J. Gen. Appl. Microbiol., **64**, 1–8 (2018)
doi 10.2323/jgam.2017.02.009

jGAM

Full Paper

Bacterial glycerol oxidation coupled to sulfate reduction at neutral and acidic pH

(Received September 6, 2016; Accepted February 28, 2017; J-STAGE Advance publication date: November 29, 2017)

Sidnei C. Santos,[1,2,*] Martin G. Liebensteiner,[1] Antonie H. van Gelder,[1] Mauricio R. Dimitrov,[1] Paulo F. Almeida,[3] Cristina M. Quintella,[4] Alfons J. M. Stams,[1,5] and Irene Sánchez-Andrea[1]

[1] *Laboratory of Microbiology, Wageningen University, P.O. Box 8033, 6700 HB Wageningen, the Netherlands*
[2] *Studies Institute in Health and Biological, Federal University of South and Southeast of Pará, Av. dos Ipês, Cidade Jardim, 68500-00, Marabá, Pará, Brasil*
[3] *Laboratory of Biotechnology and Ecology of Microorganisms, Department of Bio-Interaction Sciences, Federal University of Bahia, Av. Reitor Miguel Calmon, Vale do Canela, 40110-902, Salvador, Bahia, Brazil*
[4] *Laboratory of Kinetic and Molecular Dynamic, Institute of Chemistry, Federal University of Bahia, Campus de Ondina, 40170-290, Salvador, Bahia, Brazil*
[5] *Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal*

Glycerol is a main co-product of biodiesel production. Crude glycerol may serve as a cheap and attractive substrate in biotechnological applications, e.g. for the production of valuable chemicals or as an electron donor for reduction processes. In this work, sulfate reduction with glycerol was studied at neutral and acidic pH using bioreactor sludge samples and Tinto River sediments as a source of inoculum, respectively. Communities of sulfate-reducing bacteria (SRB) and fermentative bacteria were co-enriched at both pH values. Molecular analyses revealed that sequences belonging to *Desulfomicrobium* genus were dominant in the cultures enriched at pH 7, while *Desulfosporosinus* sequences dominated in the culture enriched at pH 4. Glycerol conversion was coupled to sulfate reduction, but the substrate was incompletely oxidized to acetate in the neutrophilic enrichments, and acetate, lactate, and 1,3-propanediol under low pH conditions. Two strains belonging to *Desulfomicrobium* and *Proteiniphilum* genera were isolated from the neutrophilic enrichments, but the first isolate was not able to use glycerol, which suggests a syntrophic relationship between glycerol-degrading fermentative bacteria and SRB. A *Clostridium* strain able to grow with glycerol was isolated from the low pH enrichment. Our data indicate that glycerol promotes the growth of sulfate-reducing communities to form sulfide, which can be used to precipitate and recover heavy metals.

Key Words: fermentative bacteria; glycerol; metal recovery; sulfate-reducing bacteria; syntrophy

Introduction

Biodiesel is a biofuel that is produced by the transesterification of vegetable oils or animal fats with ethanol or methanol, resulting in the formation of crude glycerol as a main by-product (Silva et al., 2009). Massive production of biodiesel has created opportunities for the biotechnological application of glycerol, as its market price has dropped considerably in the past decade (Gholami et al., 2014; Yang et al., 2012). Currently, crude glycerol is considered as a waste product due to the presence of impurities such as alcohol, salts, and heavy metals (Johnson and Taconi, 2007). Innovative processes to apply glycerol are under development, such as the catalytic conversion of glycerol to propylene glycol (Chiu et al., 2006), methyl acetate (García et al., 2008), hydrogen (Slinn et al., 2008) or syngas (Yoon et al., 2010).

Several microorganisms are able to grow anaerobically with glycerol as the sole carbon and energy source (Biebl et al., 1999; Seifert et al., 2001). Microbial glycerol fermentation is an interesting way to produce valuable compounds such as 1,3-propanediol (1,3-PDO), acetic acid, butyric acid, acetone, ethanol, 2,3-butanediol, lactic acid,

*Corresponding author: Sidnei C. Santos, Studies Institute in Health and Biological, Federal University of South and Southeast of Pará, Av. dos Ipês, Cidade Jardim, 68500-00, Marabá, Pará, Brasil.
Tel: +55-94-2101-7116 E-mail: sidnei.cerqueira@unifesspa.edu.br

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

succinic acid, formate, or H_2 (Biebl et al., 1999; Ito et al., 2005; Kivistö et al., 2010; Wischral et al., 2016; Yang et al., 2007). Glycerol fermentation has been observed in species belonging to the genera Citrobacter, *Clostridium*, *Enterobacter*, *Klebsiella*, *Propionibacterium*, *Propionispora* and *Trichococcus* (Abou-Zeid et al., 2004; Biebl et al., 1999; Himmi et al., 2000; Johnson and Taconi, 2007; van Gelder et al., 2011). Glycerol may also be used as an electron donor for reduction processes such as sulfate reduction. Sulfate reduction is a process that can be applied to the removal of heavy metals from waste streams (Bertolino et al., 2014; Hulshoff Pol et al., 2001). Sulfate-reducing bacteria occur in anoxic habitats, where they reduce sulfate to sulfide with organic compounds or hydrogen as an electron donor (Muyzer and Stams, 2008). Several *Desulfovibrio* species were reported to grow with glycerol (Kremer and Hansen, 1987; Nanninga and Gottschal, 1987; Qatibi et al., 1991; Stams et al., 1985). In the presence of sulfate, *D. carbinolicus* and *D. fructosovorans* oxidize glycerol to 3-hydroxypropionate and acetate, respectively, while both strains ferment glycerol to 1,3-propanediol and 3-hydroxypropionate in the absence of sulfate (Nanninga and Gottschal, 1987). The ability to use glycerol was also shown for members of the genus *Desulfosporosinus* (Alazard et al., 2010; Sánchez-Andrea et al., 2013, 2015). The aim of the current study was to investigate the potential use of glycerol as an electron donor for sulfate reduction, using neutrophilic and acidophilic inocula as sources of microorganisms.

Materials and Methods

Inocula. Three different inocula were used for the enrichments: two neutrophilic sludge samples from bioreactors and one sample taken from a sulfate-reducing enrichment with a low pH sediment (Tinto River). The neutrophilic sludges were obtained from anaerobic bioreactors for biological sulfate reduction to produce sulfide for metal removal. One sludge originated from a chemical plant in Emmen, The Netherlands (Dar et al., 2007) and the other sludge from a zinc smelter in Budel, The Netherlands (Sipma et al., 2003). These sludges were termed Paques sludge and Budel sludge, respectively. The sludges were crushed as described by Oude Elferink et al. (1995). The third sample was a mixture of acidic sediments that were collected from the Tinto River Basin (Huelva) in September 2011 at two sampling sites: JL (37.691207°N, 6.560587°W) and SN (37.72173°N, 6.557465°W) dams. Detailed information of the sampling sites was presented recently (Sánchez-Andrea et al., 2012).

Medium composition and growth conditions. The basal medium that was used for enrichment contained the following (g/l): 0.53 $Na_2HPO_4{\cdot}2H_2O$, 0.41 KH_2PO_4, 0.3 NH_4Cl, 0.11 $CaCl_2{\cdot}2H_2O$, 0.1 $MgCl_2{\cdot}6H_2O$, 0.3 NaCl and 0.48 $Na_2S{\cdot}9H_2O$, trace elements and a vitamin solution prepared as previously described (Stams et al., 1993). The basal medium was supplemented with 0.1 g/l yeast extract (BBL, Becton Dickinson, Cockeysville, MD). Two versions of this medium were made, a neutral (pH 7) and an acidic (pH 4) version. For the neutral medium, 4 g/l $NaHCO_3$ were added as a buffer. Sulfate (20 mM) and glycerol (20 mM) were added as electron acceptor and donor, respectively. Finally, neutralised or slightly acidified sodium sulphide (2 mM) was added as a reducing agent. All compounds were heat-sterilized at 121°C for 20 min except for the vitamins and the Na_2S solution, which were filter-sterilized. The 120-ml serum bottles filled with 50 ml medium were sealed with butyl rubber stoppers (Rubber BV, Hilversum, the Netherlands). The gas phase was 1.5 atm N_2/CO_2 (80:20, v/v). Primary neutrophilic and acidophilic enrichments were incubated statically at 30°C for 11 and 35 days, respectively. Biological duplicates were made in all the enrichments.

Isolation of bacteria. To obtain axenic cultures, serial dilutions from primary enrichments were made in fresh medium. Bacteria were isolated from the highest dilution showing growth by spreading 0.1 ml of diluted samples on agar plates (agar 1.5%, w/v) prepared with medium that contained glycerol as substrate and sulfate as electron acceptor. These handlings were done inside an anaerobic chamber and the plates were incubated in anaerobic jars. One percent of Wilkins-Chalgren anaerobe broth (CM0643, Thermo Scientific, Waltham, MA), containing tryptone (10 g/l), gelatin peptone (10 g/l), yeast extract (5 g/l), glucose (1 g/l) and sodium pyruvate (1 g/l) as a typical formula, was also used as substrate to isolate bacteria that are unable to use glycerol as substrate. Individual colonies that became visible between 3 to 7 days of incubation were streaked on new plates again. The streaking procedure was repeated two times more. Finally, single colonies were picked up and inoculated into serum bottles. The purity of the cultures was checked by microscopic examination after growth, and by molecular biological analyses. A phase contrast microscope (Leica DM 2000) equipped with a digital camera (Leica DFC 420) was used to examine the morphology of the cultures.

Bacterial strains. *Desulfomicrobium baculatum* (DSM 4028^T) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. Bacteria were first cultured in medium number 63 (DSMZ) and transferred into serum bottles containing basal medium with glycerol (20 mM) as substrate and sulfate (20 mM) as electron acceptor. This strain was also tested for hydrogen utilization as electron donor in vials flushed with H_2/CO_2 as headspace.

Analytical methods. Sulfate reduction activity of cultures was monitored by measuring sulfide colorimetrically with the methylene blue method (Trüper and Schlegel, 1964). Bacterial growth was assessed by measuring the optical densities at 600 nm (OD_{600}). Organic acids and alcohols were analysed by high-pressure liquid chromatography (Thermo Scientific SpectraSystem HPLC, Waltham, MA) equipped with a Metacarb 67H column (300 × 6.5 mm) (Varian, Walnut Creek, CA), kept at 30°C, and a refractive index detector. The mobile phase was 0.005 M sulfuric acid with a flow rate of 0.8 ml/min. Gases were measured with a Gas Chromatograph (Shimadzu GC-2014, Kyoto, Japan) equipped with a Molsieve 13X column (2 m × 3 mm) and hold at 100°C. Injections were direct on column via an injection block held at 100°C. The carrier gas was argon. The detector was a TCD detector, hold at 130°C

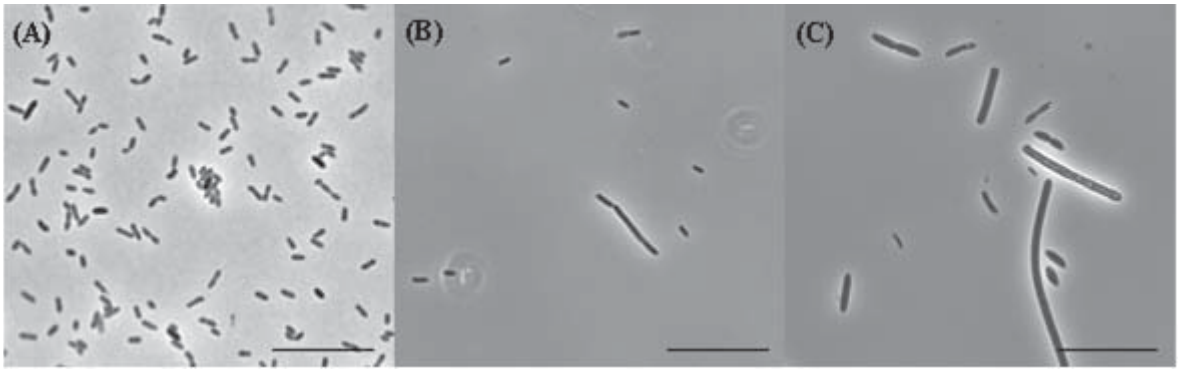

Fig. 1. Cell morphology of bacteria from Paques (A), Budel (B), and Tinto River (C) enrichments with glycerol and sulfate. Bar represents 10 μm.

with a current of 70 mA. For the quantification of sulfate, an ion chromatograph (Thermo Scientific SpectraSystem HPLC, Waltham, MA) equipped with IonPac AS22 analytical column (4 × 250 mm) and Ed40 electrochemical detector (Thermo Scientific, Waltham, MA) was used. The eluent was a mixture of 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate and the eluent flow rate was set at 1.0 ml/min. All the instruments' control and data collection were performed with ChromQuest 5.0 software (Thermo Scientific, Waltham, MA).

DNA extraction and PCR amplification. Total DNA from enrichments and pure cultures were extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. Polymerase chain reaction (PCR) amplifications were performed using the isolated DNA and the forward primers Bact27-f (Weisberg et al., 1991) and Arch109-f (Genthner et al., 1997) targeting the bacterial and archaeal 16S rRNA gene, respectively, and the universal reverse primer Uni1492-r (Weisberg et al., 1991). PCR products were checked by electrophoresis on a 1% agarose gel (w/v). Gel was stained with Sybr® Safe (Invitrogen, Carlsbad, CA) and visualized on a gel-doc system (Bio-Rad, Hercules, CA).

DGGE analysis. Extracted DNA from enriched and pure cultures was amplified by PCR using the primer F968-GC set that targets the V6-V8 regions of the 16S rRNA (Nübel et al., 1996). Forward primer 1401R had attached to its 5′ end a GC clamp (Nübel et al., 1996). PCR products were analysed by Denaturing Gradient Gel Electrophoresis (DGGE) using a Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA) (Muyzer et al., 1993). DGGE was performed on polyacrylamide gels with a denaturant gradient from 30% to 60% for the separation of 16S rRNA gene amplicons (100% denaturing acrylamide was defined as 7 M urea and 40%, v/v, formamide). Aliquots of PCR products were loaded on the gel and electrophoresis was carried out with 0.5 X Tris-acetate-EDTA buffer at 60°C and at 85 V for 16 h. After the completion of the electrophoresis, gels were silver-stained (Sanguinetti et al., 1994) and scanned.

Cloning and sequencing of microbial 16S rRNA. 16S rRNA gene fragments amplified by PCR using the primer pairs Bact27-f and Uni1492-r (Weisberg et al., 1991) were purified using the High Pure PCR Cleanup Micro kit (Roche Applied Science, Almere, The Netherlands). PCR products were cloned in *Escherichia coli* DH5α competent cells by using the pGEM-T vector (Promega, Fitchburg, WI) according to the manufacturer's instructions. Individual colonies were screened by PCR using the primers Sp6 and T7 (Promega). PCR products were sequenced with primers mentioned above at GATC Biotech (Konstanz, Germany). Archael PCR was also done using amplification products obtained with archaeon DNA from *Archaeoglobus fulgidus* VC-16 (obtained from the DSMZ) (Genthner et al., 1997).

Phylogenetic analysis of 16S rRNA gene sequences. Sequences were assembled using the DNABaser (v3.4.3) program, and prior to phylogenetic analysis, vector sequences flanking the 16S rRNA gene inserts were removed. Similarity searches were performed using BLAST (Altschul et al., 1990) and the Ribosomal Database Project (Maidak et al., 2001) to identify most closely related sequences. Clone sequences were checked for chimeras using the program Chimera Check from green genes (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi). A total of 88 complete sequences (>1400 bp) were obtained in this study.

Nucleotide sequence accession numbers. Sequences of the 16S rRNA gene clones and isolated bacteria were deposited in the GenBank database under accession numbers: KC215456 to KC215458, KC215460 to KC215464 and KP178480 to KP178483.

Results

Enrichment of bacteria with glycerol and sulfate

Bacteria were enriched, at pH 4 from acidic mixed sediment samples and at pH 7 from two neutrophilic sludges, with glycerol and sulfate. The cultures were obtained by repeated transfers of full-grown cultures to the same fresh medium. Routinely, when using an inoculum size of 1% full-grown cultures were obtained in about 10 and 30 days for neutral and acid enrichments, respectively.

Upon microscopic observation, the neutrophilic enrichments consisted of different kinds of rod-shaped cells (Figs. 1A and B). Spores were also observed. The Tinto River enrichment also contained different forms of cells (Fig. 1C).

Table 1. Main products formed by glycerol (20 mM) degradation in enrichments in the presence (20 mM), or absence, of sulfate.

Inocula	Products (mM)							pH_{start}	pH_{end}	OD (600 nm)
	glycerol converted	lactate	acetate	formate	1,3-PDO	H_2	sulfide			
Tinto River	15.1	1.6	1.1	—	4.5	—	2.5	4.0	5.5	0.25
	17.7	—	—	—	4.8	2.7	na	4.0	4.0	0.10
Paques	16.0	—	13.5	—	—	—	3.8	7.3	7.5	0.17
	10.9	—	4.2	2.5	—	16.6	na	7.3	7.4	0.10
Budel	11.9	—	9.0	—	—	—	3.3	7.4	7.5	0.11

—, Not detected; na, not applicable as no sulfate was present.

Table 2. Bacterial clone libraries from enrichments grown on glycerol and sulfate.

Two samples derived from enrichments at neutral pH (Paques, Budel) and one at acidic pH (Tinto River). The abundance, GenBank accession number, the phylogenetic affiliation of the closest cultivated relative and rRNA gene similarity are indicated.

Inocula	Clones		*Phylogenetic affiliation	Similarity (%)
	Number	Accession No.	Accession No.	
Paques	31	KC215457	*Desulfomicrobium norvegicum*	99
			Desulfomicrobium baculatum	99
	7	KP178482	*Sphaerochaeta*	98
	4	KP178481	*Proteiniphilum*	96
	3	KP178483	*Porphyromonadaceae bacterium*	96
Budel	12	KC215456	*Desulfomicrobium norvegicum*	99
			Desulfomicrobium baculatum	99
Tinto River	25	KC215458	*Desulfosporosinus acididurans*	99
	3	KC215460	*Microbacter margulisiae*	99

*Similarity to nearest neighbour in the GenBank nucleotide database as determined by BLAST results.

Glycerol-degrading enrichments from all three different sources (Paques, Budel, Rio Tinto) showed growth in the presence of sulfate to the medium (Table 1). The acidic enrichments (Rio Tinto) differed from the ones with neutral pH (Budel and Paques) regarding the spectrum of fermentation products. Acetate was the main product at neutral pH, whereas at acidic pH mostly 1,3-PDO accumulated besides traces of lactate and acetate (Table 1). In the absence of sulfate the accumulation of acetate, formate, and hydrogen was visible in the pH neutral enrichment (Paques), whereas 1,3-PDO and H_2 accumulated in the acidic enrichment when sulfate was left out (Table 1). The Budel enrichment was not further analysed in the absence of sulfate. The unbalanced stoichiometries for both, setups with and without sulfate (Table 1), indicate that reduced metabolites (e.g. alcohols, fatty acids) must have remained undetermined in this study. Methane was not detected in any of the enrichment cultures. Also the formation of sulfide was lower than theoretically expected and was apparently only formed by the oxidation of compounds intermediately formed during the conversion of glycerol.

Community analysis of the enrichments

With archaeal primers, no PCR products were obtained, suggesting that Archaea were not enriched under the selected conditions. This is consistent with the observation that methane was not formed in any of the enrichments.

To obtain an insight into which bacteria were involved in the conversion of glycerol with sulfate, clone libraries were made. From the 45 clones of the Paques enrichment, 31 belonged to a sulfate reducer closely related to *Desulfomicrobium norvegicum* and *D. baculatum* (both 99% sequence similarity), 7 were associated with the genus *Sphaerochaeta*, 4 with the genus *Proteiniphilum* and 3 with an unclassified member of the family Porphyromonadaceae (Table 2). All 12 clones of the Budel enrichment belonged to the genus *Desulfomicrobium* (Table 2). DGGE analysis of the enriched cultures showed two dominant bands (1–4) in both Budel and Paques enrichments (Fig. 2). However, also a single species can show different bands in DGGE (Nübel et al., 1996; Satokari et al., 2001). From the Tinto River enrichments, 28 clones were obtained of which 25 had 99% sequence identity with *Desulfosporosinus acididurans* (Table 2). Three other clones were related to *Microbacter margulisiae* (99% sequence similarity), recently isolated from the same environment (Sánchez-Andrea et al., 2014) (Table 2).

Isolation of strains

Attempts were made to isolate glycerol-degrading

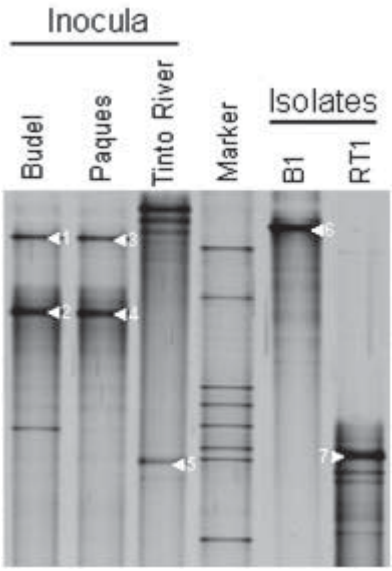

Fig. 2. DGGE profiles of bacterial PCR-amplified 16S rRNA gene fragments obtained from enrichments: Budel, Paques, and Tinto River, and pure cultures: B1 isolated from Budel enrichment and RT1 isolated from Tinto River enrichment.

Numbers (1 to 7) indicate the most representative bands of each DGGE profile.

sulfate-reducing bacteria from the Budel and Paques enrichments. Colonies were formed when enrichments were plated on media with glycerol and sulfate. However, the colonies that were obtained always consisted of two bacteria with the morphologies that were observed in the enrichments. By using Wilkins-Chalgren as substrate, a sulfate-reducer (strain B1) was isolated from the sulfate-reducing Budel enrichment. The 16S rRNA sequence of strain B1 (Accession No. KC215462) had high similarity (99%) with two *Desulfomicrobium* species: *D. baculatum* and *D. norvegicum* (Table 2). These two sulfate-reducing bacteria are non-spore-forming, Gram-negative, obligately anaerobic rods (Azabou et al., 2007; Genthner et al., 1997). Isolate B1 cells were rod-shaped (0.5–1 μm × 3–5 μm) with rounded ends and occurred singly or in pairs, motile and non-spore forming (Fig. 3A). Although strain B1 was isolated from an enrichment with glycerol and sulfate, it did not use glycerol as the electron donor for sulfate reduction. The closely related *D. baculatum* (DSM 4028^T) was also tested, but it was not able to use glycerol. This strain as well as our strain B1 are capable to use lactate, pyruvate, and H_2 coupled to the reduction of sulfate. Band 6 in the DGGE from strain B1 showed amplicons with almost the same mobility as the predominant DGGE Bands 1 and 3 from Budel and Paques enrichments, respectively (Fig. 2). The clones related to *Desulfomicrobium* from Budel and Paques showed 100% sequence similarity and 99% when compared to 16S rRNA sequence of strain B1.

Strain P2 was isolated from the Paques enrichments using glycerol as substrate. The rRNA gene sequence (Accession No. KP178480) showed low similarity (96%) with *Proteiniphilum acetatigenes*. *P. acetatigenes* cells are rod-shaped, 0.6–0.9 μm in diameter and 1.9–2.2 μm in length, non-spore-forming and motile. Yeast extract, peptone, pyruvate, glycine, and L-arginine could be used as carbon and energy sources. Acetate and NH_3 are produced from yeast extract, peptone and L-arginine, and propionate was also produced from yeast extract. Pyruvate was converted to acetate and CO_2 (Chen and Dong, 2005). This isolate forms succinate, propionate, and acetate as end products from glycerol and it does not reduce sulfate. Strain P2 cells were rod-shaped (0.2–0.3 × 0.3–0.6) and occurred singly or in pairs (Fig. 3B).

Strain RT1 was isolated from the Tinto River enrichments using medium without sulfate. The rRNA gene sequence (Accession No. KC215464) showed high similarity (98%) with *Clostridium aciditolerans*. *C. aciditolerans* cells are straight to slightly curved rods, 0.5–1 μm in diameter and 3–9 μm in length. Spores are subterminal and oval in shape and cells do not get swollen. Tryptone, peptone, pyruvate, and glucose are used as substrates, and glucose is fermented to acetate, butyrate, and ethanol as end products (Lee et al., 2007). No data are available regarding glycerol utilization by *C. aciditolerans* (Lee et al., 2007). Isolate RT1 cells were straight to slightly curved rods (1–1.5 μm × 3–15 μm) and occurred singly, in pairs or short chains. Spores are subterminal and oval in shape (Fig. 3C). This isolate forms acetate and butyrate as end products from glycerol, but it did not form the typical products that we detected in the low pH enrichment cultures. Sulfate is not used by strain RT1 and *C. aciditolerans* (Lee et al., 2007). Bands in the DGGE from strain RT1 (5) and the Tinto River enrichments (7) were at the same position (Fig. 2). However, the rRNA gene sequence from acidic enrichments (*M. margulisiae*) and strain RT1 showed low similarity (76%).

Discussion

Glycerol as electron donor for sulfate reduction

Glycerol, a cheap and readily available by-product of biodiesel production is a promising electron donor for biological reduction of sulfate to sulfide. The sulfate reduction ability can be exploited to immobilize heavy metals in sediments or to recover heavy metals in metallurgical and mining industry (Muyzer and Stams, 2008). Biogenic production of sulfide has been proposed as an economic and environmentally-friendly alternative for the treatment of areas impacted by heavy metals, such as acid mine drainage (Icgen and Harrison, 2006). We investigated glycerol as an electron donor for sulfate reduction at neutral and acidic pH. Sulfate-reducing communities were enriched with glycerol as an electron donor, but glycerol was not completely degraded when sulfate was supplied as an electron acceptor. By using 20 mM glycerol theoretically 15 mM sulfate can be reduced when glycerol is converted to acetate, while 35 mM sulfate can be reduced when glycerol is completely oxidized to CO_2. In our study, only up to 4 mM of sulfide was formed in the degradation of approximately 15 mM of glycerol. In the enrichments, glycerol conversion led to the formation of organic compounds that apparently were not degraded further. This indicates that additional procedures are needed to achieve complete glycerol degradation to CO_2 by selecting sulfates reducers that can degrade the organic compounds formed. It is known that acetate is a poor electron donor for sulfate re-

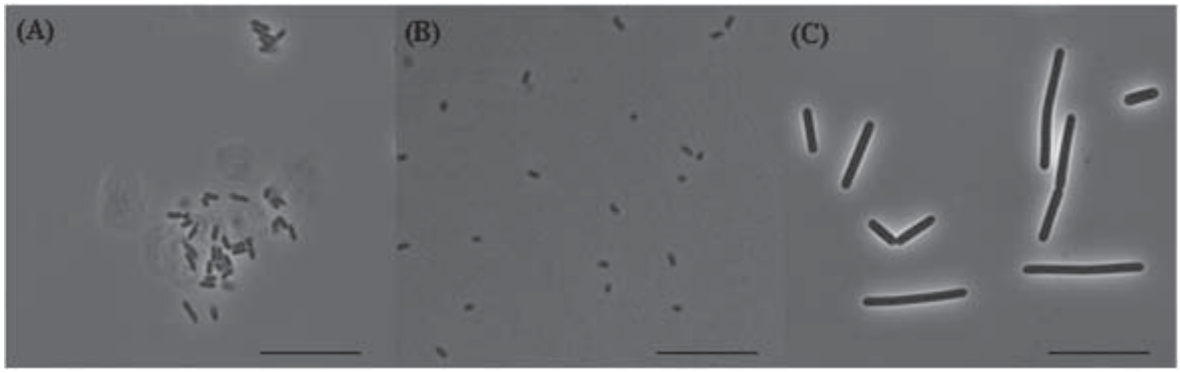

Fig. 3. Cell morphology of B1 (A), P2 (B), and RT1 (C) isolates from Budel, Paques, and Tinto River enrichments, respectively. Bar represents 10 μm.

duction ($\Delta G^{\circ\prime}$ = –48 kJ/mol) and that the growth rate of acetate-degrading sulfate-reducing bacteria is very low (Muyzer and Stams, 2008; Oude Elferink et al., 1995; Thauer et al., 1977). Likely, batch enrichment is not the optimal way to get complete conversion of glycerol coupled to sulfate reduction. Rather enrichment in continuous flow systems that are monitored for low acetate concentrations are more ideal for low pH application due to the toxicity of organic acids (Sánchez-Andrea et al., 2013). Further research efforts are needed to use glycerol more efficiently for sulfate reduction.

Bacteria in the neutral pH enrichments

In our neutrophilic enrichments *Desulfomicrobium* was dominant. The clones obtained from the enrichments at neutral pH (Budel and Paques) exhibited high 16S rRNA sequence identity (99%) to two sulfate-reducing species: *Desulfomicrobium baculatum* and *Desulfomicrobium norvegicum* (Azabou et al., 2007; Genthner et al., 1997). *D. baculatum* differs from *D. norvegicum* by its chemoautotrophic growth on H_2/CO_2 (Azabou et al., 2007). The genome from *D. baculatum* (DSM 4028) has a whole cluster of genes coding for enzymes involved in glycerol metabolism (Dbac_1434–Dbac_1446) (Copeland et al., 2009). However, this strain, as well as our isolated *Desulfomicrobium* strain, did not use glycerol as electron donor for sulfate reduction. We hypothesize that these strains are able to use glycerol, but that the strains cannot simply switch to glycerol as substrate. In the gene cluster a gene (Dbac_1439) is present that codes for a glycerol-3-phosphate regulon repressor. In addition, Dbac_1433 is a transposon, which indicates that the glycerol gene cluster might not be genetically stable. The other two detected bacteria from neutrophilic enrichment (Paques) clone library exhibited 16S rRNA sequence identity of 98% to the *Sphaerochaeta* species, *S. globosa* and *S. associate*, and 96% to the *Proteiniphilum* genus. The genus *Sphaerochaeta* was recently proposed by Ritalahti et al. (2012), and members of that genus are common in anoxic environments (Troshina et al., 2015). The *Sphaerochaeta* species generally use carbohydrates as substrate and form acetate, formate and ethanol as major end products from glucose fermentation. Glycerol is not used as substrate and sulfate is not used as electron acceptor by the described *Sphaerochaeta* species (Ritalahti et al., 2012; Troshina et al., 2015). *Proteiniphilum* species usually produce acetate or propionate as the main product from fermentation using different substrates (Chen and Dong, 2005). In this study, a microorganism (strain P2) was isolated that is moderately related with *Proteiniphilum acetatigenes*. Strain P2 was able to use glycerol, forming succinate, propionate, and acetate as end products. Succinate is not a common fermentation product of *Proteiniphilum* species. Succinate production could be a specific physiology feature of the isolated *Proteiniphilum* strain.

Most likely, in the enrichments, glycerol was used by fermentative bacteria that formed products that could be utilized by sulfate-reducing bacteria. In this work, hydrogen and formate were detected in the incubations without sulfate (Paques enrichment). These are excellent electron donors for sulfate reducers such as *Desulfomicrobium* species, and they are key components in the syntrophic degradation of organic compounds (Stams and Plugge, 2009). According to Widdel (1988) some SRB can grow autotrophically using hydrogen as an electron donor and fixing carbon dioxide, but most SRB require organic carbon, such as acetate, when growing with hydrogen and sulfate as energy substrates. Cayol et al. (2002) showed that glycerol in saline environments can be oxidized via interspecies hydrogen transfer by communities of a sulfate reducer, *Desulfohalobium retbaense*, and two fermentative bacteria, *Halanaerobium saccharolytica* subsp. *senegalense* and *Halanaerobium* sp. strain FR1H. Most likely, interspecies hydrogen transfer has played a role in our neutrophilic enrichments. The metabolic interaction of the glycerol-fermenting bacterium (*P. acetatigenes*) and *Desulfomicrobium* is not yet clear. Hydrogen and formate can be used by *Desulfomicrobium* for sulfate reduction. However, there is no indication that succinate or propionate, which are formed by *P. acetatigenes*, can be used by *Desulfomicrobium*. It might be that hydrogen-consumption by the sulfate reducer affects glycerol fermentation by *Proteiniphilum*, resulting in lower levels of reduced products.

Bacteria in the acidic pH enrichments

From mine streams and lakes with a low pH, sulfate-reducing bacteria have been isolated, which could not grow below pH 5 (Küsel et al., 2001; Lee et al., 2009). Pure cultures obtained from mixed cultures capable of reduc-

ing sulfate at pH 3 were not able to reduce sulfate below pH 5.5 (Gyure et al., 1990). However, true acidophilic sulfate-reducing bacteria belonging to the *Desulfosporosinus* genus have been reported (Alazard et al., 2010; Sánchez-Andrea et al., 2013, 2015). The process of sulfate reduction in low pH can be applied to precipitate heavy metals from acid mine drainage (Muyzer and Stams, 2008). Almost 89% of the sequences from the Tinto River clone library showed high similarity to *D. acididurans*, a novel species isolated from the same environment (Sánchez-Andrea et al., 2015) (Table 2). *D. acididurans* is an obligatory anaerobic, spore-forming, Gram-positive rod that stain Gram-negative, and is able to use glycerol as an electron donor for sulfate reduction (Sánchez-Andrea et al., 2015). Members of this genus convert organic compounds incompletely to acetate (Alazard et al., 2010; Sánchez-Andrea et al., 2015). The other detected bacteria have a high 16S rRNA gene sequence similarity with *M. margulisiae* (99% similarity) (Sánchez-Andrea et al., 2014). This bacterium is related to *Paludibacter propionicigenes* and has been recently described as a novel species in a novel genus. *M. margulisiae* is a strictly anaerobic, non-spore-forming, Gram-negative bacterium. It uses a variety of sugars, but not glycerol, as substrate. Propionate, lactate, and acetate are the major products of fermentation (Sánchez-Andrea et al., 2014). However, in our enrichment culture propionate was not detected as a product of glycerol fermentation. In this study, a *Clostridium* strain (strain RT1) was isolated that can use glycerol, but that bacterium did not appear in the clone library, and it formed butyrate, which was not detected as a product in the enrichment cultures.

Glycerol conversion to 1,3-PDO, and other alcohols and organic acids, is known to occur in *Clostridia* (*C. butyricum* and *C. pasteurianum*), *Enterobacteriaceae* (*Klebsiella pneumonia*, *Enterobacter agglomerans*, *Citrobacter freundii*) and *Lactobacillaceae* (*Lactobacilli brevis* and *L. buchneri*) (Barbirato et al., 1995; Plugmacher and Gottschalk, 1994; Schutz and Radler, 1984; Wischral et al., 2016; Yang et al., 2007). Kivistö et al. (2010) described halophilic fermentative bacteria, *Halanaerobium saccharolyticum* subspecies *saccharolyticum* and *senegalensis*, that use glycerol and form hydrogen, carbon dioxide and acetate as the main products, and the *saccharolyticum* subspecies also produces 1,3-PDO. Interestingly, in our enrichments without sulfate only 1,3-PDO and hydrogen were detected as products (Table 1), but to produce these products from glycerol an oxidized compound needs to be formed. As we were not able to detect any other organic compounds, it seems that bacteria with the ability to convert glycerol completely to CO_2 or degrade acetate to CO_2 are present in the enrichment. Syntrophic acetate conversion was suggested in glycerol-degrading acidophilic cultures (Kimura et al., 2006). Currently, we do not know which bacterium in the enrichment performs the complete oxidation of glycerol or acetate.

Acknowledgments

The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the Spanish Ministerio de Ciencia e Innovación (grant CTM2009-10521), the European Research Council (project 323009) and the graduate school WIMEK SENSE for support. The research was part of the PhD program of the Northeast Brazil Biotechnology Network (RENORBIO).

References

Abou-Zeid, D. M., Biebl, H., Spröer, C., and Müller, R.-J. (2004) *Propionispora hippei* sp. nov., a novel Gram-negative, spore-forming anaerobe that produces propionic acid. *Int. J. Syst. Evol. Microbiol.*, **54**, 951–954.

Alazard, D., Joseph, M., Battaglia-Brunet, F., Cayol, J.-L., and Ollivier, B. (2010) *Desulfosporosinus acidiphilus* sp. nov.: a moderately acidophilic sulfate-reducing bacterium isolated from acid mining drainage sediments. *Extremophiles*, **14**, 305–312.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410.

Azabou, S., Mechichi, T., Patel, B. K. C., and Sayadi, S. (2007) Isolation and characterization of a mesophilic heavy-metals-tolerant sulfate-reducing bacterium *Desulfomicrobium* sp. from an enrichment culture using phosphogypsum as a sulfate source. *J. Hazard. Mater.*, **140**, 264–270.

Barbirato, F., Camarasa, C., Claret Grivet, J. P., and Bories, A. (1995) Glycerol fermentation of 1,3-propanediol producing microorganism: *Enterobacter agglomerans*. *Appl. Microbiol. Biotechnol.*, **43**, 786–793.

Bertolino, S. M., Melgaço, L. A., Sá, R. G., and Leão, V. A. (2014) Comparing lactate and glycerol as a single-electron donor for sulfate reduction in fluidized bed reactors. *Biodegradation*, **25**, 719–733.

Biebl, H., Menzel, K., Zeng, A.-P., and Deckwer, W. D. (1999) Microbial production of 1,3-propanediol. *Appl. Microbiol. Biotechnol.*, **52**, 289–297.

Cayol, J. L., Fardeau, M. L., Garcia, J. L., and Ollivier, B. (2002) Evidence of interspecies hydrogen transfer from glycerol in saline environments. *Extremophiles*, **6**, 131–134.

Chen, S. and Dong, X. (2005) *Proteiniphilum acetatigenes* gen. nov., sp. nov., from a UASB reactor treating brewery wastewater. *Int. J. Syst. Evol. Microbiol.*, **55**, 2257–2261.

Chiu, C. W., Dasari, M. A., Sutterlin, W. R., and Suppes, G. J. (2006) Removal of residual catalyst from simulated biodiesel's crude glycerol for glycerol hydrogenolysis to propylene glycol. *Ind. Eng. Chem. Res.*, **45**, 791–795.

Copeland, A., Spring, S., Göker, M., Schneider, S., Lapidus, A. et al. (2009) Complete genome sequence of *Desulfomicrobium baculatum* type strain (X^T). *Stand. Genomic Sci.*, **1**, 29–37.

Dar, S. A., Stams, A. J. M., Kuenen, J. G., and Muyzer, G. (2007) Coexistence of physiologically similar sulfate-reducing bacteria in a full-scale sulfidogenic bioreactor fed with a single organic electron donor. *Appl. Microbiol. Biotechnol.*, **75**, 1463–1472.

García, E., Laca, M., Pérez, E., Garrido, A., and Peinado, J. (2008) New class of acetal derived from glycerin as a biodiesel fuel component. *Energy Fuels*, **22**, 4274–4280.

Genthner, B. R. S., Friedman, S. D., and Devereux, R. (1997) Reclassification of *Desulfovibrio desulfuricans* Norway 4 as *Desulfomicrobium norvegicum* comb. nov. and confirmation of *Desulfomicrobium escambiense* (corrig., formerly 'escambium') as a new species in the genus *Desulfomicrobium*. *Int. J. Syst. Bacteriol.*, **47**, 889–892.

Gholami, Z., Abdullah, A. Z., and Lee, K. T. (2014) Dealing with the surplus of glycerol production from biodiesel industry through catalytic upgrading to polyglycerols and other value-added products. *Renew. Sust. Energ. Rev.*, **39**, 327–341.

Gyure, R. A., Konopka, A., Brooks, A., and Doemel, W. (1990) Microbial sulfate reduction in acidic (pH 3) strip mine lakes. *FEMS Microbiol. Ecol.*, **73**, 193–202.

Himmi, E. H., Bories, A., Boussaid, A., and Hassani, L. (2000) Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. Shermanii. *Appl. Microbiol. Biotechnol.*, **53**, 435–440.

Hulshoff Pol, L. W., Lens, P. N. L., Weijma, J., and Stams, A. J. M. (2001) New developments in reactor and process technology for sulfate reduction. *Water Sci. Technol.*, **44**, 67–76.

Icgen, B. and Harrison, S. (2006) Exposure to sulphide causes populations shifts in sulphate-reducing consortia. *Res. Microbiol.*, **157**, 784–791.

Ito, T., Nakashimada, Y., Senba, K., Matsui, T., and Nishio, N. (2005) Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. *J. Biosci. Bioeng.*, **100**, 260–265.

Johnson, D. T. and Taconi, K. A. (2007) The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environ. Prog. Sustain. Energy*, **26**, 338–348.

Kimura, S., Hallberg, K. B., and Johnson, B. (2006) Sulfidogenesis in low pH (3.8–4.2) media by a mixed population of acidophilic bacteria. *Biodegradation*, **17**, 159–167.

Kivistö, A., Santala, V., and Karp, M. (2010) Hydrogen production from glycerol using halophilic fermentative bacteria. *Bioresour. Technol.*, **101**, 8671–8677.

Kremer, D. R. and Hansen, T. A. (1987) Glycerol and dihydroxyacetone dissimilation in *Desulfovibrio* strains. *Arch. Microbiol.*, **147**, 249–256.

Küsel, K. A., Roth, U., Trinkwalter, T., and Peiffer, S. (2001) Effect of pH on the anaerobic microbial cycling of sulfur in mining-impacted freshwater lake sediments. *Environ. Exp. Bot.*, **46**, 213–223.

Lee, Y.-J., Romanek, C. S., and Wiegel, J. (2007) *Clostridium aciditolerans* sp. nov., an acid-tolerant spore-forming anaerobic bacterium from constructed wetland sediment. *Int. J. Syst. Evol. Microbiol.*, **57**, 311–315.

Lee, Y.-J., Romanek, C. S., and Wiegel, J. (2009) *Desulfosporosinus youngiae* sp. nov., a spore-forming, sulfate-reducing bacterium isolated from a constructed wetland treating acid mine drainage. *Int. J. Syst. Evol. Microbiol.*, **59**, 2743–2746.

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T., Saxman, P. R. et al. (2001) The RDP-II (Ribosomal Database Project). *Nucleic Acids Res.*, **29**, 173–174.

Muyzer, G., Waal, E. C., and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*, **59**, 695–700.

Muyzer, G. and Stams, A. J. M. (2008) The ecology and biotechnology of sulfate-reducing bacteria. *Nat. Rev. Microbiol.*, **6**, 441–454.

Nanninga, H. J. and Gottschal, J. C. (1987) Properties of *Desulfovibrio carbinolicus* sp. nov. and other sulfate-reducing bacteria isolated from an anaerobic-purification plant. *Appl. Environ. Microbiol.*, **51**, 572–579.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A. et al. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.*, **178**, 5636–5643.

Oude Elferink, S. J., Maas, R. N., Harmsen, H. J., and Stams, A. J. (1995) *Desulforhabdus amnigenus* gen. nov. sp. nov. a sulfate reducer isolated from anaerobic granular sludge. *Arch. Microbiol.*, **164**, 119–124.

Plugmacher, U. and Gottschalk, G. (1994) Development of an immobilized cell reactor for the production of 1,3-propanediol by *Citrobacter freundii*. *Appl. Microbiol. Biotechnol.*, **41**, 313–316.

Qatibi, A.-I., Bories, A., and Garcia, J.-L. (1991) Sulfate reduction and anaerobic glycerol degradation by a mixed microbial culture. *Current Microbiol.*, **22**, 47–52.

Ritalahti, K. M., Justicia-Leon, S. D., Cusick, K. D., Ramos-Hernandez, N., Rubin, M. et al. (2012) *Sphaerochaeta globosa* gen. nov., sp. nov. and *Sphaerochaeta pleomorpha* sp. nov., free-living, spherical spirochaetes. *Int. J. Syst. Evol. Microbiol.*, **62**, 210–216.

Sánchez-Andrea, I., Knittel, K., Amann, R., Amils, R., and Sanz, J. L. (2012) Quantification of Tinto River sediment microbial communities: importance of sulfate-reducing bacteria and their role in attenuating acid mine drainage. *Appl. Environ. Microbiol.*, **78**, 4638–4645.

Sánchez-Andrea, I., Stams, A. J. M., Amils, R., and Sanz, J. L. (2013) Enrichment and isolation of acidophilic sulfate-reducing bacteria from Tinto River sediments. *Environ. Microbiol. Rep.*, **5**, 672–678.

Sánchez-Andrea, I., Sanz, J. L., and Stams, A. J. M. (2014) *Microbacter margulisiae* gen. nov., sp. nov., a novel propionigenic bacterium isolated from sediments of an acid rock drainage pond. *Int. J. Syst. Evol. Microbiol.*, **64**, 3936–3942.

Sánchez-Andrea, I., Stams, A. J. M., Hedrich, S., Nancucheo, I., and Johnson, D. B. (2015) *Desulfosporosinus acididurans* sp. nov.: an acidophilic sulfate-reducing bacterium isolated from acidic sediments. *Extremophiles*, **19**, 39–47.

Sanguinetti, C. J., Dias Neto, E., and Simpson, A. J. G. (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques*, **17**, 915–919.

Satokari, R. M., Vaughan, E. E., Akkermans, A. D. L., Saarela, M., and de Vos, W. M. (2001) Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.*, **67**, 504–513.

Schutz, H. and Radler, F. (1984) Anaerobic reduction of glycerol to 1,3-propanediol by *Lactobacillus brevis* and *Lactobacillus buchneri*. *Syst. Appl. Microbiol.*, **5**, 169–178.

Seifert, C., Bowien, S., Gottschalk, G., and Daniel, R. (2001) Identification and expression of the genes and purification and characterization of the gene products involved in reactivation of coenzyme B12-dependent glycerol dehydratase of *Citrobacter freundii*. *Eur. J. Biochem.*, **268**, 2369–2378.

Silva, G. P., Mack, M., and Contiero, J. (2009) Glycerol: a promising and abundant carbon source for industrial microbiology. *Biotechnol. Adv.*, **27**, 30–39.

Sipma, J., Lens, P. N., Stams, A. J., and Lettinga, G. (2003) Carbon monoxide conversion by anaerobic bioreactor sludges. *FEMS Microbiol. Ecol.*, **44**, 271–277.

Slinn, M., Kendall, K., Mallo, C., and Andrews, J. (2008) Steam reforming of biodiesel byproduct to make renewable hydrogen. *Bioresour. Technol.*, **99**, 5851–5858.

Stams, A. J. M. and Plugge, C. M. (2009) Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat. Rev. Microbiol.*, **7**, 568–577.

Stams, A. J. M., Hansen, T. A., and Skyring, G. W. (1985) Utilization of amino acids as energy substrates by two marine *Desulfovibrio* strains. *FEMS Microbiol. Ecol.*, **31**, 11–15.

Stams, A. J. M., van Dijk, J. B., Dijkema, C., and Plugge, C. M. (1993) Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. *Appl. Environ. Microbiol.*, **59**, 1114–1119.

Thauer, R. K., Jungermann, K., and Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.*, **41**, 100–180.

Troshina, O., Oshurkova, V., Suzina, N., Machulin, A., Ariskina, E. et al. (2015) *Sphaerochaeta associata* sp. nov., a spherical spirochaete isolated from cultures of *Methanosarcina mazei* JL01. *Int. J. Syst. Evol. Microbiol.*, **65**, 4315–4322.

Trüper, H. G. and Schlegel, H. G. (1964) Sulphur metabolism in Thiorhodeaceae. I. Quantitative measurements on growing cells of *Chromatium okenii*. *Antonie van Leeuwenhoek*, **30**, 225–238.

van Gelder, A. H., Aydin, R., Alves, M. M., and Stams, A. J. M. (2011) 1,3-Propanediol production from glycerol by a newly isolated *Trichococcus* strain. *Microb. Biotechnol.*, **5**, 573–578.

Weisberg, W. G., Barns, S. M., Pelletier, B. A., and Lane, D. J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, **173**, 697–703.

Widdel, F. (1988) Microbiology and ecology of sulfate- and sulfur reducing bacteria. *In* Biology of Anaerobic Microorganisms, ed. by Zehnder, A. J. B., Wiley, New York, pp. 469–585.

Wischral, D., Zhang, J., Cheng, C., Lin, M., De Souza, L. M. G. et al. (2016) Production of 1,3-propanediol by *Clostridium beijerinckii* DSM 791 from crude glycerol and corn steep liquor: Process optimization and metabolic engineering. *Bioresour. Technol.*, **212**, 100–110.

Yang, F., Hanna, M. A., and Sun, R. (2012) Value-added uses for crude glycerol—a byproduct of biodiesel production. *Biotechnol. Biofuels*, **5**, 1–10.

Yang, G., Tian, J., and Li, J. (2007) Fermentation of 1,3-propanediol by a lactate deficient mutant of *Klebsiella oxytoca* under microaerobic conditions. *Appl. Microbiol. Biotechnol.*, **73**, 1017–1024.

Yoon, S. J., Choi, Y., Son, Y., Lee, S. H., and Lee, J. G. (2010) Gasification of biodiesel by product with air or oxygen to make syngas. *Bioresour. Technol.*, **101**, 1227–1232.