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ORIGINAL PAPER

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Isolation and characterization of a desulforubidin-containing sulfate-reducing bacterium growing with glycolate

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Abstract Sulfate-dependent degradation of glycolate was studied with a new sulfate-reducing bacterium, strain PerGlyS, enriched and isolated from marine anoxic sediment. Cells were gram-negative, motile rods with a DNA G+C content of 56.2 ± 0.2 mol%. Cytochromes of the *b*- and *c*-type and menaquinone-5 were detected. A sulfite reductase of the desulforubidin-type was identified by characteristic absorption maxima at 279, 396, 545, and 580 nm. The purified desulforubidin is a heteropolymer consisting of three subunits with molecular masses of 42.5 (α), 38.5 (β), and 13 kDa (γ). Strain PerGlyS oxidized glycolate completely to CO_2. Lactate, malate, and fumarate were oxidized incompletely, yielding more sulfide and less acetate than expected for typical incomplete oxidation of these substrates. Part of the acetate residues formed was oxidized through the CO-dehydrogenase pathway. The biochemistry of glycolate degradation was investigated in cell-free extracts. A membrane-bound glycolate dehydrogenase, but no glyoxylate-metabolizing enzyme activity was detected; the further degradation pathway is unclear.

Key words Dissimilatory sulfate reduction · Glycolate Incomplete oxidation · Desulforubidin · Glycolate dehydrogenase · CO dehydrogenase · Menaquinone

Abbreviation *DTE* Dithioerythritol

Dedicated to Prof. Norbert Pfennig on the occasion of his 70th birthday

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Introduction

A major source of glycolate in nature is excretion by algae and other photoautographs and chemoautotrophs (Whittingham and Pritchard 1963; Codd and Smith 1974; Codd et al. 1976; Beudeker et al. 1981). Due to the oxygenase activity of the D-ribulose-1,5-bisphosphate carboxylase at low CO_2 and high O_2 concentrations, 3-phosphoglycerate and phosphoglycolate are formed in these organisms, the latter being subsequently dephosphorylated to glycolate (Beck 1979). Excreted glycolate accumulates up to 4 µM during the diurnal cycle in the surface water of a eutrophic lake (Plußsee, northern Germany; U. Münster, personal communication), and up to 1.3 µM in marine coastal waters (New York Bight; Edenborn and Litchfield 1987). Glycolate formation has also been observed in the upper 2-mm layer of a cyanobacterial mat in a hot spring in Yellowstone National Park, USA; up to 7% of the photosynthetate is excreted as glycolate (Bateson and Ward 1988). Recently, Fründ and Cohen (1992) found evidence for sulfate-dependent glycolate oxidation in a similar environment: the sulfate reduction rate in the upper, hyperoxic layer of a cyanobacterial mat in an experimental hypersaline pond system is stimulated by addition of glycolate.

Anaerobic glycolate degradation has been studied so far only with fermenting bacteria. Janssen (1990) reported on a ternary mixed culture that ferments glycolate to acetate, propionate, and carbon dioxide in the presence of yeast extract. A new pathway of anaerobic glycolate degradation by a defined methanogenic syntrophic coculture was discovered recently (Friedrich et al. 1991; Friedrich and Schink 1993; Schink and Friedrich 1994); this pathway comprises glycolate oxidation to CO_2 and H_2 via malyl-CoA by a fermenting bacterium, combined with interspecies hydrogen transfer to the methanogenic partner. Due to the high mid-point potential of the redox couple glyoxylate/glycolate ($E^{0\prime}$ = –92 mV), glycolate degradation by the fermenting bacterium depends on a low hydrogen partial pressure that is maintained by the methanogenic hydrogen-scavenging partner. In addition, reversed

electron transport is necessary to release H_2 from glycolate oxidation to glyoxylate (Friedrich and Schink 1993).

The present study was initiated to investigate the degradation of glycolate in the presence of sulfate. We report on the isolation and characterization of a new sulfate-reducing bacterium from an anoxic marine sediment that grows with glycolate as sole carbon source.

Materials and methods

Bacteria and cultivation

Strain PerGlyS was isolated from an enrichment culture inoculated with anoxic black marine sediment of Rio della Pergola (Venice, Italy). *Methanospirillum hungatei* strain SK was obtained from Prof. F. Widdel (Bremen, Germany). *Acetobacterium woodii* (DSM 1030), *Desulfovibrio vulgaris* strain Marburg (DSM 2119), *Desulfovibrio desulfuricans* strain CSN, *Desulfobotulus sapovorans* (DSM 1083), and *Desulfobulbus propionicus* (DSM 2032) were from our own culture collection.

Bacteria were grown in bicarbonate-buffered, sulfide-reduced (1 mM) freshwater or saltwater medium, as described earlier (Friedrich and Schink 1991). Growth experiments were carried out at 28°C in 50-ml screw-capped bottles, in 120-ml serum flasks filled with 50 ml mineral medium under an atmosphere of N_2/CO_2 (80:20, v/v), or H_2/CO_2 (80:20, v/v), or in 22-ml screw-capped tubes.

Isolation and cytological characterization

The agar-shake dilution method (Widdel and Pfennig 1984) was applied for isolation. Purity of cultures was checked microscopically after growth in mineral medium with sodium glycolate (10 mM) and sodium sulfate (20 mM), or in semi-solid complex medium (AC medium, Difco, Ann Arbor, Mich., USA) at standard concentration and fivefold diluted. The Gram type was determined according to Bartholomew (1962) with *Acetobacterium woodii* and *Desulfovibrio vulgaris* as controls and by the KOH method (Gregersen 1978). Flagella were stained using the method of Blenden and Goldberg (1965).

DNA base composition

The G+C content of the DNA was determined by direct quantitation of the nucleosides by HPLC after enzymatic degradation of the DNA (Mesbah et al. 1989), using phage λDNA as standard. DNA was isolated from a 1-l culture of glycolate-plus-sulfate-grown cells of strain PerGlyS using the NaOH method for cell disruption (Mesbah et al. 1989).

Preparation of cell-free extracts

Strain PerGlyS was grown with glycolate (10 mM) and sulfate (20 mM) in 1.25-l infusion bottles filled with 1 l saltwater medium. Cells were harvested anoxically in the late exponential growth phase by centrifugation (13,700 × *g*; 20 min), as described previously (Friedrich and Schink 1993), and washed twice with 50 mM potassium phosphate (pH 7.0), reduced with 2.5 mM dithioerythritol (DTE). Crude extracts were prepared by French pressure cell treatment (4–5 runs) at 136 MPa. Cell debris was removed by centrifugation at 3,300 × *g* for 20 min at 5C, and the final cell-free extract was stored on ice prior to use. Subcellular fractions were obtained by ultracentrifugation at 417,000 × *g* for 30 min at 5°C.

Enzyme determinations

Spectrophotometric enzyme assays were performed under an anoxic atmosphere in rubber-stoppered cuvettes, as described earlier (Friedrich and Schink 1991, 1993). Unless mentioned otherwise, enzyme reactions were started with protein, and as a control alternatively with substrate. With coupled assays, controls were run employing reaction intermediates when available.

Glycolate dehydrogenase (EC 1.1.99.-) activity was determined according to Friedrich and Schink (1993), and *malyl-CoA lyase* (EC 4.1.3.24)/*malate:CoA ligase* (EC 6.2.1.9) was determined according to Friedrich et al. (1991).

Glyoxylate reductase (EC 1.1.1.26) was analyzed with NAD(P)H (ε_{365nm} = 3.5 mM^{-1} cm^{-1}) as electron donor. The assay mixture contained 50 mM potassium phosphate (pH 6.5) 2.5 mM dithioerythritol (DTE), 5 mM $MgCl_2$, 0.3 mM NAD(P)H, and 10 mM glyoxylate.

Glyoxylate carboligase (EC 4.1.1.6)/*tartronic semialdehyde reductase* (EC 1.1.1.81) was determined in a coupled assay. D-Glycerate formation from glyoxylate was measured via tartronic semialdehyde reduction with NAD(P)H as electron donor modified according to Kornberg and Gotto (1961). The assay mixture contained 100 mM potassium phosphate (pH 7.0), 2.5 mM DTE, 0.3 mM NAD(P)H, 0.5 mM thiamine pyrophosphate, 5 mM $MgCl_2$, and 5 mM glyoxylate.

Erythro-β-hydroxyaspartate dehydratase (EC 4.2.1.38) was checked by following oxaloacetate formation from erythro-β-hydroxyaspartate according to Kornberg and Morris (1965). Oxaloacetate formation was determined using malate dehydrogenase present in crude extracts.

Isocitrate lyase (EC 4.1.3.1*) and* malate synthase (EC 4.1.3.2) were determined according to Dixon and Kornberg (1959).

ATP-citrate lyase (EC 4.1.3.8) was analyzed by following oxaloacetate formation from citrate in the presence of ATP and CoASH. Oxaloacetate formation was measured using malate dehydrogenase present in crude extracts. The assay mixture contained 50 mM Tris-HCl (pH 8.5), 0.3 mM NADH, 2.5 mM $MgCl_2$, 2 mM ATP, 0.5 mM CoASH, and 5 mM ammonium citrate.

Citrate synthase(EC 4.1.3.7) and *isocitrate dehydrogenase* (EC 1.1.1.42) were determined according to Brune and Schink (1990). With the latter enzyme, $NAD(P)^+$ (1 mM), benzyl viologen (5 mM), and methylene blue (0.1 mM) were employed as electron acceptors.

2-Oxoglutarate dehydrogenase (EC 1.2.7.3) was measured according to Brandis-Heep et al. (1983). The modified assay mixture contained 50 mM Tris-HCl (pH 8.5), 5 mM benzyl viologen, 5 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate, and 2 mM 2-oxoglutarate. Alternatively, $NAD(P)^+$ (1 mM) was checked as electron acceptor.

Succinate dehydrogenase (EC 1.3.99.1) was measured with ferricyanide (1 mM) as electron acceptor (Stams et al. 1984) with and without 0.1 mM phenazine methosulfate (PMS), and Triton X-100 (0.01%, v/v). Alternatively, methylene blue was used as electron acceptor.

Fumarase (EC 4.2.1.2) was analyzed following fumarate conversion to malate (Brandis-Heep et al. 1983) or fumarate formation from malate (Stams et al. 1984) by monitoring changes in fumarate concentration (ε_{250nm} = 1.45 mM^{-1} cm^{-1}). Assay mixtures contained 50 mM potassium phosphate (pH 7.5), 2.5 mM L-malate or 50 mM Tris-HCl (pH 8.0), and 0.5 mM fumarate.

Malate dehydrogenase (EC 1.1.1.37) was determined according to Stams et al. (1984) following either NAD(P)H oxidation with oxaloacetate as electron acceptor or malate oxidation coupled to $APAD^+$ ($E^{0\prime}$ = –248 mV; Bergmeyer 1974) reduction.

Acetate kinase (EC 2.7.2.1) was measured as described by Bergmeyer (1974).

Phosphotransacetylase (EC 2.3.1.8) was analyzed by following conversion of acetyl phosphate and CoASH to acetyl CoA ($\varepsilon_{233\ nm}$ = 4.44 mM^{-1} cm^{-1}) (modification of the procedure of Bergmeyer 1974). The assay mixture contained 100 mM Tris-HCl (pH 7.1), 0.25 mM CoASH, 6 mM NH_4Cl, and 2 mM acetyl phosphate.

Hydrogenase (EC 1.12.1.2) and *CO dehydrogenase* (EC 1.2.99.2) were measured according to Diekert and Thauer (1978); *malic enzyme* (EC 1.1.1.40) and *lactate dehydrogenase* were measured as described by Stams et al. (1984), and *pyruvate synthase* (EC 1.2.7.1) was analyzed according to Odom and Peck (1981). *Formyltetrahydrofolate synthetase* (EC 6.3.4.3) and *formate dehydrogenase* (EC 1.2.1.43 ?) were determined according to Spormann and Thauer (1988); the latter enzyme was checked either with benzyl viologen (5 mM) or with $NAD(P)^+$ (1 mM) as electron acceptor. *Oxaloacetate decarboxylase* (EC 4.1.1.2) activity was recorded following oxaloacetate decrease ($\varepsilon_{265\ nm}$ = 0.95 mM^{-1} cm^{-1}) as described by Dimroth (1981). *ATP synthase* (EC 3.6.1.34) was analyzed according to Vogel and Steinhart (1976).

NAD(P)H:acceptor oxidoreductase (EC 1.6.99.1) was measured following the reduction of benzyl viologen ($\varepsilon_{578\ nm}$ = 8.65 mM^{-1} cm^{-1}) or methylene blue ($\varepsilon_{570\ nm}$ = 13.1 mM^{-1} cm^{-1}) with NAD(P)H. The assay mixture contained 50 mM potassium phosphate (pH 6.5), 2.5 mM DTE, 0.3 mM NAD(P)H, and 5 mM benzyl viologen or 0.1 mM methylene blue.

Cytochromes

Cytochromes were detected by redox-difference spectroscopy (dithionite-reduced *minus* air-oxidized) of subcellular fractions at 400 to 600 nm using either a Uvikon 860 or a Type 930 double-beam spectrophotometer (Kontron, Zürich, Switzerland). Cytochromes were identified by their typical absorption maxima (Dickerson and Timkovich 1975; Widdel 1980).

Sulfite reductases

Desulfoviridin was detected by recording absorption spectra of cell-free extracts between 400 and 700 nm. Sulfite reductase P-582 was analyzed by recording CO-difference spectra of subcellular fractions as described by Trudinger (1970) and Widdel (1980).

Desulforubidin was purified chromatographically and identified by absorption spectroscopy. The cytoplasmic fraction (830 mg protein) of strain PerGlyS was loaded on a 5-ml High Trap Q anion-exchange column (Pharmacia, Freiburg, Germany) equilibrated with 20 mM Tris-HCl, (pH 8.0; buffer A). Sulfite reductase was eluted with a NaCl step-gradient (200, 250, 300, and 400 mM, and 1 M NaCl) at 400 mM NaCl. Fractions were analyzed spectroscopically, and the red fractions with the highest 398 nm/545 nm ratio were pooled. To concentrate these fractions, the protein was loaded on a 1-ml High Trap Q column and eluted with 1 M NaCl. The concentrated sample was then applied to a Superose 12 gel-filtration column (10 × 300 mm, Pharmacia) equilibrated with buffer A containing 150 mM NaCl. The fractions with the highest 398 nm/545 nm ratio were concentrated using a 1-ml High Trap Q column, and then applied to a Superdex 200 preparatory-grade gel filtration column (16 × 600 mm, Pharmacia) equilibrated with buffer A containing 150 mM NaCl. Finally, the sulfite reductase-containing fractions were loaded on a Mono Q anion-exchange column (10 × 50 mm, Pharmacia) equilibrated with buffer A. The red sulfite-reductase-containing fractions were collected at a NaCl concentration between 340 and 420 mM.

Quinones

Lyophilized cells (105 mg) of strain PerGlyS were extracted with chloroform/methanol (2:1, v/v) for 2 h, and subsequently with petroleum ether/methanol (1:1, v/v), as described previously (Friedrich and Schink 1993). Quinones were identified by thin-layer chromatography (silica gel 60 F_{254}; Merck, Darmstadt, Germany). Dr. R. M. Kroppenstedt (Braunschweig, Germany) determined the menaquinone type by HPLC analysis (Kroppenstedt 1985).

Analytical methods

Protein was determined by the microprotein assay (Bradford 1976) with bovine serum albumin as standard. SDS-PAGE with a discontinuous buffer system according to Laemmli (1970) was performed in the Mini Protean II vertical electrophoresis system (Bio-Rad, Munich, Germany). Prior to loading, samples were boiled for 5 min in the presence of 1% SDS and 5% β-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue R250 (0.25%, w/v, in methanol/glacial acetic acid/H_2O, 45:10:45, by vol).

Methane and hydrogen were quantified by gas chromatography (Platen and Schink 1987; Friedrich and Schink 1993); samples containing less than 0.2 nmol hydrogen were analyzed on a RGD2 reduction gas detector (Techmation, Düsseldorf, Germany) (Friedrich and Schink 1993).

Organic acids were measured by HPLC as described previously (Friedrich et al. 1991), using an ORH-801 organic acids column (300 × 6.5 mm; Interaction Chemical, Mountain View, Calif., USA) with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml/min at 50°C, and a refractive index detector (Erma, Tokyo, Japan). Sulfide was determined according the method of Cline (1969).

Chemicals

All chemicals were of analytical or reagent grade and were obtained from Biomol (Ilvesheim, Germany), Boehringer (Mannheim, Germany), Eastman Kodak (Rochester, NY, USA), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Pharmacia (Freiburg, Germany), Serva (Heidelberg, Germany), or Sigma (Deisenhofen, Germany). Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

Enrichment and isolation

Strain PerGlyS was enriched from anoxic black sediment of Rio della Pergola, a channel in Venice, Italy, with glycolate (10 mM) and sulfate (10 mM) in sulfide-reduced saltwater mineral medium. Growth was followed as sulfide formation or turbidity increase, and started after 2–4 weeks of incubation. After repeated transfers (10% inoculum size), a slightly curved, motile rod dominated in the enrichment culture. Strain PerGlyS was isolated by two subsequent agar-shake dilutions with glycolate and sulfate as substrates.

Cytological and physiological characterization

Cells of strain PerGlyS were gram-negative, slightly curved rods, 2.0–4.5 × 0.55 m in size, and were motile by one subterminally inserted flagellum (Fig. 1 a, b). Spore formation was never observed, not even after addition of 100 μM $MnSO_4$ or several months of starvation. The G + C content of the DNA was 56.20.1 mol%.

Cytochromes of the *b*- and *c*-type were present in the cytoplasm and in the membrane fraction, as revealed by absorption maxima at 420, 523, and 552 nm and at 422, 524, and 554 nm wavelength, respectively (Bartsch 1968).

Desulfoviridin was not detected in cell-free extracts by its typical absorption band at 630 nm wavelength (Post-

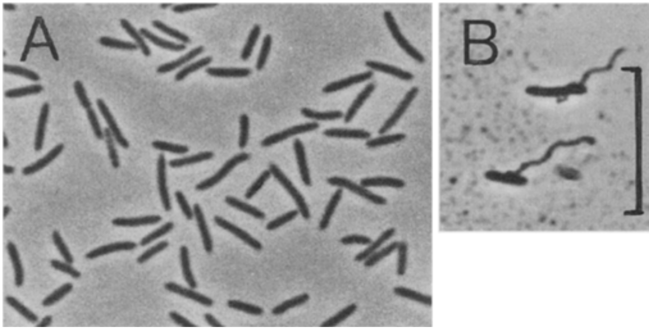


Fig. 1 **A** Phase contrast photomicrograph of strain PerGlyS, and **B** flagella staining of strain PerGlyS cells. *Bar* 10 m for both panels

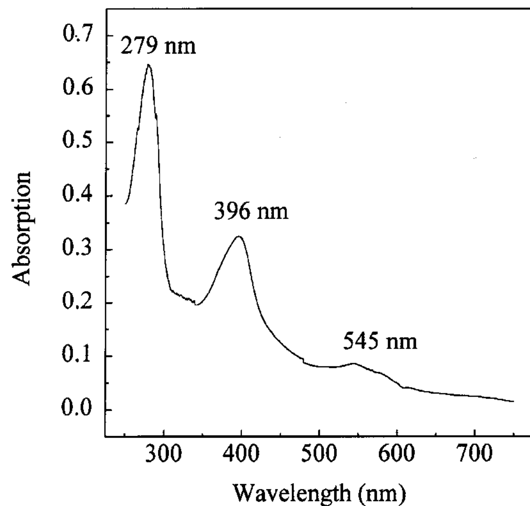


Fig. 2 UV/Vis spectrum of desulforubidin purified from strain PerGlyS. Protein concentration 0.21 mg ml^{-1}; buffer, 20 mM Tris-HCl (pH 8.0) containing 350 mM NaCl

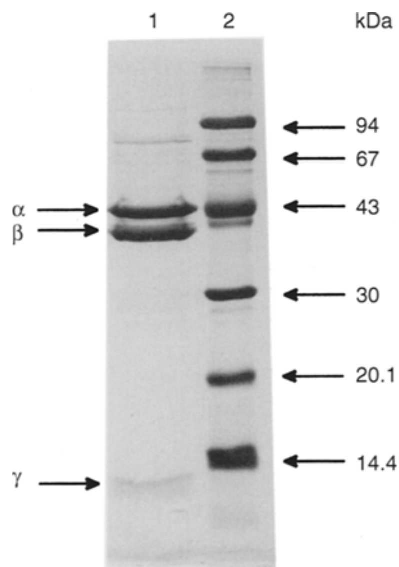


Fig. 3 SDS-PAGE (13.5% acrylamide gel) of desulforubidin from strain PerGlyS. *Lane 1* 4 µg desulforubidin, *lane 2* molecular mass marker mixture (Pharmacia, Freiburg, Germany); molecular masses are indicated

gate 1956). Sulfite reductase P-582 could not be demonstrated by CO-difference spectroscopy in subcellular fractions because of interfering cytochrome *c* bands (Trudinger 1970; Weston and Knowles 1973). However, a sulfite reductase of the desulforubidin-type was detected spectroscopically in the cytoplasmic fraction after separation from interfering cytochromes by chromatography. The absorption spectrum of the purified protein exhibited a maximum at 545 nm, typical of sulfite reductases of the desulforubidin-type (Lee et al. 1973; Arendsen et al. 1993); in addition, further characteristic peaks were observed at 279, 396, and 580 nm (Fig. 2). After the final purification step, we found mainly two protein bands with molecular masses of 42.5 and 38.5 kDa, and two minor bands of 78 and 13 kDa, as shown by denaturing PAGE (Fig. 3). The 78-kDa band may be disregarded because it is present only at a low molar ratio and is, therefore, probably a contaminant. The molecular mass of the holoprotein as determined by gel filtration under non-denaturing conditions was approximately 260 kDa. Densitometric analyses (ImageQuant software, Molecular Dynamics, Sunnyvale, Calif., USA) of Coomassie-stained gels (Fig. 3), revealed a 0.9 : 1 : 0.4 ($\alpha:\beta:\gamma$) stoichiometry after correction for the molecular mass. According to this analysis, the enzyme was 98% pure. The enzyme preparation was highly purified also on the basis of UV/Vis spectroscopy; the purity indices were 3.77 (398 nm/545 nm), and 0.5 (398 nm/280 nm), in comparison to 3.65 (398 nm/545 nm), and 0.35 (398 nm/545 nm) for desulforubidin from *Desulfosarcina variabilis* (Arendsen et al. 1993). Desulfoviridins of two *Desulfovibrio vulgaris* strains have been shown previously to resolve into two (Seki et al. 1979) or three (Wolfe et al. 1994) distinct forms during anion-exchange chromatography. Interestingly, also the desulforubidin of strain PerGlyS resolved into three distinct peaks on a Mono Q anion-exchange column in the final purification step; however, the protein pattern in fractions of the three distinct peaks was identical in each peak, as revealed by SDS-PAGE. Cells of strain PerGlyS contained menaquinone-5 (H_2), as revealed by HPLC analysis.

Table 1 Substrate range of the sulfate-reducing strain PerGlyS with 20 mM sulfate as electron acceptor. Concentrations of the substrates tested are given in parentheses (mM)

Substrates tested supporting growth:
glycolate (10), glyoxylate (5), L-lactate (10), L-malate (10), fumarate (10), succinate (10), yeast extract (0.1%), H_2/CO_2 (80:20, v/v, in the gas phase) plus 2 mM acetate
Substrates tested not supporting growth:
fructose (5), glucose (5)
formate (10), acetate (10), propionate (10), butyrate (10), isobutyrate (10), caproate (10), valerate (10), 3-methyl butyrate (10)
glycolaldehyde (5), glyceraldehyde (5)
oxalate (10), pimelate (10), glutarate (10)
methanol (10), ethanol (10), butanol (5)
betaine (10), choline (10), cysteine (10), triethanolamine (10), glycine (10), indole (5)
benzoate (5)

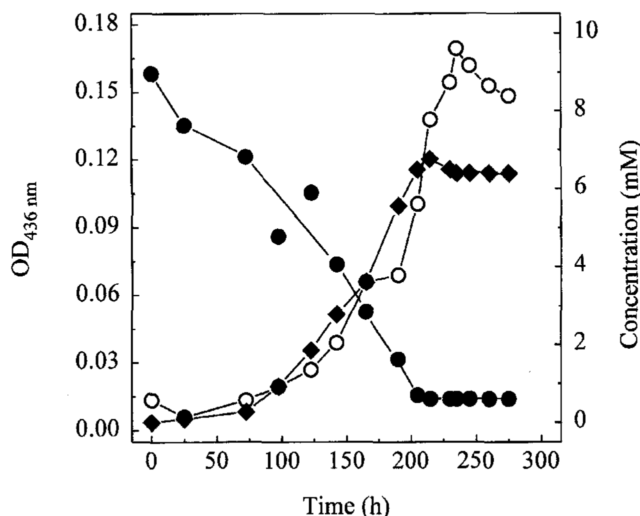
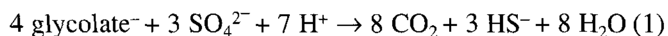


Fig. 4 Time course of glycolate degradation by strain PerGlyS. OD_{436} (*open circles*), sulfide (*filled diamonds*), and glycolate (*filled circles*)

Strain PerGlyS grew in saltwater (340 mM NaCl; 14 mM $MgCl_2$) and in brackish-water medium (120 mM NaCl; 6.4 mM $MgCl_2$). Growth was observed between 15 and 37°C, in the range of pH 6.7 to 8.3, and was optimal at 28°C and pH 7.3. In tubes with semi-solid AC medium (nutrients 1:5 diluted) supplemented with or without glycolate or sulfate, growth ceased only 0.5 cm below the air interface, indicating a comparably high oxygen tolerance. Besides glycolate, other substrates were oxidized with sulfate as electron acceptor (Table 1). Glyoxylate-grown cultures exhibited long and unpredictable lag phases upon transfer on the same substrate. In the presence of 2 mM acetate, strain PerGlyS grew with H_2/CO_2, but not with formate or methanol. Growth occurred also with sulfite or elemental sulfur as alternative electron acceptors and glycolate as electron donor; however, thiosulfate, nitrate, and fumarate were not reduced. Other sulfate-reducing bacteria, such as *Desulfovibrio desulfuricans, D. vulgaris, Desulfobotulus sapovorans, Desulfobulbus propionicus,* and *Desulfomicrobium apsheronum* (A. Galouchko, personal communication) did not grow with glycolate (10 mM) and sulfate (20 mM).

Strain PerGlyS grew with glycolate and sulfate with a doubling time of 46 h (μ = 0.015 h^{-1}; (Fig. 4). Glycolate was oxidized to CO_2 according to the following equation (Table 2):


$$4\ \text{glycolate}^- + 3\ SO_4^{2-} + 7\ H^+ \rightarrow 8\ CO_2 + 3\ HS^- + 8\ H_2O \quad (1)$$

Hydrogen or formate formation during glycolate degradation was not detected. Attempts to grow strain PerGlyS syntrophically with glycolate in the presence of *Methanospirillum hungatei* as hydrogen- or formate-scavenging partner failed. Lactate, malate, and fumarate were not completely oxidized to CO_2. However, less acetate and more HS^- was found than expected for typical incomplete oxidation of these compounds (Table 2).

Biochemistry

Glycolate degradation was studied with cell-free extracts of glycolate/sulfate-grown cells. All enzymes detected exhibited specific activities in the range of the in vivo glycolate degradation rate of 112 nmol min^{-1} (mg protein)$^{-1}$. A glycolate dehydrogenase activity reacting with methylene blue or benzyl viologen as electron acceptor was detected mainly in the membrane fraction after subcellular fractionation (Table 3). Also, methylene-blue-dependent succinate dehydrogenase was found to be membrane-associated. As a control, malate dehydrogenase was found largely in the cytoplasm, indicating successful separation of the fractions. Malate dehydrogenase activity was specific for NAD^+ or $APAD^+$, but did not react with $NADP^+$ or methylene blue. Other enzymes of the citric acid cycle detected in cell-free extracts of strain PerGlyS are listed in Table 3.

Since glycolate dehydrogenase was detected in strain PerGlyS, we tried to track the fate of glyoxylate by checking for enzymes of known glyoxylate oxidation pathways. All attempts to measure key enzymes such as *erythro*-β-hydroxyaspartate dehydratase (β-hydroxyaspartate pathway, Kornberg and Morris 1965), glyoxylate carboligase/tartronic semialdehyde dehydrogenase (glycerate pathway, Kornberg and Gotto 1961), malate synthase (dicarboxylic acid cycle; Kornberg and Sadler 1960), and ma-

Table 2 Stoichiometry of substrate conversion and growth yields of strain PerGlyS. Growth experiments were performed in 22-ml screw-capped tubes in duplicate. All tubes contained 20 mM Na_2SO_4

Substrate	Concentration (mM)	Substrate degraded (μmol)	Cell dry mass formed[a] (mg)	Substrate assimilated[b] (μmol)	Products formed (μmol)		Electron recovery (%)	Molar growth yield (g mol^{-1})
					Acetate	H_2S		
Glycolate	9.65	212	0.98	26.9	–	128	92	5.3
Lactate	9.8	216	0.87	11.9	141	127	88	4.3
Malate	4.85	107	0.72	9.9	33	90	85	7.4
Fumarate	4.15	91	0.76	10.5	24	90	95	9.4

[a] Cell dry matter values were calculated via cell density using an experimentally determined conversion factor (0.1 $OD_{500\ nm}$ = 19.2 mg dry matter l^{-1})
[b] The amount of substrate assimilated was calculated according to the following formulas:

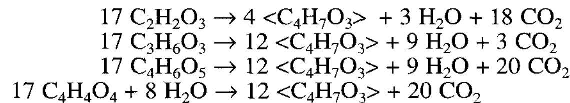

$$17\ C_2H_2O_3 \rightarrow 4\ \langle C_4H_7O_3\rangle + 3\ H_2O + 18\ CO_2$$
$$17\ C_3H_6O_3 \rightarrow 12\ \langle C_4H_7O_3\rangle + 9\ H_2O + 3\ CO_2$$
$$17\ C_4H_6O_5 \rightarrow 12\ \langle C_4H_7O_3\rangle + 9\ H_2O + 20\ CO_2$$
$$17\ C_4H_4O_4 + 8\ H_2O \rightarrow 12\ \langle C_4H_7O_3\rangle + 20\ CO_2$$

Table 3 Enzyme activities in cell-free extracts and in the membrane fraction of strain PerGlyS grown with glycolate and sulfate. Electron acceptors for redox reactions are given in *parentheses* (*BV* benzyl viologen, *MB* methylene blue, *n.d.* not determined)

Enzyme activity	Specific activity [μmol min^{-1} (mg $protein)^{-1}$]		
	Glycolate/sulfate-grown cells		Lactate/sulfate grown cells
	Cell-free extract	Membrane fraction	Cell-free extract
Glycolate dehydrogenase (MB)	0.2–0.3	6.4	0.14
L-Malate dehydrogenase (NAD^+)[a]	5.5–32.3	1.8	19.7
Oxaloacetate decarboxylase	1.2	n.d.	n.d.
Pyruvate synthease	0.56	n.d.	0.9
Fumarase	1.64	n.d.	n.d.
Succinate dehydrogenase (MB)	0.16–0.40	0.93	0.07
Isocitrate dehydrogenase (MB)	0.03–0.36	n.d.	n.d.
Formate dehydrogenase (BV)	0.21	n.d	0.60
CO dehydrogenase (BV)	0.83–0.94	n.d.	3.0
Hydrogenase (BV)	0.32–0.42	n.d.	0.82
ATPase	0.60	n.d.	n.d.
Lactate dehydrogenase (NAD^+)	< 0.001	n.d.	0.19

[a] Determined as oxaloacetate reduction

lyl-CoA lyase/malate:CoA ligase (malyl-CoA pathway, Friedrich and Schink 1991, Friedrich et al. 1991) by employing standard protocols or by identifying tentative products of anticipated enzymatic reactions failed. In addition, we tried to measure isocitrate lyase, which could operate as a glyoxylate-condensing enzyme in the reverse direction; however, attempts were unsuccessful. We checked for activities of acetate kinase, phosphotransacetylase, fumarate reductase, ATP-citrate lyase, citrate synthase, 2-oxoglutarate dehydrogenase, and formyltetrahydrofolate synthetase; however, all attempts were without success.

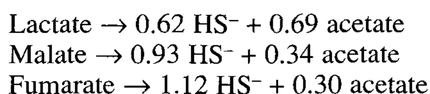
To elucidate the unusual degradation stoichiometry of lactate, fumarate, and malate (see above; Table 2), we studied the enzymes of lactate metabolism in cell-free extracts of cells grown with lactate/sulfate. An NAD^+-dependent lactate dehydrogenase was detected at activities sufficient to account for dissimilatory turnover of lactate by an exponentially growing culture; pyruvate synthase, malate dehydrogenase, and succinate dehydrogenase were also detected (Table 2). The latter was significantly lower in activity in lactate-sulfate-grown cells than in glycolate-sulfate-grown cells, indicating a possible involvement of succinate dehydrogenase in glycolate metabolism. Interestingly, glycolate dehydrogenase could be measured in lactate sulfate-grown cells at activities comparable to growth on glycolate, indicating a constitutive expression of this activity. On the other hand, lactate dehydrogenase was not found in glycolate sulfate-grown cells.

Phosphotransacetylase and acetate kinase could account for acetate excretion from lactate, but we were unable to detect either one. Since part of lactate was oxidized to CO_2 as revealed by the degradation balance, we checked for key enzymes of acetate oxidation. 2-Oxoglutarate dehydrogenase, the key enzyme of acetate oxidation via the citric acid cycle, was not detected. Instead, we found CO dehydrogenase [3 mol min^{-1} (mg $protein)^{-1}$] at activities sufficient to account for the observed lactate degradation rate and at a significantly higher activity than in glycolate-sulfate-grown cells.

Discussion

In this communication, we report on the isolation of a new sulfate-reducing bacterium, strain PerGlyS, that oxidizes glycolate completely to CO_2. So far, the substrate range of dissimilatory sulfate-reducing bacteria includes electron donors such as H_2, fatty acids, other monocarboxylic acids, dicarboxylic acids, alcohols, polyols, acetone, amino acids, a few sugars, aromatic compounds, and long-chain alkanes (Widdel 1988; Widdel and Bak 1992; Widdel and Hansen 1992; Hansen 1994) This is the first description of glycolate degradation by a sulfate-reducing bacterium.

Known sulfate-reducing bacteria oxidize their substrates either completely to CO_2 or incompletely to CO_2 and acetate. Strain PerGlyS oxidized glycolate and glyoxylate completely. Other substrates such as lactate, malate, and fumarate were degraded to acetate, HS^-, and CO_2, yielding the following product patterns (CO_2 not determined; Table 2):



Lactate $\rightarrow$ 0.62 HS^- + 0.69 acetate
Malate $\rightarrow$ 0.93 HS^- + 0.34 acetate
Fumarate $\rightarrow$ 1.12 HS^- + 0.30 acetate

Obviously, these substrates were neither degraded completely to CO_2 nor incompletely to one mol acetate per mol lactate, malate, or fumarate, respectively. Especially with malate and fumarate, only one third of the acetate expected for incomplete substrate oxidation was found, but the stoichiometries were balanced by increased sulfide formation. Some completely oxidizing sulfate reducers excrete acetate during growth on more complex substrates and may use it up later (Widdel 1988). It was assumed that these substrates are oxidized faster to the level of acetyl-CoA than they are oxidized further, thus favoring acetate excretion via acetylphosphate and substrate level phosphorylation. CO dehydrogenase activities of 3 mol min^{-1} (mg $protein)^{-1}$ of lactate/sulfate-grown cells of strain PerGlyS demonstrate its capability to oxidize acetate

completely via the CO dehydrogenase pathway. Lack of phosphotransacetylase and acetate kinase, however, indicates that this strain employs different enzymes for acetate excretion or activation.

Desulforubidin

Strain PerGlyS contains a sulfite reductase of the desulforubidin type, as identified by the characteristic absorption maxima at 396, 545, and 580 nm of the purified protein. Dissimilatory sulfite reductases known so far all comprise a heteropolymeric $\alpha_2\beta_2\gamma_2$structure with molecular masses in the range of 150 to 230 kDa and with approximate subunit molecular masses of 50 (α), 40 (β), and 12 kDa (γ) (Pierik et al. 1992; Hansen 1994); it was only recently that the small subunit was discovered (Pierik et al. 1992). The desulforubidin purified previously from *Desulfosarcina variabilis* has been reported to have subunits of 50 (α), 42.5 (β), and 12 kDa (γ) molecular mass (Arendsen et al. 1993). As revealed by SDS-PAGE of the purified desulforubidin from strain PerGlyS, we found three subunits of 42.5, 38.5, and 13 kDa corresponding to the $\alpha\beta\gamma$-composition of dissimilatory sulfite reductases, but the sizes of the α and β subunits differ significantly from those of other sulfite reductases. Densitometric scans revealed a 0.9:1:0.4 stoichiometry of the $\alpha\beta\gamma$-complex, suggesting either that part of the γ subunit was lost during purification, that the subunit stoichiometry differs from the typical $\alpha_2\beta_2\gamma_2$ composition (Pierik et al. 1992), or that the subunits stain with Coomassie blue with different intensity. However, the molecular mass of approximately 260 kDa that we found for the holoprotein and the densitometric analysis support rather a $\alpha_3\beta_3\gamma_?$ composition for the desulforubidin complex in strain PerGlyS.

Biochemistry of glycolate degradation

The biochemistry of glycolate degradation by strain PerGlyS was studied in cell-free extracts. Analogous to the pathway of glycolate degradation in the fermenting bacterium of the syntrophic coculture strain FlGlyM (Friedrich et al. 1991; Friedrich and Schink 1993, 1995) glycolate was oxidized to glyoxylate by a membrane-bound glycolate dehydrogenase. This enzyme was measured with methylene blue as electron acceptor, but it reacted also with benzyl viologen. The natural electron acceptor of this enzyme is presently unknown, as is also true with the enzyme of the fermenting bacterium, but both bacteria contain menaquinones as possible electron acceptors for this reaction. The presence of an NAD^+-dependent malate dehydrogenase, oxaloacetate decarboxylase, and pyruvate synthase supports the concept that in strain PerGlyS, glycolate is oxidized via the malyl-CoA pathway, as found in the fermenting bacterium of the coculture. This pathway includes condensation of glyoxylate with acetyl-CoA to malyl-CoA, ATP formation by malyl-CoA synthetase, malate oxidation and decarboxylation by malic enzyme, and oxidative decarboxylation of pyruvate to acetyl-CoA (Friedrich et al. 1991). However, we could not demonstrate the key enzymes of this pathway, malyl-CoA lyase/malate:CoA ligase. All other attempts to demonstrate key enzymes of known glyoxylate-degrading pathways, such as the glycerate pathway (Kornberg and Gotto 1961), the β-hydroxyaspartate pathway (Kornberg and Morris 1965), or the dicarboxylic acid cycle (malate synthase; Kornberg and Sadler 1960) failed. Since we found enzymes of the citric acid cycle in glycolate/sulfate-grown cells of strain PerGlyS, such as isocitrate dehydrogenase, succinate dehydrogenase, and fumarase, we also checked for a possible glyoxylate condensation with succinate to isocitrate; however, isocitrate lyase could not be detected using a standard protocol. Another possible route for glyoxylate oxidation that we checked was oxidation through the C_1 pathway. Glyoxylate as a reactive aldehyde could possibly react with tetrahydrofolate and simultaneously be decarboxylated. Further oxidation would lead via formyltetrahydrofolate to formate, but lack of formyltetrahydrofolate synthetase in glycolate/sulfate-grown cells renders also this hypothesis unlikely. Thus, further studies are required to elucidate the biochemistry of glyoxylate degradation by this strain.

Energetics

Oxidation of glycolate with sulfate as electron acceptor according to Eq. 1 yields -78 kJ per mol glycolate under standard conditions (calculated according to Thauer et al. 1977). This amount of free energy could account for synthesis of one mol ATP per mol glycolate, assuming consumption of 70 kJ mol^{-1} for irreversible ATP synthesis in a living cell (Schink 1992). The observed molar growth yield of 4.5 g per mol glycolate is lower than expected, assuming that 10 g cell dry mass is formed per mol ATP (Stouthamer 1979). It has to be considered that growth with this substrate is slow and that cell matter synthesis from glycolate may require more energetical effort than from, for example malate or fumarate. A comparable cell yield of 3.7 g per mol glycolate has been reported earlier for the syntrophically glycolate-degrading coculture FlGlyM (Friedrich et al. 1991) in which two bacteria share the energy derived from methanogenic glycolate degradation ($\Delta G_0' = -63$ kJ per mol glycolate). On the basis of our current results, we have to assume that energy conservation by strain PerGlyS depends exclusively on chemiosmotic mechanisms.

A forthcoming study will define the phylogenetic status of strain PerGlyS and the fermenting strain FlGlyM on the basis of 16S rRNA analysis. Strain PerGlyS has been deposited with the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany), under DSM 9705.

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