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Fermentative degradation of glyoxylate by a new strictly anaerobic bacterium

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Abstract. A new strictly anaerobic, gram-negative, non-sporeforming bacterium, Strain PerGlx1, was enriched and isolated from marine sediment samples with glyoxylate as sole carbon and energy source. The guanine-plus-cytosine content of the DNA was 44.1 ± 0.2 mol %. Glyoxylate was utilized as the only substrate and was stoichiometrically degraded to carbon dioxide, hydrogen, and glycolate. An acetyl-CoA and ADP-dependent glyoxylate converting enzyme activity, malic enzyme, and pyruvate synthase were found at activities sufficient for growth (≥ 0.25 U × mg protein^{-1}). These findings allow to design a new degradation pathway for glyoxylate: glyoxylate is condensed with acetyl-CoA to form malyl-CoA; the free energy of the thioester linkage in malyl-CoA is conserved by substrate level phosphorylation. Part of the electrons released during glyoxylate oxidation to CO_2 reduce a small fraction of glyoxylate to glycolate.

Key words: Glyoxylate – Anaerobic degradation – Malic enzyme – Substrate level phosphorylation

Glyoxylic acid is an important monocarboxylic acid found in unripe fruits as well as in green leaves of many plants, e.g. apple, plum, rhubarb, currant, gooseberry, and wine (Brunner and Chuard 1886) and in young sugar beets (von Lippmann 1894). Furthermore, glyoxylate is excreted by certain algal species (Stewart and Codd 1981). As intermediate, glyoxylate is involved in various metabolic pathways: e.g. in purine degradation by *Pseudomonas aeruginosa* and *P. acidovorans*, in the serine-isocitrate-lyase pathway of many methylotrophic microorganisms, and the glyoxylate cycle in aerobic and nitrate-reducing bacteria (Gottschalk 1986).

Metabolism of glyoxylate under aerobic conditions is well documented, and several different pathways are known: the glycerate pathway (Hansen and Hayashi 1962), a dicarboxylic acid pathway with malate as key intermediate (Kornberg and Sadler 1960), and the β-hydroxyaspartate pathway (Kornberg and Morris 1965). Glyoxylate degradation under strictly anaerobic conditions has not yet been studied. This paper reports for the first time on fermentative degradation of glyoxylate by a new strictly anaerobic bacterium to carbon dioxide, hydrogen, and glycolate. All enzymes involved in glyoxylate degradation were measured, and a catabolic pathway is proposed.

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Materials and methods

Source of organisms

Strain PerGlx1 was isolated from an enrichment culture inoculated with anoxic black marine sediment of Rio della Pergola, a canal located in the city of Venice, Italy.

Methanospirillum hungatei strain SK was obtained from Prof. Dr. F. Widdel, München, FRG.

Media and growth conditions

All procedures for cultivation and isolation were as previously described (Widdel and Pfennig 1981; Schink and Pfennig 1982; Schink 1984). The mineral medium for enrichment, isolation and cultivation contained 30 mM sodium bicarbonate as buffer, 1 mM sodium sulfide as reducing agent, 20 nM selenite and tungstate, the trace element solution SL 10 (Widdel et al. 1983), and 7-vitamin solution (Widdel and Pfennig 1984). The pH was adjusted to 7.2–7.4. Freshwater medium contained 0.5 g NaCl and 0.4 g $MgCl_2 \times 6\ H_2O$, saltwater medium 20 g NaCl and 3 g $MgCl_2 \times 6\ H_2O$, respectively, per liter. Growth experiments were carried out at 28° C. Growth was determined by turbidity measurement at 500 nm wavelength either in a Spectronic 20 Spectrophotometer (Bausch & Lomb, Rochester, NY, USA) or in a Hitachi 100-40 spectrophotometer (Hitachi, Tokyo, Japan).

Methanospirillum hungatei strain SK was grown in freshwater medium containing 5 mM sodium acetate in 120-ml serum flasks filled with 50 ml medium under an atmosphere of 80% H_2/20% CO_2.

Isolation and characterization

For isolation of pure cultures, the agar shake dilution method was applied (Pfennig 1978). Culture purity was checked microscopically after growth in defined medium and after growth tests in complex media containing yeast extract, fumarate, pyruvate, and glucose. Gram staining was carried out according to Bartholomew (1962) with *Acetobacterium woodii* and *Escherichia coli* as controls. In addition, the Gram type was checked by the KOH method (Gregersen 1978).

Detection of cytochromes was carried out as described previously (Dörner and Schink 1990). The guanine-plus-cytosine content was determined by the thermal denaturation method (DeLey 1970, Marmur 1961).

Chemical analyses

Glyoxylate was determined colorimetrically by a colour reaction with dinitrophenylhydrazine (Yamada and Jakoby 1958). Hydrogen was assayed by gas chromatography as described earlier (Matthies et al. 1989). Glycolate was analyzed with a Beckman System Gold high-pressure liquid chromatograph (München, FRG) equipped with a LiChrospher 100 RP-18 encapped 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 phosphate 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, The 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.

Formation of nitrite from nitrate was assayed by azo dye formation with sulfanilic acid and α-naphthylamine (Procházková 1959). Sulfide formation from sulfur or sulfate was analyzed qualitatively by a rapid detection test (Cord-Ruwisch 1985). Protein was determined after Bradford (1976) with bovine serum albumine as standard.

Enzyme measurements

Cells were grown in 100 ml freshwater mineral medium with Na-glyoxylate added successively in 10 mM steps up to a final concentration of 50 mM glyoxylate.

Cell suspensions were harvested in the late logarithmic growth phase by centrifugation at 3,300 × *g* for 20 min, washed, and resuspended in 125 mM potassium phosphate buffer (pH 7.0) prereduced with 2.5 mM dithioerythritol. Crude extracts were prepared anoxically by French Press treatment at 140 MPa. After removal of cell debris at 5,000 × *g* for 20 min, the crude extract was stored on ice.

Cuvettes (1.5 ml; d = 1 cm) sealed with rubber stoppers and gassed with N_2 were employed to determine enzyme activities spectrophotometrically in a Hitachi 100-40 spectrophotometer (Hitachi, Tokyo, Japan) at 25° C. All buffers and stock solutions were anoxic. Assay mixtures contained cell extract with 20–120 µg protein. Dependence of reaction rates on protein contents was checked in all cases. Enzyme nomenclature and EC-numbers are based on the International Union of Biochemistry (1984).

Glyoxylate reductase (EC 1.1.1.26) was measured following NADH oxidation at 365 nm wavelength. The assay was modified after Zeltich (1953) and contained potassium phosphate buffer, pH 7.0, 120 mM; NADH, 0.3 mM. The reaction was started with Na-glyoxylate, 2.5 mM.

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. 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 phenylhydrazone 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, 4 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. The assay was modified after Stams et al. (1984), and 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 reduction of benzyl viologen with pyruvate ($\varepsilon_{578} = 8.65\ mM^{-1}\ cm^{-1}$) according to Odom and Peck (1981).

Formate:ferredoxin oxidoreductase was assayed following the reduction of benzyl viologen or methyl viologen ($\varepsilon_{578} = 9.7\ mM^{-1}\ cm^{-1}$) with formate (Spormann and Thauer 1988).

Hydrogenase (EC 1.12.1.2) activity was analyzed following the reduction of benzyl viologen or methyl viologen with hydrogen (after Diekert and Thauer 1978).

NAD^+/$NADP^+$:ferredoxin oxidoreductase was monitored following the oxidation of benzyl viologen or methyl viologen at 578 nm wavelength with NAD^+/$NADP^+$. The assay mixture contained: potassium phosphate buffer, pH 7.5, 100 mM; benzyl or methyl viologen, 5 mM, and traces of $Na_2S_2O_4$. The reaction was started with NAD^+/$NADP^+$, 0.5 mM.

The reverse reaction was assayed as reduction of benzyl or methyl viologen with NADH/NADPH. The reaction was started with NADH/NADPH, 0.15 mM.

Chemicals

All chemicals were of analytical or reagent grade quality and were obtained from Merck, Darmstadt, Sigma, Deisenhofen, and Fluka, Neu-Ulm, FRG. Gases were obtained from Messer-Griesheim, Darmstadt, FRG.

Results

Enrichments and isolation

Enrichment cultures with freshwater or saltwater medium containing 2 mM glyoxylate as substrate were inoculated with 5 ml sediment and sewage sludge from various sources. Gas production and turbidity formation started after 4 weeks in an enrichment culture with saltwater medium and anoxic sediment from Rio della Pergola. Subcultures inoculated with 5 ml culture fluid tolerated glyoxylate concentrations up to 15 mM. After 4 to 5 transfers, mainly a thin, long rod was observed.

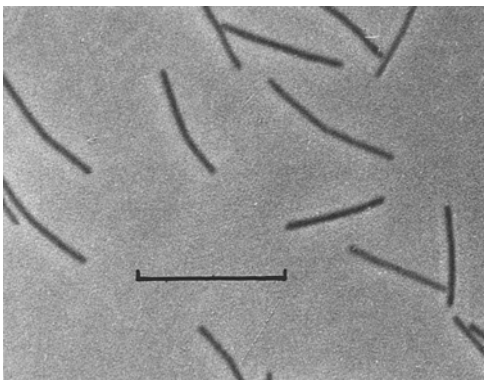

Fig. 1. Phase-contrast photomicrograph of strain PerGlx1. *Bar* equals 10 µm

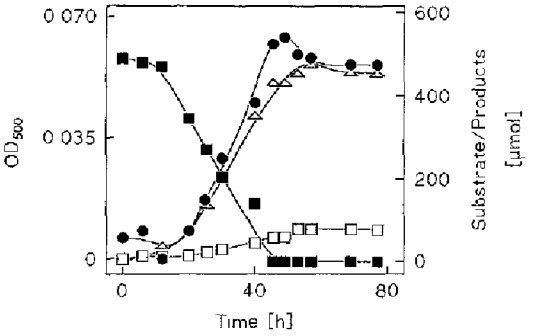

Fig. 2. Fermentation time course of strain PerGlx1 growing with 10 mM glyoxylate. Experiments were performed at 28°C in 120 ml serum bottles filled with 50 ml medium under an N_2/CO_2 atmosphere, and samples were taken with syringes. OD_{500}: optical density at 500 nm wavelength (●), glyoxylate (■), products: glycolate (□), and hydrogen (△)

A pure culture of this bacterium (strain PerGlx1) was obtained by two subsequent agar shake dilution series with 5 mM glyoxylate as substrate. Enrichment cultures with freshwater medium inoculated with sewage sludge from a municipal wastewater plant in Tübingen, FRG, started growing after 6 weeks, however, there was no clearly prevailing bacterium in this culture and growth was very slow.

Morphological and cytological properties

Cells of strain PerGlx1 were non-motile, long, thin rods, $0.5 \times 7-11$ µm in size (Fig. 1). Gram staining as well as the KOH test indicated a gram-negative cell wall architecture. Spore formation was never observed, not even after 6 months of starvation. The guanosine-plus-cytosine content of the DNA was 44.1 ± 0.2 mol %. No cytochromes could be detected by redox difference spectroscopy of crude cell extracts.

Physiology, growth yield, and fermentation balance

Strain PerGlx1 grew well in mineral medium containing NaCl from 30 to 370 mM and $MgCl_2$ from 1.9 to 15 mM; growth was possible up to 700 mM NaCl and 30 mM $MgCl_2$. Phosphate inhibited growth at concentrations > 20 mM. The temperature limits of growth were 12 and 35°C. Optimal growth was observed at 32°C. The pH range of growth was 6.7–8.3, with an optimum of pH 7.7. Strain PerGlx1 grew at a maximum growth rate of $\mu = 0.028\ h^{-1}$ ($t_d = 25$ h) (Fig. 2). Addition of traces of dithionite (≤ 50 µM) helped subcultures to resume growth. Glyoxylate was the only substrate utilized; yeast extract was not required for growth and did not enhance the growth yield in combination with glyoxylate (Table 1). Neither inorganic electron acceptors (nitrate, sulfate, sulfite, thiosulfate, elemental sulfur) nor fumarate was reduced during glyoxylate oxidation. Glyoxylate was fermented completely to CO_2, hydrogen, and glycolate (Table 2); CO_2 was not determined. Cell matter formation was proportional to provided substrate up to 30 mM glyoxylate (Table 2).

Table 1. Substrates tested for growth with strain PerGlx1; concentration of substrates added was 10 mM, unless indicated otherwise (in parentheses)

Compound utilized:
glyoxylate
Compounds not utilized:
formate, acetate, propionate, glycolate, DL-lactate, DL-glycerate, thioglycolate
oxalate, malonate, DL-malate, succinate, fumarate, tartrate
methanol, ethanol, glycerol, ethylene glycol
glucose (5), fructose (5), arabinose (5), xylose (5)
formaldehyde (1; 2), glycolaldehyde (2; 5), glyceraldehyde (2; 5), pyruvate (5)
glycine (5), alanine (5)
yeast extract (0.1%)
Compounds not utilized in combination with 10 mM glyoxylate:
acetate (5; 10), glycine (5; 10), pyruvate (5), succinate (5), DL-malate (5), fumarate (10), yeast extract (0.1%)

In coculture experiments with *Methanospirillum hungatei* as hydrogen consuming partner, no shift of the fermentation balance towards less glycolate production could be observed (data not shown). Such cocultures were not able to grow either with glycolate as substrate.

Enzymes involved in glyoxylate degradation

Enzymes were assayed in cell-free extracts of strain PerGlx1. The enzymes detected (Table 3) were present at activities sufficient to account for the dissimilatory turnover of glyoxylate (0.52 µmol · min^{-1} · mg^{-1} protein). Glyoxylate reductase was specific for NADH; NADPH was not oxidized, and hydroxypyruvate was not reduced. Glycolate oxidation was not observed, neither with NAD^+ nor with APAD (acetyl pyrimidine adenine dinucleotide, $E^{\circ\prime} = -248$ mV; Bergmeyer 1974) as electron acceptor, nor with dinitrophenylhydrazine as glyoxylate trap. Instead, a malyl-CoA lyase/malate:CoA ligase enzyme system was detected at significant activity. The forward reaction was ADP-dependent, the backward

Table 2. Stoichiometry of fermentation and growth yields of strain PerGlx1

Glyoxylate concentration (mM)	Glyoxylate degraded (μmol)	Optical density reached E_{500}	Cell dry mass formed[a] (mg)	Substrate assimilated[b] (μmol)	Products formed (μmol)		Electron recovery (%)	Molar growth yield (g/mol)
					glycolate	H_2		
5	231	0.026	0.31	11	54	235	92	1.3
10	512	0.069	0.92	41	140	547	103	1.8
15	751	0.105	1.34	60	156	774	90	1.8
30	1430	0.230	2.8	125	328	1378	90	2.0

Growth experiments were carried out in 120 ml serum flasks filled with 50 ml mineral medium under a N_2/CO_2 (90%/10%) atmosphere

[a] Cell dry matter values were calculated via cell density using an experimentally determined conversion factor (0.1 $OD_{500} = 25$ mg dry matter/l)

[b] Glyoxylate assimilated was calculated according to the formula: $17\ C_2H_2O_3 \rightarrow 4\ \langle C_4H_7O_3 \rangle + 3\ H_2O + 18\ CO_2$

Table 3. Enzymes involved in glyoxylate degradation by strain PerGlx1

Enzyme	EC number	Specific activity (μmol · min^{-1} · mg protein^{-1})
1 Glyoxylate reductase (NAD)	1.1.1.26	0.9–1.9
2 Malyl-CoA lyase plus 3 Malate:CoA ligase	–	0.25
4 Malic enzyme ($NADP^+$)	1.1.1.40	0.7
5 Pyruvate synthase	1.2.7.1	2.2 (BV)
6 Hydrogenase	1.12.1.2	1.7 (BV) 0.7 (MV)
7 NAD^+:ferredoxin oxidoreductase	–	0.17 (BV) 1.1 (MV)
7 NADH:ferredoxin oxidoreductase	–	0.7 (BV)
8 $NADP^+$:ferredoxin oxidoreductase	–	n.d.
8 NADPH:ferredoxin oxidoreductase	–	1.36 (BV)
9 Formate:ferredoxin oxidoreductase	–	0.13 (BV) 0.16 (MV)

Benzyl viologen (BV) or methyl viologen (MV) were used as electron donor or acceptors; n.d. = not determined

reaction, forming acetyl-CoA and glyoxylate from L-malate, was CoASH and ATP-dependent. Malic enzyme activity was $NADP^+$-dependent, but oxaloacetate was not decarboxylated to pyruvate as described for malic enzyme, EC 1.1.1.40.

The following enzymes were checked for, but could not be detected by the methods indicated: malate synthase, EC 4.1.3.2 (Dixon and Kornberg 1959), malate $NAD^+/NADP^+$ oxidoreductase, EC 1.1.1.37/1.1.1.38 (Stams et al. 1984), phosphotransacetylase, EC 2.3.1.8 (Bergmeyer 1974), isocitrate lyase, EC 4.1.3.1 (Dixon and Kornberg 1959), carbonmonoxide dehydrogenase, EC 1.2.99.2 (Diekert and Thauer 1978), D-glycerate $NAD^+/NADP^+$ oxidoreductase, EC 1.1.1.81 (Kohn and Jakoby 1968), formate NAD^+ oxidoreductase, EC 1.2.1.2 (Spormann and Thauer 1988), pyruvate NAD^+ oxidoreductase (Bergmeyer 1974), and oxaloacetate decarboxylase, EC 4.1.1.3 (Dimroth 1981).

Discussion

Physiology and taxonomic status of strain PerGlx1

In the present study, fermentative degradation of glyoxylate is described for the first time. The newly isolated strain PerGlx1 ferments glyoxylate to carbon dioxide, hydrogen, and glycolate, approximately according to the following equation:

$$4\,\text{glyoxylate}^- + 3\,H^+ + 3\,H_2O \rightarrow 6\,CO_2 + 5\,H_2 + \text{glycolate}^- \quad (1)$$

Glyoxylate was found to be the only substrate metabolized by this new strictly anaerobic, gram-negative isolate. Gram type, morphology, lack of spore formation, and the guanine-plus-cytosine content of the DNA (44.1 ± 0.2 mol %) of strain PerGlx1 give first indications to affiliate this highly specialized isolate with the family Bacteroidaceae. Further studies are required to define a new species for this bacterium.

Our isolate was sensitive to phosphate at concentrations higher than 20 mM; enrichment and isolation was therefore possible only in a low-phosphate medium as used here.

Enrichment cultures with anaerobic sewage sludge did not select for a single prevailing bacterium indicating that glyoxylate degradation in these enrichments depended on a complex interaction of various bacteria.

Biochemistry

The pathway of glyoxylate degradation was studied by assays for key enzymes of known glyoxylate degradation pathways. Neither enzymes of the glycerate pathway such as glycerate:$NAD(P)^+$ oxidoreductase, EC 1.1.1.60 (Hansen and Hayashi 1962) nor of the β-hydroxyaspartate pathway or the dicarboxylic acid pathway via malate (e.g. malate synthase, malate $NAD^+/NADP^+$ oxidoreductase, or oxaloacetate decarboxylase; Kornberg and Morris 1965; Kornberg and Sadler 1960) were

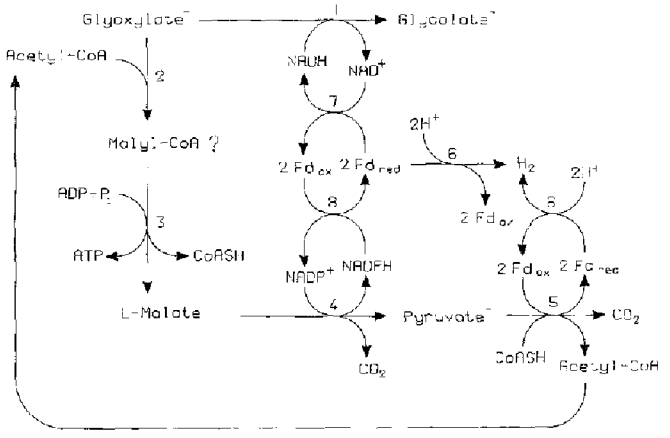

Fig. 3. Hypothetical pathway of glyoxylate degradation by strain PerGlx1 as suggested by our results. Numbers refer to the following enzyme activities detected in cell-free extracts: (1) glyoxylate reductase, (2) malyl-CoA lyase, (3) malate:CoA ligase, (4) malic enzyme, (5) pyruvate synthase, (6) hydrogenase, (7) NAD^+/NADH: ferredoxin oxidoreductase, (8) NADPH:ferredoxin oxidoreductase. *Abbreviations:* $Fd_{ox/red}$, oxidized/reduced ferredoxin

detected. We also checked for a glyoxylate oxidizing enzyme activity, for formate formation from glyoxylate, and for a methylene tetrahydrofolate oxidizing enzyme, however, without success.

Instead, enzymes of a new pathway were detected. The reaction sequence (Fig. 3) starts with the condensation of glyoxylate and acetyl-CoA, probably to form malyl-CoA by malyl-CoA lyase; malyl-CoA is apparently converted to malate by malate:CoA ligase. The free energy of this assumed thioester bond is used to phosphorylate ADP through substrate level phosphorylation. Establishment of this pathway was based on a coupled enzyme assay in both reaction directions: the formation of malate from acetyl-CoA and glyoxylate was ADP-dependent. Oxidative decarboxylation of malate by an $NADP^+$-dependent malic enzyme yields pyruvate which is decarboxylated oxidatively by pyruvate synthase.

Finally acetyl-CoA, the acceptor for glyoxylate in this pathway, is regenerated to complete the cycle. This new degradation pathway resembles the malate pathway characteristic of certain *Pseudomonas* sp. growing aerobically with glycolate (Kornberg and Sadler 1960), however, an activated form of malate, probably malyl-CoA, is the key intermediate of this new pathway. It conserves the free energy of the acetyl-CoA thioester linkage in the condensation reaction ($\Delta G^{\circ\prime} = -7.1$ kJ/mol), and synthesizes 1 ATP by substrate level phosphorylation. So far, malyl-CoA lyase and malate:CoA ligase are known only as part of the serine-isocitrate-lyase pathway used by certain aerobic methylotrophic bacteria (Gottschalk 1986). *Rhodopseudomonas sphaeroides* growing anaerobically in the light with malate or glutamate uses the same enzymes (Tuboi and Kikuchi 1965). However, unlike these pathways, metabolism in strain PerGlx1 proceeds in the opposite direction: acetyl-CoA serves as glyoxylate acceptor, and malyl-CoA appears to be formed.

Energetics

Through the reaction sequence above, the major part (about 75%) of glyoxylate is oxidized to CO_2, and two pairs of electrons are released per glyoxylate molecule. Malic enzyme ($E^{\circ\prime} = -331$ mV) transfers electrons to NADPH ($E^{\circ\prime} = -324$ mV), and pyruvate oxidation to acetyl-CoA + CO_2 ($E^{\circ\prime} = -470$ mV) reduces a low-potential electron carrier, probably ferredoxin.

A hydrogenase enzyme reacts with viologens and reoxidizes reduced ferredoxin to H_2 ($E^{\circ\prime} = -414$ mV). Ferredoxin appears to play a role also as an electron carrier between NADPH generated in the malic enzyme reaction ($NADP^+$:ferredoxin oxidoreductase) and NADH which is reoxidized in glyoxylate reductase reaction to glycolate (see Fig. 3). Direct transhydrogenation from NADPH to NAD^+ or vice versa was not detected.

The experimentally determined stoichiometry of glyoxylate conversion (Eq. (1)) indicates that a comparably small fraction of the total electron pool is used to reduce glyoxylate to glycolate ($E^{\circ\prime} = -92$ mV; Thauer 1988). If we assume that the low-potential electrons from pyruvate oxidation are released entirely as H_2, the NADPH electrons from malate oxidation obviously go two ways: two thirds are released as H_2 while one third flows into glyoxylate reduction. A similar dismutation is known for the NADH electrons obtained in the glyceraldehyde phosphate dehydrogenase reaction: Pure cultures of *Clostridium pasteurianum* channel part of these electrons into butyrate formation from acetyl-CoA, and another part is released as H_2 (Thauer et al. 1977).

Glyoxylate fermentation according to Eq. (1) releases 35.7 kJ per mol glyoxylate (Thauer et al. 1977). Three mol ATP are synthesized by substrate level phosphorylation per 4 mol glyoxylate metabolized; only 47.6 kJ is available per mol ATP, therefore. This is hardly enough for irreversible ATP synthesis in a growing cell; usually at least 70 kJ is calculated per mol ATP synthesized (Thauer et al. 1977), and the molar growth yield observed fits into this frame. Thus it has to be assumed that part of the ATP synthesized by substrate level phosphorylation is invested either in substrate uptake or in hydrogen release against the electrochemical potential. Similar ATP-requiring shifts of thermodynamic reaction equilibria have been postulated especially for syntrophic oxidations of alcohols and fatty acids (Schink 1991). Reduction of part of glyoxylate to glycolate helps to improve the energy balance of glyoxylate fermentation; complete oxidation to CO_2 and H_2 only releases even less energy, and only 26.4 kJ would be available per ATP synthesized:

$$\text{glyoxylate}^- + H^+ + H_2O \rightarrow 2\,CO_2 + 2\,H_2 \qquad (2)$$
$$\Delta G^{\circ\prime} = -26.4 \text{ kJ per mol glyoxylate.}$$

The ratio of glyoxylate oxidized over glyoxylate reduced is very stable with our isolate, and could not be influenced significantly in cocultures with hydrogen-scavening partner bacteria. Reduction of glyoxylate to glycolate with NADH electrons is a nearly irreversible reaction ($\Delta E^{\circ\prime} = -228$ mV), and it was impossible to measure a reverse reaction in cell-free extracts of strain PerGlx1, not even in the presence of dinitrophenylhydrazine as aldehyde

trap. The high gain of overall reaction entropy by glycolate formation has to be strictly controlled by the bacterium to maintain optimal efficiency of ATP synthesis. Oxidation of glycolate via glyoxylate to CO_2 is not possible with strain PerGlx1, neither in pure nor in mixed culture with methanogenic bacteria. This oxidation requires a special energy-dependent electron shift in the first step which appears to operate in syntrophic glycolate-oxidizing cultures isolated recently in our laboratory (Friedrich et al. 1991).

Strain PerGlx1 has been deposited with Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG.

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