Selection for Novel, Acid-Tolerant *Desulfovibrio* spp. from a Closed Transbaikal Mine Site in a Temporal pH-Gradient Bioreactor

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Abstract

Almost all the known isolates of acidophilic or acid-tolerant sulphate-reducing bacteria (SRB) belong to the spore-forming genus *Desulfosporosinus*. The objective of this study was to isolate acidophilic/acid-tolerant members of the genus *Desulfovibrio* belonging to deltaproteobacterial SRB. The sample material originated from microbial mat biomass submerged in mine water was enriched for sulphate reducers by cultivation in anaerobic medium with lactate as electron donor. A stirred tank bioreactor with the same medium composition was inoculated with the sulphidogenic enrichment. The bioreactor was operated with a temporal pH gradient, changing daily, of initial pH 7.3 and final pH 3.7. Among the bacteria in the bioreactor culture, *Desulfovibrio* was the only SRB group retrieved from the bioreactor consortium as observed by 16S rRNA-targeted denaturing gradient gel electrophoresis. Moderately acidophilic/acid-tolerant isolates belonged to *D. aerotolerans-D. carbinophilus-D. magneticus* and *D. idahonensis-D. mexicanus* clades within the genus *Desulfovibrio*. A moderately acidophilic strain, *Desulfovibrio* sp. VK (pH optimum 5.7) and acid-tolerant *Desulfovibrio* sp. ED (pH optimum 6.6) dominated in the bioreactor consortium at different time points and were isolated in pure culture.

Keywords *acid tolerant; Deltaproteobacteria; Desulfovibrio; mine waste; sulphate-reducing bacteria*

Introduction

Dissimilatory sulphate reduction in low pH environments such as sulphide ore and coal mine sites has been recognised for almost 50 years (Gyure et al. 1990; Tuttle et al. 1969a,b). To date, relatively few acidophilic or acid-tolerant sulphate-reducing bacteria (SRB) have been isolated in pure culture. Hitherto, two acidophilic SRB, *Desulfosporosinus acidiphilus* and *Desulfosporosinus acididurans*, have been characterised by Alazard et al. (2010) and Sánchez-Andrea et al. (2015). Several undescribed members of this genus have been reported that grow in acidic media (Abicht et al. 2011; Jameson et al. 2010; Karnachuk et al. 2009, 2015a, 2017; Küsel et al. 2001; Senko et al. 2009). Another member of the *Firmicutes* phylum, the thermophilic SRB *Thermodesulfobium narugense* isolated from a hot spring, has a slightly acidic pH optimum of 5.5-6.0 for growth (Mori et al. 2003). A moderately acidophilic *Thermodesulfobium acidiphilum* with pH optimum of 4.8-5.0 has been recently described (Frolov et al., 2017). The isolation of the only known acid tolerant deltaproteobacterial *Desulfovibrio* strain TomC, capable of growing at initial pH 2.5, was recently reported (Karnachuk et al. 2015b). The strain is, however, relatively sensitive to copper and cadmium. The strain TomC could not grow at concentrations exceeding 1.6 mM Cu and 0.5 mM Cd (Karnachuk et al., unpublished), a trait which may be a disadvantage for bioremediation purposes. While spore-forming *Desulfosporosinus* spp. have already been identified from acid-impacted habitats, a search of acidic environments for novel desulfovibrios is warranted because they may have better traits for bioremediation.

Acidophilic/acid-tolerant SRB are important in biological treatment of acidic metal- and sulphate-rich waste streams such as acid mine drainage in constructed wetlands or bioreactor processes (Koschorreck 2008; Kaksonen et al. 2008; Sánchez-Andrea et al. 2014). Spore-forming *Desulfosporosinus* spp. are disadvantaged due to relatively slow growth (Sánchez-Andrea et al. 2014) and low resistance to oxygen (Alazard et al. 2010; Stackebrandt et al. 2003) as compared to the deltaproteobacterial *Desulfovibrio* spp. (Cypionka, 2000; Ramel et al. 2015). Tolerance to oxygen is of advantage in the bioremediation of oxidised mine waste and acid mine water. Various bioremediation schemes may include SRB contact with ambient environments, such as the fixed-bed in-lake bioreactor exploiting sulphate reduction to treat acidic mine pit lake described by Koschorreck et al. (2010).

Despite the absence of pure cultures, several studies have reported the occurrence of Deltaproteobacteria, including members of *Desulfovibrio*, in bench-scale bioreactor and

biobarrier systems treating acidic mine water (Bijmans et al. 2009, 2010; Cole et al. 2011; Kaksonen et al. 2004, 2008; Pruden et al. 2007; Rampinelli et al. 2008). Biodiversity studies have indicated that acidophilic/acid-tolerant deltaproteobacterial SRB related to *Desulfovibrio* have been detected by pyroseqencing in the subsurface bacterial community of a uranium contamination plum at the pH range from 4.5 to 5.7 (Cardenas et al. 2010). The deltaproteobacterial *Desulfomonile*-related clones occurred in the acidic pit lake associated with the Iberian Pyrite Belt at pH 3.0 (Falagán et al. 2014). The occurrence of Deltaproteobacteria has been demonstrated by pyrosequencing in pilot-scale flow-through bioreactors for remediation of coal mine-generated acid drainage at pH 4.5 (Lefticariu et al., 2015). *Desulfovibrio* were present in a small-scale biofilm reactor seeded with acid mine drainage water from anthracite mining in the pH range from 3.3 to 5.8 (Cole et al. 2011).

In our previous attempts we did not detect deltaproteobacterial SRB in the metagenome of acidic water (pH 2.6) from an abandoned open cast tin mine at the Sherlovaya Gora in the Transbaikal area (Kadnikov et al. 2016). Cultivation attempts from Sherlovaya Gora samples and a tungsten mine site in the Transbaikal area, Bom-Gorkhon (pH 3.0) yielded isolates of acidohilic *Desulfosporosinus* spp. (Karnachuk et al. 2015a). Members of the *Desulfosporosinus* genus were the only sulphate-reducers detected by pyrosequencing, PCR-denaturing gradient gel electrophoresis (PCR-DGGE), and cultivation in gold mine waste at the pH range varying from 2.4 to 3.6 in Novii Berikul, Kuznetsk mining area area, Siberia (Karnachuk et al. 2009, Mardanov et al. 2016). However, our preliminary studies by PCR-DGGE indicated that a number of *Desulfovibrio* spp. could grow at pH values as low as 3.0 in sulphidogenic enrichments from a microbial mat sampled at an abandoned mine site in the Transbaikal area (O.V. Karnachuk et al. unpublished). This sample was used for isolation of acid-tolerant deltaproteobacterial SRB in the present study. We hypothesised that shifts in dominant SRB may be resolved over time in a bioreactor with a temporal pH gradient, which can select for acidophilic or acid-tolerant bacteria transiently for pure culture isolation. The purpose of this study was to use an anaerobic bioreactor with an enrichment culture that was subjected to gradual changes in pH over time. The use of a bioreactor temporal gradient has not been previously applied to isolation of acidophilic/acid-tolerant SRB.

Materials and methods

Sample site

Sample material was collected on July 29, 2014 from the tailings area in the abandoned Novii Akatui Pb-Zn mine (Transbaikal area, Russia; N51°02', E117°47'). The mine has been exploited for silver-lead sulphide ore since the 19th century. More recently, the carbonate country rocks have been mined for Pb-Zn (sphalerite-galena) ores via underground mining. The Novii Akatui mine was closed in 2002. At present, the underground workings are flooded and overflow via a horizontal tunnel (adit) into the tailings area. Flow of mine water emerges from a major mine tunnel, dated 1959. Water sample for chemical analyses (ShG14-5-water) was taken from the mouth of the adit close to the submerged log with microbial mats. The mat sample (ShG14-5-mat) was collected aseptically from a log submerged in the mine water. A grab sample of tailings material, ShG14-6-waste, for mineralogical analysis was collected from the tailings area located downstream the mine adit. Temperature, pH, and Eh of the water sample were determined on site using a HANNA HI 8314 F pH meter (Hanna Instruments Deutschland GmbH, Vöhringen). Water for chemical analysis was filtered into 50 ml polyethylene flasks with a Millipore filter (0.2 μm pore size) and polypropylene syringe and transported to the British Geological Survey for elemental and ion analyses by inductively coupled plasma mass spectrometry (ICP-MS) and ion chromatography as previously described (Mardanov et al. 2016). The mineralogical analysis of the tailings material was carried out by X-ray diffraction (XRD) using a Shimadzu XRD-6000 (Shimadzu Corporation, Kyoto, Japan) difractometer as previously described (Ikkert et al., 2013).

Bioreactor culture and temporal pH gradient

The mat sample was inoculated into headspace free vials with screw caps filled with sterile liquid freshwater medium contained (per litre) 4.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 1.0 g NaCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.5 g KCl, 0.113 g $CaCl_2$, 2 ml of vitamin solution, 1 ml of microelement solution, 1 ml each of Na_2SeO_3 (final concentration 23.6 μM) and Na_2WO_4 (24.2 μM) solutions (Widdel and Bak 1992). $Na_2S \cdot 9H_2O$ was used as a reducing agent. Each vial received a piece of iron wire (100% Fe) as an additional micro source of iron (Karnachuk et al. 2006). 18 mM lactate was supplied as electron donor and the initial pH was in the range of 4.7-5.0. Carbonate buffer was excluded from the medium as recommended for acidophilic SRB isolation (Sánchez-Andrea et al. 2015). Vials were incubated at 28 °C. The lactate fed SRB

enrichment was used as the starter culture for a Biostat B plus benchtop bioreactor (Sartorius Stedim Biotech GmbH, Göttingen) with an initial working volume of 1 l, agitation at 100 rpm, and with a pH and temperature control. Ultra-pure (99.9%) N_2 was sparged at 25 ml min^{-1} through a diffuser at the bottom of the bioreactor to remove the H_2S produced in the bioreactor and to maintain an O_2-free headspace. The bioreactor was operated at 28 °C by recirculating water through the reactor mantle. The initial pH was 4.6 and was not controlled for the first 20 days of operation. At 474 h the pH in the bioreactor culture was decreased by 0.1 unit per day by pH stats with 0.5 M HCl or 1 M $NaHCO_3$ solutions.

At intervals samples were withdrawn from the bioreactor to measure H_2S and protein, to isolate DNA for PCR-DGGE analysis, and to observe bacterial morphology. Because of sparging with N_2, the production of H_2S was only monitored as an indication of the culture response to changes in the pH over time. Protein was analysed by modified Lowry method as previously described (Karnachuk et al. 2008). H_2S was measured colorimetrically with N,N-dimethyl-p-phenylenediamine (dihydrochloride salt) as the chromophore (Cline, 1969). Both parameters were analysed in triplicate using a Smart Spec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Cells were observed by phase contrast microscopy using Axio Imager A1 microscope.

Following microscopic examination, samples were serially diluted for bacterial isolation. Samples were removed 79 times (total volume 1189 ml) for analysis of H_2S and protein and 11 times (total volume of 1100 ml) for cultivation and DNA isolation. The bioreactor was operated in the semi-batch culture mode: 0.5 l aliquots of fresh lactate medium were added at 1172, 1311, 1505, 1745 and 1865 h (total volume replacement of 2500 ml) to make up for the volumes removed for culture analyses.

Samples from the bioreactor culture were serially diluted in the Widdel and Bak (1992) medium supplemented with lactate, the same as used for the initial enrichment, and incubated at 28 °C at same pH as in temporal pH gradient in the bioreactor culture at the time of the sampling. Serial dilution procedures were repeated until culture purity could be verified. Culture purity was determined by microscopic examination of the bacterial morphology, by the absence of growth on Plate Count Agar (per litre: 1.0 g dextrose, 5.0 g tryptone, and 2.5 g yeast extract; aerobic incubation) and Anaerobic Agar (per litre: 5.0 g caseine, 2.5 g yeast extract, and 1.0 g dextrose; anaerobic incubation), and by amplifying the 16S rRNA gene fragment with the subsequent analysis by PCR-DGGE to verify a single band on the gel.

The specific growth rates were determined in batch cultures by monitoring the cell numbers. The cultures were grown in the Widdel and Bak (1992) medium with lactate as electron donor at 28 °C. Three replicate cultures were analysed at each time point. Specific growth rates were calculated by taking the slope of the exponential phase of growth of a semilogarithmic plot of cell number versus time.

PCR-DGGE and phylogenetic analysis of 16S rRNA gene fragments

DNA from the SRB cultures was isolated using the MO BIO Power Soil DNA Kit (MO BIO Laboratories, Qiagen Carlsbad, Carlsbad, CA) per manufacturer's recommendations. 16S rRNA gene-targeted PCR-DGGE was used to monitor the dominating phylotypes in the bioreactor and pure culture isolation. Nested PCR was applied to amplify 16S rRNA gene fragments as previously described (Frank et al. 2016). For sequencing of the 16S rRNA genes of the SRB isolates, amplification was carried out with the primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (DeLong 1992; Weisburg et al. 1991). The PCR-products obtained from the reaction with outer primers was used as a template for the subsequent reaction with primers BacV3f (Muyzer et al., 1996) and 907r (Lane 1991). The genomic DNA isolated from *Desulfovibri*o sp. TomC was diluted with nuclease-free water (Fermentas, EU) to a concentration of ca. 100 ng ml-1 and used as a positive control in PCR reactions. A negative control, in which the template was replaced by sterile deionized water, was included for each set of reaction. Each PCR product was applied onto 8% (w/v) polyacrylamide gel in duplicates. The bands were cut from the gels and sequenced at the Bioengineering Centre of the Russian Academy of Science. The sequences were analysed using the BioEdit software package and the BLAST GenBank tool (http://www.ncbi.nlm.nih.gov/) (Altschul 1997). Phylogenetic analyses were carried out using the ARB software (Ludwig et al. 2004). To determine the branching order, bootstrap analysis was carried out using 1000 alternative trees. The 16S rRNA gene sequences of the two pure cultures were deposited to GenBank NCBI under accession numbers KU201950- KU201951.

Results

Mine water characteristics

The sample ShG14-5-water collected at the mine site was analysed for dissolved metals and ions (Table 1). The data on geochemical characteristics of drainage from the abandoned

mine site suggest continuing oxidation of sulphidic phases. Sulphate was the major anion with the concentration of 223 mg SO_4^{2-} l^{-1}, which is a hydrochemical fingerprint of sulphide oxidation. The mine water contained slightly elevated concentrations of metals, including iron (141 μg Fe l^{-1}), manganese (178 μg Mn l^{-1}), and zinc (2,720 μg Zn l^{-1}). The iron concentration in the minewater is relatively modest and related to redox conditions – as Eh increases, iron decreases – suggesting iron in the minewater is largely in ferrous (Fe^{2+}) form and is precipitated out by oxidation to ferric (Fe^{3+}) iron. Other dissolved metals were present in much more modest concentrations. The mine water is of the Ca-(Mg)-SO_4 type, suggesting that acid generated by sulphide oxidation is neutralised by Ca(Mg)-carbonates in the host rocks. The XRD-analysis of the ShG14-6-waste confirmed the carbonates occurrence, namely dolomite $CaMg(CO_3)_2$ and calcite, $CaCO_3$ (data are not shown). The pH of the mine water sample was 7.1. Circumneutral and alkaline mine sites are known to have microniches with sulphidic phases, which are subject to acid production due to chemical and biological weathering (Banks et al. 2002).

Temporal pH gradient bioreactor performance

The SRB enrichment culture from ShG14-5-mat was initially grown at pH 5.0 with lactate as electron donor and was then used to inoculate the bioreactor. The culture in the bioreactor was initially adjusted to pH 4.6. The performance of the reactor as a function of time is shown in Fig. 1. The concentration of protein started to increase after 375 h of cultivation while H_2S remained below the detection limit. Concurrently, the pH increased to pH 5.7 at this point. The pH increased to 7.7 within the subsequent week.

Starting at 474 h, the temporal pH gradient was initiated in the bioreactor culture by lowering the pH by 0.1 unit day^{-1}. A substantial increase in protein (264 mg protein l^{-1}) and H_2S (133 mg l^{-1}) was observed at pH 7.3 after 642 h. Subsequently the gradual pH adjustment caused a decrease in protein and H_2S concentrations. Both parameters fluctuated at low levels, on average 100 mg protein l^{-1} and 10 mg H_2S l^{-1}, as compared to the maximum of 718±53 mg protein l^{-1} observed at 623 h and 514±14 mg H_2S l^{-1} at 642 h. A substantial increase in sulphidogenesis and protein in the bioreactor culture occurred after the culture reached the pH 5.1 at 1,300 h (Fig. 1). Both parameters declined as the pH decreased to below 4.0. The bioreactor experiment was terminated when the cells were found to lyse at pH 3.7.

DGGE analysis of pH gradient culture samples from the bioreactor

Samples were taken from the bioreactor over a period of 50 days for DGGE analysis of 16S rRNA gene amplicons to examine dominant phylotypes in the bioreactor consortium. The sampling times for DNA isolation were chosen on the basis of microscopic observations, when a distinct morphotype prevailed in the culture. Aliquots of the samples were diluted serially in order to isolate dominant SRB in pure culture. The closest relatives based on the 16S rRNA gene sequences of the phylotypes retrieved from the bioreactor consortium are shown in Fig. 2. All retrieved phylotypes with known capability to reduce sulphate were members of the genus *Desulfovibrio*. The SRB phylotypes fell into two separate clades within *Desulfovibrio*: (1) a group related to *D. aerotolerans*-*D. carbinophilus*-*D. magneticus*, and (2) a group related to *D. idahonensis*-*D. mexicanus* (Fig. 3). SRB related to *D. mexicanus* were the most stable components of the consortium and were detected at nearly all sampling times. This latter group of *Desulfovibrio* spp. was the only SRB phylotype, which was retrieved from the initial enrichment used to inoculate the bioreactor.

Non-SRB phylotypes in the bioreactor consortium were *Cupriavidus basilensis* (β-Proteobacteria), *Sulfurospirillum multivorans* (ε-Proteobacteria) and *Terrabacter terrae* (Actinobacteria). *Acinetobacter*-related phylotypes (γ-Proteobacteria) were detected at pH ≥5.7, whereas 16S rRNA gene fragments matching with *Cellulomonas persica* (Actinobacteria) were only observed at the most acidic time point (pH 4.7) of sampling at 1407 h. The initial enrichment contained bacteria close to *Paludibacter propionicigenes* (Bacteroidetes) but they were not detected in the pH gradient samples.

Desulfovibrio strains ED and VK

Samples for pure culture isolation were taken from the bioreactor at different time points spanning a period of 50 d between the first and last sampling. Serial dilutions of the samples from time points 212 h (pH 5.3) and 357 h (pH 5.7) yielded two SRB isolates designated strain VK and strain ED, respectively. These isolates were obtained via subcultures of repeated serial dilutions and their purity was confirmed by PCR-DGGE analysis and absence of growth on Plate Count Agar and Anaerobic Agar. Phylogenetic analysis of nearly full 16S rRNA gene sequences placed both isolates in the genus *Desulfovibrio*. The closest relative of strain VK was *D. carbinoliphilus* with the sequence similarity of 98.8%. Strain ED fell into another clade on the phylogenetic tree with the closest relative of *D. idahonensis* with the sequence similarity

of 99.6%. Both isolates were able to grow at low pH values (Fig. 4), starting from pH 2.8 (*Desulfovibrio* strain VK) to pH 3.5 (*Desulfovibrio* strain ED). The highest growth rates were observed at pH 5.7 for strain VK and at pH 6.6 for strain ED. Growth was limited at circumneutral pH and neither strain was able to grow above initial pH 7.8. Strain VK formed cell aggregates when grown in liquid medium at neutral pH value (Fig. 5). When the strains were grown in culture vials without pH control, the initial acidic pH increased by several pH units; for example, the initial pH 2.4 in the culture VK increased to pH 5.9 after 18 days of incubation.

Discussion

The temporal pH gradient was used successfully to resolve phylotypes in the bioreactor consortium, but samples taken from the pH gradient failed to yield pure cultures upon direct cultivation using the repeated serial dilution approach. The PCR-DGGE results showed that SRB assigned to the *D. mexicanus* group were abundant over a broad, moderately low pH range but they were not recovered upon cultivation in repeated serial dilutions. The members of the *D. aerotolerans-magneticus* group dominated in bioreactor at pH 5.3. The *D. idahonensis* group was detected only during the early phase of the bioreactor operation at pH 5.7 and *Desulfovibrio* strain ED of this group was successfully isolated at this phase of the time course. Strain ED can be considered as acid-tolerant with the pH optimum of 6.6. *D. carbinoliphilus*-related phylotypes were also detected only at the early phase of the bioreactor at sampling point of 357 h at pH 5.7 and one phylotype was subsequently isolated as strain VK.

Non-SRB phylotypes in the bioreactor included *Acinetobacter calcoaceticus*. This bacterium is part of the *A. calcoaceticus-baumannii* (Acb) complex that includes human intestinal strains. *A. calcoaceticus* has been isolated from various environmental samples and a strain was recently reported as a sulphate-reducer (Han et al. 2015). The available sequences from GenBank *Acinetobacter* genomes do not contain *dsrAB* genes, the key enzyme of dissimilatory sulphate reduction. It is plausible that *Acinetobacter* occurs in consortia with sulphate-reducers, as was the case in the present bioreactor study, and their separation as pure cultures may be deceivingly difficult.

Sulphate reduction is a proton-consuming process at acidic pH values, near neutral at circumneutral pH (*p*Ka 6.95 for HS^-/H_2S), and proton generating at alkaline pH values. In the bioreactor the initial pH values increased without pH control. The acidophilic SRB differ

304 contrastingly from the acid-producing iron- and sulphur-oxidizing acidophiles due to the proton
305 consumption in the course of sulphate reduction. Thus the tolerance to the low pH conditions
306 is important for the SRB that grow optimally at moderately low pH values. In this study,
307 however, the pH control in the SRB batch cultures was not maintained with pH stats and exact
308 pH ranges cannot, therefore, be presented for the isolates.

309 Lactic acid has been suggested to be an inappropriate substrate for acid-tolerant SRB due
310 to its inhibitory effect at low pH (Alazard et al. 2006, Meier et al. 2012; Sánchez-Andrea et al.
311 2015). The *p*Ka of lactic acid is 3.86. At pH 4.5 and 5.0 about 80% and 90%, respectively, of
312 lactic acid is dissociated as lactate, greatly alleviating the potential toxicity of undissociated
313 lactic acid. Our results confirm, however, that lactic acid can be successfully used as a carbon
314 source and electron donor to isolate at least moderately acid tolerant (pH 4.5-5.5) SRB. Acetic
315 acid is the end product of incomplete oxidation of lactic acid by *Desulfovibrio* spp. The
316 observed cell lysis of the bioreactor culture at pH 3.7 suggests that lactic acid (*p*Ka 3.86) or
317 acetic acid (*p*Ka 4.75) may account at least partially for the deleterious effect on SRB. Our
318 experiments on growing the *Desulfovibrio* isolates at the initial pH range of 2.8 to 4.0 in capped
319 vials did not, however, suggest severe inhibitory effect by lactic acid or metabolites.

320 The temporal pH gradient changing daily over extended time demonstrated shifts in
321 bacterial phylotypes and can be developed to a selection technique for bioprocesses requiring
322 narrow ranges for culture parameters or isolation. Similar outcomes can be accomplished with
323 continuous culture techniques. The approach in this study has the advantage that low density
324 or slow growth population is not washed out because the dilution rate concept does not apply
325 to this technique.

326

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Conflict of interests

The authors declare that they have no conflict of interests.

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494

495 Table 1. Analysis of the ShG-14-5-water sample.

496

Parameter	Units	Value
T	°C	2.8
pH	-	7.1
Eh	mV	+ 210
Cl^-	mg l^{-1}	0.57
SO_4	mg l^{-1}	223
NO_3	mg l^{-1}	1.4
Ca	mg l^{-1}	136
Mg	mg l^{-1}	33.6
Na	mg l^{-1}	2.9
K	mg l^{-1}	1.0
Fe	µg l^{-1}	141
Zn	µg l^{-1}	2720
Mn	µg l^{-1}	178
Al	µg l^{-1}	5
Cu	µg l^{-1}	0.6
Cd	µg l^{-1}	3.96
Co	µg l^{-1}	0.81
Ni	µg l^{-1}	7.2
Pb	µg l^{-1}	0.7
Sb	µg l^{-1}	5.42
As	µg l^{-1}	52.8
Sr	µg l^{-1}	508
U	µg l^{-1}	3.76
Cr	µg l^{-1}	<0.09

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Legends to figures

Fig. 1 Bioreactor performance over time. The sampling points and the corresponding pH values are shown by arrows. The concentrations of protein and H_2S are expressed as the means of three replicates. The vertical bars show standard deviation.

Fig. 2 Dominant bacterial phylotypes in the bioreactor revealed by PCR-DGGE analysis. All SRB were members of the genus *Desulfovibrio*. Non-SRB-phylotypes are shown in shadowed boxes. Data are also shown for the initial enrichment that was used for bioreactor inoculation. The numbers at the phylotypes indicate % similarity with the closest validly described species.

Fig. 3 Neighbor-joining 16S rRNA gene tree showing the affiliations of strains ED, DV, and VK and the *Desulfovibrio* phylotypes (in bold) retrieved from PRC-DGGE analyses. The original phylogenetic tree was constructed for nearly complete 16S rDNA sequences. Partial sequences (550 bp) obtained from DGGE-bands were inserted into the tree using the ARB parsimony analysis tool. Bootstrap analysis, based on 1000 replications, was carried out to determine the confidence level for each node. The first three/four digits in the phylotype designation indicate the sampling point in h, followed by the DGGE band number. The phylotypes, which start with "Enrich" were retrieved from the enrichment culture used as the inoculum for the bioreactor. The scale bar represents an estimated 10% sequence divergence. *Thermodesulfovibrio yellowstonii* was used as an outgroup (not shown).

Fig. 4 Effect of pH on the specific growth rates of *Desulfovibrio* isolates. The cultures were grown on 18 mM lactate at 28 °C. Data are expressed as the means of three replicates and the vertical bars show standard deviation.

Fig. 5 Photomicrographs of *Desulfovibrio* sp. VK grown (A) at pH 4.3 and (B, C) at pH 7.8. Scale bars = 5 μm, 1200x magnification.

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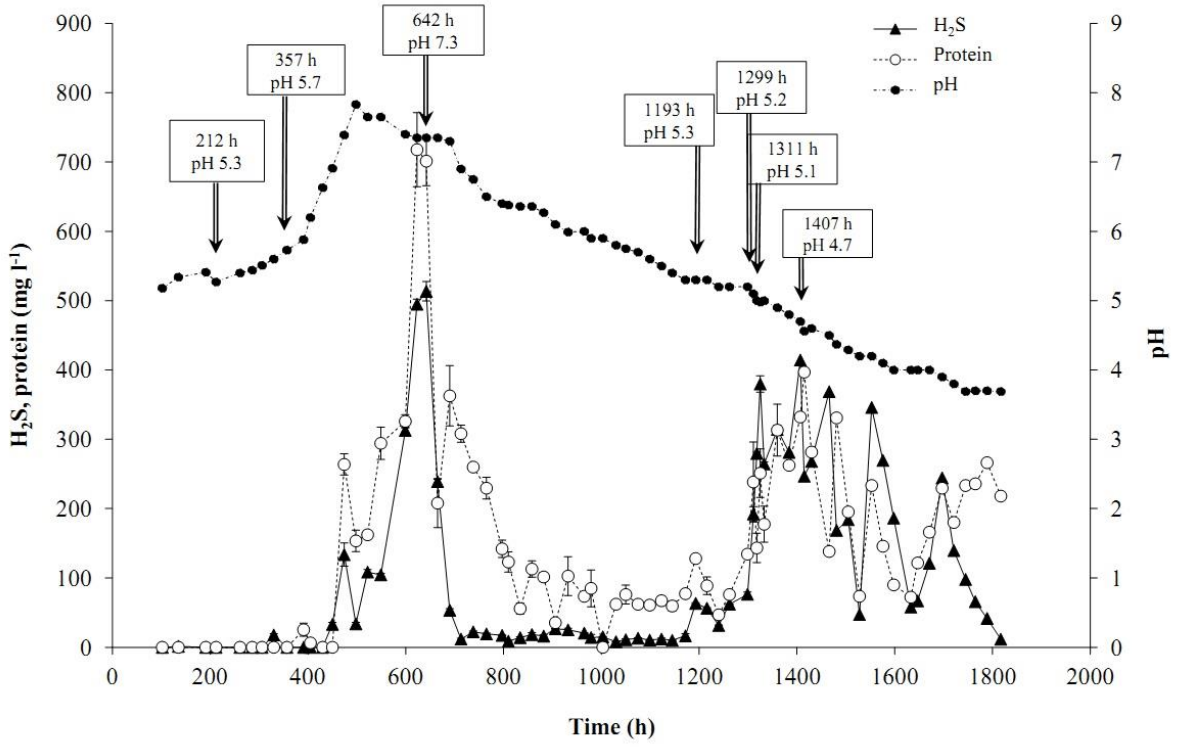
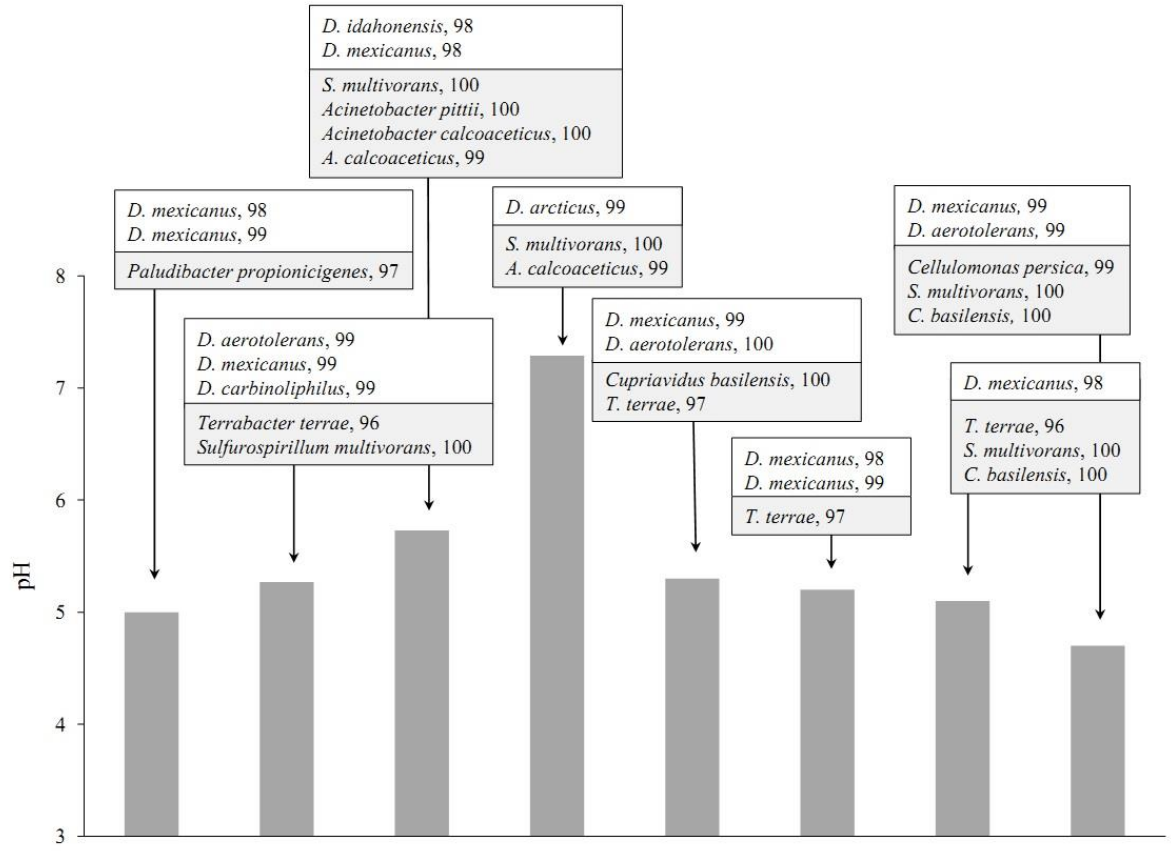


530

531

532 Figure 1.

533



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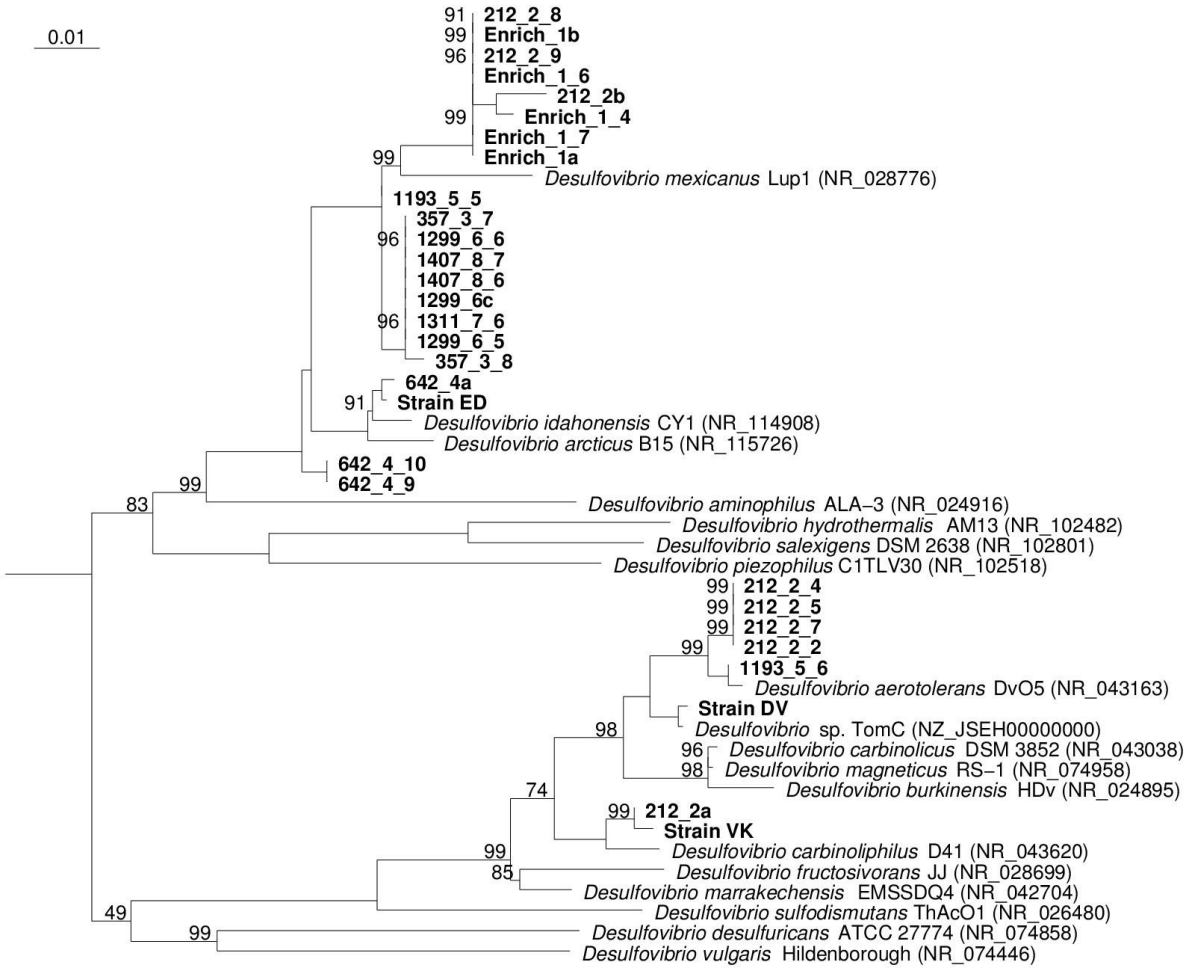
536 Figure 2.


0.01
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Enrich_1b
212_2_9
Enrich_1_6
212_2b
Enrich_1_4
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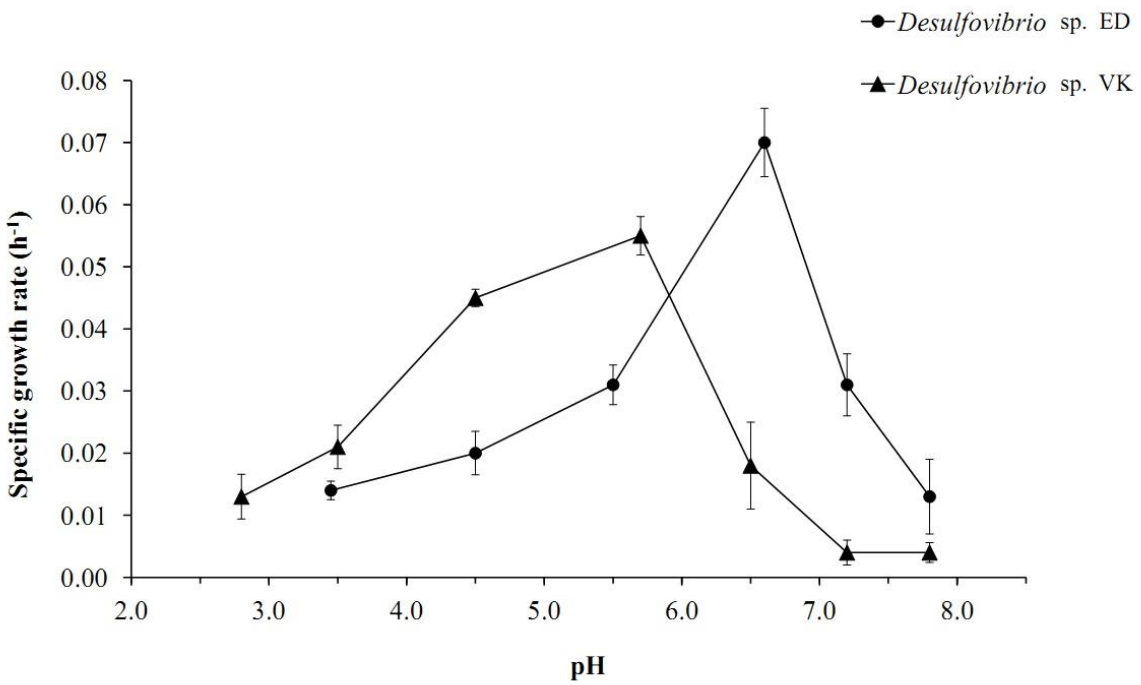
Desulfovibrio mexicanus Lup1 (NR_028776)
1193_5_5
357_3_7
1299_6_6
1407_8_7
1407_8_6
1299_6c
1311_7_6
1299_6_5
357_3_8
642_4a
Strain ED
Desulfovibrio idahonensis CY1 (NR_114908)
Desulfovibrio arcticus B15 (NR_115726)
642_4_10
642_4_9
Desulfovibrio aminophilus ALA–3 (NR_024916)
Desulfovibrio hydrothermalis AM13 (NR_102482)
Desulfovibrio salexigens DSM 2638 (NR_102801)
Desulfovibrio piezophilus C1TLV30 (NR_102518)
212_2_4
212_2_5
212_2_7
212_2_2
1193_5_6
Desulfovibrio aerotolerans DvO5 (NR_043163)
Strain DV
Desulfovibrio sp. TomC (NZ_JSEH00000000)
Desulfovibrio carbinolicus DSM 3852 (NR_043038)
Desulfovibrio magneticus RS–1 (NR_074958)
Desulfovibrio burkinensis HDv (NR_024895)
212_2a
Strain VK
Desulfovibrio carbinoliphilus D41 (NR_043620)
Desulfovibrio fructosivorans JJ (NR_028699)
Desulfovibrio marrakechensis EMSSDQ4 (NR_042704)
Desulfovibrio sulfodismutans ThAcO1 (NR_026480)
Desulfovibrio desulfuricans ATCC 27774 (NR_074858)
Desulfovibrio vulgaris Hildenborough (NR_074446)

Figure 3.



541

542 Figure 4.

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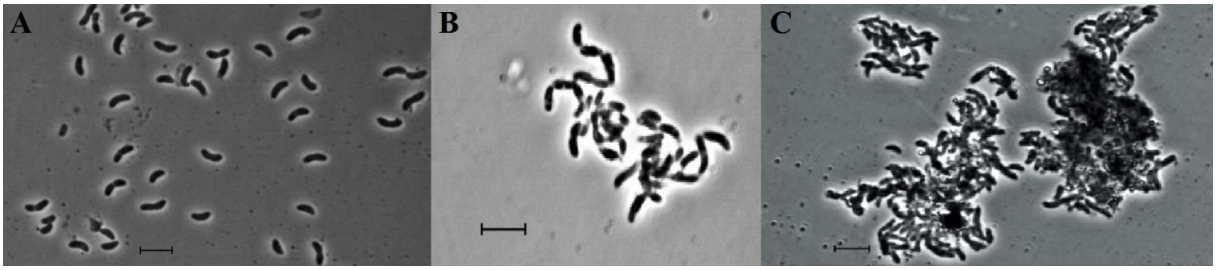

Figure 5.