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Anaerobic metabolism of resorcyclic acids (*m*-dihydroxybenzoic acids) and resorcinol (1,3-benzenediol) in a fermenting and in a denitrifying bacterium

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Abstract. The anaerobic metabolism of 2,4- and 2,6-dihydroxybenzoic acid (beta- and gamma-resorcyclic acid) and 1,3-benzenediol (resorcinol) was investigated in a fermenting coculture of a *Clostridium* sp. with a *Campylobacter* sp. (Tschech A and Schink B (1985) Arch Microbiol 143:52–59) and in a newly isolated denitrifying gram-negative bacterium. The enzymes of this pathway were searched for and partly characterized in vitro. It is shown that resorcyclic acids are decarboxylated in both organisms by specific enzymes, 2,4- or 2,6-dihydroxybenzoic acid decarboxylase. In the fermenting bacterium, the aromatic product, 1,3-benzenediol, is reduced by 1,3-benzenediol (resorcinol) reductase to the non-aromatic 1,3-cyclohexanedione; the novel enzyme which catalyzes the two-electron-reduction of the aromatic nucleus is oxygen-sensitive and uses reduced methyl viologen as artificial electron donor. The cyclic dione is then hydrolytically cleaved to 5-oxocaproic acid by 1,3-cyclohexanedione hydrolase. The denitrifying bacterium did not metabolize 1,3-cyclohexanedione, and the enzymes metabolizing 1,3-benzenediol or 1,3-cyclohexanedione were not detected. It is concluded that two different pathways of anaerobic 1,3-benzenediol metabolism exist.

Key words: Aromatic compounds – Resorcinol – Resorcyclic acids – 1,3-Benzenediol – 1,3-Cyclohexanedione – Resorcinol reductase – 2,4-Dihydroxybenzoic acid decarboxylase – 2,6-Dihydroxybenzoic acid decarboxylase – 1,3-Cyclohexanedione hydrolase – 5-Oxocaproic acid – Clostridium – Denitrifying – Anaerobic aromatic metabolism

Resorcinol (1,3-benzenediol) and resorcyclic acids (*m*-dihydroxybenzoic acids) are widely being used in industry. They are produced in large quantities in petrochemical and coal industry and therefore are of concern as environmental pollutants. They are also used in medicine and daily life chemicals (Windholz 1983). As constituents of plant phenolic compounds (Harborne 1980) they are part of our diet and are excreted as resorcinol sulfate (Curzon and Pratt 1964). Resorcyclic acids are decarboxylated to resorcinol by heating, the latter can be reduced to 1,3-cyclohexanedione in aqueous solution (Beilstein 1967).

The aerobic metabolism of resorcinol proceeds via hydroxylation either to 1,2,4- or to 1,2,3-trihydroxybenzene followed by dioxygenase-catalyzed ring cleavage (Chapman and Ribbons 1976a, b; Groseclose and Ribbons 1981). The anaerobic metabolism of dihydroxybenzenes cannot rely on oxygenase-catalyzed reactions, and enzymes were not investigated (Tschech and Schink 1985; Szewzyk and Schink 1989; Schnell et al. 1989; for review see Schink and Tschech 1988).

The anaerobic metabolism of 1,3,5-trihydroxybenzene (phloroglucinol) represents a good model for the degradation of 1,3-benzenediol, since both compounds have meta-hydroxyl functions which destabilize the aromatic ring structure. Phloroglucinol therefore is easily reduced chemically or by an NADPH-dependent enzyme to 1,3-dioxo-5-hydroxycyclohexane (Patel et al. 1981; Haddock and Ferry 1989). In contrast to the direct reduction of this polyphenolic compound, phenol is first carboxylated in a biological Kolbe-Schmitt reaction to 4-hydroxybenzoate and then converted to benzoyl-CoA (Tschech and Fuchs 1989), although a direct reduction to cyclohexanol may occur as well (Balba and Evans 1980). Also, 1,4-benzenediol (hydroquinone) is metabolized via benzoate (Szewzyk and Schink 1989).

Tschech and Schink (1985) studied resorcinol and resorcyclic acid metabolism in a fermenting coculture of a *Clostridium* sp. with a *Campylobacter* sp., the latter being unable to metabolize aromatic compounds. In dense cell suspensions resorcyclic acids were decarboxylated to resorcinol. Besides these aromatic compounds, also 1,3-cyclohexanedione was used as growth substrate.

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It was proposed that the resorcyclic acids were enzymatically decarboxylated and resorcinol was reduced to the non-aromatic product. Recently, resorcinol-degrading pure cultures of sulfate reducing bacteria were obtained which also metabolized 1,3-cyclohexanedione (Schnell et al. 1989).

This investigation was aimed at studying in vitro the anaerobic metabolism of these compounds using the *Clostridium* coculture. Since studies with pure faster growing cultures would be preferable, we isolated denitrifying bacteria which grow on resorcyclic acids, resorcinol or benzoate plus nitrate as sole energy and carbon sources.

Materials and methods

Materials

The *Clostridium* coculture KN 245 was that described by Tschech and Schink (1985). *Desulfobacterium autotrophicum* was the original strain (Brysch et al. 1987). Biochemicals were from Boehringer (Mannheim, FRG), chemicals from Fluka (Neu-Ulm, FRG), Merck (Darmstadt, FRG), Serva (Heidelberg, FRG), and Aldrich (Steinheim, FRG). Yeast extract and agar were from Difco (Detroit, USA). Gases were from Linde (München, FRG).

Enrichment, isolation, characterization, growth, and cell harvest of bacteria

Growth was measured as $A_{578\ nm}$ (light path 1 cm) against medium as a blank. *Desulfobacterium autotrophicum* was grown autotrophically on a mineral salt medium with H_2, CO_2, and sulfate (Schauder et al. 1986). The *Clostridium* KN 245 coculture was grown on a mineral salt medium (Widdel and Pfennig 1981) supplemented with 7 vitamins (Pfennig 1978) and the aromatic compound; the coculture was harvested as soon as initiation of spore formation could be seen microscopically. The denitrifying bacteria were enriched at 28° C, isolated, and cultivated as described for phenol degrading bacteria (Tschech and Fuchs 1987), microphotographs were taken (Pfennig and Wagener 1986), and the Gram-type was determined (Magee et al. 1975; as modified by Widdel 1980; Gregersen 1978). Enrichment cultures were set up with 2.5 mM of the aromatic compound and 7.5 mM nitrate and 15% inoculum from the anaerobic part of the sewage treatment plant at Ulm; for strain characterization 2.5 times lower substrate concentrations were used. The gas phase was N_2 when cells were grown anaerobically, and the cultures were slightly stirred. Aerobic cultures were shaken at 145 rpm, nitrate was omitted and ammonia was used as nitrogen source. When larger amounts of cells were grown trace elements and vitamins (Pfennig 1978) were fed back at $\Delta A_{578\ nm} = 0.3$ and nitrate and/or the aromatic substrate were repeatedly fed when the aromatic compounds could not be detected anymore spectroscopically. Cells were harvested at $A_{578\ nm} = 0.4-0.6$ by centrifugation. The generation time at 28° C was 8 h, the molar growth yield in mineral medium with 2,4-dihydroxybenzoic acid and nitrate was 38 g cell dry matter formed per mol of 2,4-dihydroxybenzoic acid metabolized.

Enzyme assays and characterization of the enzyme reactions

Cell extracts were prepared using the French press (Tschech and Fuchs 1989). The enzyme activities in the extract were measured at 28° C spectrophotometrically in stoppered cuvettes with a N_2 head space, or in an anaerobic hood with 95% N_2/5% H_2 gas space. The assay mixtures (1 ml total volume) were anaerobic and additions were made by microliter syringes. After the linear protein-dependence and the approximate pH-optimum were determined the following standard assays and conditions were used:

2,4-Dihydroxybenzoic acid decarboxylase and 2,6-dihydroxybenzoic acid decarboxylase

Assay mixture: 100 mM potassium phosphate buffer pH 5.8; 0.2 mM 2,4- or 0.3 mM 2,6-dihydroxybenzoate; 20 µl extract which was four-fold diluted in buffer (0.2 mg protein). Below pH 5.2 the spectrophotometric assays were disturbed by increasing turbidity due to precipitates. The reaction was started by addition of substrate. The wavelengths used for measuring decarboxylation of these and other compounds (0.1 – 0.2 mM) are given in Table 1. For determination of the pH-dependence 100 mM of sodium 2-(*N*-morpholino) ethanesulfonate buffer and other buffers were used (Fig. 1). 2,4-Dihydroxybenzoic acid, $\varepsilon_{290\ nm} = 3.6 \cdot 10^3\ cm^{-1} \cdot M^{-1}$, pH 7; 2,6-dihydroxybenzoic aicd, $\varepsilon_{305\ nm} = 2.9 \cdot 10^3\ cm^{-1} \cdot M^{-1}$, pH 7; the product resorcinol does not absorb at these wavelengths.

Resorcinol reductase

Assay mixture: 100 mM potassium phosphate buffer pH 7.0; 1 mM dithioerythritol; 0.4 mM methyl viologen; 0.6 mM resorcinol; sodium dithionite to keep the absorbance at approximately 2; 30 µl extract. The reaction was started by adding resorcinol. The activity was hardly detected at pH 8, and at pH 6 unspecific methyl viologen oxidation prevented the assay. Methyl viologen oxidation was followed at 578 nm ($\varepsilon_{578\ nm} = 9.7 \cdot 10^3\ M^{-1} \cdot cm^{-1}$).

1,3-Cyclohexanedione hydrolase

Assay mixture: 100 mM potassium phosphate buffer pH 6.7; 60 µM 1,3-cyclohexanedione; 10 µl extract four-fold diluted in buffer (0.1 mg protein). The reaction was started with the substrate. Succinate/borate buffer pH 6.5 – 7 was inhibitory if the protein content was low. The absorbance decrease at 277 nm was followed. The absorption coefficient strongly depends on the pH (Meek et al. 1953); $\varepsilon_{277} = 25 \cdot 10^3\ cm^{-1} \cdot M^{-1}$ (pH 6.7). Due to the high absorption coefficient of the dione substrate saturation conditions could not be achieved when measuring at this wavelength.

Analytical methods

Protein was determined by the method of Bradford (1976). Nitrate and nitrite were determined according to Rider and Mellon (1946). Aromatic compounds were normally analyzed by their UV absorption spectra in 50 mM K-phosphate buffer pH 7. Acetate, butyrate and the methyl ester of 5-oxocaproic acid were identified and quantitated by gas chromatography.

Conditions: Glass column (3 mm × 1.8 m), 10% SP 1000 + 1% H_3PO_4 on chromosorb WAW (80 – 100 mesh) (Macherey-Nagel, Düren). Carrier gas 24 ml · N_2 × min^{-1}; flame ionization detection. Oven temperature 120° C or 140° C. Injector and detector temperature 260° C. Sample size 2 µl.

Retention times: Acetate 3.5 min (at 120° C), butyrate 3.4 min (at 140° C), 5-oxocaproic acid methyl ester 8.4 min (at 140° C). Samples were deproteinized with perchloric acid and centrifuged, or were

Table 1. List of suitable wavelengths for the spectrophotometric assays of aromatic decarboxylases based on the UV absorbance difference between the substrate (phenolic acid) and the decarboxylated product (phenolic compound). The values in parentheses are the absorption minima

Aromatic acid		Decarboxylation product	
2-Hydroxybenzoate	294 nm (255)	Phenol	268 nm (235)
3-Hydroxybenzoate	285 nm (258)	Phenol	268 nm (235)
4-Hydroxybenzoate	244 nm (223)	Phenol	268 nm (235)
2,4-Dihydroxybenzoate	290 nm (267)	Resorcinol	272 nm (243)
2,6-Dihydroxybenzoate	305 nm (267)	Resorcinol	272 nm (243)
3,5-Dihydroxybenzoate	293 nm (266)	Resorcinol	272 nm (243)
2,5-Dihydroxybenzoate	318 nm (263)	1,4-Dihydroxybenzene (Hydroquinone)	287 nm (250)
2,3-Dihydroxybenzoate	304 nm (263)	1,2-Dihydroxybenzene	274 nm (243)
3,4-Dihydroxybenzoate	285 nm (271)	1,2-Dihydroxybenzene	274 nm (243)
3,4,5-Trihydroxybenzoate	257 nm (234)	1,2,3-Trihydroxybenzene (Pyrogallol)	265 nm (248)
2,4,6-Trihydroxybenzoate	254 nm (240)	1,3,5-Trihydroxybenzene (Phloroglucinol)	265 nm (249)

directly acidified with phosphoric acid prior to analysis. Methylation procedure was according to Laanbroek et al. (1977). 2,4-Dihydroxybenzoic acid, resorcinol, and 1,3-cyclohexanedione were determined by HPLC on (RP) C18 "Li Chrospher" (Merck, Darmstadt, FRG). The solvent was 15% by vol. methanol/85% by vol. 0.1% acetic acid at a flow rate of 1 ml · min^{-1}. Photometrical detection at 272 nm (resorcinol, 1,3-cyclohexanedione) or 290 nm (dihydroxybenzoate).

Retention times: Resorcinol 8.8 min, 1,3-cyclohexanedione 12.8 min, 2,4-dihydroxybenzoate (19.2 min).

Results

Detection of enzymes involved in anaerobic metabolism of resorcyclic acids and resorcinol in the fermenting Clostridium coculture and determination of their catalytic properties

Enzymes of anaerobic metabolism of resorcyclic acids and resorcinol were searched for in cells of the *Clostridium* coculture grown on beta- or gamma-resorcyclic acid or on resorcinol. All enzymes to be described were oxygen-sensitive and were found in the soluble cell fraction (100000 g supernatant of cell extract). Extracts could be stored frozen at -20° C for several days without significant loss of enzyme activity, except for resorcinol reductase. The assays were performed at the growth temperature of 28° C.

2,4-Dihydroxybenzoic acid decarboxylase (E.C. 4.1.1.–) and 2,6-dihydroxybenzoic acid decarboxylase (E.C. 4.1.1.–)

Detection and specific activity: Extracts of the *Clostridium* coculture, when grown on beta- or gamma-resorcylic acid, catalyzed the decarboxylation of the respective acid to resorcinol. The specific activity was 200 nmol aromatic acid decarboxylated · min^{-1} · mg^{-1} cell protein (pH 5.8). Two different inducible decarboxylases appear to exist: 2,4-dihydroxybenzoic acid was almost exclusively decarboxylated only when the cells were grown on this substrate, and vice versa, 2,6-dihydroxybenzoic acid was decarboxylated rather specifically by extracts of 2,6-dihydroxybenzoic acid grown cells. Extracts of cells grown on resorcinol were almost inactive towards either one of the two aromatic carboxylic acids. Decarboxylation of these (and other hydroxylated) aromatic acids at pH 5.8 was followed spectrophotometrically in the near UV region where the phenolic acids but not the phenolic products exhibit an absorption maximum (Table 1).

Stoichiometry: The product of both reactions on resorcyclic acids was identified as resorcinol and was quantitated by HPLC. Both aromatic acids were completely and stoichiometrically decarboxylated.

Kinetic properties: Both decarboxylase activities exhibited similar though not identical pH-dependence; they were highest around pH 5.2 – 5.4 in 100 mM phosphate buffer (Fig. 1). Both activities decreased almost linearly with increasing pH, independent of the buffer used. The reaction was not stimulated by monovalent (K^+, Na^+) or divalent (Mg^{2+}, Mn^{2+}) cations, nor was it inhibited by 4 mM EDTA. Citrate buffer (100 mM, pH 5.8), however, completely inhibited 2,6-dihydroxybenzoic acid decarboxylase, but only little (30%) 2,4-dihydroxybenzoic acid decarboxylase. This is further circumstantial evidence for the existence of two different enzymes.

K_m-Values: K_m-value of 44 μM for 2,4-dihydroxybenzoic acid (pH 5.8) and ≤ 5 μM for 2,6-dihydroxybenzoic acid (pH 5.8), respectively, was estimated from linear

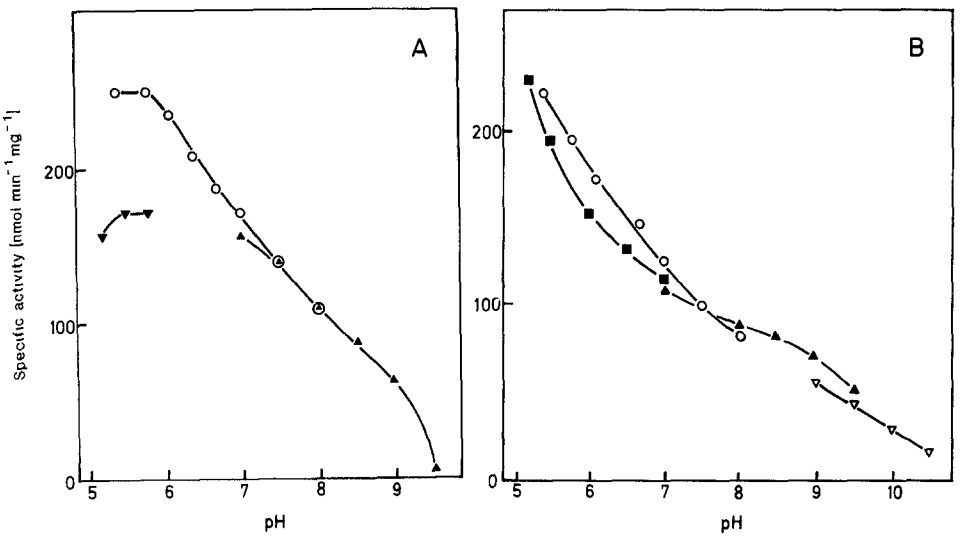

Fig. 1 A, B. Dependence on the pH of (**A**) 2,4-dihydroxybenzoic acid decarboxylase and (**B**) 2,6-dihydroxybenzoic acid decarboxylase activity in extracts of the *Clostridium* coculture. The buffers used were (○) K-phosphate, (▲) *Tris*/HCl, (▼) Na-citrate, (■) succinate/borate, (▽) glycine buffer, each 100 mM

Table 2. Comparison of the substrate specificity of 2,4-dihydroxybenzoic acid decarboxylase (specific activity 250 nmol · min^{-1} · mg^{-1} protein) and 2,6-dihydroxybenzoic acid decarboxylase (specific activity 220 nmol · min^{-1} · mg^{-1} protein). Not metabolized were: 2-, 3-, 4-hydroxybenzoic acid; 2,3-, 2,5-, 3,4-, 3,5-dihydroxybenzoic acid; 3,4,5-trihydroxybenzoic acid. The relative enzyme activities refer to the specific rates with the physiological substrate (= 100%)

Substrate [0.2 mM]	2,4-Dihydroxybenzoate Decarboxylase (%)	2,6-Dihydroxybenzoate Decarboxylase (%)
2,4-Dihydroxybenzoate	100	12
2,6-Dihydroxybenzoate	1.5	100
2,4,6-Trihydroxybenzoate	17	220

Table 3. Identification of the products and stoichiometry of the reduced methyl viologen dependent resorcinol reductase reaction. Note that the extracts contained active 1,3-cyclohexanedione hydrolase which immediately hydrolyzed the product of the reaction, 1,3-cyclohexanedione. The products of the assays were methylated and determined by gas chromatography. Standard compounds were treated the same way and were used for identification and quantification. In order to avoid the detrimental effect of excess dithionite a regenerating system for reduced methyl viologen was used in this case consisting of: H_2 or CO, hydrogenase and CO dehydrogenase containing extract (5 µl) of *Desulfobacterium autotrophicum* which contained none of the enzymes to be tested (controls not shown), methyl viologen

Resorcinol added [µmoles]	1,3-Cyclohexanedione found [µmol]	5-Oxocaproic acid (methyl ester) formed [µmol]
0.25	0	0.26
0.50	0	0.48
1.00	0.05	1.01

Lineweaver-Burk plots. If the actual substrate is the undissociated acid the true K_m-values would be more than one order of magnitude lower.

Substrate specificity: The two decarboxylases present in extracts of cells grown on the respective aromatic acid were rather specific for their individual substrate (Table 2). 2,6-Dihydroxybenzoic acid decarboxylase also decarboxylated 2,4,6-trihydroxybenzoic acid, but the apparent K_m-value of 240 µM (pH 5.8) was approximately 50 times higher than for the natural substrate.

1,3-Benzenediol (resorcinol) reductase (E.C. 1.–.–.–)

Detection, specific activity, and stoichiometry: Extracts of cells which were grown on 2,4- or 2,6-dihydroxybenzoic acid or 1,3-benzenediol (resorcinol) catalyzed the resorcinol-dependent oxidation of reduced methyl viologen; NAD(P)H, $FADH_2$, or reduced benzyl viologen were not oxidized. The decrease of resorcinol was shown by HPLC. The product of the reaction, 1,3-cyclohexanedione, was immediately hydrolyzed to 5-oxocaproic acid by 1,3-cyclohexanedione hydrolase present in the extracts (see below). The almost stoichiometric formation of this secondary product was demonstrated by gas chromatography (Table 3). Furthermore, when 15 µM resorcinol was added to the assay, the final absorption decrease at 578 nm (light path 1 cm) due to the oxidation of reduced methyl viologen ($\varepsilon_{578\ nm} = 9.7 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) was 0.3 which corresponds to the oxidation of 30 µM of this one-electron transferring dye. At pH 7 the specific activity was 15 nmol resorcinol reduced · min^{-1} · mg^{-1} protein. Oxidation of 1,3-cyclohexanedione to resorcinol could not be measured with different electron acceptors $(NAD(P)^+$, FAD, methylene blue, or 2,6-dichlorophenolindophenol in combination with phenazine methosulfate).

Kinetic properties: Since excess of dithionite and therefore of reduced methyl viologen was inhibitory the K_m-values could not be determined accurately. With non-saturating concentrations of the electron donor (0.2 mM) half maximal activity was observed at ≤ 5 µM resorcinol and

Table 4. Identification of the product and stoichiometry of 1,3-cyclohexanedione hydrolase reaction

1,3-Cyclohexanedione added [μmol]	5-Oxocaproic acid (Methylester) formed [μmol]	Stoichiometry
0.25	0.26	1.04
0.50	0.46	0.92
1.00	1.05	1.05

70 μM reduced methyl viologen. The reaction was not stimulated by Mg^{2+} nor inhibited by EDTA.

Substrate specificity: Besides resorcinol the enzyme also reduced 1,3-dihydroxy-5-methyl-benzene (5-methyl-resorcinol, orcinol) (20% relative activity) and 1,2,4-trihydroxybenzene (hydroxyhydroquinone) (67% relative rate), each measured with 1 mM substrate. 1,3,5-Trihydroxybenzene (phloroglucinol) was not reduced.

1,3-Cyclohexanedione hydrolase (E.C. 3.7.–.–)

Detection, specific activity, and stoichiometry: Extracts of cells grown on any of the aromatic compounds catalyzed the hydrolytic cleavage of 1,3-cyclohexanedione to 5-oxocaproic acid. The conversion was complete and stoichiometric (Table 4). The product was determined by gas chromatography as its methyl ester. Since addition of coenzyme A to the assay did not stimulate the reaction rate it is concluded that the cleavage was hydrolytic rather than thiolytic. The maximum specific activity was 880 nmol 1,3-cyclohexanedione hydrolyzed · min^{-1} · mg^{-1} protein (pH 6.7).

Kinetic properties: The enzyme exhibited a pronounced bell-shaped pH dependence with an optimum at pH 6.7; half maximal rates were observed at pH 6 and pH 7.6 (Tris/HCl or K-phosphate buffer), and the activity could be followed up to pH 9. Divalent metal ions or EDTA were without any effect on the enzyme activity. The K_m-value was extrapolated from a linear Lineweaver-Burk plot as 44 μM 1,3-cyclohexanedione.

Substrate specificity: The enzyme reacted specifically with the natural substrate. 1,2-Cyclohexanedione (20 μM), 1,3-cyclopentanedione (300 μM) or 1,3-dioxo-5-hydroxycyclohexane (dihydrophloroglucinol) was not cleaved nor was any of these compounds inhibitory in the standard assay (70 μM substrate).

Regulation of enzyme activity level

The level of enzyme activities was determined in extracts of cells grown on the different aromatic substrates to understand the regulation of this metabolic pathway.

The results (Table 5) demonstrate that the enzymes are regulated probably by induction/repression as one would expect: the decarboxylase activities were induced by their specific substrates whereas hydrolase activity and reductase activity were present under any growth conditions tested.

Control experiment to suggest that the enzymes of aromatic metabolism were formed by the Clostridium sp. and not by the Campylobacter sp.

In the *Clostridium* coculture the *Campylobacter* sp. always represented only a small fraction (5% estimated). It could be grown separately in pure culture, since it was able to grow by fermenting fumarate, but not aromatic compounds. When grown on 20 mM fumarate in the presence of a mixture of the two dihydroxybenzoic acids and resorcinol (each 1 mM), the aromatic compounds were not degraded. In extracts of the cells none of the described aromatic metabolizing enzymes could be detected, even at high protein concentrations.

Isolation of a denitrifying bacterium with resorcyclic acids or resorcinol as substrates and search for aromatic metabolizing enzymes

To obtain pure cultures more suitable for future enzyme work denitrifying bacteria were enriched on mineral salt medium with 1,3-benzenediol and nitrate. A rod-shaped gram-negative bacterium was isolated which could use oxygen, nitrate, or nitrite as terminal electron acceptor. It grew anaerobically on 2,4-dihydroxybenzoic acid, benzoate, and 1,3-benzenediol, but not on 1,3-cyclohexanedione; nitrate was reduced to N_2, and nitrite accumulated transiently in the medium. β-Resorcylate was first decarboxylated to resorcinol which was excreted in almost stoichiometric amounts and was then completely oxidized resulting in a biphasic growth curve (data not shown). This strain, LG 245, did not grow anaerobically on 2,6- or 3,5-dihydroxybenzoic acid, 1,2,4-trihydroxybenzene, 4-hydroxybenzoate, or phenol. It grew aerobically with acetate, but not with aromatic acids such as benzoate or 2,4-dihydroxybenzoate. The generation time was 6 times shorter and the cell density which could be reached reproducible was 4 times higher than with the *Clostridium* coculture.

Extracts of the strain LG 245, which was grown on 2,4-dihydroxybenzoic acid and nitrate, catalyzed the decarboxylation of that aromatic acid to resorcinol at a specific activity of around 800 nmol · min^{-1} · mg^{-1} protein. However, no resorcinol or phloroglucinol reductase activity could be detected; different electron donors such as NAD(P)H or dithionite reduced methyl or benzyl viologen were tried. No hydrolytic or coenzyme A-dependent thiolytic cleavage of 1,3-cyclohexanedione could be detected.

Discussion

The data presented support the pathway proposed in Fig. 2 and the conclusions derived from studies with the

Table 5. Specific activities of the enzymes catalyzing the initial reactions in the anaerobic metabolism of resorcylic acids and resorcinol in the *Clostridium* coculture. The cells were grown on the substrates indicated

Enzymes	Specific activities [μmol · min^{-1} · mg^{-1} protein] in extracts of cells grown on		
	2,4-Dihydroxybenzoate	2,6-Dihydroxybenzoate	Resorcinol
2,4-Dihydroxybenzoate decarboxylase (E.C. 4.1.1.–.)	0.25	0.026	0.01
2,6-Dihydroxybenzoate decarboxylase (E.C. 4.1.1.–.)	0.043	0.22	0.034
Resorcinol reductase (E.C. 1.–.–.–.)	0.015	0.01	0.015
1,3-Cyclohexanedione hydrolase (E.C. 3.7.–.–.)	0.49	0.24	0.45

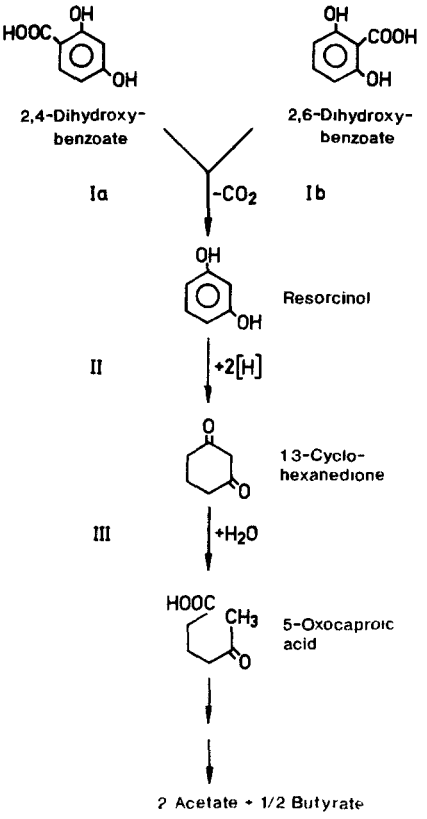

Fig. 2. Proposed initial reactions in the anaerobic degradation of resorcylic acids and of resorcinol by the *Clostridium* coculture. Ia = 2,4-dihydroxybenzoic acid decarboxylase, Ib = 2,6-dihydroxybenzoic acid decarboxylase, II = resorcinol (1,3-benzenediol) reductase, III = 1,3-cyclohexanedione hydrolase

Clostridium coculture (Tschech and Schink 1985). 2,4- and 2,6-Dihydroxybenzoic acids are decarboxylated by specific enzymes. It is shown that the aromatic nucleus of 1,3-benzenediol (resorcinol) is reduced in a 2 electrons-step, rather than 4 electrons- or 6 electrons-step, to a cyclic β-dione which is easily amenable to β-cleavage. The same intermediate, 1,3-cyclohexanedione, is formed in anaerobic metabolism of alicyclic compounds originating from cyclohexanol (Dangel et al. 1989). The decarboxylase activities are strictly regulated probably on the transcription level, and this may also be expected for the reductase and hydrolase. In general, the specific activity, the substrate affinity, and the specificity of the enzymes were sufficiently high to explain anaerobic growth of the coculture on aromatic compounds (estimated rate of 2,4-dihydroxybenzoic acid metabolized, 60 nmol · min^{-1} · mg^{-1} protein).

However, it may be pointed out that this degradation pathway for resorcinol is probably not the only strategy of anaerobes to cope with this problem. By no means could similar enzymes be detected in the denitrifying bacterium which, unlike the fermenting bacterium, appeared not to be able to metabolize 1,3-cyclohexanedione.

In the *Clostridium* sp. the metabolic fate of resorcylic acids and resorcinol resembles the anaerobic metabolism of gallic acid and phloroglucinol in fermenting bacteria (Patel et al. 1981; Schink and Pfennig 1982; Samain et al. 1986; Krumholz and Bryant 1986; Krumholz et al. 1987; Haddock and Ferry 1989). Trihydroxybenzoates are decarboxylated to trihydroxybenzene which may be isomerized to phloroglucinol (1,3,5-trihydroxybenzene) (Krumholz and Bryant 1988; Brune and Schink 1990). The subsequent ring reduction and alicyclic ring cleavage reactions are analogous.

The two aromatic acid decarboxylases are characterized by a strongly acidic pH optimum. Similar pH optima have been reported for 2,5-dihydroxybenzoate (gentisate) decarboxylase, 3,4-dihydroxybenzoate (protocatechuate) decarboxylase, and 3,4,5-trihydroxybenzoate (gallate) decarboxylase in *Klebsiella (Aerobacter) aerogenes* (Grant and Patel 1969). The observed pH-dependence might indicate that the undissociated acids with the hydroxyl groups still being protonated are the actual substrates of the enzymes [2,4-dihydroxybenzoic acid: pK of COOH, 3.3; of 4-OH, 9.1; 2-OH, 15.6 (Mattoo 1959)].

The enzyme resorcinol reductase was oxygen-sensitive and did not react with pyridine nucleotides and thus differs from phloroglucinol reductase. The reduction of resorcinol is chemically more challenging and therefore may require a reductant with a more negative redox potential and different enzyme active site. Phloroglucinol reductase from *Eubacterium oxidoreducens* exhibited no special prosthetic groups or unusual properties (Haddock and Ferry 1989). The purified enzyme was specific for phloroglucinol, whereas extracts of *Pelobacter acidigallici* were reported to reduce with NADH not only phloroglucinol but also resorcinol (Samain et al. 1986). A phenol reducing enzyme has not been demonstrated so far.

Resorcinol reductase from *Clostridium* sp. KN 245 also reduced orcinol (5-methylresorcinol) and 1,2,4-trihydroxybenzene (this compound was used as growth

substrate, data not shown). The enzyme catalyzing cleavage of the non-aromatic carbon ring, 1,3-cyclohexanedione hydrolase, was specific for this substrate and did not cleave 1,3-dioxo-5-hydroxycyclohexanone (dihydrophloroglucinol). Dihydrophloroglucinol hydrolase from *E. oxidoreducens* appeared to be specific for its 1,3-dione substrate (Krumholz et al. 1987). The 1,3-cyclohexanedione hydrolase involved in cyclohexanol metabolism by denitrifying bacteria also was specific for its substrate, but unlike the enzyme reported here, was oxygen stable and was inhibited by 1,2-cyclohexanedione (Dangel et al. 1989). All these enzymes appear to catalyze a hydrolytic rather than thiolytic C-C cleavage of the alicyclic ring.

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