First publ. in: Archives of Microbiology, 143 (1985) 1, pp.52-59

Fermentative degradation of resorcinol and resorcylic acids

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Abstract. Anaerobic fermentative degradation of resorcinol and resorcylates was studied in enrichment cultures inoculated with marine or freshwater sediments or digested sludge. α-Resorcylate (3,5-dihydroxybenzoate) was degraded very rapidly to acetate and methane by enrichment cultures inoculated with freshwater sediment or sewage sludge, but degradation was slow in enrichments from marine habitats. The freshwater cultures did not degrade any other related phenolic substrates. Inhibition of methanogenic bacteria by bromoethanesulfonate and acetylene led to enhanced acetate formation indicating homoacetogenic hydrogen oxidation. With resorcinol (1,3-dihydroxybenzene) and β- and γ-resorcylate (2,4- and 2,6-dihydroxybenzoate), two different types of Gram-positive spore-forming strict anaerobes were isolated, which both did not grow with α-resorcylate. Both were assigned to the genus *Clostridium*. From freshwater enrichments, six strains were isolated in defined coculture with *Campylobacter* sp. They fermented resorcinol and β- and γ-resorcylate stoichiometrically to acetate and butyrate. No interspecies hydrogen transfer to methanogenic or other anaerobic bacteria was found. None out of numerous organic nutrients tested substituted for *Campylobacter* sp. as partner in defined cultures; the nutritive dependence of this bacterium could not be elucidated. Isolates from marine sediments formed acetate and hydrogen from resorcyclic compounds, and depended on syntrophic association with hydrogen-scavenging anaerobes such as methanogens.

Key words: Anaerobic phenol degradation – Aromatic compounds – Resorcinol – Resorcylic acids – *Clostridium* sp. – Interspecies hydrogen transfer – Butyrate fermentation – *Campylobacter* sp.

Methoxylated and hydroxylated phenylpropanoate and benzoate derivatives are the basic constituents of lignin and various plant phenols (Young 1984; Pridham 1965). Whereas aerobic degradation of natural and synthetic benzene-derivatives has been studied extensively (Dagley 1975) our knowledge on anaerobic degradation of these compounds is very poor. Complete methanogenic degradation of several methoxylated and hydroxylated benzoate and phenylpropanoate derivatives was demonstrated first in 1934 (Tarvin and Buswell 1934) and lateron again with more refined techniques (Healy and Young 1978, 1979; Healy et al. 1980; Chou et al. 1978; Kaiser and Hanselmann 1982). Methoxyl groups are transformed into hydroxyl groups by some homoacetogenic bacteria such as *Acetobacterium woodii* (Bache and Pfennig 1981).

The further fate of hydroxybenzoates and phenols is largely unknown. If the aromatic ring carries three hydroxyl substituents as e.g. in gallic acid, pyrogallol, or phloroglucinol, it can be fermented to acetate by pure cultures of *Pelobacter acidigallici* (Schink and Pfennig 1982). Hydroquinone, catechol (1,4- and 1,2-dihydroxybenzene) and some of their carboxylated derivatives are degraded via reductive dehydroxylation (and decarboxylation) to phenol as demonstrated in enrichment culture studies (Szewzyk et al. 1985). The bacteria degrading the phenol ring could not yet be isolated, and appear to depend on syntrophic cooperation with e.g. methanogens.

Resorcinol (1,3-dihydroxybenzene) and resorcylic acids (resorcinolmonocarboxylic acids) are no common natural products, and do not occur as lignin constituents. They are used in the pharmaceutical and dye-stuff industry and as intestinal antiseptics (Windholz et al. 1976). During aerobic degradation, resorcinol is hydroxylated by a monooxygenase reaction prior to ortho- or meta-cleavage (Groseclose and Ribbons 1981). Anaerobic degradation of resorcinol to methane and carbon dioxide in sludge and sediment samples has been reported but the bacteria involved and the intermediate products formed were not characterized (Chmielowsky et al. 1965; Chou et al. 1978). In the present paper, quantitative fermentation of resorcinol and resorcylates by enrichment cultures from various origins and by defined cultures is described. The organisms involved are characterized and degradation pathways for the resorcyclic compounds are proposed.

Materials and methods

Sources of organisms

Freshwater enrichments were inoculated with anaerobic digested sludge from the municipal sewage plants in Konstanz and Göttingen, and with sediment samples from a polluted creek near Konstanz, FRG. Marine enrichments were inoculated with anaerobic sediments from channels in Venice, Italy. *Campylobacter* sp. strain spirillum 5175 (Wolfe and Pfennig 1977) was obtained from N. Pfennig, Konstanz, *Desulfovibrio vulgaris* strain Marburg from R. K. Thauer, Marburg. *Methanospirillum hungatei* strain M1h was isolated from digested sludge and uses hydrogen and formate as sole substrates. *Desulfovibrio* sp. strain Cuxhaven

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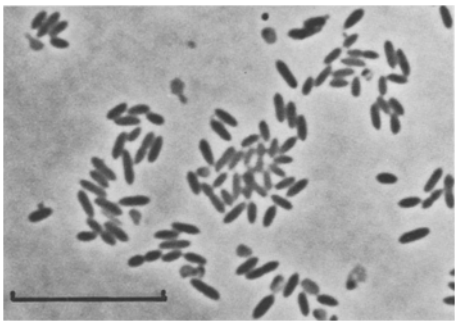

Fig. 1. Phase contrast photomicrograph of enrichment culture Gö 3.5 grown on α-resorcylic acid. Bar equals 10 μm

was isolated by F. Widdel, Konstanz, with H_2/CO_2 and acetate as substrates. It incompletely oxidized lactate, pyruvate, fumarate, and formate with sulfate as electron acceptor.

Media and cultivation conditions

All procedures for cultivation and isolation as well as all methods for analysis of metabolic products were essentially as described in earlier papers (Widdel and Pfennig 1981; Schink and Pfennig 1982). The mineral medium for enrichment and further cultivation contained 30 mM sodium bicarbonate buffer and sodium sulfide, trace element solution SL 10 (Widdel et al. 1983) and selenite/tungstate solution (Tschech and Pfennig 1984) at one half of the concentrations given in the respective original descriptions. The pH was 7.1 – 7.3. Neutral stock solutions (0.25 or 0.5 M) of di- and trihydroxybenzenoid substrates were prepared in oxygen-free water or NaOH solution under nitrogen gas, and were filter-sterilized into gassed sterile serum bottles. Most phenolic compounds were more stable in slightly acidic than in alkaline solution.

Growth of defined cultures was followed in 20 ml tubes in a Bausch and Lomb Spectronic 70 spectrophotometer at 600 nm. Growth of enrichment cultures was followed by measurement of optical density in 1 cm cuvettes. For syntrophic growth tests either *Methanospirillum hungatei* or *Desulfovibrio vulgaris* was added to the cultures, in the latter case with additional sodium sulfate (10 mM final concentration). For further characterization, also commercial media systems (API 20A, BioMerieux, Nürtingen, FRG) were applied. Aerobic growth was tested in agar shake gradient cultures under air/CO_2 mixture (80%/20%). All growth tests were carried out at least in triplicates at 28° C.

Isolation

Defined cultures were obtained by repeated application of the agar shake culture method as described (Pfennig 1978). For isolation of strains from freshwater enrichments with resorcinol, β- and γ-resorcylate, 5 ml of a fresh *Campylobacter* culture (either spirillum 5175 or strain 245) was added to 50 ml culture medium. 5 ml culture of a marine *Desulfovibrio* sp. as hydrogen scavenger was added for isolation of marine resorcylate degrading bacteria. Tubes were gassed with N_2/CO_2 (80/20) and sealed wih butyl rubber stoppers. Purity was checked microscopically after growth in defined as well as in complex medium (AC-medium Difco Laboratories, Detroit, MI, USA).

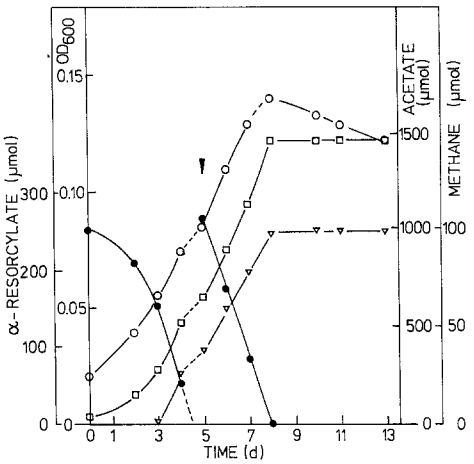

Fig. 2. Fermentation time course of enrichment culture Ott 3.5 after 60 transfers. Experiments were performed at 28° C in 120 ml serum bottles sealed with Bellco butyl rubber septa. Samples were taken by syringes at times indicated. The headspace contained N_2/CO_2 (80%/20%) gas mixture. ○ Cell density; □ acetate; ▽ methane; ● α-resorcylate. *Arrow* indicates addition of substrate

Growth tests with inhibition of methanogenic bacteria were carried out in 120 ml serum bottles with 50 ml culture fluid under an N_2/CO_2 (80%/20%) atmosphere. Either 2-bromoethanesulfonate (0.5 or 2.5 mM), ethylene (5% of culture headspace), or acetylene (1% of culture headspace) were added to the cultures. Development of turbidity as well as substrate disappearance and product formation were taken as indicators of microbial activity.

Chemical analyses

Fatty acids, alcohols and methane were determined by gas chromatography (Schink and Pfennig 1982), sulfide after Cline (1969). Aromatic substrates were identified and quantified by their absorption spectra. Medium samples were diluted 1:100 in 10 mM potassium phosphate buffer, pH 7.0, and spectra were taken at 200 to 350 nm wavelength with a Shimadzu UV 300 spectrophotometer.

Chemicals

All chemicals were of reagent grade quality and obtained from Merck, Darmstadt, FRG. Aromatic compounds were purchased from Fluka, Buchs, Switzerland.

Results

Enrichments with α-resorcylate (3,5-dihydroxybenzoate)

Enrichment cultures from freshwater habitats with α-resorcylate as sole energy and carbon source started methane formation after 10 to 14 days of incubation. In all four enrichments from various freshwater origins, the same type of tiny, rod-shaped cells developed, and acetate and methane were detected as sole fermentation products. Two out of four enrichment cultures were characterized further, one from digested sludge (Gö 3.5) and one from creek sediment (Ott 3.5). After 10 to 12 transfers, these cultures fermented 5 mM α-resorcylate within 3 to 4 days to acetate and methane. Fluorescing methanogenic bacteria observed in the cultures were all very tiny rods, about 0.5 × 1.0 μm in

Table 1. Stoichiometry of α-resorcylate fermentation by enrichment cultures Gö 3.5 and Ott 3.5
Cultures were grown at 28° C in 120 ml serum bottles sealed with butyl rubber septa under a headspace of N_2/CO_2 (80%/20%) gas mixture. Initial substrate concentrations were 5 mM or 10 mM. Fermentation products were quantified after growth had ceased and no aromatic substrate was detected any more by UV-spectroscopy. All values given are means of at least two independent assays

Culture	Amount of substrate degraded (μmol)	Acetate produced (μmol)	CH_4 produced (μmol)	Cell density obtained ($OD_{650}^{1\,cm}$)	Cell material formed (mg)	Carbon recovery (%)
Gö 3.5	250	693	45.6	0.075	0.93	90.3
	500	1453	104.1	0.14	1.75	95.4
Ott 3.5	250	675	43.7	0.08	1.0	88.7
	500	1358	114.6	0.15	1.9	90.0

size. The prevalent bacterial cells were Gram-negative, slightly bigger ($0.5 \times 1.5-3.0$ μm) in size, with pointed ends, and tended to form aggregates (Fig. 1). Unfortunately, these cells used to lyze within few hours after substrate consumption. Subcultures started growth immediately if freshly grown cell material was used as inoculum. Elder inocula exhibited lag phases of 1 to 2 weeks before growth recuperated.

The correlation of growth, substrate decomposition, and product formation by enrichment culture Ott 3.5 after 60 transfers is shown in Fig. 2. No qualitative modification of the UV-spectrum of the culture fluid was detected during growth indicating that unsaturated intermediates were not excreted to significant concentrations. Fatty acids other than acetate were not detected. The products measured accounted for 89% to 95% of the carbon provided as substrate (Table 1). Substrate assimilation was estimated from turbidity measurements to account for 5%−10% of the substrate provided. Thus, the results obtained were in good aggreement with the theoretical fermentation equation:

$$4\,C_6H_3(OH)_2COO^- + 17\,H_2O \rightarrow 12\,CH_3COO^- + 11\,H^+ + 3\,HCO_3^- + CH_4.$$

Several compounds related to α-resorcylate were tested for utilization by the enrichment cultures after 49 transfers. No growth was found with β- and γ-resorcylate, resorcinol, dihydroresorcinol (1,3-cyclohexanedione), 1,3-cyclohexanediol, m-hydroxybenzoate, or benzoate (each added to a final concentration of 2.5 mM). All attempts to isolate the ring-degrading bacterium in pure or defined culture failed so far. Inhibition of methanogenic bacteria by 2-bromoethanesulfonate, acetylene, or ethylene, led to increased acetate formation as compared to non-inhibited cultures, but neither hydrogen nor fatty acids other than acetate accumulated.

Enrichment cultures from marine sediments required 2 months and longer for degradation of 5 mM α-resorcylate. These cultures were not characterized any further.

Enrichments from freshwater habitats with resorcinol, β- or γ-resorcylate (2,4- or 2,6-dihydroxybenzoate)

In freshwater enrichment cultures with resorcinol, β- or γ-resorcylate, gas production started after 15 to 20 days of incubation. As soon as turbidity developed cultures were transferred in order to outdilute acetate-utilizing methanogens. After 4 to 5 transfers, methane production ceased and acetate and butyrate were found as sole fermentation products. In all cases the same type of big, refractile, sporeforming rods was present in high numbers. Isolation of these bacteria was tried in agar shake dilution series. After 15 to 20 days of incubation, white lens-shaped colonies appeared in the first 3 to 4 dilution tubes. In all colonies very small, actively motile spirilloid cells accompanied the big refractile rods. These two types of bacteria could not be separated in subsequent agar dilution series with and without growth factors such as yeast extract (0.05%), rumen fluid (5%), phenylpropionate (2.5 μM), phenylacetate (2.5 μM), or mixtures of various fatty acids. Spore preparations obtained by pasteurizing enrichment cultures (10 min, 80° C) did not germinate in liquid or agar shake cultures unless the motile spirilloid bacterium was added to the medium.

Isolation and characterization of the spirilloid bacteria

The spirilloid bacterium was isolated in agar dilution series with fumarate as sole energy and carbon source. Cells grown with fumarate were thicker ($0.4-0.5$ μm $\times$ $1.5-2.5$ μm) than when grown in coculture with the resorcinol- or resorcylate degrading bacterium ($0.2-0.3 \times 1.5-2.5$ μm). They were actively motile by a single polar flagellum, fermented fumarate and malate, and oxidized hydrogen, formate, pyruvate and succinate with concomitant reduction of nitrate. The guanine plus cytosine content of the DNA was 41.2 ± 2.5% as determined by thermal denaturation with *Escherichia coli* strain K12, DSM 498, as reference strain. These properties allowed to assign this bacterium, strain 245, to the genus *Campylobacter* (Laanbroek et al. 1977). It did not degrade or modify any aromatic compounds. *Campylobacter* strain 245 or spirillum 5175 (Wolfe and Pfennig 1977) was added to further agar dilution series in order to isolate the resorcinol-degrading bacterium in defined coculture. Now colonies of the resorcinol degrader grew down to the seventh tube. The resorcinol- or resorcylate degrading bacteria were isolated in coculture with *Campylobacter* sp. strain 245 and spirillum 5175 from all enrichment cultures initiated wih freshwater inocula and resorcinol as well as resorcylic acids as substrate.

Properties of freshwater resorcinol degraders

Cells of all isolated strains were long, fat, straight rods with rounded ends, $1.2 \times 3.0-6.0$ μm in size (Fig. 3a). Cells were motile in fresh cultures in a tumbling manner, and subpolar flagella were observed in negatively stained cells by electron microscopy. Motility was lost after several transfers. In ageing cultures, subterminal, ellipsoidal spores, 1.0×2.0 μm

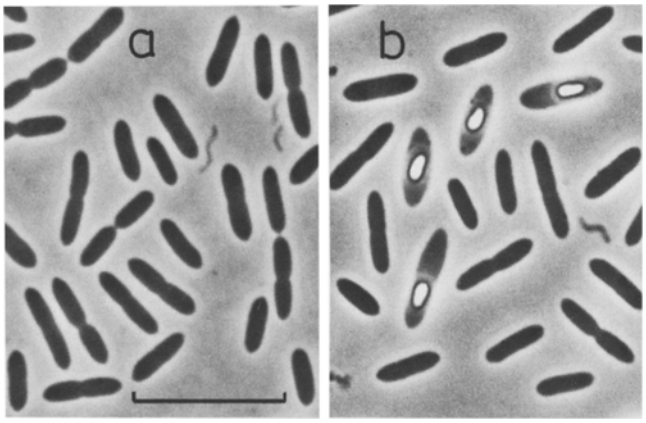

Fig. 3a, b. Phase contrast photomicrographs of defined freshwater cultures degrading resorcinol. **a** Strain KN 245 during exponential growth; **b** sporeforming cells of the same strain in the stationary growth phase. Note small spirilloid *Campylobacter* cells. Bar equals 10 μm for both prints

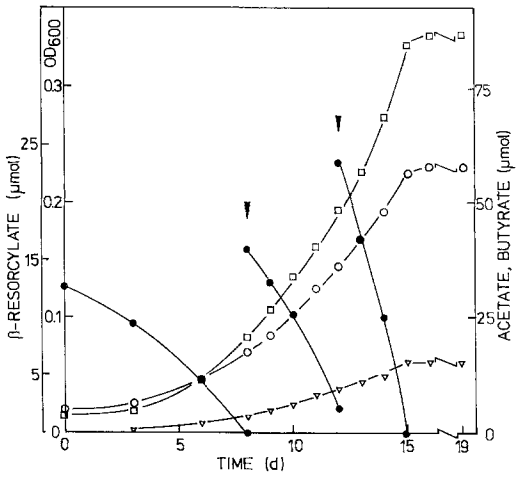

Fig. 4. Fermentation time course of strain KN 245 growing with β-resorcylate. Experiments were performed at 28° C in 120 ml serum bottles sealed with Bellco butyl rubber septa. Samples were taken by syringes at times indicated. The headspace gas was N_2/CO_2 (80%/20%) gas mixture. ○ Cell density; □ acetate; ▽ butyrate; ● β-resorcylate. *Arrows* indicate additions of substrate

in size, were formed (Fig. 3b). Cells stained Gram-negative to weakly Gram-positive. Thick layers of extracellular capsular material were visible in Indian Ink preparations (not shown). The thickness of this capsule varied from strain to strain and decreased with culture age.

Resorcinol, dihydroresorcinol, and β- and γ-resorcylate were the only substrates degraded, and were converted to acetate and butyrate (and carbon dioxide) as sole products (Fig. 4). Resorcinol was degraded according to the equation:

$$2\,C_6H_4(OH)_2 + 6\,H_2O \rightarrow 4\,C_2H_3O_2^- + C_4H_7O_2^- + 5\,H^+.$$

Substrate conversion was incomplete with dihydroresorcinol which was partly transformed into an unidentified product exhibiting UV absorption maxima at 286 and 238 nm. The yield of cell material formed was 7.5 to 8.0 g dry matter per mol of resorcinol and resorcylic acids, and more than 10 g during incomplete dihydroresorcinol utilization (Table 2). No growth was found with any of the following substrates and no qualitative modification of the UV-spectrum of the culture fluid was detected after 4 weeks of incubation. The substrates were provided at the concentrations given in brackets: 2,3-, 2,5-, 3,4-, 3,5-dihydroxybenzoate, catechol, hydroquinone, 4-hydroxybenzoate, phenol, cyclohexanol, 1,3-cyclohexanediol, cyclohexanone (each 1 mM), cyclohexane carboxylate, adipate, pimelate, caproate, heptanoate, 3-hydroxybutyrate, gallate, phloroglucinol, pyrogallol, 3-hydroxybenzoate, salicylate, benzoate, pyruvate, glycerol, methanol, ethanol, malate, succinate, citrate, fumarate, crotonate (each 5.0 mM), formate (20 mM), hydrogen (in excess), glucose, fructose (each 2.5 mM), and several other sugars supplied in the API 20A substrate series. In the presence of *Desulfovibrio vulgaris* as a hydrogen sink, no further substrates were utilized and no sulfide was formed. Sulfate, thiosulfate, sulfite, nitrate, and fumarate did not serve as electron acceptors. During growth with resorcinol or resorcylic acid, no qualitative change of the UV-spectrum of the culture fluid was observed. Decarboxylation of resorcylic acids to resorcinol was demonstrated with dense cell suspension. A 1 l culture of strain KN 245 grown with β-resorcylate was centrifuged and resuspended in freshwater medium to an optical density of 12 to 13. β- or γ-resorcylate or resorcinol was added to this cell suspension to a final concentration of 15 mM. After 10 h of incubation at 34° C resorcinol was formed up to 5 mM concentration as recorded by the culture fluid absorption spectrum, and disappeared again after further 10–20 h. At the end of the experiment, all three resorcylic compounds were quantitatively degraded to acetate and butyrate. With resorcinol as substrate, no qualitative change in the UV-spectrum of the culture fluid could be detected in dense cell suspensions.

Enrichment cultures and defined cocultures exhibited lag phases of varying length after transfer into fresh culture medium. Addition of yeast extract (0.05%) to the defined cocultures shortened the lag period considerably. Yeast extract did not serve as growth substrate but enhanced growth yields. Salt concentrations higher than 1% (w/v) NaCl inhibited or prevented growth of freshwater isolates; phosphate was inhibitory at more than 10 mM concentration. Optimal growth was found at 28° C and pH 7.0–7.5. Under these conditions, the doubling time of the defined culture was 48 ± 10 h. The growth limits were 20° and 40° C and pH 6.5 and 8.0, respectively. Cysteine or ascorbate as reducing agents allowed only poor growth; sodium dithionite additions to fresh transfers helped to overcome lag phases.

Enrichments from marine sediments with β- or γ-resorcylate

In saltwalter enrichment cultures with β- or γ-resorcylate, gas production started after 25 to 30 days of incubation. After 5 to 6 transfers, the same type of big, straight, rod-shaped bacteria predominated in all four enrichment cultures. Acetate and methane were the only products formed. Very small rods predominated among the fluorescent methanogenic bacteria observed. Inhibition experiments with 2-bromoethanesulfonate, acetylene, or ethylene led to enhanced acetate production indicating homoacetogenic hydrogen oxidation. Isolation of resorcylic acid-degrading bacteria was therefore tried in the presence

Table 2. Growth yields and fermentation stoichiometry of strain KN 245

Substrate	Amount of substrate degraded (μmol)	E_{600}	Cell dry weight formed (mg)	Substrate assimilated (μmol)	Products formed (μmol)		Molar yield (g cell material per mol substrate)	Carbon recovery (%)
					Acetate	Butyrate		
Resorcinol	100	0.16	0.80	5.0	208	42	8.0	102
β-Resorcylate	100	0.16	0.80	5.0	206	42	8.0	101
γ-Resorcylate	100	0.15	0.75	4.7	204	44	7.5	102
Dihydroresorcinol	100	0.20	1.00	6.3	80	54	10.0	~50[c]

[a] Cell dry weights were determined directly with β-resorcylate in 500 ml cultures. The other dry weight values were calculated by comparison of cell densities reached in 20 ml culture tubes. The conversion factor used was 0.1 OD_{600} ≙ 25.0 mg dry cell matter per 1

[b] Assimilated resorcylic compounds for cell material were calculated by the equation:

$17\ C_6H_6O_2 + 2\ HCO_3^- + 38\ H_2O + 2\ H^+ \rightarrow 26\ \langle C_4H_7O_3\rangle$;

thus 6.34 μmol resorcinol compound is required to form 1 mg of cell dry matter. All figures are means of at least five independent assays

[c] Carbon recovery as acetate + butyrate; other products could not be identified

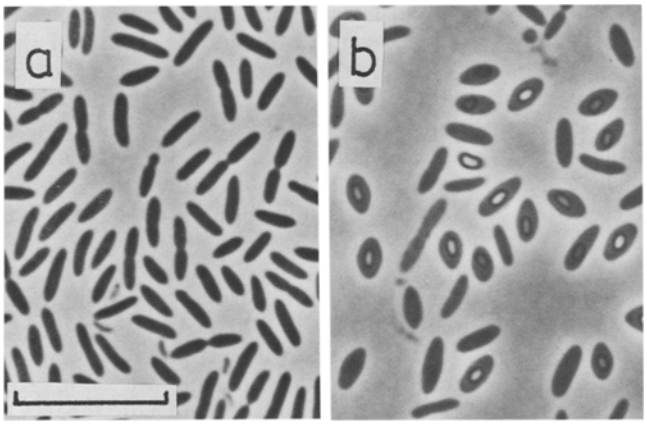

Fig. 5a, b. Phase contrast photomicrograph of syntrophic saltwater cultures degrading resorcinol. **a** Strain Ma 266 during exponential growth; **b** sporeforming cells of the same strain in the stationary growth phase. Note vibrioid syntrophic *Desulfovibrio* cells. Bar equals 10 μm for both prints

of hydrogen-oxidizing *Desulfovibrio* lawns. Two strains were obtained, one from a β- and one from a γ-resorcylic acid enrichment culture. Both were smaller in size than the freshwater resorcinol isolates, 0.8 – 1.0 × 2.0 – 3.5 μm, and formed central to subterminal ellipsoidal spores (Fig. 5a, b). The Gram reaction was positive. Motility was observed in young cultures. Resorcinol, β- and γ-resorcylate were the only substrates degraded, and were converted to acetate and sulfide or acetate and methane depending on whether *Desulfovibrio* sp. or *Methanospirillum hungatei* served as syntrophic partner. The bacteria degrading resorcylic compounds depended on syntrophic hydrogen oxidation: no growth was observed with *Desulfovibrio* as partner in the absence of sulfate. Resorcinol was fermented according to the equation:

$$4\ C_6H_4(OH)_2 + HCO_3^- + 13\ H_2O \rightarrow 12\ CH_3COO^- + 11\ H^+ + CH_4.$$

In experiments, 250 μmol resorcinol was fermented to 695 μmol acetate and 56 μmol methane. This corresponds to a 93% recovery of reducing equivalents as fermentation products. The rest was probably assimilated into cell carbon.

No further substrate was used for growth, neither dihydroresorcinol nor any of those substrates tested in the foregoing chapter with the freshwater resorcinol isolates.

The defined mixed culture with *Desulfovibrio* as partner required 0.05% yeast extract to shorten lag phases and doubling times. Growth was optimal at 28° C and pH 7.5; the limits were 20° and 45° C and pH 6.5 and 8.5, respectively. Cultures required a minimum salt concentration of 1% (w/v) NaCl and 0.15% (w/v) $MgCl_2$, but growth rates and yields were higher in complete saltwater medium.

Discussion

Physiology of enrichment and defined cultures

The defined and enrichment cultures described in the present paper had some properties in common, but also differed by some basic characteristics. The bacteria degrading resorcylic compounds were all strictly anaerobic specialists with respect to substrate utilization and did not grow with common substrates such as sugars, organic acids, alcohols, or yeast extract. The bacteria degrading 3,5-dihydroxybenzoate (α-resorcylate) were Gram-negative and substrate degradation apparently depended on syntrophic cooperation with methanogenic or homoacetogenic bacteria. No other resorcinol derivatives were used for growth. Both types of anaerobes obtained with resorcinol 2,4- or 2,6-dihydroxybenzoate (β- or γ-resorcylate) were Gram-positive sporeformers assigned to the genus *Clostridium*. Both did not degrade α-resorcylate. Whereas the marine isolates converted the substrates to acetate and (probably) hydrogen in syntrophic cooperation with hydrogen-scavenging anaerobes, the freshwater isolates fermented the substrates to acetate and butyrate and were not able to participate in interspecies hydrogen transfer.

Nonetheless, the freshwater resorcinol isolates could not be grown in pure culture but depended on the presence of a small spirilloid anaerobe which was identified as a *Campylobacter* sp. The relationship between these two bacteria is not yet understood. Since neither methanogens nor sulfate reducing bacteria could substitute for *Campylobacter* sp. a syntrophic cooperation with hydrogen or formate transfer can be ruled out. *Campylobacter* sp. could not be replaced by a series of nutrients, growth factors, chelating agents, or inorganic salts added, and filter-sterilized *Campylobacter* culture supernatant did not help either to allow growth of the resorcinol-fermenting isolates. Either a very labile growth factor required by the resorcinol fermenter is excreted by the *Campylobacter* cells, or the latter

inactivates a toxic agent present in the medium or produced by the former. For the moment, we have to grow the freshwater resorcinol strains in defined coculture with known *Campylobacter* strains, however, growth is not always easy to reproduce.

Physiology and biochemistry of anaerobic degradation of resorcylic compounds

The pathways of anaerobic degradation of aromatic compounds were studied in the past with benzoate as model substrate. For the anaerobic phototroph *Rhodopseudomonas palustris* a reductive degradation pathway was proposed in which the aromatic ring is saturated to cyclohexane carboxylate prior to ring fission by a reaction analogous to the β-oxidation of fatty acids (Dutton and Evans 1969). Basically the same path was suggested for nitrate-dependent (Williams and Evans 1975) and methanogenic benzoate degradation (Evans 1977). Whereas the former two processes are catalyzed by pure bacterial cultures, methanogenic benzoate degradation depends on syntrophic cooperation of fermenting bacteria and hydrogen-oxidizing methanogens (Ferry and Wolfe 1976) because benzoate fermentation to acetate, hydrogen and carbon dioxide is an endergonic reaction under standard conditions (calculations after Thauer et al. 1977):

$$C_6H_5COO^- + 7\,H_2O \rightarrow 3\,CH_3COO^- + 3\,H^+ + HCO_3^- + 3\,H_2.$$
$$\Delta G'_0 = +70.6\ \text{kJ}.$$

If the hydrogen produced is oxidized by methanogens, the overall reaction becomes exergonic:

$$4\,C_6H_5COO^- + 19\,H_2O \rightarrow 12\,CH_3COO^- + 9\,H^+ + HCO_3^- + 3\,CH_4.$$
$$\Delta G'_0 = -31.1\ \text{kJ per mol benzoate}.$$

Benzoate-fermenting anaerobes were recently isolated in defined mixed culture with hydrogen-oxidizing sulfate reducers and methanogens (Mountfort and Bryant 1982).

Fermentation of hydroxylated benzoates becomes more exergonic with increasing number of hydroxyl substituents:

$$C_6H_4(OH)COO^- + 6\,H_2O \rightarrow 3\,CH_3COO^- + 3\,H^+ + HCO_3^- + 2\,H_2.$$
$$\Delta G'_0 = +6.0\ \text{kJ}.$$

$$C_6H_3(OH)_2COO^- + 5\,H_2O \rightarrow 3\,CH_3COO^- + 3\,H^+ + HCO_3^- + H_2.$$
$$\Delta G'_0 = -77.8\ \text{kJ}.$$

$$C_6H_2(OH)_3COO^- + 4\,H_2O \rightarrow 3\,CH_3COO^- + 3\,H^+ + HCO_3^-.$$
$$\Delta G'_0 = -160.0\ \text{kJ}.$$

Gibb's free energy changes for fermentation of the non-carboxylated hydroxybenzenes differ only negligibly from the respective values given. Thus, monohydroxybenzoate and phenol fermentation to acetate and hydrogen depend on syntrophic product removal, and this was found to be true with phenol-degrading enrichment cultures (Szewzyk et al. 1985). Trihydroxybenzoates such as gallic acids are fermented to acetate by pure cultures (Schink and Pfennig 1982). Although dihydroxybenzoate (and dihydroxybenzenes) could theoretically be fermented to acetate and hydrogen in pure culture, no bacterium catalyzing such a reaction has been isolated so far. The enrichments and defined cultures obtained in the present study either cooperated with hydrogen scavengers or fermented the substrates to acetate and butyrate. The latter reaction is slightly more exergonic ($\Delta G'_0 = -102.3\ \text{kJ} \cdot \text{mol}^{-1}$) than fermentation to acetate and hydrogen (see above), however, the cell can conserve less ATP via acetate kinase. From this point of view, the butyrate-forming resorcinol degraders are even in a disadvantageous situation compared to the other bacteria since they cannot participate in interspecies hydrogen transfer in their natural environment which is probably as low in dissolved hydrogen as any other anoxic sediment is.

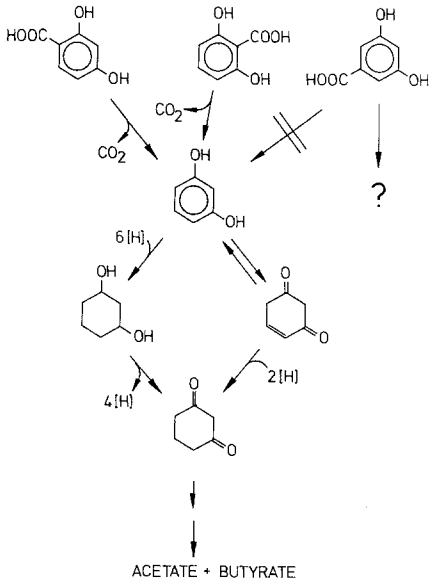

Fig. 6. Proposed pathway of anaerobic degradation of resorcinol and β- and γ-resorcylate. β-Resorcylate (*upper left*) and γ-resorcylate (*upper mid*) are decarboxylated to resorcinol, α-resorcylate (*upper right*) takes a different, so far unknown pathway. Further explanations are given in the text

The degradation pathways of resorcinol and the three resorcinol carboxylates deserve some discussion. Obviously, α-resorcylate is degraded by bacteria different from those degrading the two other resorcylates and resorcinol. In β- and γ-resorcylate, the carboxylic group is comparably labile and both can be decarboxylated chemically by boiling in aqueous solution (Deutsche Chemische Gesellschaft 1932). The carboxylic group in α-resorcylate, on the other hand, is bound very strongly, and stands heating to 300°C for several hours. This may explain why the latter compound takes a path of degradation different from resorcinol and those resorcylates which can be easily transformed to resorcinol. Decarboxylation of β- and γ-resorcylate to resorcinol could be demonstrated in dense cell suspensions. It appears justified, therefore, to assume that β- and γ-resorcylate are degraded via resorcinol, as this was assumed to occur in a similar manner during degradation of carboxylated trihydroxybenzenes (Schink and Pfennig 1982). The further degradation path does not necessarily require complete saturation to cyclohexanediol as the "classical" theory postulates (Evans 1977; Young 1984; see Fig. 6, lower part left). The two hydroxyl functions influence the π-electron system sufficiently to stabilize a cyclohexenedione tautomer (Ruske 1968) which after reduction to cyclohexanedione should be easily accessible to hydrolysis (Fig. 6,

lower part right). It is interesting to note that cyclohexanedione, a possible intermediate of resorcinol-degradation, served as growth substrate for our freshwater resorcinol isolates, although the metabolism of this compound is not yet completely understood. Hydrolytic or thiolytic cleavage to 3-oxocaproate and further to acetate and butyrate or acetate residues alone does not pose basic problems with respect to the degradation mechanism any more.

The cell yields obtained with the freshwater resorcinol strains (about 8 g dry matter per mol of substrate; Table 2) are in the same range as those measured with *Pelobacter acidigallici* grown on gallic acid (10 g per mol; Schink and Pfennig 1982). Both values indicate that about 1 mol ATP per mol of substrate is conserved during substrate degradation (Stouthamer 1979). It has to be assumed, therefore, that not all of the theoretically possible phosphorylation sites are exploited by the bacteria.

Since the two hydroxyl functions of resorcinol are in a relative position to each other which is well suited for thiolytic or hydrolytic cleavage of the ring skeleton to acetate or butyrate residues no reductive dehydroxylation reactions are necessary and do not appear to be involved, either. Reductive removal of hydroxyl groups was recently described in our lab as the primary step in hydroquinone and catechol degradation (Szewzyk et al. 1985).

The degradation of α-resorcylic acid proceeds via a different and so far unknown pathway. Feeding experiments gave no indication of a possible intermediate benzoate or 3-hydroxybenzoate formation, but these results are only of limited value since suited transport systems may be lacking.

Resorcinol and resorcylates are produced in huge amounts by the chemical industry and enter oxic and anoxic habitats. Moreover, resorcinol was shown to be an intermediary product of pyrogallol and gallic acid metabolism by rumen or intestinal microflora (Scheline 1966; Martin 1982). Resorcinol sulfate esters are excreted in trace amounts in the urine of some humans (Curzon and Pratt 1964). Since aromatic compounds with hydroxyl groups in meta-position to each other are not produced by higher plants the question remains open on how the highly specialized organisms described in this study survive in nature.

Acknowledgements. The authors are indebted to Prof. Dr. N. Pfennig for support and stimulating discussions, and to Regine Szewzyk for helpful discussions and enrichment cultures with resorcinol. Electron microscopy by Waltraud Dilling is gratefully acknowledged. This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

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Received February 8, 1985/Accepted June 25, 1985