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Anaerobic degradation of protocatechuate (3,4-dihydroxybenzoate) by *Thauera aromatica* strain AR-1

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Abstract

The denitrifying bacterium *Thauera aromatica* strain AR 1 grows anaerobically with protocatechuate (3,4 dihydroxybenzoate (DHB)) as sole energy and carbon source. This bacterium harbors two distinct pathways for degradation of aromatic compounds, the benzoyl coenzyme A (CoA) pathway for benzoate degradation and the hydroxyhydroquinone (HHQ) pathway for degradation of 3,5 DHB. In order to elucidate whether protocatechuate is degraded via the benzoyl CoA or the HHQ pathway, induction experiments were carried out. Dense suspensions of cells grown on protocatechuate or benzoate readily degraded benzoate and protocatechuate but not 3,5 DHB. Dense suspensions of 3,5 DHB grown cells degraded 3,4 and 3,5 DHB at similar rates, but benzoate was not degraded. 3,5 DHB hydroxylating activity was found only in cells grown with this substrate. HHQ dehydrogenase activity was found in extracts of cells grown with 3,5 DHB and at a low rate also in protocatechuate grown cells, but not in extracts of cells grown with benzoate. Activities of protocatechuyl CoA synthetase and protocatechuyl CoA reductase leading to 3 hydroxybenzoyl CoA were found in extracts of cells grown with protocatechuate. There was no repression of the HHQ pathway by the presence of protocatechuate, unlike by degradation of benzoate. We conclude that protocatechuate is not degraded via the HHQ pathway because there was no evidence of a hydroxylation reaction involved in this process. Instead, our results strongly suggest that protocatechuate is degraded via a pathway which connects to the benzoyl CoA route of degradation.

Keywords: Anaerobic degradation; Aromatic compound; Protocatechuate; 3,5-Dihydroxybenzoate; Benzoyl-coenzyme A; Hydroxyhydroquinone; *Thauera aromatica*

1. Introduction

Degradation of aromatic compounds by micro-organisms is an essential contribution to the global carbon cycle and is also a biochemical challenge due to their chemical stability. Under air, degradation is carried out by oxygenases with molecular oxygen as co-substrate. In the absence of oxygen, bacteria have to use alternative strategies to overcome the stability of aromatic rings. The most common mechanism here is the reduction of aromatic rings [1,2]. The denitrifying bacterium *Thauera aromatica* transforms various aromatic compounds to benzoyl-coenzyme A (CoA), which is subject to ring reduction by benzoyl-CoA reductase. The resulting cyclohex-1,5-diene-1-carboxyl-CoA is further oxidized to three acetyl moieties and CO_2. The benzoyl-CoA pathway is found in fermenting, sulfate-, nitrate- and iron-reducing as well as in anoxygenic phototrophic bacteria and constitutes thus a central pathway in anaerobic degradation of aromatic compounds.

Denitrifying bacteria have a further option for degradation of certain phenolic compounds, which does not involve a reductive mechanism [2]. Degradation of resorcinol (1,3-dihydroxybenzene) and 3,5-dihydroxybenzoate

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(3,5-DHB) is initiated by hydroxylation to form hydroxyhydroquinone (HHQ, 1,2,4-trihydroxybenzene) as the product, which is further oxidized to hydroxybenzoquinone [3 5]. Thus, oxidations are employed to overcome the stability of the aromatic ring. Ring cleavage and further metabolization of this compound have not yet been fully elucidated.

Protocatechuate (3,4-DHB) is a key intermediate in aerobic degradation of aromatic acids, e.g. benzoate [6]. In the absence of oxygen, this compound is degraded only very slowly, and anaerobic degradation has been documented with fermenting [7], sulfate-reducing [8] and nitrate-reducing bacteria [9]. Whereas degradation by a sulfate-reducing bacterium proceeds via benzoyl-CoA, protocatechuate breakdown in the nitrate-reducing bacterium *T. aromatica* strain AR-1 could also proceed via hydroxylation and decarboxylation to HHQ. This pathway could be energetically more favorable because it avoids the energy-expensive activation and de-aromatization reactions involved in the benzoyl-CoA pathway. The present study was aimed at finding out which pathway is used for anaerobic degradation of protocatechuate by *T. aromatica*.

2. Materials and methods

2.1. Organism and culture conditions

T. aromatica strain AR-1 (DSM 11528) was cultivated as described [3,5,9]. For biochemical assays, strain AR-1 was cultivated in infusion bottles with 2 3 mM of the aromatic substrate and 10 15 mM $NaNO_3$ at 28°C in the dark.

2.2. Determination of growth parameters

Growth yields were determined in 100-ml batch cultures. Optical density (OD) was monitored in 1-cm light path cuvettes at 578 nm wavelength. The OD-dry matter conversion factor ($OD_{578\,nm}$ $1 = 265$ mg l^{-1}) was determined gravimetrically after filtering a 100-ml batch culture through a polyamide filter (pore size 0.2 μm, Macherey-Nagel, Düren, Germany). Substrate utilization, nitrate and nitrite decrease were analyzed by high-performance liquid chromatography (HPLC) as described below. Substrate assimilated into cell material was calculated after the equation $17\ C_7H_6O_4 + 40\ H_2O \rightarrow 26\ \langle C_4H_7O_3\rangle + 15\ CO_2$.

2.3. Preparation of cell suspensions and cell-free extracts

Cell suspensions and extracts were obtained from cultures in the late exponential phase grown with the respective substrate under strictly anoxic conditions as described previously [3,4,10]. Cell-free extracts were prepared by French press treatment (138 MPa, three passages) followed by two centrifugation steps ($17\,500 \times g$ for 20 min at 4°C) to remove cell debris.

2.4. Cell suspension experiments

For degradation experiments, cell pellets were resuspended in 50 mM anoxic potassium phosphate buffer, pH 7.2, to a final OD of 5 10 (equivalent to approximately 1.4 2.8 mg dry cell mass per ml). Experiments were performed under nitrogen gas in butyl rubber-sealed 5-ml Hungate tubes containing 1 ml N_2O (equivalent to 5 mM in the liquid phase) at 28°C. All additions and samplings were carried out with gas-tight Unimetrix microliter syringes (Macherey-Nagel, Düren, Germany). Reactions were started by addition of the aromatic substrates. Samples (100 μl) were taken immediately after starting the reaction and at regular intervals. Samples containing protocatechuate were analyzed immediately, samples containing 3,5-DHB were transferred into 100 μl ice-cold H_2O and kept on ice until analysis [3]. Samples containing benzoate only were added to 100 μl phosphoric acid (100 mM) and kept at 20°C until analysis. All samples were centrifuged quickly at $15\,000 \times g$ before HPLC analysis.

2.5. Enzyme assays

Enzyme activities in cell-free extracts were measured under strictly anoxic conditions at 28°C in 1.5-ml cuvettes or 5-ml Hungate tubes using anoxic buffers and solutions. Cuvettes and tubes were flushed with N_2 and closed with butyl rubber stoppers. All additions were made with gas-tight Unimetrix microliter syringes (Macherey-Nagel, Düren, Germany). Linear correlations between reaction rates and protein contents were checked for in all assays.

3,5-DHB oxidizing activity catalyzes the hydroxylation of 3,5-DHB to 2,3,5-trihydroxybenzoate which is subsequently decarboxylated to HHQ; this activity was measured continuously by a photometric assay [3]. HHQ dehydrogenase catalyzes the conversion of HHQ to hydroxybenzoquinone with concomitant reduction of nitrate to nitrite and was measured as described [4].

Benzoyl-CoA reductase was measured as described previously [11]. Protocatechuyl-CoA synthetase was measured discontinuously by HPLC analysis. The reaction mixture contained potassium phosphate buffer (100 mM, pH 7.1), $MgCl_2$ (10 mM), CoA (1 mM), ATP (1 mM), and protocatechuate (1 mM). Samples were taken at regular intervals and transferred into H_3PO_4 (100 mM) before HPLC analysis.

Protocatechuyl-CoA-reducing activity (dehydroxylating) with benzyl viologen or methyl viologen as electron donor was measured continuously by a photometric assay at 578 nm coupled to the protocatechuyl-CoA synthetase assay. This reaction mixture contained dithiothreitol (2 mM) and reduced benzyl or methyl viologen (0.3 1 mM) as additional components. Viologens were partly reduced by ad-

Table 1
Specific rates of protocatechuate, 3,5-DHB and benzoate degradation by dense cell suspensions of *T. aromatica* strain AR-1 after growth with different substrates

Growth substrate (2 mM in mineral medium)	Assay substrate (1 mM in phosphate buffer)	Specific degradation rate (nmol min ^{1} (mg cell dry matter) ^{1})
Protocatechuate	Protocatechuate	8.5
	3,5-DHB	0.1
	Benzoate	4.8
3,5-DHB	Protocatechuate	7.8
	3,5-DHB	5.9
	Benzoate	0.1
Benzoate	Protocatechuate	6.8
	3,5-DHB	0.2
	Benzoate	5.4

dition of sodium dithionite to keep the absorbance at 578 nm approximately between 1 and 1.5.

2.6. SDS PAGE and immunoblotting

SDS PAGE was carried out according to Laemmli [12] using gels containing 10% acrylamide. In each experiment, identical amounts of protein (20 40 μg) were applied to each lane. For immunoblot analysis of benzoyl-CoA reductase [11], cell-free extracts were separated on SDS gels and subsequently transferred to nitrocellulose filters (Bio-Blot-NC, Costar®, Cambridge, MA, USA) with the Semiphor™ system (Hoefer Scientific Instruments, San Francisco, CA, USA). Benzoyl-CoA reductase was detected immunologically using the CSPD® system (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using the commercial BCA protein assay (Pierce, Rockford, IL, USA).

2.7. HPLC analysis

HPLC analysis was performed as described [4,13]. Concentrations were calculated via external standards. Compounds were identified by comparison of retention times and on-line scanning of absorption spectra with a Beckman 168 diode array detector.

2.8. Chemicals

Antiserum against benzoyl-CoA reductase was kindly provided by Dr. G. Fuchs (Freiburg, Germany). All chemicals and gases were of analytical grade and the highest purity available.

3. Results

3.1. Growth with protocatechuate

T. aromatica AR-1 oxidized protocatechuate with nitrate as electron acceptor; cells grew after a lag phase of 1 2 days with 21 h doubling time. Nitrate was reduced via nitrite to N_2 concomitant with substrate utilization and an increase in OD; the electron recovery was 78%. From the growth rate (0.033 h ^{1}) and the molar growth yield (51 g dry matter mol ^{1}), the in vivo substrate turnover rate was calculated to be 11 nmol min ^{1} (mg dry matter) ^{1}.

3.2. Induction experiments with cell suspensions

Dense suspensions of cells grown with protocatechuate did not degrade 3,5-DHB but degraded benzoate, whereas suspensions of 3,5-DHB-grown cells degraded protocatechuate but not benzoate. Cells grown with benzoate were induced for degradation of protocatechuate but not for degradation of 3,5-DHB (Table 1). Protocatechuate-grown cells were also induced for degradation of 3-hydroxybenzoate and 4-hydroxybenzoate (not shown).

3.3. Induction of enzymes

Activity of benzoyl-CoA reductase could not be measured in extracts of cells grown with any of the substrates. However, benzoyl-CoA reductase was detected in cell-free extracts of cells grown with protocatechuate and in benzoate-grown cells, using antibodies raised against benzoyl-CoA reductase of *T. aromatica* strain K-172. The immunoblot signal was always slightly lower in cells grown with 3,5-DHB (not shown).

3,5-DHB hydroxylating and HHQ dehydrogenase activities were detected in extracts of cells grown with 3,5-DHB (Table 2) in a similar range as previously determined [3]. In extracts of cells grown with benzoate or protocatechuate, 3,5-DHB hydroxylating activity was below the detection limit. HHQ dehydrogenase activity was very low in extracts of benzoate-grown cells and intermediate in extracts of protocatechuate-grown cells (Table 2). No protocatechuate oxidizing activity with nitrate or $K_3Fe(CN)_6$ as

Table 2
Specific activities of key enzymes of the HHQ pathway in *T. aromatica* strain AR-1 in extracts of cells grown with different substrates

Cell extract	3,5-DHB hydroxylating activity (nmol min ^{1} (mg protein) ^{1})	HHQ dehydrogenating activity (nmol min ^{1} (mg protein) ^{1})
3,5-DHB-grown cells	4.4	8.3
Protocatechuate-grown cells	>0.1	2.0
Benzoate-grown cells	>0.1	0.1

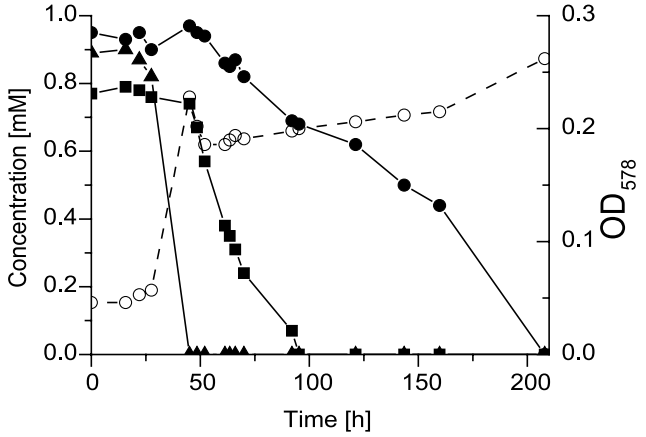

Fig. 1. Growth shown as increase in OD at 578 nm (○) of *T. aromatica* strain AR-1 with a mixture of benzoate (▲), protocatechuate (■) and 3,5-DHB (●).

electron acceptors could be detected in extracts of cells grown with either protocatechuate, 3,5-DHB, or benzoate.

Protocatechuyl-CoA synthetase activity was detected in extracts of cells grown with protocatechuate (specific activity 18 nmol min ^{1} (mg protein) ^{1}). In the presence of reduced benzyl viologen or methyl viologen, protocatechuyl-CoA was reduced to 3-hydroxybenzoyl-CoA (specific activities 14 19 nmol min ^{1} (mg protein) ^{1} with methyl viologen and 7 9 nmol min ^{1} (mg protein) ^{1} with benzyl viologen).

3.4. Growth experiments

Cells grown with protocatechuate were transferred to a medium containing protocatechuate plus benzoate. After a lag phase of 1 day, the culture showed diauxic growth (not shown). Benzoate was consumed first; after a slight decline in OD and a short lag phase, the growth rate increased again and protocatechuate was utilized. Growth with protocatechuate plus 3,5-DHB was not biphasic. Utilization of 3,5-DHB started slightly earlier and at a higher rate than degradation of protocatechuate (not shown). If cells were transferred to medium containing a mixture of protocatechuate, benzoate and 3,5-DHB, growth started similar to that described for protocatechuate and benzoate, with a growth rate of $\mu = 0.06$ h ^{1} (Fig. 1). After depletion of benzoate, the OD always declined slightly. Growth continued at a much lower rate (less than one duplication within 150 h) after benzoate depletion concomitant with simultaneous protocatechuate and 3,5-DHB degradation. The rate of protocatechuate utilization was higher than the rate of 3,5-DHB consumption.

4. Discussion

The denitrifying bacterium *T. aromatica* AR-1 possesses two distinct pathways for anaerobic degradation of aromatic compounds, the benzoyl-CoA pathway and the HHQ pathway [3]. Either pathway is inducible and, in addition, the HHQ pathway is repressed in the presence of benzoate. *T. aromatica* AR-1 utilizes a broad variety of aromatic substrates including benzoate, hydroxybenzoates, phenol, phenylalanine and toluene [9]. All compounds tested so far are degraded via the benzoyl-CoA pathway [1] except for 3,5-DHB, which is degraded via the HHQ pathway [3,5]. The route of protocatechuate degradation by this organism was unknown so far. Regarding the metabolic capacity of strain AR-1 and the chemical properties of the compound, degradation via either pathway is feasible. In analogy to 4-hydroxybenzoate, protocatechuate could be esterified with CoA and then be reductively dehydroxylated to 3-hydroxybenzoyl-CoA. Alternatively, hydroxylation of protocatechuate to 2,4,5-trihydroxybenzoate and subsequent decarboxylation to HHQ appear conceivable as well. Our induction experiments showed that cells grown with protocatechuate were induced for benzoate metabolism and not for 3,5-DHB metabolism, suggesting that protocatechuate is degraded via the benzoyl-CoA pathway. Induction of benzoate-grown cells for protocatechuate and immunological detection of benzoyl-CoA reductase in protocatechuate-grown cells support this conclusion. Furthermore, 3,5-DHB hydroxylating activity was not induced in protocatechuate-grown cells, explaining why 3,5-DHB could not be degraded in such cell suspensions. In general, we could not obtain any evidence that a hydroxylation is involved in protocatechuate degradation at all. However, 3,5-DHB-grown cells could degrade protocatechuate but were not induced for benzoate, suggesting that protocatechuate is degraded via the HHQ pathway. Activity of HHQ dehydrogenase, the de-aromatizing enzyme of the HHQ pathway, was detectable in protocatechuate-grown cells, although only at a low level. As a further level of regulation, we have previously found repression of the HHQ pathway by the presence of benzoate [3]. Benzoate repressed protocatechuate degradation as well, leading to diauxic growth whereas protocatechuate

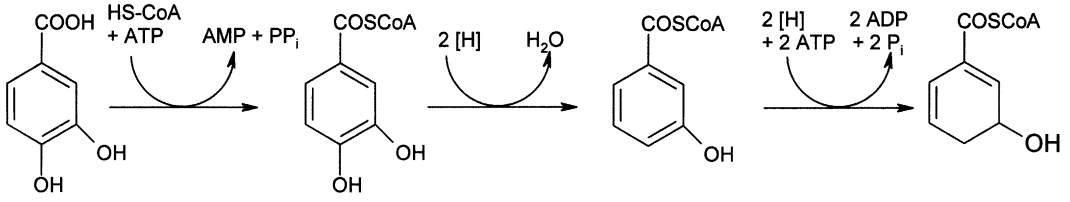

Fig. 2. Proposed initial reactions in anaerobic degradation of protocatechuate by *T. aromatica* strain AR-1.

and 3,5-DHB were degraded simultaneously, although at different rates.

Summarizing these findings we draw the following conclusions: protocatechuate degradation does not proceed via the HHQ pathway because no hydroxylation reactions appear to be involved. Rather, reactions related to the benzoyl-CoA pathway are employed since we found an activation to protocatechuyl-CoA and subsequent reductive dehydroxylation to 3-hydroxybenzoyl-CoA (Fig. 2). Further degradation of 3-hydroxybenzoyl-CoA by *T. aromatica* strain K-172 was recently described [14]. This strain reduces 3-hydroxybenzoyl-CoA directly by benzoyl-CoA reductase without removal of the hydroxyl group.

Utilization of protocatechuate unlike utilization of benzoate does not repress the HHQ pathway. This may indicate that degradation of benzoate but not of protocatechuate leads to an intermediate which may activate a repressor of the HHQ pathway. Our proposed pathway of protocatechuate breakdown does not involve benzoyl-CoA. Therefore, benzoyl-CoA itself could be the intermediate which leads to repression of the HHQ pathway during benzoate degradation.

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