Phylogenetic Positions of *Desulfofustis glycolicus* gen. nov., sp. nov., and *Syntrophobotulus glycolicus* gen. nov., sp. nov., Two New Strict Anaerobes Growing with Glycolic Acid

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The glycolate-oxidizing, sulfate-reducing bacterium strain PerGlyS and the syntrophically glycolate-oxidizing bacterium strain FIGlyR were studied with respect to their phylogenetic relationships on the basis of *in vitro* amplification and direct sequencing of 16S rRNA-encoding DNA. Strain PerGlyS clustered with representatives of the δ subclass of the class *Proteobacteria*, close to "*Desulforhopalus vacuolatus*" but sufficiently distinct to preclude its assignment to this genus. These organisms, together with *Desulfobulbus propionicus*, represent a phylogenetic subgroup among members of the δ subclass of *Proteobacteria*. Strain FIGlyR was found to cluster with the gram-positive bacteria with low-G+C DNA, and *Desulfotomaculum dehalogenans* and *Desulfotomaculum orientis* are its closest relatives. Other species of the genus *Desulfotomaculum* are phylogenetically only moderately closely related to these organisms. These results necessitate the establishment of new genera and species for these two strains. Strain PerGlyS was designated the type strain of *Desulfofustis glycolicus* gen. nov., sp. nov., and strain FIGlyR was designated the type strain of *Syntrophobotulus glycolicus* gen. nov., sp. nov.

Glycolate is an important constituent of fruits and sugar cane (15, 23) and is excreted by algae and autotrophic prokaryotes under conditions of carbon dioxide limitation and excess oxygen (1–3). Aerobic degradation of glycolate has been studied in detail; its anaerobic degradation has aroused interest only recently (9, 12). Defined cocultures of fermenting bacteria with homoacetogenic or methanogenic bacteria were isolated which converted glycolate completely to carbon dioxide and hydrogen, with concomitant reduction of carbon dioxide to either acetate or methane (9). The primary fermenting partner in these cocultures was isolated later in pure culture with glyoxylate as the substrate (strain FIGlyR; 11). This strain has been studied in detail with respect to its biochemistry; glycolate oxidation to glyoxylate and vice versa is coupled to a membrane-bound electron transport system that catalyzes either a proton potential-driven reversed electron transport from glycolate to hydrogen or a hydrogen-dependent glyoxylate reduction coupled to ATP synthesis by electron transport phosphorylation (10, 11). Also a glycolate-oxidizing sulfate-reducing bacterium, strain PerGlyS, was isolated that deserves attention because of its rather high desulforubidin content and the fact that only some part of acetyl intermediates is oxidized to CO₂ (12).

The present communication reports on the phylogenetic positions of these two strains and is based on sequence data obtained by direct sequencing of 16S rRNA-encoding DNA.

MATERIALS AND METHODS

Pure cultures of strains FIGlyR (DSM 8271) and PerGlyS (DSM 9705) were taken from our laboratory collection. Both have been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, under the reference numbers indicated. Strain FIGlyR was originally isolated from anoxic sewage sludge, and strain PerGlyS was from marine sediment obtained from a channel in Venice, Italy.

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Both strains were cultivated in a bicarbonate-buffered, sulfide- or cysteine-reduced mineral medium (21, 30) which contained trace element solution SL10 (29), selenite-tungstate solution (29), and seven-vitamin solution (30). Details of cultivation and characterization are given in the original descriptions (9, 12).

In vitro amplification and direct sequencing of 16S rRNA encoding DNA were performed as described earlier (24). The new sequences were added to an alignment of about 5,000 complete or partial 16S rRNA primary structures from bacteria (17, 27) by using the alignment tool of the ARB program package (25). Phylogenetic analyses were performed by applying the distance matrix (ARB, PHYLIP; 8), maximum-parsimony (ARB, PHYLIP), and maximum-likelihood (fastDNAml; 17) methods to different sets of data. Distance matrix and maximum-parsimony analyses were performed with sets of data comprising all available 16S rRNA primary structures from gram-positive bacteria with low-G+C DNA (about 850 sequences) and the δ subclass of the class *Proteobacteria* (66 sequences), as well as selected reference sequences from each of the remaining major phylogenetic groups of bacteria. Maximum-likelihood methods were used to analyze subsets of about 50 sequences comprising the homologous sequences of the nearest neighbors of the organisms studied here and selected outgroup references from other phylogenetic groups. The sets of data varied with respect to the reference sequences, as well as the alignment positions included. The variabilities of the individual alignment positions were determined by using the respective tool of the ARB package and used as a criterion to successively remove highly variable positions from the set of data. This was done to recognize and minimize treeing artifacts resulting from alignment errors, database inconsistencies, and false identities (multiple base changes) in highly variable regions.

Nucleotide sequence accession numbers. The 16S rRNA-encoding DNA sequences of strains PerGlyS and FIGlyR were entered into the Ribosomal Database under accession numbers 99707 and 99706, respectively.

RESULTS AND DISCUSSION

The 16S rRNA gene sequences from glycolate-utilizing strains PerGlyS and FIGlyR were determined by direct sequencing of *in vitro*-amplified rRNA-encoding DNA. The moderate overall similarity (78%) of the two sequences indicates that the strains represent different lines of descent. The isolates PerGlyS and FIGlyR could unambiguously be assigned to the major bacterial phylogenetic groups comprising the δ subclass of *Proteobacteria* and the gram-positive bacteria with low-G+C DNA, respectively (18).

The phylogenetic relationships of strains PerGlyS and FIGlyR and a selection of related bacteria are shown in Fig. 1. The tree is based on the results obtained by performing a distance matrix analysis of a set of data comprising all currently

FIG. 1. 16S rRNA-based phylogenetic tree reflecting the relationships of glycolate-utilizing strains PerGlyS (*Desulfobulbus propionicus*) and FIGlyR (*Syntrophobacter*). The tree topology was derived by performing distance matrix analyses and corrected in accordance with the results of maximum-parsimony and maximum-likelihood analyses. The subtrees of *Proteobacteria* and gram-positive bacteria were optimized independently by including sequence positions which contain identical residues in at least 50% of all available 16S rRNA sequences from the δ subclass of *Proteobacteria* and gram-positive bacteria with DNA with low G+C content, respectively. The triangles indicate groups of phylogenetically related species or genera. The bar indicates 10% estimated sequence divergence. 1, *Desulfobacter* spp. plus *Desulfobacterium*, *Desulfobacula*, *Desulfobutulus*, *Desulfococcus*, and *Desulfosarcina* spp.; 2, *Syntrophobacter* spp. plus *Desulfohalobium* and *Desulfococcus* spp.; 3, *Pelobacter* spp. plus *Desulfuromonas* and *Geobacter* spp.; 4, *Desulfovibrio* spp. plus *Bilophila* and *Desulfomicrobium* spp.; 5, *Selenomonas* spp. plus *Acidaminococcus*, *Acetonebacter*, *Dialister*, *Megasphaera*, *Pectinatus*, *Phascolarctobacterium*, *Selenomonas*, *Veillonella*, and *Zymophilus* spp.

available 16S rRNA sequences from the (phylogenetically) gram-positive bacteria with low-G+C DNA and the δ subclass of *Proteobacteria*. Homologous primary structures from selected representatives of all major phylogenetic groups (17, 32) were included as references. Only sequence positions which contain identical residues in at least 50% of all available sequences from gram-positive bacteria with low-G+C DNA were used for tree construction. This was done to reduce potential treeing artifacts which may result from multiple base changes at highly variable positions (16). Given that no closely related organisms are known or have been analyzed with respect to their 16S rRNAs, deletion of highly variable sequence positions should not reduce the phylogenetic information content of the sets of data (16). Changing the selection of positions by applying the 50% invariance criterion to the set of data of available δ -subclass proteobacterial sequences did not change the respective subtree. The tree topology was evaluated by performing maximum-parsimony analyses of the same sets of data, as well as maximum-likelihood analyses of reduced sets of data (about 50 sequences) comprising the selection shown in Fig. 1 and a varying collection of additional reference sequences. The subtrees of *Proteobacteria* and gram-positive bacteria were optimized independently, including sequence posi-

tions which contain identical residues in at least 50% of all available 16S rRNA sequences from the δ subclass of *Proteobacteria* and gram-positive bacteria with low-G+C DNA, respectively.

Multifurcations in the tree of Fig. 1 indicate branchings for which a relative order could not unambiguously be determined or was not supported by (at least the majority of) the analyses performed by applying different treeing methods. *Escherichia coli* and *Bacillus subtilis* were included in Fig. 1 to indicate the phylogenetic depths of *Proteobacteria* and gram-positive bacteria with low-G+C DNA, respectively.

Among the *Proteobacteria* of the δ subclass, strain PerGlyS, "*Desulforhopalus*" sp. (14), and *Desulfobulbus* sp. (6) represent a phylogenetic cluster as also indicated by similarity values of 90.6 and 86.5%, respectively (Table 1). The corresponding values shared by strain PerGlyS and the other representatives of the δ subclass are 80.6 and 85.4%. No closer relationship between this cluster and *Syntrophobacter* (13), *Syntrophus* (28), or *Desulfobulbus* spp. was supported by results obtained by applying different treeing analyses.

Within the phylogenetic group of gram-positive bacteria with low-G+C DNA, *Desulfotomaculum dehalogenans* (26) and *Desulfotomaculum orientis* (6) share 91.2% and 88.6% 16S

TABLE 1. Overall 16S rRNA sequence similarities for strain PerGlyS and related reference organisms^a

Organism(s)	% Similarity to:										
	PGS	Dva	Dpr	Sbu	Sge	Dbu	Sba	Pba	Mba	Des	Eco
PGS											
Dva	90.6										
Dpr	86.5	87.6									
Sbu	84.7	84.1	86.0								
Sge	85.4	85.0	86.3	98.3							
Dbu	82.2	83.5	83.6	84.9	85.2						
Sba	83.8	83.4	84.2	83.8	84.0	82.6					
Pba	83.4	84.5	84.3	85.0	85.4	83.9	84.5				
Mba	80.6	81.0	82.3	82.7	82.8	80.4	82.1	83.0			
Des	81.8	81.9	82.1	81.3	81.7	81.4	81.3	82.4	80.0		
Eco	79.7	79.8	79.8	80.3	80.1	78.8	78.8	81.0	79.0	78.9	
Gpl	78.3	78.9	79.3	79.5	79.7	78.6	79.6	80.7	78.3	79.2	77.4

^a The values indicate the fraction of identical residues within the respective sequence pairs calculated by including only positions present in both sequences. Mean values are given for phylogenetic groups. Abbreviations: Dba, *Desulfobacter*, *Desulfobacterium*, *Desulfobacula*, *Desulfobutulus*, *Desulfococcus*, and *Desulfosarcina* spp.; Des, *Desulfovibrio* spp.; Dpr, *Desulfobulbus propionicus*; Dva, *Desulforhopalus vacuolatus*; Eco, *Escherichia coli*; Mba, myxobacteria; Pba, *Pelobacter*, *Desulfuromonas*, *Desulfuromusa*, and *Geobacter* spp.; Gpl, gram-positive bacteria with DNA with low G+C content; PGS, PerGlyS; Sba, *Syntrophobacter*, *Desulforhabdus*, and *Desulfoarculus* spp.; Sbu, *Syntrophus buswellii*; Sge, *Syntrophus gentianae*.

rRNA sequence similarity, respectively, with strain FIGlyR (Table 2). The corresponding values for these organisms and other members of the major phylogenetic group of gram-positive bacteria with DNA with a low G+C content are slightly lower (83.8 to 85.9%). Most analyses supported a moderate relationship of this group to *Heliobacterium* spp. (33), the remaining *Desulfotomaculum* species (6, 7, 19, 20), and other phylogenetic gram positives with gram-negative cell walls such as *Acidaminococcus*, *Acetonea*, *Dialister*, *Megasphaera*, *Pectinatus*, *Phascolarctobacterium*, *Selenomonas*, *Veillonella*, and *Zymophilus* spp. (4, 5, 22, 31, 34). However, the significance of such a grouping is low, as indicated by the short internode distance of the branch containing the former organisms or groups and the *Syntrophospora*-*Syntrophomonas* (34) branch.

The results presented here justify the establishment of new genera and species for strains PerGlyS and FIGlyR, for which we propose the names *Desulfofustis glycolicus* and *Syntrophobutulus glycolicus*, respectively. The descriptions are based on references 9 and 12.

Description of *Desulfofustis* gen. nov. *Desulfofustis* (De.sul .fo.fus'tis. L. prefix *de*, off, L. n. *sulfur*, sulfur, *fustis*, club). M. L. m. n. *Desulfofustis*, a sulfate-reducing club. The description of this new genus is identical to that of its only species, *D. glycolicus* sp. nov.

Description of *Desulfofustis glycolicus* sp. nov. *glycolicus* (gly.co'li.cus. M. L. n. *acidum glycolicum*, glycolic acid). *glycolicus*, referring to glycolic acid as the key substrate of this species. Gram-negative, rod-shaped bacteria, straight to slightly curved, 2.0 to 4.5 by 0.55 μ m in size, occurring typically as single cells. Motile by one subterminally inserted flagellum, no spore formation.

Strictly anaerobic bacteria, growing chemotrophically by oxidation of glycolate, glyoxylate, L-lactate, L-malate, fumarate, succinate, yeast extract, or hydrogen (in the presence of carbon dioxide and traces of acetate), with sulfate as an electron acceptor. Other substrates (formate, acetate, propionate, butyrate, isobutyrate, isovalerate, valerate, caproate, 3-methylbutyrate, fructose, glucose, oxalate, pimelate, glutarate, glycolal-

TABLE 2. Overall 16S rRNA sequence similarities for strains FIGlyR and related reference organisms^a

Organism(s)	% Similarity to:												
	FGR	Ddh	Dor	Hel	Sel	Dni	Dru	Dau	Dge	Dth	Sbr	Swf	Bsu
FGR													
Ddh	91.2												
Dor	88.6	92.1											
Hel	86.5	87.5	84.8										
Sel	82.6	83.0	82.2	82.9									
Dni	84.7	85.5	83.1	86.3	82.2								
Dru	84.8	85.9	84.5	85.2	82.6	91.5							
Dau	83.3	84.5	83.6	83.2	80.8	85.5	83.7						
Dge	83.3	86.3	84.1	84.4	82.2	87.1	86.8	87.3					
Dth	83.6	85.3	84.2	84.4	81.9	86.7	84.6	90.1	88.5				
Sbr	84.6	84.7	83.5	84.6	80.8	83.1	83.4	84.0	83.0	84.5			
Swf	83.6	84.0	83.5	83.5	80.2	82.2	82.6	84.6	82.6	84.0	92.8		
Bsu	83.7	84.3	83.7	83.9	81.5	83.4	83.6	83.1	83.5	84.5	82.9	82.6	
Pbd	78.6	79.4	78.4	79.9	77.3	78.8	78.9	78.6	79.5	79.3	79.6	79.1	79.2

^a The values indicate the fraction of identical residues within the respective sequence pairs calculated by including only positions present in both sequences. Mean values are given for phylogenetic groups. Abbreviations: Bsu, *Bacillus subtilis*; Dau, *Desulfotomaculum australicum*; Ddh, *Desulfotomaculum dehalogenans*; Dge, *Desulfotomaculum geothermicum*; Dor, *Desulfotomaculum orientis*; Dni, *Desulfotomaculum nigrificans*; Dru, *Desulfotomaculum ruminis*; Dth, *Desulfotomaculum thermobenzoicum*; FGR, FIGlyR; Hel, *Heliobacterium chlorum*, *H. fasciatum*, *H. gestii*, *H. mobilis*, and *H. modestocaldum*; Pbd, δ subclass of *Proteobacteria*; Sbr, *Syntrophospora bryantii*; Sel, *Selenomonas*, *Acidaminococcus*, *Acetonea*, *Dialister*, *Megasphaera*, *Pectinatus*, *Phascolarctobacterium*, *Veillonella*, and *Zymophilus* spp.; Swf, *Syntrophomonas wolfei*.

dehyde, glyceraldehyde, simple aliphatic alcohols, betaine, choline, cysteine, triethanolamine, glycine, indol, and benzoate) not oxidized. Sulfite and elemental sulfur reduced with glycolate as electron donor, thiosulfate or nitrate not reduced. Cells contain menaquinone-5(H₂), desulfurubidin, and cytochromes *b* and *c*, no desulfoviridin.

Organic substrates other than glycolate are typically incompletely oxidized, but part of acetyl intermediates may be oxidized, as well, to various extents.

pH range, 6.7 to 8.3; optimum pH, 7.3. Temperature range, 15 to 37°C; optimum temperature, 28°C. Growth optimal in marine medium with 340 mM NaCl–14 mM MgCl₂. Growth possible also in brackish-water medium with one-third of this salt concentration; no growth in freshwater medium. DNA base ratio, 56.2 ± 0.1 mol% G+C (determined by high-pressure liquid chromatography analysis). Habitat, anoxic marine sediments.

Type strain, PerGlyS (DSM 9705), deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Description of *Syntrophobotulus* gen. nov. *Syntrophobotulus* (Syn.tro.pho.bo'tu.lus. Gr. prefix *syn*, together, gr. v. *trephein*, to nourish, L. *n. botulus*, sausage. M. L. m. n. *Syntrophobotulus*, a syntrophic, sausage-like bacterium. The description of this new genus is identical to that of its only species, *S. glycolicus* sp. nov.

Description of *Syntrophobotulus glycolicus* sp. nov. *glycolicus* (gly.co'li.cus. M. L. n. *acidum glycolicum*, glycolic acid.) *glycolicus*, referring to glycolic acid, the key substrate of this species.

Rod-shaped bacterium, slightly curved, 2.5 to 3.5 by 0.5 µm in size, occurring typically as single cells or in small aggregates. Nonmotile. Oval spores formed in ageing cultures at the cell ends. Gram staining is negative, ultrastructural analysis shows gram-positive cell wall architecture.

Strictly anaerobic bacteria, growing chemotrophically in pure culture by fermentative oxidation of glyoxylate. Glycolate is oxidized in syntrophic coculture with, e.g., *Methanospirillum hungatei* or *Acetobacterium woodii* as a partner. No other organic or inorganic substrates used. Glycolic acid is converted to carbon dioxide and hydrogen in syntrophic culture; glyoxylic acid is fermented in pure culture to carbon dioxide, hydrogen, and glycolic acid. Neither sulfate, sulfite, thiosulfate, elemental sulfur, nor nitrate reduced. Cells contain menaquinone-7-10, with MK-9 as major fraction. No cytochromes present. pH range, 6.7 to 8.3; optimum pH, 7.3. Temperature range, 15 to 37°C; optimum temperature, 28°C. Growth optimal in freshwater medium. Growth possible also in brackish-water medium with 110 mM NaCl–5 mM MgCl₂; no growth in marine medium. DNA base ratio, 46.7 ± 0.15 mol% G+C (determined by high-pressure liquid chromatography analysis). Habitat, sewage sludge and anoxic freshwater sediments. Type strain, FIGlyR (DSM 8271), deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

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