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Anaerobic degradation of α-resorcylate by *Thauera aromatica* strain AR-1 proceeds via oxidation and decarboxylation to hydroxyhydroquinone

Abstract Anaerobic degradation of α-resorcylate (3,5-dihydroxybenzoate) was studied with the denitrifying strain AR-1, which was assigned to the described species *Thauera aromatica*. α-Resorcylate degradation does not proceed via the benzoyl-CoA, the resorcinol, or the phloroglucinol pathway. Instead, α-resorcylate is converted to hydroxyhydroquinone (1,2,4-trihydroxybenzene) by dehydrogenative oxidation and decarboxylation. Nitrate, $K_3[Fe(CN)_6]$, dichlorophenol indophenol, and the NAD^+ analogue 3-acetylpyridine adeninedinucleotide were suitable electron acceptors for the oxidation reaction; NAD^+ did not function as an electron acceptor. The oxidation reaction was strongly accelerated by the additional presence of the redox carrier phenazine methosulfate, which could also be used as sole electron acceptor. Oxidation of α-resorcylate with molecular oxygen in cell suspensions or in cell-free extracts of α-resorcylate- and nitrate-grown cells was also detected although this bacterium did not grow with α-resorcylate under an air atmosphere. α-Resorcylate degradation to hydroxyhydroquinone proceeded in two steps. The α-resorcylate-oxidizing enzyme activity was membrane-associated and exhibited maximal activity at pH 8.0. The primary oxidation product was not hydroxyhydroquinone. Rather, formation of hydroxyhydroquinone by decarboxylation of the unknown intermediate in addition required the cytoplasmic fraction and needed lower pH values since hydroxyhydroquinone was not stable at alkaline pH.

Key words α-resorcylate (3,5-dihydroxybenzoate) · Hydroxyhydroquinone (1,2,4-trihydroxybenzene) · Aromatic compounds · Anaerobic degradation

Abbreviations *$APAD^+$* 3-Acetylpyridine adenine dinucleotide

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Introduction

In recent years, anaerobic degradation pathways for many aromatic compounds have been described (Schink et al. 1992; Fuchs et al. 1994). Currently, three different pathways for the anaerobic degradation of aromatic compounds are known; these involve either benzoyl-CoA, resorcinol, or phloroglucinol as key intermediates. All monocyclic aromatic substrates for which degradation pathways under anoxic conditions have been described are transformed by modification reactions to one of these three key intermediates. Most widespread is degradation via benzoyl-CoA. The first reaction in benzoate degradation is the activation of the free aromatic acid to the CoA thioester, which was demonstrated for the first time by Hutber and Ribbons (1983). The thioesterification helps to facilitate subsequent reduction of the benzene ring (Koch et al. 1993). Resorcinol and phloroglucinol are also degraded via reduction (Samain et al. 1986; Krumholz et al. 1987; Kluge et al. 1990). These two compounds do not need activation because the two or three *meta* hydroxy groups cause a destabilization of the α-electron system by tautomerization.

The common feature of the three pathways described is the transformation of an aromatic substrate to a less stable derivative that is amenable to reduction. The only exception known to date for which this mechanism has not been able to be confirmed is resorcinol degradation by nitrate-reducing bacteria (Gorny et al. 1992). On the basis of experiments with dense cell suspensions, a hydrolytic cleavage of resorcinol has been postulated, although this reaction has not yet been verified in cell-free extracts.

Recently, we have reported the anaerobic degradation of α-resorcylate (3,5-dihydroxybenzoate) by *Thauera aromatica* strain AR-1 (Gallus et al. 1997). In the present study, we demonstrate that this bacterium uses a new pathway that is fundamentally different from those described to date.

Materials and methods

Media and cultivation

T. aromatica strain AR-1 (DSM 11528) was grown in a bicarbonate-buffered mineral salts medium without reducing agent as described previously (Gallus et al. 1997). α-Resorcylate was stored as stock solution in sterile infusion bottles under nitrogen gas and was added to cultures with syringes. $NaNO_3$ was added from a sterile stock solution. For biochemical assays, strain AR-1 was cultivated in 1.2-l infusion bottles with 2–3 mM α-resorcylate and 4–6 mM $NaNO_3$ under an N_2/CO_2 (80:20, v/v) atmosphere at 30° C in the dark.

Preparation of cell-free extracts

Cells were harvested in the late exponential growth phase under anoxic conditions by centrifugation in a Du Pont Sorvall RC-5B centrifuge at 13,000 × *g* for 30 min at 4° C. Cells were washed and resuspended either with 100 mM potassium phosphate buffer (pH 7.0) or with 100 mM Tris-HCl (pH 8.0). For separating membranes from cytoplasm, crude extract was centrifuged at 420,000 × *g* for 30 min in a Beckman Optima TL ultracentrifuge. Membranes were washed once and resuspended in one of the two buffers mentioned above. All manipulations were carried out in an anoxic chamber (Coy, Ann Arbor, Mich., USA) under N_2/H_2 (95:5, v/v). Cell-free extracts were prepared by passing cells through a French press at 136 MPa (5–7 times) under anoxic conditions. Cell debris was removed by centrifugation at 5,000 × *g* for 30 min at 4° C.

Experiments with cell suspensions

For experiments with cell suspensions, cells were harvested immediately before use. The cell density was adjusted to OD_{578} = 4. Assays were performed either aerobically in 1-ml reaction cups or anaerobically in N_2-gassed, 2-ml glass tubes sealed with rubber septa. For tests with nitrate as the electron acceptor, cell suspensions were washed with buffer containing 5 mM $NaNO_3$. In some assays, protein biosynthesis was inhibited by incubating cell suspensions with chloramphenicol [30 μg (mg protein)$^{-1}$] for 20 min at room temperature before starting the experiment by substrate addition. Samples were taken with gas-tight syringes (Unimetrics; Macherey-Nagel, Düren, Germany), and the reaction was stopped by dilution (1:5) in 0.1 M H_3PO_4. Substrate concentration was determined by reversed-phase HPLC.

Enzyme assays

Enzyme assays were performed under anoxic conditions at 25° C, either spectrophotometrically or in discontinuous tests analyzed by HPLC. Each reaction assay (1 ml total volume) contained 10–200 μl cell extract and was started with the key substrate specific for each reaction.

Phloroglucinol reductase (EC 1.1.1.-) was measured recording NAD(P)H oxidation at 365 nm (ε_{365} = 3.4 mM^{-1} cm^{-1}) (Brune and Schink 1992).

Dihydrophloroglucinol hydrolase (EC 3.7.1.-) was assayed discontinuously as described by Brune and Schink (1992). Other hypothetical degradation reactions of dihydrophloroglucinol were checked by addition of 3 mM $K_3[Fe(CN)_6]$ plus 0.2 mM phenazine methosulfate, 2.5 mM 3-acetylpyridine adenine dinucleotide, 1.5 mM NAD(P)$^+$, or 1 mM CoASH plus 1 mM ATP. In these assay mixtures, 1,4-dithiothreitol was omitted.

Reduction of α-resorcylate was assayed by spectrophotometry or HPLC analyses. The reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.2) or 100 mM Tris-HCl (pH 8.0), 2 mM 1,4-dithioerythritol, 5 mM $MgCl_2$, 0.2–1.0 mM NADH, and 0.5–1.0 mM α-resorcylate. Instead of NADH, the following possible electron donors were tested: NADPH, $FADH_2$, $FMNH_2$, titanium(III) citrate, methyl viologen, benzyl viologen, methylene blue, 3-acetylpyridine adenine dinucleotide, and dichlorophenol indophenol. The electron donors were prereduced by sodium dithionite if needed.

The assay for acyl-CoA-synthetase (EC 6.2.1.-) was performed discontinuously following thioester formation and coenzyme A disappearance by HPLC analysis. The assay mixture contained: 20 mM triethanolamine buffer (pH 8.0) or 100 mM potassium phosphate buffer (pH 7.2), 10 mM $MgCl_2$, 1 mM ATP, 1 mM CoASH, and 1 mM α-resorcylate, 3-hydroxybenzoate, or benzoate. Alternatively, acyl-CoA-synthetase was measured in a continuous coupled assay using pyruvate kinase (EC 2.7.1.40), myokinase (EC 2.7.4.3), and lactate dehydrogenase (EC 1.1.1.27) as auxiliary enzymes (Geissler et al. 1988).

Acyl-CoA: acceptor-CoA transferase (EC 2.8.3.-) was measured analogous to the discontinuous assay for acyl-CoA synthetase except that (1) free coenzyme A was substituted by acetyl-CoA, succinyl-CoA, or benzoyl-CoA, and (2) ATP was omitted.

3-Hydroxyacyl-CoA dehydratase (EC 4.2.1.17), 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157), and β-ketothiolase (EC 2.3.1.16) were measured according to standard methods (Bergmeyer 1983).

α-Resorcylate oxidation was measured either in discontinuous assays analyzed by HPLC, or in continuous photometric assays. Except for those with O_2 as the electron acceptor, all tests were performed under anoxic conditions. The following buffers were tested: 50 mM potassium phosphate (pH 6.0), 50 mM potassium phosphate (pH 7.2), 100 mM potassium phosphate (pH 7.0), 100 mM potassium phosphate (pH 7.2), 100 mM 2-(*N*-morpholino)-ethane sulfonic acid (pH 6.0), 100 mM 3-(*N*-morpholino)-propane sulfonic acid (pH 7.2), 100 mM 3-(*N*-morpholino)-propane sulfonic acid (pH 8.0), 100 mM Tris-HCl (pH 8.0), 100 mM Tris-HCl (pH 8.5), and 100 mM Tris-HCl (pH 9.0). For each buffer, parallels were run with 5 mM $MgCl_2$. α-Resorcylate was added to a final concentration of 1.0 mM. $NaNO_3$, $K_3[Fe(CN)_6]$, dichlorophenol indophenol, phenazine methosulfate, FMN, FAD, 3-acetylpyridine adeninedinucleotide (APAD$^+$), and NAD(P)$^+$ were tested as electron acceptors for the oxidation. To avoid possible inhibition of the reaction by NADH, some assays contained 0.5–2.0 mM pyruvate and 2 U lactate dehydrogenase (EC 1.1.1.27) in addition to NAD$^+$ to regenerate the oxidized pyridine dinucleotide if necessary.

Analytical methods

Aromatic compounds, coenzyme A, and thioesters were analyzed by reversed-phase HPLC (Brune and Schink 1990a). Hydroxyhydroquinone was identified by comparing UV spectra of the respective samples after preparation by HPLC with a reference treated in the same way. CO_2 was analyzed with a flame ionization detector after conversion to CH_4 by a methanizer (NiCr-Ni catalyzer; Chrompack, Frankfurt/Main, Germany). Protein was determined by the bicinchoninic acid method (Smith et al. 1985) using a test kit (Pierce, Rockford, Ill., USA) with bovine serum albumin as the standard.

Chemicals

Ti(III) citrate was prepared according to Zehnder and Wuhrmann (1976), and α-resorcylyl-CoA was prepared according to the procedure described for pimelyl-CoA synthesis (Gallus and Schink 1994). Dihydrophloroglucinol was obtained from A. Brune (Konstanz, Germany). All other chemicals were of analytical grade and were obtained from Aldrich (Steinheim, Germany), Biomol (Ilvesheim, Germany), Boehringer (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Riedel-de Haen (Seelze, Germany), Serva (Heidelberg, Germany), and Sigma (Deisenhofen, Germany). Gases were purchased from Messer-Griesheim (Ludwigshafen, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

Assays for substrate activation

A possible activation of α-resorcylate was checked in continuous and discontinuous enzyme assays. α-Resorcylyl-CoA was not formed with free coenzyme A plus ATP or with acetyl-CoA, succinyl-CoA, or benzoyl-CoA as activated precursors. 3-Hydroxybenzoate was also not activated by extracts of cells grown with α-resorcylate. Benzoyl-CoA synthetase activity was measured in cells after growth with α-resorcylate or benzoate [0.016 μmol min^{-1} (mg protein)$^{-1}$]. However, this enzyme activity was constitutive since it was also expressed in cells grown with malate.

Assays for α-resorcylate reduction

NADH, NADPH, $FADH_2$, $FMNH_2$, titanium(III) citrate, reduced methyl viologen, benzyl viologen, methylene blue, $APAD^+$, and dichlorphenolindophenol were tested as electron donors for a possible primary reduction of α-resorcylate. No reduction was observed in continuous or discontinuous assays within 6 h.

Assays for α-resorcylate degradation via the phloroglucinol pathway

Oxidative decarboxylation of α-resorcylate should yield phloroglucinol as the first intermediate, and we postulated that phloroglucinol reductase would necessarily be involved in further degradation. No such activity was detected in cell-free extracts of strain AR-1. Dihydrophloroglucinol, an intermediate in phloroglucinol degradation by the fermenting bacterium *Pelobacter acidigallici* (Brune and Schink 1992), was not hydrolyzed or oxidized.

Oxidation of α-resorcylate

The influence of nitrate and molecular oxygen on the degradation of α-resorcylate was studied in cell suspensions (OD_{578} = 4) of strain AR-1 after growth with α-resorcylate and nitrate. Without nitrate, no significant decrease of the α-resorcylate concentration was observed under anoxic conditions (Fig. 1). If the same assay was incubated aerobically, 1 mM α-resorcylate was completely degraded within 4 h at an initial rate of 8.7 μM min^{-1} [0.28 nmol min^{-1} (mg protein)$^{-1}$]. Degradation was strongly accelerated by the addition of nitrate. In the presence of 5 mM nitrate, 1 mM α-resorcylate was degraded within 90 min at an initial rate of 17.0 μM min^{-1} [0.54 nmol min^{-1} (mg protein)$^{-1}$], independent of oxic or anoxic incubation. Turnover rates were the same when chloramphenicol, a specific inhibitor of peptidyltransferase, was added.

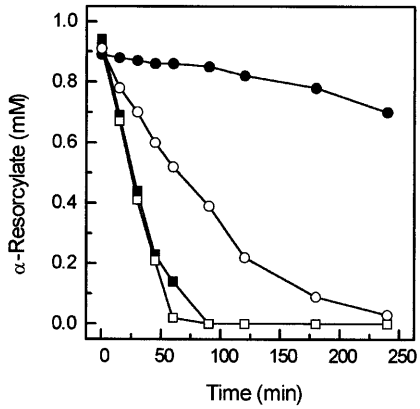

Fig. 1 Degradation of α-resorcylate by cell suspensions of strain AR-1. Incubation conditions: anoxic without nitrate (●) and with 5 mM nitrate (■); oxic without nitrate (○) and with 5 mM nitrate (□)

Table 1 Oxidation of α-resorcylate by the membrane fraction of strain AR-1 in the presence of phenazine methosulfate plus 3-acetylpyridine adenine dinucleotide ($APAD^+$) at different concentrations

Phenazine methosulfate (mM)	$APAD^+$ (mM)	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]
0.2	–	18.1
0.2	2.5	16.1
0.2	3.0	18.0
0.2	4.0	18.1
0.5	–	32.5
0.5	2.5	28.9
0.75	–	21.7

α-Resorcylate oxidation was further investigated using cell-free extracts. Besides nitrate and oxygen, several other oxidants such as $K_3[Fe(CN)_6]$, dichlorophenol indophenol, or $APAD^+$ served as the electron acceptor. Oxidation was accelerated by the additional presence of phenazine methosulfate. The specific activity of α-resorcylate oxidation depended only on the concentration of phenazine methosulfate (Table 1); in the presence of additional $APAD^+$, a higher amount of α-resorcylate was oxidized. The highest specific activity of 33 nmol min^{-1} (mg protein)$^{-1}$ achieved in the presence of 0.5 mM phenazine methosulfate (Table 1) is in the same range as the in vivo activity [35 nmol min^{-1} (mg protein)$^{-1}$]. FAD, FMN, NAD^+, and $NADP^+$ were not suitable for α-resorcylate oxidation. NAD^+ was not used as an electron acceptor even when lactate dehydrogenase and pyruvate were added to regenerate NAD^+. Decrease of the α-resorcylate concentration depended strictly on cell-free extract, demonstrating that this oxidation was not a purely chemical process.

Nitrate-dependent α-resorcylate oxidation exhibited the highest specific activity at pH 8.0. Oxidation of α-resorcylate was stimulated by 5 mM $MgCl_2$, whereas the ionic strength of the buffer had no influence.

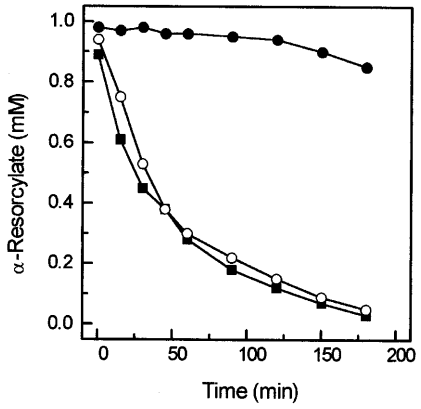

Fig. 2 Degradation of α-resorcylate in the presence of 3-acetylpyridine adenine dinucleotide (APAD+) plus phenazine methosulfate by ■ cell-free extract (2.5 mg protein), ● the soluble fraction (1.1 mg protein), and ○ the membrane fraction (1.1 mg protein)

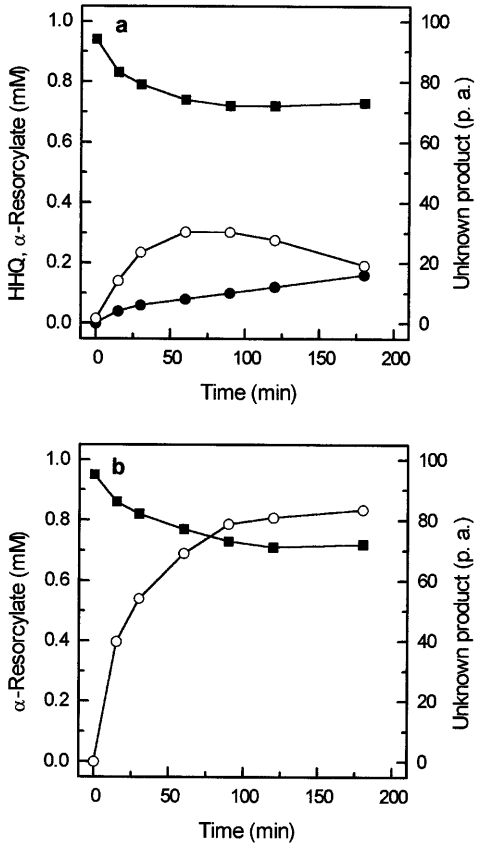

Fig. 3 Degradation of α-resorcylate with 0.2 mM phenazine methosulfate at pH 7.0 **a** by cell-free extract and **b** by the membrane fraction. ■ α-Resorcylate, ● hydroxyhydroquinone, and ○ unidentified product (*p.a.* peak area)

α-Resorcylate oxidation was assayed in membrane preparations and in the cytoplasmic fraction obtained by ultracentrifugation of cell-free extract (Fig. 2). Eighty-seven percent of the α-resorcylate oxidizing activity measured with $APAD^+$ plus phenazine methosulfate as electron acceptors was recovered within the membrane fraction; only 4% was recorded within the soluble fraction.

Products of α-resorcylate degradation

Cell-free extracts degraded α-resorcylate at pH 7.0 to an unknown compound that was detected by HPLC. When complete cell-free extract was used, this intermediate accumulated only transiently and a further product was formed; the latter was identified as hydroxyhydroquinone by comparing its retention time in HPLC analysis and its UV spectrum with the retention time and UV spectrum of authentic hydroxyhydroquinone (Fig. 3a). In assays with the membrane fraction only, the unidentified compound accumulated to higher amounts and no further degradation occurred (Fig. 3b). When the cytoplasmic fraction was added later, the unknown compound disappeared and hydroxyhydroquinone was formed concomitant with CO_2 release.

Enzymes of fatty acid β-oxidation

Enzyme activities for β-oxidation of short-chain fatty acids were detected in α-resorcylate-grown cells of strain AR-1. The specific activities of 3-hydroxyacyl-CoA dehydratase [2.3 μmol min^{-1} (mg protein)$^{-1}$], 3-hydroxybutyryl-CoA dehydrogenase [0.19 μmol min^{-1} (mg protein)$^{-1}$], and β-ketothiolase [0.99 μmol min^{-1} (mg protein)$^{-1}$] were almost equal to those measured in cell-free extracts after growth with benzoate and nitrate.

Discussion

Monocyclic aromatic compounds are most often degraded via benzoyl-CoA (Fuchs et al. 1994). We investigated whether also α-resorcylate was degraded by our strain AR-1 through this pathway. Formation of α-resorcylyl-CoA or one of the dehydroxylation products 3-hydroxybenzoyl-CoA or benzoyl-CoA was not observed. Reductive dehydroxylation of free α-resorcylate to 3-hydroxybenzoate or benzoate was also not detected. Enzymes of fatty acid β-oxidation catalyzing the last reactions in benzoate breakdown from crotonyl-CoA to acetyl-CoA were present at equal specific activities after growth with α-resorcylate or benzoate. Constitutive expression of these enzymes in other bacteria has been reported before (Hutber and Ribbons 1983; Gallus and Schink 1994). Decarboxylation as the first step in degradation of aromatic acids has been shown for β- and γ-resorcylate (Kluge et al. 1990), and for gallate (3,4,5-trihydroxybenzoate) and phloroglucinate (2,4,6-trihydroxybenzoate) (Brune and Schink 1990a, 1992). Our experiments gave no indications of a decarboxylation or of the involvement of resorcinol in α-resorcylate degradation. A possible direct reduction of α-resorcylate without preceding decarboxylation (analogous to the reduction of resorcinol by the fermenting coculture *Clostridium* sp. KN 245; Kluge et al. 1990) was investigated. No such reduction was detected in cell-free extracts of strain AR-1 with any of the electron donors tested. Finally, α-resorcylate degradation could merge into the

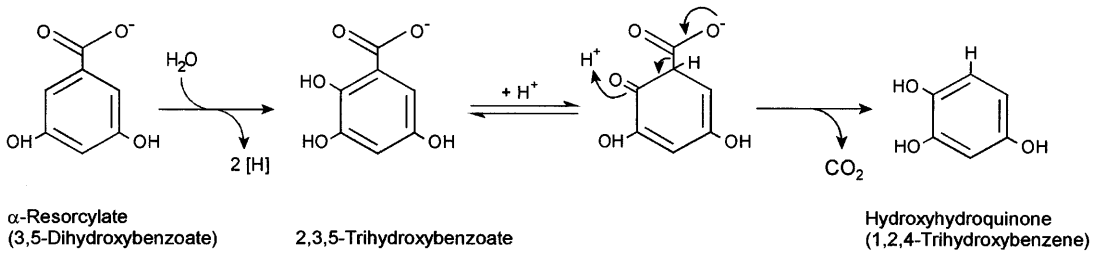

Fig. 4 Postulated pathway of a-resorcylate degradation to hydroxyhydroquinone by *Thauera aromatica* strain AR-1

phloroglucinol pathway (Brune and Schink 1990b, 1992; Schnell et al. 1991), e.g., by oxidative decarboxylation to phloroglucinol or by hydration and decarboxylation to dihydrophloroglucinol. However, no growth of strain AR-1 was found with phloroglucinol (Gallus et al. 1997), no phloroglucinol-reducing activity with NADPH or with NADH was measured, and dihydrophloroglucinol was not degraded in cell-free extracts. We conclude that α-resorcylate degradation by strain AR-1 does not proceed through benzoyl-CoA, resorcinol, or phloroglucinol, the key intermediates of the three pathways known for anaerobic degradation of aromatic compounds. Instead, we found that this strain degrades α-resorcylate via oxidation and decarboxylation to hydroxyhydroquinone according to the pathway suggested in Fig. 4.

α-Resorcylate was oxidized in cell suspensions and in cell-free extracts with various electron acceptors of comparably high redox potential, the lowermost one being that of the NAD^+ analogue $APAD^+$ (E_0' = –248 mV; Bergmeyer 1983). Phenazine methosulfate acted as an intermediate redox carrier, as it does in similar reactions catalyzed by other membrane-associated enzymes such as glycolate dehydrogenase (Friedrich and Schink 1993) and butyryl-CoA dehydrogenase (Wallrabenstein and Schink 1994). The redox couple 2,3,5-trihydroxybenzoate/α-resorcylate has an E_0' of –0.027 V as calculated by the increment method (Mavrovouniotis 1991); the analogous couple 2-hydroxybenzoate/benzoate is in the same range (E_0' = –0.102 V; Thauer et al. 1977). Taking the subsequent decarboxylation with a free energy change of –20 to –25 kJ mol^{-1} into account, the redox potential of the overall reaction from α-resorcylate to hydroxyhydroquinone shifts to –0.150 to –0.230 V. At pH 8.0, where the highest activity was measured, the redox potential of the $APAD^+$/APADH + H^+ couple is –0.218 V. Even then, the equilibrium of the total reaction is on the substrate side and explains why the electron acceptor was needed in excess. In our experiments, α-resorcylate degradation never reached equilibrium, probably because the hydroxylated derivatives underwent further polymerization. The physiological electron acceptor could be a quinone, a cytochrome, or pyrroloquinoline quinone (PQQ: +90 mV; Stanier et al. 1987). Spontaneous reoxidation of these carriers with molecular oxygen may explain why α-resorcylate oxidation in cell suspensions or in cell-free extracts could be coupled to oxygen reduction although this strain did not grow aerobically with this substrate.

Unfortunately, the postulated intermediate, 2,3,5-trihydroxybenzoate, could not be identified because no reference compound was available. As an alternative to the pathway depicted in Fig. 4, hydration and oxidation could proceed in two subsequent steps. It should be noticed that α-resorcylate was oxidized at highest activity by the membrane fraction at pH 8.0. The resulting 2,3,5-trihydroxybenzoate was further degraded in the cytoplasm; detection of the product hydroxyhydroquinone required pH ≤ 7 in the assay because hydroxyhydroquinone polymerizes fast under alkaline conditions.

Formation of hydroxyhydroquinone as an intermediate in anaerobic degradation of an aromatic compound has never been observed before. Hydroxyhydroquinone contains a 1,3-diol group, as do resorcinol and phloroglucinol; this group decreases the molecule's aromaticity and facilitates a reductive attack on the benzene nucleus. Thus, the common principle of the three known pathways, the conversion of a stable aromatic substrate to an intermediate with decreased aromaticity, is also realized in the case of α-resorcylate degradation.

The further pathway of hydroxyhydroquinone degradation is still unclear. Conversion to phloroglucinol by transhydroxylations as observed with *Pelobacter massiliensis* (Schnell et al. 1991) can be ruled out since strain AR-1 does not degrade phloroglucinol (Gallus et al. 1997). The sulfate-reducing bacterium strain HHQ 20 reduces hydroxyhydroquinone with NADH or NADPH electrons (Reichenbecher and Schink 1997), but the reduction product and its further fate are still unknown. In cell-free extracts of strain AR-1, no such reductive activity was detected. Hydroxyhydroquinone could be cleaved hydrolytically or thiolytically, yielding 3-oxoadipic semialdehyde or its CoA derivative. The oxidation product of the semialdehyde is 3-oxoadipate which is a common intermediate in aerobic benzoate degradation by *ortho* cleavage and leads to formation of succinyl-CoA and acetyl-CoA. Further investigations are required to examine this hypothesis.

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