore, dehydroxylation of 3-hydroxybenzoyl–CoA catalyzed by these strains either required an electron donor we did not test or involved a different reaction, e.g., a direct reduction of the aromatic nucleus. The latter alternative cannot be ruled out because in former investigations concerning anaerobic 3-hydroxybenzoate degradation by denitrifying bacteria, no indication of reductive dehydroxylation to benzoate or benzoyl-CoA was found (17).

On the basis of the results of this study, we propose a new pathway of anaerobic catechol degradation by *Desulfobacterium* sp. strain Cat2 as depicted in Fig. 3.

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