Fermentative degradation of glycolic acid by defined syntrophic cocultures

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Abstract. Three different defined cocultures of glycolate-degrading strictly anaerobic bacteria were isolated from enrichment cultures inoculated with freshwater sediment samples. Each culture contained a primary fermenting bacterium which used only glycolate as growth substrate. These cells were gram-positive, formed terminal oval spores, and did not contain cytochromes. Growth with glycolate was possible only in coculture with either a homoacetogenic bacterium or a hydrogen-utilizing methanogenic bacterium; the overall fermentation balance was either $4 \text{ glycolate} \rightarrow 3 \text{ acetate} + 2\text{CO}_2$, or $4 \text{ glycolate} \rightarrow 3 \text{ CH}_4 + 5 \text{ CO}_2$. These transformations indicate that glycolate was converted by the primary fermenting bacterium entirely to CO_2 and reducing equivalents which were transferred to the partner organisms, probably through interspecies hydrogen transfer. The key enzymes of fermentative glycolate degradation were identified in cell-free extracts. An acetyl-CoA and ADP-dependent glyoxylate-converting enzyme activity, malic enzyme, pyruvate synthase, and methyl viologen-dependent hydrogenase were found at comparably high activities suggesting that these bacteria oxidize glycolate through a new pathway via malyl-CoA, and that ATP is synthesized by substrate-level phosphorylation, in a similar manner as found in a recently isolated glyoxylate-fermenting anaerobe.

Key words: Interspecies hydrogen transfer — Methanogenesis — Homoacetogenesis — Malic enzyme — Substrate level phosphorylation

Glycolate is an important constituent of unripe grapes, sugar beets, or sugar cane (Karrer 1958), and can make up to 75–80% of the total acidity of sugar cane juice (Shorey 1899). It is also produced by algae and other aerobic photo- and chemoautotrophs during periods of

CO_2 limitation and oxygen oversaturation as a side product of the ribulose biphosphate carboxylase/oxygenase reaction, and subsequent dephosphorylation (Whittingham and Pritchard 1963; Codd et al. 1976; Beudeker et al. 1981; Beck 1979). Glycolate can also be formed through an oxygenase-dependent hydroxylation of acetate, e.g. for synthesis of glycolyl neuraminic acid (Schauer 1982).

Glycolate production up to 40% of the total CO_2 fixation was observed in laboratory cultures of algae and phytoplankton (Tolbert and Zill 1956; Watt 1966; Fogg et al. 1965; Stewart and Codd 1981) as well as in natural waters. Glycolate accumulates up to $4 \mu\text{M}$ during the diurnal cycle in surface waters of a eutrophic lake (Plußsee, Northern Germany; U. Münster, Plön, personal communication) and up to $0.5 \mu\text{M}$ in marine coastal waters (New York Bight; Edenborn and Litchfield 1987). An unusually short turnover time ($< 1 \text{ h}$) and a high heterotrophic degradative capacity for glycolate indicate that aerobic bacteria actively oxidize this substrate in the same water layers (Edenborn and Litchfield 1987; Wright 1975; Wright and Shah 1975; 1977). In the upper 2 mm layer of a hot spring (55°C) cyanobacterial mat in Yellowstone National Park, up to 7% of the total photosynthetic CO_2 fixation was excreted as glycolate (Bateson and Ward 1988).

Chemically synthesized glycolic acid is used as a cheap organic acid in many technical processes (Windholz et al. 1983). Glycolic acid was also detected as an intermediate of anaerobic chloroacetate degradation (Egli et al. 1989), as a side product during anaerobic treatment of 2-methoxyethanol-containing wastewaters (Tanaka et al. 1986), and as a major byproduct of carboxy methyl cellulose production with chloroacetic acid.

Several aerobic bacteria have been isolated which can feed on glycolate as sole substrate (Kurz and LaRue 1973; Edenborn and Litchfield 1985). The biochemistry of aerobic glycolate degradation appears to be closely related to that of glyoxylate degradation. Oxidation of glycolate leads to glyoxylate, for which three different degradation pathways have been described: the glycerate

pathway via tartronic semialdehyde (Hansen and Hayashi 1962), the β -hydroxyaspartate pathway starting with condensation of glyoxylate and glycine (Kornberg and Morris 1965), and a dicarboxylic acid pathway with malate as key intermediate (Kornberg and Sadler 1960).

No information existed so far on the microbiology and biochemistry of anaerobic glycolate degradation. The comparably high oxidation state of this compound should make it easily susceptible to fermentative transformations. A ternary mixed culture of fermenting bacteria was recently reported which degrades glycolate in the presence of yeast extract to acetate, propionate, and carbon dioxide (Janssen 1990). The present study was initiated to elucidate how glycolic acid is degraded by strictly anaerobic bacteria in mineral media, and which enzymes are involved in this process.

Materials and methods

Organisms and cultivation

Enrichment cultures were inoculated either with anoxic sewage sludge obtained from the municipal sewage treatment plant at Konstanz, FRG, (enrichment FI), or with samples obtained from freshwater creeks near Konstanz (enrichments As) and Hannover (enrichments Ott), or from marine sediment samples taken at Norddeich, FRG, or in Rio Marin, a canal in the city of Venice, Italy. A sample of the chloroacetate-degrading "methanogenic mixed culture" (Egli et al. 1989) was kindly supplied by Prof. Dr. Th. Leisinger, Zürich, Switzerland.

Methanospirillum hungatei strain SK was obtained from Prof. Dr. F. Widdel, München, FRG. *Acetobacterium woodii* (DSM 1030) and *Desulfovibrio vulgaris* strain Marburg (DSM 2119) were from our own strain collection.

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, isolation, and cultivation contained 30 mM sodium bicarbonate buffer, 1 mM sodium sulfide as reducing agent, the trace element solution SL 10 (Widdel et al. 1983), selenite-tungstate solution (Tschech and Pfennig 1984) and a 7 vitamin solution (Pfennig 1978). In some experiments, the selenite concentration was enhanced ten-fold. Freshwater medium contained 0.5 g NaCl and 0.4 g $\text{MgCl}_2 \times 6 \text{H}_2\text{O}$, saltwater medium 20.0 g and 3.0 g, respectively, per liter. The pH was 7.1–7.3, and the incubation temperature 28–30°C. *Methanospirillum hungatei* strain SK was grown in the same medium (ca. 50 ml) containing 5 mM sodium acetate in 120 ml serum bottles under an atmosphere of 80% H_2 /20% CO_2 . For isolation of pure cultures, the agar shake dilution method was applied (Pfennig 1978). Gram typing was carried out by staining after Magee et al. (1975) and by the KOI test (Grogersen 1978) with *Acetobacterium woodii* and *Escherichia coli* as reference organisms. Purity of cultures was checked microscopically after growth either in defined mineral medium with glycolate as substrate, or in semisolid complex medium (AC-medium, Difco Co., Ann Arbor, Mich., USA) at normal and at five-fold diluted concentration.

For enzyme assays, the methanogenic coculture FI/GlyM was grown in 125 ml infusion bottles filled with 100 ml mineral medium containing 10 mM glycolate and 5 mM acetate, and gassed with N_2/CO_2 (80/20%). Cells were harvested in the late log phase at 0.22 OD_{578} by centrifugation at 3300 $\times g$ for 20 min in 125 ml infusion bottles under N_2/CO_2 at 10°C in a Sorvall centrifuge rotor equipped with rubber adapters.

Preparation of cell-free extracts

All manipulations of cell material were performed under a nitrogen atmosphere, preventing any access of air. Cells of 100 ml culture medium were washed in 50 ml 125 mM triethanolamine buffer, pH 7.4, containing 2.5 mM dithioerythritol, and were resuspended in 2 ml of the same buffer. Cells were broken by 4–5 runs through an anoxic French press at 140 MPa pressure. The cell homogenate was centrifuged in oxygen-free vials for 20 min at 5000 $\times g$ to remove intact cells.

Cytochromes were checked for in cell extracts of a 1-l culture by redox difference spectroscopy (dithionite-reduced minus air-oxidized) in a Kontron Uvicon spectrophotometer (Kontron, Zürich, Switzerland).

Enzyme assays

All enzyme assays were carried out with a Model 100-40 variable wavelength spectrophotometer (Hitachi, Tokyo, Japan) at 22°C in rubber-stoppered cuvettes under nitrogen atmosphere. Buffers and stock solutions were kept anoxic prior to addition by hypodermic syringes.

Carbon monoxide dehydrogenase was assayed with methyl viologen as electron acceptor (Diekert and Thauer 1978).

Malyl-CoA lyase (EC 4.1.3.24)/malate:CoA ligase (EC 6.2.1.9) activities were determined as a reaction chain:

(i) by coupling the conversion of acetyl-CoA + glyoxylate to L-malate via malyl-CoA with malyl-CoA lyase, malate:CoA ligase and malic enzyme reactions. The cell extract contained all three enzyme activities and the activity of both, malyl-CoA lyase and malate:CoA ligase could be followed as formation of NADPH from NADP^+ at 365 nm wavelength (malic enzyme reaction). The assay mixture contained: potassium phosphate buffer, pH 7.5, 100 mM; DTE, 2.0 mM; MgCl_2 , 10 mM; NADP^+ , 1 mM; Na_2ADP , 5 mM and Na-glyoxylate, 15 mM. Li_3 -acetyl-CoA, 0.5 mM, was added to start the reaction.

(ii) by coupling the conversion of L-malate to acetyl-CoA and glyoxylate via malyl-CoA with malate:CoA ligase and malyl-CoA lyase (Tuboi and Kikuchi 1965), and monitoring phenylhydrazine formation at 324 nm wavelength. The modified assay mixture contained: Tris/glycine, pH 7.4, 140 mM; DTE, 2 mM; MgCl_2 , 10 mM; Li_3 -CoA, 0.63 mM; Na_2ATP , 5 mM and phenylhydrazine/HCl, pH 6.0, 3 mM. The reaction was started with Na-L-malate, 25 mM.

Malic enzyme (EC 1.1.1.40) activity was determined following formation of NADPH from NADP^+ at 365 nm wavelength upon addition of malate (modified after Stams et al. 1984). The assay mixture contained: Tris/glycine, pH 7.4; 150 mM; MgCl_2 , 12.5 mM; MnCl_2 , 12.5 mM; NADP^+ , 2 mM; the reaction was started with Na-L-malate, 15 mM. To avoid precipitation of Mg- or Mn-phosphates the cell extract was prepared with triethanolamine buffer, pH 7.4, 125 mM.

Pyruvate synthase (EC 1.2.7.1.) was measured following benzyl viologen reduction with pyruvate ($\epsilon_{578} = 8.65 \text{ mM}^{-1} \text{ cm}^{-1}$) according to Odom and Peck (1981).

Hydrogenase (EC 1.12.1.2) activity was analyzed following the reduction of benzyl viologen or methyl viologen ($\epsilon_{578} = 9.7 \text{ mM}^{-1} \text{ cm}^{-1}$) with hydrogen (Diekert and Thauer 1978).

Chemical analyses

Alcohols and short-chain fatty acids were identified and quantified by gas chromatography (Dehning et al. 1989). Glycolate was identified and quantified with a System Gold high-pressure liquid chromatograph (Beckman, München, FRG) equipped with a LiChrospher 100 RP-18 encapsulated column (4 by 250 mm; Merck, Darmstadt, FRG) connected to an Ultrasphere-ODS column (4.6 by 150 mm; Beckman Instruments, Mervue, Galway, Ireland) with

4.5 mM potassium buffer (pH 2.5). Samples of culture supernatant (20 µl) were acidified with phosphoric acid (final concentration 100 mM), injected with a Spark Promise II autosampler (Spark Holland BV, Emmen, Netherlands) and eluted at a flow rate of 1 ml/min. Peak elution was followed with a Beckman 167 detector at 205 nm wavelength. Chromatograms were analyzed by a computer program and the amount of glycolate in samples was measured by comparison with external standards of known concentration.

Protein was quantified by a micro-protein assay after Bradford (1976) with bovine serum albumin as standard.

Chemicals

All chemicals used were of analytical grade quality and obtained from Fluka, Neu-Ulm, and Merck, Darmstadt, FRG. Biochemicals were obtained from Boehringer, Mannheim, and from Sigma, München, FRG. N₂ and N₂/CO₂ gas mixtures were obtained from Messer-Griesheim, Ludwigshafen, FRG.

Results

Enrichment cultures and isolations

Enrichment cultures were started in 120-ml serum bottles containing 50 ml mineral medium with 10 mM glycolate as sole carbon and energy source and sodium selenite at normal (10^{-8} M) or at tenfold increased (10^{-7} M) concentration. These cultures were inoculated with about 5 ml of sludge or sediment samples from freshwater and marine origin. Gas production was observed only after incubation for more than 8 weeks and resumed in subcultures not before another 3 weeks. Subcultures were inoculated with 5 ml preculture, and care was taken to transfer also part of the sedimentary material. Also after repeated transfers, the subcultures needed at least 1–2 weeks to start growth again. These lag phases could be shortened with the freshwater enrichments by frequent transfers. The marine cultures continued to grow extremely slowly and were discarded after more than 1 year of cultivation.

Different bacterial populations developed in the enrichments. Coccoid cells and thin rods prevailed in the marine enrichment cultures, whereas only rod-shaped cells were observed in the enrichment cultures obtained from freshwater sediment (OttGly and AsGly) and from sewage sludge (FIGly). Gas production ceased in these cultures, and acetate and traces of ethanol were found as fermentation products. There was no difference in growth rate or population composition between cultures with normal or with enhanced selenite concentration. After more than 10 transfers, basically two types of cells dominated at about even amounts in these cultures, a thin, slightly curved rod sometimes forming short cell chains, and a thicker rod with slightly pointed ends. All efforts to separate the two different cell types in agar shake dilution series with glycolate as substrate failed. Since the cell shape of the latter bacterial type reminded of *Acetobacterium* sp., a series of agar shakes was run with 10 mM ethylene glycol, a substrate typically used by these homoacetogenic bacteria (Schink and Bomar 1991). After two subsequent agar shake dilution series, strain OttEG1 was isolated in pure culture. This bacterium was further

cultivated in pure culture with ethylene glycol as substrate. It did not grow with glycolate, and was therefore used as partner organism to isolate the primary glycolate-fermenting bacteria. Strain OttEG1 was inoculated as a background lawn of about 10^6 cells per ml in further agar shakes in which glycolate was provided as substrate. Two to three subsequent dilution series of this kind allowed finally the isolation of three different defined cocultures of glycolate-degrading bacteria, cultures FIGly1, OttGly1, and AsGly1. These three cultures from different origins were morphologically identical (Fig. 1a) and behaved identical also in the further physiological characterization.

Characterization of defined cultures

Cells of strain OttEG1 were straight rods with slightly pointed ends (Fig. 1a), 1.0×1.5 – 2.5 µm in size, and stained gram-positive. Spores were never observed. This strain grew with various substrates including ethylene glycol, formate, H₂/CO₂, trimethoxybenzoate, sinapinate, betain, fructose, glycerol, pyruvate, and lactate. All these substrates were fermented stoichiometrically to acetate as sole product; betain and the methoxyl aromatics were demethylated (data not shown). No growth was observed with any of the following substrates: Glyoxylate, glycolate, oxalate, malonate, glycerate, glycine, methanol, ethanol, acetoin, 2,3-butanediol, malate, aspartate, fumarate, glucose, xylose, arabinose, erythritol, nitrilotriacetate (NTA), ethylene diamine tetraacetate (EDTA), malt extract, casamino acids, or yeast extract. The guanine-plus-cytosine content of the DNA was 42.9 ± 1.0 mol %. The pattern of utilized substrates, formation of acetate as primary fermentation product, and presence of carbon monoxide dehydrogenase at high activity in cell extracts (2.4 µmol CO oxidized per min and mg protein) all indicated that this bacterium was a homoacetogenic bacterium.

The second bacterium appearing in the defined cocultures FIGly1, OttGly1, and AsGly1 was a thin, slightly curved rod, 0.5×2.5 – 3.5 µm in size (Fig. 1a). Cells stained gram-negative but did not form slime in the KOH test as this is typical of gram-negative bacteria. Electron microscopic examination of ultrathin sections revealed a typical cell wall structure of gram-positive bacteria. Oval spores were formed in ageing cultures at the cell ends (Fig. 1b). This bacterium could be grown only with glycolate and only in the presence of a partner organism such as strain OttEG1 with which it cooperated in a syntrophic manner. This hypothesis was further substantiated by exchange of the partner organism: a well-sporulated, aged culture of strain FIGly1 was pasteurized (10 min at 80°C) and then transferred into cultures of *Acetobacterium woodii* or *Methanospirillum hungatei*, in the latter case in the presence of 2 mM acetate for assimilatory metabolism. Also these new defined cocultures grew with glycolate as substrate. Cocultures with the sulfate reducer *Desulfovibrio vulgaris* showed no significant growth. All attempts to grow the glycolate-degrading bacterium in pure culture by transfer of pasteurized

Fig. 1. Phase-contrast photomicrographs of cocultures (a) FIGly1; (b) FIGly1 forming endospores; arrow indicates beginning spore-formation; (c) FIGlyM. Bar equals 10 μm of all panels

Table 1. Stoichiometry of fermentation and growth yields of cocultures FIGly1 and FIGlyM

Strain	Glycolate concentration (mM)	Glycolate degraded (μmol)	Optical density reached ΔE_{500}	Cell dry mass formed ^a (mg)	Acetate assimilated ^b (μmol)	Products formed (μmol)		Electron recovery (%)	Molar growth yield (g/mol)
						acetate	CH_4		
FIGly1 ^c	10	220	0.27	1.33	27	142	—	102	6.0
FIGly1	20	440	0.43	1.97	41	306	—	105	4.5
FIGlyM ^d	10	1059	0.19	3.91	80	—	811	102	3.7

^a Cell dry matter values were calculated via cell density using an experimentally determined conversion factor (FIGly1: 0.1 $\text{OD}_{500} = 20.8$ mg dry matter/l; FIGlyM: 0.1 $\text{OD}_{500} = 19.4$ mg dry matter/l)

^b Acetate assimilated was calculated according to the formula: $17 \text{ C}_2\text{H}_3\text{O}_2^- \rightarrow 8 \text{ C}_4\text{H}_7\text{O}_3 + 2 \text{ HCO}_3^- + 15 \text{ OH}^-$

^c Growth experiments with strain FIGly1 were carried out in 22-ml screw-cap tubes

^d Growth experiments with strain FIGlyM were carried out in 125-ml infusion flasks filled with 100 ml freshwater medium, 5 mM Na-acetate, under an N_2/CO_2 (90%/10%) atmosphere

spore preparations into various complex media with or without glycolate or glyoxylate failed. All isolated cocultures were strictly anaerobic, and were inhibited by traces of oxygen in semisolid gradient media. Neither nitrate, nor sulfate, thiosulfate, or elemental sulfur was reduced. Optimal growth of the defined cocultures was observed at 30°C (limits 12 and 37°C) and pH 7.0 (limits pH 6.0 and 7.8) in freshwater medium. Addition of traces of sodium dithionite ($\leq 50 \mu\text{M}$) helped transferred cultures resume growth. Enhanced salt concentrations impaired growth considerably, and no growth was possible in saltwater medium. Phosphate did not inhibit growth up to 50 mM concentration.

The stoichiometry of glycolate degradation by the coculture FIGly1 and the methanogenic cocultures FIGlyM derived from it (by cocultivation of the glycolate fermenter with *M. hungatei*; Fig. 1c) is documented in Table 1. Fermentation led to acetate as only organic product in the homoacetogenic coculture, or to only methane, respectively. The cell yield in the homoacetogenic cocultures was in all cases in the range of 4.5–6.0 g per mol glycolate. The kinetics of glycolate fermentation to acetate by the homoacetogenic coculture FIGly1 are documented in Fig. 2. The growth rate under optimal conditions was 0.058 h^{-1} ($t_d = 12 \text{ h}$). The coculture

Fig. 2. Fermentation time course of coculture FIGly1 growing with 8.5 mM glycolate. Experiments were performed at 28°C in 120-ml serum bottles filled with 50 ml medium under an N_2/CO_2 atmosphere, and samples were taken with syringes. OD_{546} : optical density at 546 nm wavelength (●), glycolate (□), and acetate (◆)

OttGly1 grew at a similar rate, the coculture AsGly1 only half as fast.

Enzymes involved in glycolate degradation by the glycolate-fermenting partner organism were assayed in crude extracts of the methanogenic coculture FIGlyM. High activities of an acetyl-CoA and ADP-dependent

Table 2. Enzymes involved in glycolate degradation by coculture FIGlyM

Enzyme	EC number	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ protein^{-1})
Malyl-CoA lyase plus Malate:CoA ligase	—	0.24
Malic enzyme (NADP ⁺)	1.1.1.40	0.19–0.26
Pyruvate synthase	1.2.7.1	0.14–0.4 (BV)
Hydrogenase	1.12.1.2	1.24 (MV)

Benzyl viologen (BV) or methyl viologen (MV) as electron donor or acceptor

glyoxylate utilizing enzyme, NADP-dependent malic enzyme, methyl viologen-dependent hydrogenase and benzyl viologen-dependent pyruvate synthase were detected (Table 2). The latter enzyme was observed at low activity ($0.08 \text{ U} \times \text{mg protein}^{-1}$) and methyl viologen-dependent hydrogenase at catabolic activity ($0.66 \text{ U} \times \text{mg protein}^{-1}$) also in cell-free extracts of a pure culture of *Methanospirillum hungatei* strain SK. No glycolate dehydrogenase or glyoxylate reductase activity could be observed, neither with NAD nor with artificial electron carriers such as dichlorophenol indophenol. No cytochromes were detectable in cell-free extracts of either coculture.

The same enzymes detected in our methanogenic coculture FIGlyM were also detected at similar activities in crude extracts of the chloroacetate-degrading "methanogenic mixed culture" (Egli et al 1989).

Discussion

Physiology and biochemistry

Fermentative degradation of glycolate is of interest from an ecological point of view because this compound is isoelectronic with the carbon moieties of nitrilotriacetate and ethylene diamine tetraacetate, two organic complexing agents of major environmental concern (Anderson et al. 1985) for which fermentative degradation has not yet been documented. It is also isoelectronic with glycine which can be fermented through oxidation to CO_2 and concomitant reduction to acetate (Dürre and Andreessen 1982; Dürre et al. 1983; Zindel et al. 1988). Since glycine reductase contains selenium (Stadtman 1980) and glycine reduction to acetate requires enhanced selenite concentrations in the growth medium (Dürre and Andreessen 1982) we started enrichment cultures in the present study with media with normal as well as with enhanced selenite contents. In both enrichment series, similar types of bacteria developed, and also the defined cultures turned out to be independent of selenium additions. Obviously, there was no direct reduction of glycolate to acetate, but dismutation of this substrate to CO_2 and acetate was catalyzed by a syntrophic association of two partner bacteria which cooperated by interspecies hydrogen transfer. This concept of degradation was basi-

Fig. 3. Hypothetical pathway of glycolate degradation by coculture FIGlyM as proposed by our results. Numbers refer to the following enzyme activities measured in cell-free extracts: (1) malyl-CoA lyase, (2) malate:CoA ligase, (3) malic enzyme, (4) pyruvate synthase. Abbreviations: $\text{Fd}_{\text{ox/red}}$, oxidized/reduced ferredoxin. X stands for a so far unknown electron acceptor

cally different from what we expected, and was different as well from that exhibited by a recently published ternary mixed culture of fermenting bacteria which forms acetate and propionate (Janssen 1990). Various pathways could be taken to oxidize glycolate to CO_2 . Syntrophic oxidation could proceed via glyoxylate, oxalate, and formate, or, after decarboxylation, via formaldehyde and formate to CO_2 . These ideas were followed in enzyme assays for the respective key enzymes, as well as for those enzymes characteristic of the glycerate pathway, the β -hydroxyaspartate pathway, and the malate pathway, but no significant activity of any of these enzymes could be detected in cell-free extracts of our methanogenic coculture FIGlyM. The same was true for a recently isolated, glyoxylate-fermenting bacterium (Friedrich and Schink 1991). Rather, we found in both cultures significant activities of a reaction chain probably involving malyl-CoA lyase and malate:CoA ligase, as well as NADP-dependent malic enzyme and pyruvate synthase indicating that glycolate is first oxidized to glyoxylate and then condensed with acetyl-CoA to yield malyl-CoA. The latter is converted to malate in an ADP-dependent reaction indicating that this step is used for substrate-level phosphorylation; the reverse reaction was found to be ATP-dependent. Oxidative decarboxylation of malate by malic enzyme and further oxidative decarboxylation of pyruvate by pyruvate synthase regenerates the acetyl-CoA necessary for a further reaction cycle (Fig. 3). This pathway differs from the "dicarboxylic acid cycle" with malate as key intermediate, which is used by aerobic *Pseudomonas* strains (Kornberg and Sadler 1960) in that the free energy of the acetyl-CoA ester linkage is conserved and is exploited later by phosphorylation of ADP. Obviously, this pathway is better suited for a highly

specialized, strictly anaerobic bacterium that has to use every fraction of energy available in a reaction sequence.

Energetics

Conversion of glycolate to CO_2 and H_2 is an endergonic reaction (calculations after Thauer et al. 1977):

This reaction cannot support growth of a bacterium, and the same would be true if formate rather than hydrogen would be the reduced electron carrier. The reaction becomes exergonic only if one of the reaction products is removed and kept at a low concentration, e.g. by coupling to hydrogen-dependent homoacetogenesis or methanogenesis:

It is surprising that in our original enrichment cultures a homoacetogenic rather than a methanogenic association established since methanogenic bacteria usually out-compete homoacetogens for hydrogen (Seitz et al. 1990; Schink 1991). Need for acetate as a substrate for cell matter synthesis, and toxic effects of glycolate on certain methanogenic bacteria may be reasons for this unexpected result.

Glycolate oxidation to CO_2 through the discussed pathway (Fig. 3), releases electrons in three reactions, the oxidation of glycolate to glyoxylate ($E_0' = -92$ mV), the oxidation of malate to pyruvate and CO_2 ($E_0' = -331$ mV), and the oxidation of pyruvate + CoASH to acetyl-CoA + CO_2 ($E_0' = -470$ mV). Redox potentials of -92 mV or of -331 mV are in equilibrium with hydrogen partial pressures of 10^{-11} bar or 10^{-3} bar, respectively (Schink 1991). Hydrogen-dependent acetate formation and methane formation reach thermodynamic equilibria at $10^{-4.5}$ bar and $10^{-5.8}$ bar, respectively, and stop hydrogen utilization at thresholds in the range of 10^{-3} – 10^{-4} bar (Stieb and Schink 1987; Cord-Ruwisch et al. 1988; Conrad and Wetter 1990). These calculations make clear that the electrons of pyruvate and malate oxidation can easily be released as molecular hydrogen in homoacetogenic or methanogenic cocultures, but that neither partner organism can maintain a hydrogen partial pressure low enough to allow direct proton reduction with the electrons released in glycolate oxidation to glyoxylate. It has to be postulated, therefore, that part of the energy conserved as 1 ATP through substrate level phosphorylation (see above) has to be reinvested by the glycolate-fermenting bacterium to drive a reversed electron transport to a low-potential electron carrier. Such energy-dependent potential shifts have been postulated also for syntrophic butyrate or propionate oxidation (Thauer and Morris 1984; Schink 1991), but have so far not been proven experimentally. Our failure to detect a glycolate-oxidizing enzyme in extracts of our cultures

FIGly1 or FIGlyM indicates that this reaction is not catalyzed by a simple dehydrogenase enzyme.

Taxonomy

The glycolate-oxidizing partners in the described cocultures are extremely specialized only on oxidation of this compound and do not grow with any other substrates. They definitively represent new, so far undescribed taxonomic entities. Their final taxonomic position will be defined after more data on their relatedness to other bacteria have been obtained. As gram-positive obligately anaerobic sporeformers, they should be grouped with the genus *Clostridium* (Sneath et al. 1986). However, a further obligately syntrophic bacterium has been transferred recently from this genus to a new genus *Syntrophospora* (Zhao et al. 1990) comprising such highly specialized obligately syntrophic sporeformers. This genus may be a better affiliation for our syntrophic glycolate oxidizers when direct comparisons of nucleic acid structures have been made.

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