Title

Significance of lysogeny for the metabolism of Desulfovibrio spp. strains isolated from aquatic environments of Georgia

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Identification of bacterial strains of *Desulfovibrio* spp. in various aquatic environments of Georgia and the significance of cell lysogenicity

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ABSTRACT

Ten strains of sulfate-reducing bacteria (SRB) have been identified from 22 samples collected from various aquatic environments in the country of Georgia. SRB strains were isolated from two regions of the Black Sea coast of Georgia (Batumi and Anaklia) and from riverbanks of Chkhoushi (Zugdidi region). New isolates were compared to different strains from the DSMZ collection (*Desulfovibrio vulgaris* DSM 644^T; *D. vulgaris* DSM 2119^T; *D. africanus* subsp. *africanus* DSM 2603^T; *D. africanus* subsp. *uniflagellum* DSM 23860^T) and to previously isolated cultures of *D. vulgaris* and *D. africanus*. Based on their genetic, cytological, and physiological properties, the 10 Georgian bacterial isolates were assigned to the genus *Desulfovibrio*. The strains were characterized with respect to their morphology, substrate utilization, and eco-physiological properties to validate their taxonomic position. In addition, temperate bacteriophages were induced from three of these sulfate-reducing strains by UV-exposure. Comparison of metal reduction and utilization of various carbon sources by the wild-type (lysogenic) strains and their UV-irradiated counterparts pointed to a significant role for temperate bacteriophages in metabolism and metabolic potential by this ecologically important group of microbes.

KEYWORDS*: Sulfate-reducing bacteria, aquatic environments, lysogeny, UV-induction, temperate phage, metal reduction, substrate utilization.*

INTRODUCTION

Sulfate-reducing bacteria (SRB) are mostly obligate anaerobes that gain their energy by using sulfate as the terminal electron acceptor (TEA). They play an important role in the global sulfur and carbon cycling by completely mineralizing organic matter, including simple organic acids (lactate, acetate, propionate, fatty acids), complex aromatic petroleum hydrocarbons (benzene, toluene, naphthalene, phenols), as well as alkanes like hexadecane and decane among others [9, 11]. SRB are ubiquitous in environments including mud, ponds, sewage, fresh and marine water, underground aquifers, oil reservoirs, as well as the rumen of sheep and cattle and the guts of insects (need references here).

SRB are a taxonomically diverse group of bacteria with representatives in several phyla within the domain *Bacteria.* The majority of known species to date belong to *Deltaproteobacteria*, of which, *Desulfovibrio* is a widely studied genus [13, 30]. *Desulfovibrio* spp. are fermenting, anaerobic, Gram-negative curved rods characterized

by the presence of a pigment, desulfoviridin [28, 41]. These microorganisms grow relatively slowly and can be easily missed or overgrown by other cultures [30].

SRB produce hydrogen sulfide as a respiratory end product, which is highly reduced and chemically reactive. Sulfide reacts with metal pipes (such as iron, steel, and copper), well casings, and storage tanks and causes structural damage by heavy corrosion, resulting in huge losses especially to the oil industry. These losses amount to almost $100 million dollars in the United States alone annually [3]. In petroleum reservoirs, these organisms are readily introduced into the wells during secondary oil recovery by water flooding. SRB can grow in various environments including within the aqueous phase of oil and petroleum storage systems, as well as oil tankers.

Bacterial viruses (bacteriophages) are the most abundant biological entities on the planet [4, 32, 18]. Lytic phages infect and disrupt intact microbial cells and control bacterial carbon and energy transfer within various ecosystems [17, 19, 44]. The majority of bacterial genomes contain prophage sequences, which undergo complex genetic rearrangements. Temperate phages mediate acquisition of new functions via horizontal gene transfer, benefit their host, and favor community stability [7, 8, 34, 38]. While phage-mediated microbial diversification is prevalent in the environment [42, 50], the mechanisms of phage-host interaction(s) remain largely unknown. Several temperate but only a few lytic bacteriophages have been isolated against SRB and various *Desulfovibrio* species (*e.g., D. vulgaris, D. salexigen, D. aespoeensis* [2, 17, 22, 26, 47]). The study of lytic and temperate bacteriophages is an important step towards understanding the ecology and molecular biology of SRB and may open new opportunities in using the host-specificity of phages coupled with their resistance to environmental conditions for controlling detrimental SRB biofilms on different surfaces.

In this study, we report the isolation of several sulfate-reducing bacteria from environmental samples collected near oil refineries in the Black Sea region of Georgia. We also explored the presence and impact of temperate phages in several SRB strains isolated in Georgia and previously in the USA.

MATERIALS AND METHODS

Collection of environmental samples and sources of SRB strains: To isolate indigenous bacterial strains of SRB, seawater, sewage, sludge, sediment, mud, slush, oil contaminated sediments, and fresh water from lakes and rivers were collected from different aquatic environments of Georgia. The samples were collected in Tbilisi and its vicinity, as well as from the Black Sea coastline of Georgia. River water was sampled close to the bank of Chkhoushi River (Zugdidi region) and seawater from the bottom of the Black Sea at a depth of 3 m. Other samples were collected at dry corrosion sites near the seaside city of Batumi close to an oil terminal. The sludge samples originated from a pond near the delta of the Tikori River (Anaklia region). When sampling, attention was paid to environmental contamination with oil and the accompanying smell of sulfur as indicator parameters. The samples were collected in sterile plastic bottles and kept at 5ºC until further processing.

The strains *D. vulgaris* Hildenborough [23, 36] and *D. africanus* PCS [5, 10] were obtained from the culture collection at Lawrence Berkeley National Laboratory. *D. vulgaris* DSM 644^T; *D. vulgaris* DSM 2119^T; *D. africanus* subsp. *africanus* DSM 2603^T; and *D. africanus* subsp. *uniflagellum* DSM 23860^T were kindly provided by the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany).

Media and isolation of Desulfovibrio strains: Initially, the anaerobic medium API [1] and the *Desulfovibrio* medium-87 (http://culturecollection.vtt.fi/m/html?p=mel&id=87&pr, an analog of the DSMZ medium-63; www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium63.pdf) were used for the detection and cultivation of SRB. The media were dispensed in 20-ml Hungate tubes or 125-ml serum bottles and sealed with butyl robber stoppers and aluminum crimps under

a nitrogen headspace. Inoculated samples were incubated at 30ºC under anaerobic conditions and checked periodically for up to 30 days. The bottles that turned black due to the production of iron sulfide mediated by SRB metabolism (reaction of H_2S with iron in the medium to form a black insoluble precipitate) were considered positive. Cell growth was monitored using optical density measurements at 600 nm (OD_{600}).

Positive enrichments after several transfers were harvested by centrifugation at 6,000 x *g* for 15 min at 4ºC and inoculated in three types of media to obtain pure cultures. These included: LS4D - lactate sulfate medium [29] based on Postgate's medium C [36], with 0.3% or 1.5% agar; thioglycolate medium (Sigma, St. Louis, MO) with 1.5% Na_2SO_3 and 1 mM Na_2S, and *Desulfovibrio* medium-87 [6, 24, 43]. In most experiments medium-87 was used with some modifications - sodium acetate and glucose were replaced by sodium lactate, with or without 1.5% Na_2SO_3 to inhibit unwanted microorganisms.

After several passages in liquid medium, the SRB enrichments were transferred onto soft agar (agar concentration 0.5-0.8%) amended with medium-87. The plates were incubated at 30ºC in a GasPak anaerobic jar (BD, Franklin Lake, NJ). Characteristic black colonies were obtained as indicators of sulfate reducing bacteria. Colonies were picked, serially diluted, and repeatedly transferred onto medium-87 (with glucose, 1,5% Na_2SO_3, and 0.5% agar) and Trypticase Soy Agar (TSA) modified with 0.2% $MgS0_4 \bullet 7H_20$; 0.05% $(NH_4)_2Fe(SO_4)_2 \bullet 6H_2O$, and 0.4% sodium lactate [24]. To confirm the purity of isolates, plates were incubated both aerobically and anaerobically.

Isolated bacterial strains were grown at 30°C to mid-log phase (optical density, OD_{600} of 0.3) and preserved for long-term maintenance. In short, 1-ml of culture, each, was dispensed into multiple 2-ml cryogenic vials (Nalgene, Rochester, NY) with 0.5 ml 45% glycerol (w/v) and frozen at -80°C. To minimize phenotypic divergence, each experiment was started from a frozen stock. All manipulations, inoculations, and transfers were done in an anaerobic chamber.

Desulfoviridin pigment identification and H_2S-test. The presence of the pigment desulfoviridin was checked by swiping the bacterial colonies with a cotton swab and then adding 1 drop of 2 N NaOH directly onto the swab. The reaction was immediately observed in a dark room under UV light at 365 nm. Red fluorescence indicated a positive reaction [49]. For detection of H_2S production by the microorganisms Hydrogen Sulfide Test strips (FLUKA 06728/Sigma Aldrich-Chemie, Germany) were used. The strains *D. vulgaris* Hildenborough and *D. africanus* PCS were used as positive controls and an *E. coli* strain as the negative control.

PCR amplification and gene sequencing: Genomic DNA was extracted from the bacterial isolates using the spin-column protocol supplied with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The 16S rRNA-coding genes were amplified with an AccuPrime™ Taq DNA Polymerase System (Life Technologies; Grand Island, NY) using the primers 27Fjgi (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492Rjgi (5'- GGTTACCTTACGACTT-3') with the following reaction conditions: initial denaturation at 95ºC for 15 min, followed by 40 cycles of denaturation at 95ºC for 15s, annealing at 60ºC for 30s, and extension at 72ºC for 30s, and a final extension cycle at 72ºC for 2 min. The amplicons of expected size of about 1,500 nucleotides (nt) were purified with a PCR purification kit (QIAGEN, Hilden, Germany) and sent for sequencing to the UC Berkeley DNA Sequencing Facility (Berkeley, CA). Bi-directional raw sequences were edited using 4Peaks (http://nucleobytes.com/index.php/4peaks) resulting ≈1,200-nt reads, aligned with the help of Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and compared to the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/blast) to find the best matches based on percentage sequence identity.

Pulsed field gel electrophoresis (PFGE) and SDS-PAGE: Ten-milliliter exponential phase bacterial cultures were centrifuged at 3,000 x *g* for 3 min to remove black precipitates. Supernatant was re-centrifuged at 6,000 x *g* for 15 min. Pellets were washed three times

with phosphate buffered saline (PBS), re-suspended in PBS with 2% SeaKem Gold Agarose (Lonza; Basel, Switzerland), and poured into plug molds. The agarose-embedded bacteria were lysed with lysozyme (1%; w/v) and treated with proteinase K (1%; w/v) overnight at 37°C and 55°C, respectively. Plugs were then washed several times with 1X TBE and HE (10 mM HEPES, 1 mM EDTA) buffers [14]. Slices of agarose-embedded DNA were digested with 20 U of restriction enzyme *Not*I (5'-GC/GGCCGC-3'). The plugs were then loaded onto a 1% agarose gel in 0.5X TBE. PFGE was performed on a Gene Navigator™ system (Amersham Biosciences, Piscataway, NJ). Running conditions included 0.5X TBE running buffer, 14°C running temperature, 6.0 Vcm^{-1}, and the switching time linearly ramped from 60 s - 120 s. The gel was stained with ethidium bromide and photographed under UV using Gel Logic 112 (Kodak, Rochester, NJ).

The whole-cell protein composition of the SRB strains was examined by a standard SDS-PAGE (dodecyl sulfate-polyacrylamide gel electrophoresis) method, using 10% resolving and 3% stacking gel [27].

Morphological characterization: Gram staining of bacterial cells was performed using standard technique. Phase contrast photomicrographs were prepared using a Nikon microscope equipped with ELWO (Nikon Instruments, Melville, NY). For electron microscopic examination, bacterial suspensions were centrifuged at 3,000 x *g* for 3 min to remove black precipitates. The resulting suspensions were centrifuged again at 6,000 x *g* for 5 min at 4°C and the pellet re-suspended in PBS buffer. Morphology of bacteriophage particles was examined with a JEOL 100C electron microscope (Jeol, Akishima-Shi, Tokyo, Japan). Phages were transferred onto formvar and carbon-coated copper grids for 30 s to let the particles settle and stained with 1% of uranyl acetate for 40 s. Filter paper was used to wick off excess sample. Grids were examined at different magnifications.

Influence of environmental factors: Growth rate experiments were conducted in liquid medium. A stock bacterial culture was grown at 30ºC to mid-log phase (OD_{600} of 0.3, corresponding to about 10^8 cells/ml). To establish the optimal temperature for cultivation, bacterial cells were grown at 4ºC, 15ºC, 22ºC, 30ºC, 37ºC, and 55ºC in medium-87 with pH 7.4. For determination of optimal acidity and salinity for cell growth, cultures were inoculated into medium-87 with pre-adjusted acidity (pH: 3.6; 5.5; 7.4 and 8.2) or concentration of NaCl (0; 85; 170; 255; 340, and 425 mM) and incubated at 30ºC. Bacterial growth was examined at different time points (after 24, 48, 72, and 96 h) spectrophotometrically by measuring OD_{600} values.

Phenotypic characterization: Growth kinetics of the *Desulfovibrio* strains was studied in LS4D medium with lactate as the sole electron donor and sulfate as the electron acceptor. Other times, lactate was substituted with other electron donors – glucose, pyruvate, succinate, butyrate, or acetate (at 10-20 mM concentration). As alternative electron acceptors, thiosulfate, nitrate, or sodium sulfate were tested.——

Induction of temperate phages: For phage induction the *Desulfovibrio* strains were subjected to UV irradiation. Forty-eight-hour old bacterial cultures (about 10^8 CFU/ml) were centrifuged at 6,000 x *g* for 15 min, washed with 0.9% NaCl, re-suspended in 0.01 M $MgSO_4$, and irradiated for 1 min. A LAMP UVGL-58 (UVP Upland, CA) was used emitting at 254 nm. Non-exposed bacterial cultures served as control. The UV-dose was measured with the culture diluting method [39, 40]. Two samples were examined: an LD_{50} cell suspension and another sample, after 1 min of UV irradiation. The samples were diluted 3-fold into medium-87 and incubated at 30°C. At 24, 48, and 144 h, samples were withdrawn, centrifuged, filtered (0.22 µm pore-sized filter) and verified for the presence of prophages by the spot test on the indicator strains.

OMNILOG phenotypic array: The OMNILOG Phenotype MicroAarray™ (Biolog, Hayward, CA) assesses the phenotypic response of an organism of interest on an array of substrates. Kinetic plots were generated by the OmniLog® instrument (Biolog, Hayward, CA), an incubated chamber that captures digital images every 15 min for up to 150 h.

Mid-log phase cells (OD_{600} 0.3-0.4) corresponding to about 10^8 cells/ml were prepared for inoculation in 100 µl volumes in 96-well plates. Cells were spun down at 6,000 g for 15 min and the pellet re-suspended corresponding to about 2 x 10^7 cells/ml. PM1 and PM2 MicroPlate™ Carbon Sources panels were run in duplicate. Inoculated plates were sealed in an anaerobic atmosphere in a Whirl-Pak® Retain Bag with low permeability to oxygen (Nasco, Atlanta, GA) before being transferred to the OmniLog for data collection at 37°C. Under these incubation conditions, the plates stayed anaerobic for about 5 days. An OL unit of <50 corresponded to 10^7 cells/ml, and an OL unit of >100 corresponded to 10^8 cells/ml.

The growth of the SRB culture was determined by fixing the increase in the turbidity of the sample, which correlated both with the optical density at 600 nm and with the total number of cells.

Metal reduction experiments: Iron reduction of untreated and UV-induced SRB strains was studied by the Ferrozine assay [37]. At the start of experiment the OD_{600} value for both cultures was adjusted to the same optical density. The strains were grown in triplicate in LS4D medium, harvested by centrifugation, washed, and re-suspended in PIPES. All manipulations were performed in an anaerobic chamber. The ferric to ferrous iron reduction was tested over 24 h. At predetermined times (T_0, T_2, T_4, and T_{24}), sub-samples were extracted in 0.5 N HCl prior to measuring Fe(II) concentration at 562 nm. A series of Fe(II) standards was also prepared (0.1-20 mM) to obtain a standard curve for comparison.

Similarly, Cr(VI) reduction by untreated and UV-induced strains was tested. Cells were grown in defined LS4D medium to mid-log phase (OD_{600} of 0.3), with lactate as electron donor and sulfate as electron acceptor. Cells were collected by centrifugation, and the cell pellet washed with 30 mM phosphate buffer. Centrifugation and washing were repeated to minimize potential carryover of sulfate in the Cr(VI) reduction experiment. The cell pellet was then re-suspended in phosphate buffer and sealed in anaerobic serum vials. To all the vials, 200 µM potassium dichromate was added as electron acceptor and 10 mM lactate as electron donor. Electron donor addition was left out of the control treatments. The vials were incubated in the dark at 32ºC. Changes in Cr(VI) concentration were determined colorimetrically at 540 nm using the diphenyl carbazide (DPC) assay [35].

RESULTS

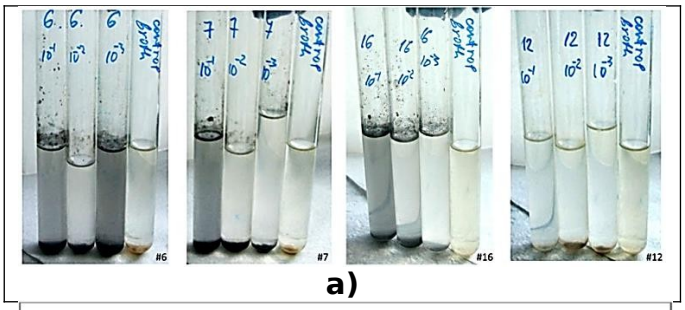
Sampling sites and identification of SRB. Distribution, diversity, and physiology of SRB from Georgia have not been previously studied extensively. Habitat of SRB can be seawater, as well as other aquatic environments, thus environmental samples were collected in Tbilisi and in its vicinity and from the Black sea coastline of different regions of Georgia (Table 1, column 2).

Table 1. Sampling sites and sequence similarities of *Desulfovibrio* strains isolated in Georgia

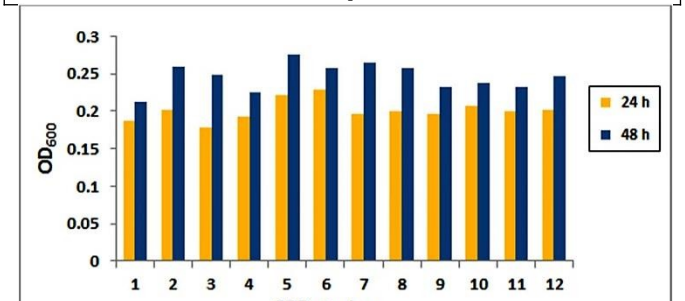
#	Environmental samples	Presence of SRB and strain name	16S rRNA sequence identity
1	**2**	**3**	**4**
1	Sewage from the main collector of the city (Tbilisi)	-	-
2	Mud from the pipes of the technical water pool /I (Eliava Institute, Tbilisi)	-	-
3	Water with sludge from the bottom of the technical water pool/II (Eliava Institute, Tbilisi)	-	-

4	Water from Bartskhana River, near the oil terminal (Batumi)	DvRCH1/GE6	100% - *Desulfovibrio vulgaris* Hildenborough
5	Water with sediment from Bartskhana River marshland estuary (Batumi)	D/PA35E4/ GE7	98.78% - *Desulfovibrio* spp. str. PA35E4
		DvH/GE7/1	99% - *Desulfovibrio vulgaris* Hildenborough
6	Water from Turtle Lake, (Tbilisi)	-	-
7	Water from Lisi Lake (Tbilisi)	-	-
8	Sewage from Saburtalo district of the city (Tbilisi)	-	-
9	Water, slush from pipes, and sludge from the walls in sulphur baths (Tbilisi)	-	-
10	Black Sea water from the canyon of the Enguri River (Anaklia)	-	-
11	Water with sediment from the bottom of the Black Sea (3 m below surface; Batumi)	DvH/GE15	99% - *Desulfovibrio vulgaris* Hildenborough
12	Sea water with sludge from the pond, near the outpour of the Tikori River, (strong hydrogen sulfide odor; Anaklia)	D/LZK1/GE16	99% - *Desulfovibrio* spp. LZK1
		DvH/GE16/1	99% - *Desulfovibrio vulgaris* Hildenborough
13	Water from the bottom of the Black Sea (2.5 m below the water surface; Anaklia)	-	-
14	Water from the coast line of the Chkhoushi River (hydrogen sulfide odor; Zugdidi)	D/JG1/GE18	99.37% - *Desulfovibrio* spp. str. JG1
15	Sea water with sediment, near the Patriot Camp (Anaklia)	D/GE19	99% *Desulfovibrio* spp. clone Ecwsrb042
16	Dry stratum of turf near the oil terminal of Batumi	D/LZK1/GE20	99% - *Desulfovibrio* spp. LZK1
		D/LZK1/ GE20/1	99% - *Desulfovibrio* spp. LZK1

For identification of SRB strains, collected samples were cultivated in anaerobic conditions using different selective media. SRB positive cultures with black precipitates were passaged in liquid and soft media until apparent pure cultures were obtained [see Material and Methods]. Generally, cultures produced black colored precipitate (FeS) and the smell of hydrogen sulfide after 48 h of incubation in liquid media at 30°C (Fig.1; a, b). When grown in soft solid agar, black precipitate appeared after 2-3 weeks of cultivation (Fig.1; c, d).



a)



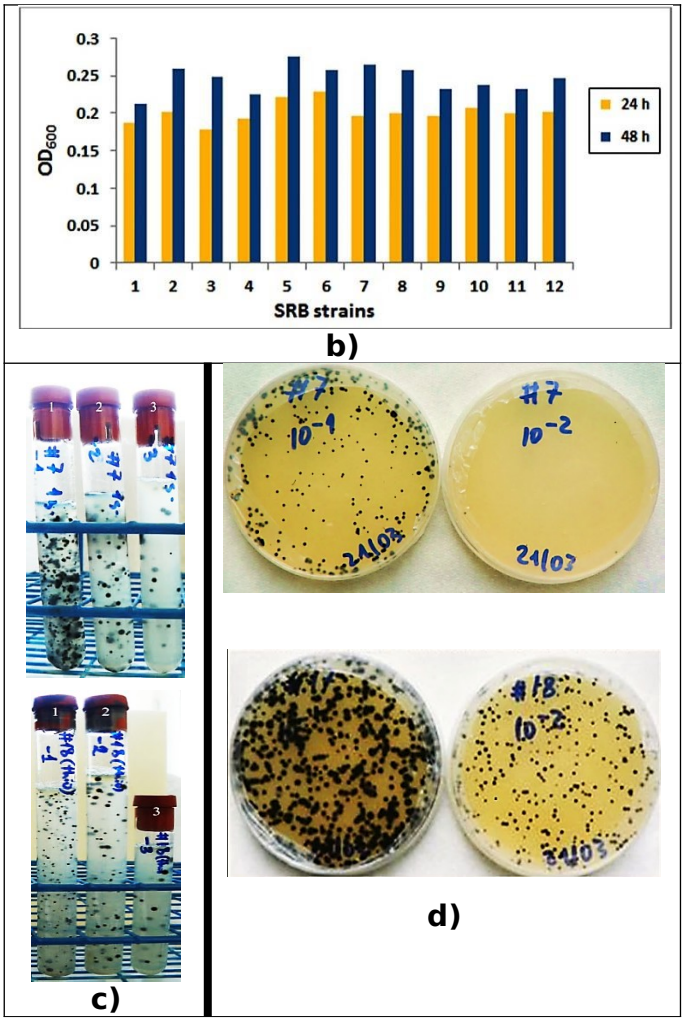


Fig.1. Identification of SRB in the environmental samples collected in Georgia
a) Cultivation in liquid medium (vials with diluted samples: #6; #7; #16; #12);
b) Optical density (OD_{600}) of bacterial cultures cultivated in liquid medium-87: 1. *D. vulgaris* Hildenborough, 2. *D. africanus* PCS, 3. DvRCH1/GE6, 4. D/PA35E4/GE7, 5. DvH/GE7/1, 6. DvH/GE15, 7. D/LZK1/GE16,
8. DvH/GE16/1, 9. D/JG1/GE18, 10. D/GE19, 11. D/LZK1/GE20, 12. D/LZK1/GE20/1;
c) Bacterial colonies in soft agar medium (samples: #7 and #18);
d) Bacterial colonies on solid medium (samples: #7 and # 18).

Among 22 various aquatic environments of Georgia, ten SRB strains were identified in 7 samples (Table 1, column 2 and 3). All SRB strains were isolated from samples from Western Georgia, in particular from the two Black Sea regions (Batumi, Anaklia) and from the bank of the Chkhoushi river (Zugdidi) (Fig.2).

Figure 2. Sampling sites (Western Georgia, Black Sea region).

Bacterial DNA was amplified by PCR using the 27Fjgi and 1492Rjgi 16S rRNA primers. Ten bacterial strains were confirmed as SRB by 16S RNA-gene sequencing [48]. All collected strains were found to belong to the genus *Desulfovibrio*. Four strains isolated in Georgia (DvRCH1/GE6, DvH/GE7/1, DvH/GE15, and DvH/GE16/1) had 99 - 100% sequence identity with *D. vulgaris* subsp. *vulgaris* Hildenborough; three strains (D/LZK1/GE16, D/LZK1/GE20, and D/LZK1/GE20/1) were 99% identical to the *Desulfovibrio* strain LZK1 (http://www.ncbi.nlm.nih.gov/blast); D/GE19 was 99% identical to *Desulfovibrio* sp. enrichment culture clone Ecwsrb042, while strains D/PA35E4/GE7 and D/JG1/GE18 were also similar to different clones of *Desulfovibrio* by more than 98% in the same database (Table 1; column 3 and 4).

Genetic relationship of SRB isolates. To determine genome similarity between *Desulfovibrio* strains isolated in Georgia the genotypes were analyzed by DNA fingerprinting and pulsed-field gel electrophoresis (PFGE). Types that varied by 1 to 2 band shifts were assigned as subtypes [33] (Figure 3; a). Clustering of strains was based on the unweighted-pair group method using average linkages (UPGMA). The scale bar indicates genetic distance. Numbers at the branching points show percentage of similarity between strains (Figure 3; b). A phylogenetic tree was generated with standard FreeTree and TreeView-based Genetic Distance similarity software.

Visual analysis of the PFGE results revealed patterns of high heterogeneity among the SRB. The isolates formed two clusters, A and B (Figure 3; b). Cluster A included 9 isolates - four strains from Georgia (D/GE19, DvH/GE16/1, DvH/GE15, and DvH/GE7/1), four strains obtained from DSMZ (D.a.23860, D.v.644, D.a.2603, and D.v.2119), and *D. vulgaris* Hildenborough. Six other strains isolated from Georgia - D/PA35E4/GE7, D/LZK1/GE16, D/LZK1/GE20, D/LZK1/GE20/1, D/JG1/GE18, and DvRCH1/GE6 - formed cluster B. Some strains isolated from the same environmental samples differed from one another significantly - *e.g.*, the pairs GE16 (cluster B) and GE16/1 (cluster A) or GE7 (cluster B) and GE7/1 (cluster A), while other strains isolated from different locations (*e.g.*, GE7/1, Ge15, and GE16/1) appeared genetically closer. The PFGE patterns of most of the Georgian strains showed differences to the standard strains of SRB (Fig.3).

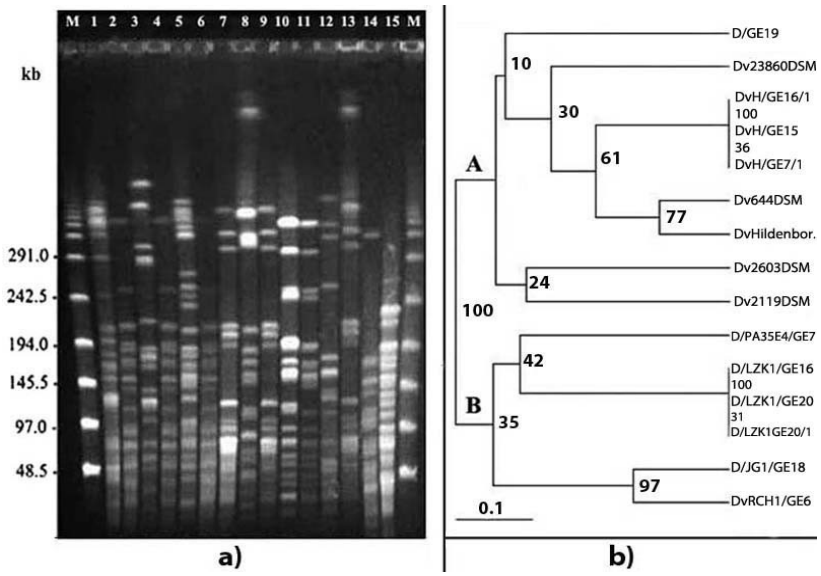


Figure 3. PFGE relatedness of SRB strains
a) PFGE-pattern of *Not*I-digested genomic DNA *Desulfovibrio* bacterial strains: Lane1 DvRCH1/GE6, Lane 2 DvH/GE7/1, Lane 3 D/PA35E4/GE7, Lane 4 DvH/GE15, Lane 5 D/JG1/GE18, Lane 6 DvH/GE16/1, Lane 7 D/LZK1/GE20, Lane 8 D/GE19, Lane 9 D/LZK1/GE20/1, Lane 10

DvHildenborough, Lane 11 DvDSM 644, Lane 12 Dv DSM 2119, Lane 13 D/LZK1/GE16, Lane 14 DaDSM 2603, and Lane 15 DaDSM 23860.
b) Phylogenetic tree based on PFGE result and constructed by UPGMA.

Cell structure. Morphological examination of bacteria by staining and phase contrast and electron microscopy showed that all novel SRB isolates are Gram-negative, non-spore forming, motile, mostly slightly curved rods (DvRCH1/GE6 - 2.1 x 0.4 µm; D/GE19 - 3.2 x 0.5 µm; DvH/GE15 - 2.1 x 0.6 µm; D/LZK1/GE20/1 - 2.3 x 1.0 µm; DvH/GE7/1 - 3.0 x 0.6 µm) with a single or lophotrichous polar flagella (Fig. 4). The strains are desulfoviridine-positive and produce H_2S. All these characteristics are common for members of the genus *Desulfovibrio* [17].

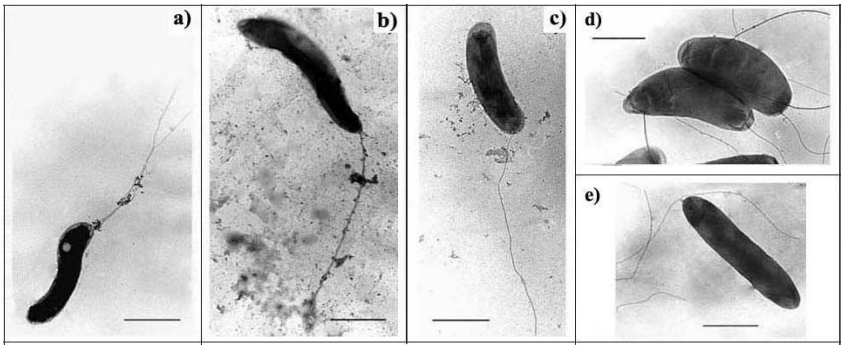


Figure 4. Electron micrographs of SRB strains, isolated in Georgia
a)DvRCH1/GE6; b)D/GE19; c)DvH/GE15; d)D/LZK1/GE20/1; e)DvH/GE7/1. Bar represents 1 µm.

Influence of eco-physiological factors on the growth of bacteria. To identify the effect of environmental factors on growth of SRB strains, cultivation was carried out in LS4D liquid medium at different temperatures, as well as at various acidity and salinity of the medium. Cell growth was determined spectrophotometrically by measuring the optical density at 600 nm.

The optimal growth temperature for most Georgian strains was in the range 30-37°C. Two strains, D/JG1/GE18 and D/GE19 grew in the range of 22-37°C, while DvH/GE15 and D/LZK1/GE20 grew best at 30°C. When tested, most strains grew well at pH 5.5, but two strains, D/LZK1/GE16 and D/JG1/GE18 grew in a broader range of pH 5.5 - 8.2. Some SRB strains, *e.g.* strains DvH/GE15 and D/JG1/GE18, grew well at wide concentrations of NaCl, ranging from 0 to 425 mM. Other strains such as DvH/GE7/1 and DvH/GE16/1 were characterized by tolerance to a relatively narrow range of salinity (Table 2). Strains D/JG1/GE18 was characterized by the widest range of acceptable growth conditions (0 to 425 mM NaCl, 22 to 37°C, and pH from 5.5 to 8.2).

Table 2. The influence of environmental factors on the growth of SRB strains isolated in Georgia

Results after 72 hours of incubation in a liquid medium LS4D

SRB bacterial strain	Temperature at pH 7.4 (T°C)	Acidity at 30°C (pH)	Salinity at 30°C, pH7.4 (mM NaCl)
DvRCH1/GE6	30 -37	5.5	85 - 425
D/PA35E4/ GE7	30 - 37	5.5	85 - 255
DvH/GE7/1	30 - 37	5.5	0 - 85
DvH/GE15	30	5.5	0 - 425
D/LZK1/GE16	30 - 37	5.5 - 8.2	85 - 425
DvH/GE16/1	30 - 37	7.4	85 - 170

D/JG1/GE18	22 - 37	5.5 - 8.2	0 - 425
D/GE19	22 - 37	5.5	85 - 425
D/LZK1/GE20	30	5.5	85 - 340
D/LZK1/ GE20/1	30 - 37	5.5	85 - 425

For phenotypic characterization of *Desulfovibrio* strains, substrate utilization by bacteria was studied. Growth rate of *Desulfovibrio* strains was examined in LS4D medium with electron acceptor sodium sulfate, and electron donors - lactate, glucose, pyruvate, succinate, butyrate, or acetate. The effect of various electron donors on the growth (OD_{600} value) of *Desulfovibrio* strains is shown on Figure 5. Based on phenotypic characteristics the isolates can transform various sugars and other substrates and use them as a carbon source for sulfate reduction similar to other reference strains of *Desulfovibrio*. The Georgian SRB strains differed from each other in their use of substrates (Fig.5).

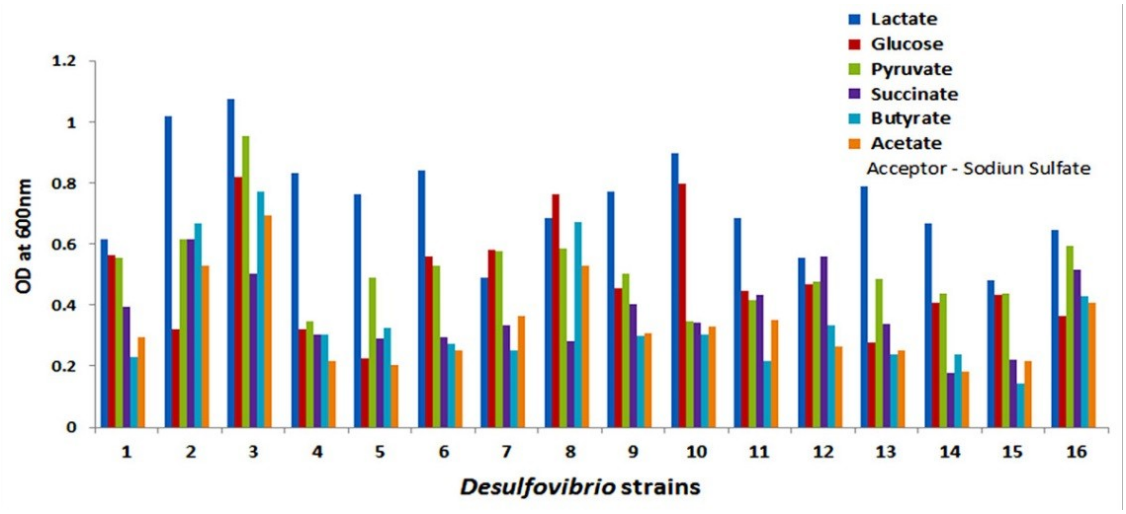


Figure 5. Growth rate of SRB strains in LS4D medium with different electron donors. OD_{600} value was determined after cultivating the strains for 72 h at 30°C.
1.*D. vulgaris* Hildenborough; 2. *D. africanus* PCS; 3. DvRCH1/GE6; 4. D/PA35E4/GE7; 5. DvH/GE7/1. 6.DvH/GE15; 7. D/LZK1/GE16; 8. DvH/GE16/1; 9. D/JG1/GE18; 10. D/GE19; 11. D/LZK1/ GE20;
12. D/LZK1/GE20/1; 13.*D. vulgaris* DSM 644; 14.*D. vulgaris* DSM 2119; 15.*D. africanus* DSM 2603; 16. *D. africanus* DSM 23860.

Lysogenic strains of SRB and temperate phages. To detect/identify the spread of lysogenicity among the isolates, they were subjected to UV irradiation. Two samples, each, were generated and examined: an LD_{50} cell suspension and another sample following 1 min UV irradiation. Seven isolates (DvRCH1/GE6, D/PA35E4/GE7, DvH/GE7/1, DvH/GE15, DvH/GE16/1, D/JG1/GE18, and D/LZK1/GE20) did not release any prophage. Three Georgian isolates (genetically closely related strains D/LZK1/GE16, D/LZK1/GE20/1, and strain D/GE19), and two previously isolated SRB strains, *D. vulgaris* Hildenborough and *D. africanus* PCS showed the presence of temperate phages. The spot test showed that the released phages infected the recipient (indicator) bacterial strains and cleared the lawns, but did not act on the donor bacteria, as these lysogenic strains were immune to the prophages they released. The induced temperate phages lysed three Georgian SRB test strains: D/LZK1/GE6, D/JG1/GE18, and strain DvRCH1/GE7 (Table 3). These test strains were used as hosts for phage plaque isolation.

Table 3 SRB test strains and titer of UV-induced temperate bacteriophages

SRB indicator test-strain	UV-induced temperate bacteriophage	Phage titer (PFU/ mL)
DvRCH1/GE6	φGE19 uv	$1x10^7$
	φGE20/1 uv	$7x10^6$
	φDvHildenborough uv	$1x10^7$
D/PA35E4/GE7	φDaPCS uv	$1x10^7$
D/JG1/GE18	φGE16 uv	$5x10^6$

Transmission electron microscopy (TEM) was used to confirm the presence of induced phages in the UV irradiated samples. Morphologically, all temperate phages belonged to the *Siphoviridae* family (Figure 6). The phage particles consisted of a head (54x54 nm) and a long, non-contractile tail (145x12 nm). A comparison of the DNA restriction patterns of induced phages did show no significant differences between the phage genomes (data not shown).

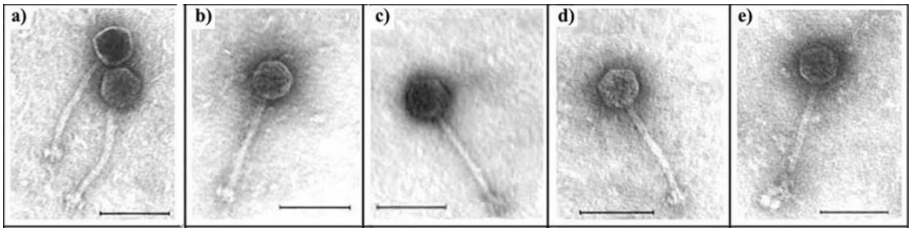


Figure 6. TEM image of temperate SRB phages. (Magnification: 240,000x).
a) φGE16 uv, b) φGE19 uv, c) φGE20/1 uv, d) φDvHildenborough uv, e) φDaPCS uv.
Bar represents 1μm.

Substrate utilization. Three Georgian lysogenic SRB isolates released temperate phages after UV exposure.
To identify the differences in the reproduction ability of untreated and UV-irradiated cultures, the bacterial cells were cultivated under identical conditions. When grown in LS4D medium, all 5 lysogenic strains' native and irradiated pairs revealed more or less different reproductive capacity (with lactate as an electron donor and sulfate as an electron acceptor). Growth of SRB cultures in LS4D before and after UV irradiation is presented in Figure 7.

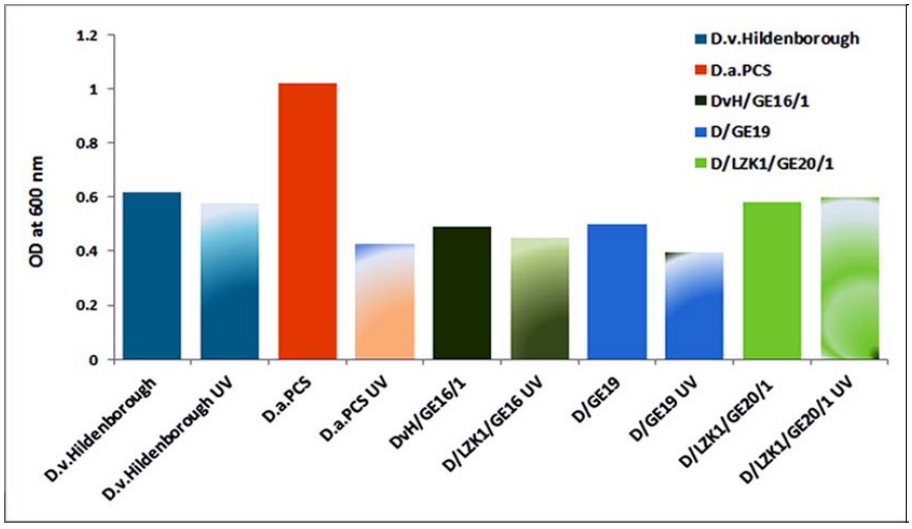


Figure 7. Growth of SRB cultures in LS4D medium before and after UV irradiation (OD_{600} value after cultivation for 72 hours at 30°C).

Metabolic panels PM1 and PM2 and a fully automated OMNILOG Phenotype MicroArray (Biolog, Hayward, CA) were used to test a series of carbon substrates [29, 46]. A comparison of these various carbon sources on untreated wild-type lysogenic and phage-induced bacterial strains showed significant differences (Table 4). In particular, the OMNILOG results showed metabolic differences: the wild-type lysogenic strain D/LZK1/GE16 utilized 44 of 161 carbon sources (27%), while its UV-treated counterpart utilized 84 carbon compounds (52%). The wild-type strains D/GE19 and D/LZK1/GE20/1 metabolized 41% and 33% of the tested substances, while their UV-irradiated counterparts utilized 58% and 92%, respectively. There was a difference between the lysogenic and induced strains in the kind of carbon sources used as well (Table 4). Such results may indicate that temperate phages incorporated in the genome of host bacteria can alter some functions in bacterial carbon metabolism.

Table 4

Comparison of metabolized carbon sources by wild-type lysogenic and UV-induced SRB (OMNILOG results)

#	Carbon Sources # 1-80	Pair of SRB strains						#	Carbon Sources # 81-161	Pair of SRB strains					
		D/LZK1/GE16	D/LZK1/GE16	D/GE19	D/GE19 UV	D/LZK1/GE20/1	D/LZK1/GE20/1			D/LZK1/GE16	D/LZK1/GE16	D/GE19	D/GE19 UV	D/LZK1/GE20/1	D/LZK1/GE20/1
1	L-Arabinose	■	■			■		81	Glycogen				■		■
2	N-Acetyl-D-Glucosamine	■	■	■	■		■	82	Inulin		■				■
3	D-Saccharic Acid			■	■		■	83	Laminarin	■					■
4	Succinic Acid		■	■			■	84	Mannan	■	■		■		■
5	D-Galactose			■	■	■	■	85	Pectin	■	■	■		■	
6	L-Aspartic Acid		■	■	■	■	■	86	N-Acetyl-D-Galactosamine				■		■
7	L-Proline		■				■	87	N-Acetyl-Neuraminic Acid		■		■		■
8	D-Alanine				■			88	b-D-Allose		■		■	■	■
9	D-Trehalose			■	■	■	■	89	Amygdalin				■		■
10	D-Mannose	■		■	■	■	■	90	D-Arabinose	■				■	
11	Dulcitol	■			■		■	91	D-Arabitol		■				■
12	D-Sorbitol		■				■	92	L-Arabitol				■	■	■
13	Glycerol		■	■	■		■	93	Arbutin		■		■	■	■
14	L-Fucose		■		■		■	94	2-Deoxy-D-Ribose	■				■	
15	D-Glucuronic Acid		■		■		■	95	i-Erythritol		■			■	■
16	D-Gluconic Acid			■	■		■	96	D-Fucose						■
17	D,L-a-Glycerol Phosphate				■		■	97	3-0-b-D-Galactopyranosyl-D-Arabinose	■			■	■	■
18	D,L-Lactic acid	■		■	■		■	98	Gentiobiose				■	■	■
19	Formic Acid			■	■	■	■	99	L-Glucose		■				■
20	D-Mannitol				■	■	■	100	D-Lactitol		■	■	■		■
21	L-Glutamic Acid	■	■	■			■	101	D-Melezitose		■		■	■	■
22	D-Glucose-6-Phosphate		■	■	■		■	102	Maltitol	■	■		■	■	■
23	D-Galactonic Acid-g-Lactone				■		■	103	a-Methyl-D-Galactoside	■	■		■	■	■
24	D,L-Malic Acid				■	■	■	104	b-Methyl-D-Galactoside		■		■		■
25	Tween 20		■	■			■	105	3-Methylglucose	■	■				■
26	L-Rhamnose		■	■	■	■	■	106	b-Methyl-D-Glucuronic Acid		■		■		■
27	D-Fructose	■		■	■	■	■	107	a-Methyl-D-Mannoside			■			■
28	Acetic Acid		■	■			■	108	b-Methyl-D-Xyloside					■	■
29	a-D-Glucose			■	■	■	■	109	Palatinose	■	■	■	■	■	■
30	Maltose				■	■	■	110	D-Raffinose		■	■	■	■	■
31	D-Melibiose	■			■	■	■	111	Salicin				■	■	■
32	Thymidine	■	■	■	■		■	112	Sedoheptulosan		■				■
33	L-Asparagine		■	■	■			113	L-Sorbose		■				■
34	D-Glucosaminic Acid		■			■	■	114	Stachyose		■		■	■	■
35	1,2-Propanediol		■				■	115	D-Tagatose				■	■	■
36	a-Ketoglutaric Acid		■	■	■		■	116	Turanose		■		■	■	■
37	a-Methyl-D-Galactoside		■		■	■	■	117	Xylitol		■	■	■	■	■
38	a-D-Lactose	■	■		■	■	■	118	N-Acetyl-D-glucosaminitol						■
39	Lactulose	■	■		■	■	■	119	g-Amino-N-Butyric Acid						■
40	Sucrose	■			■	■	■	120	d-Amino Valeric Acid				■		■
41	Uridine	■		■	■		■	121	Butyric Acid	■			■	■	■
42	L-Glutamine		■	■	■			122	Caproic Acid						■
43	D-Fructose-6-Phosphate		■	■	■		■	123	Citraconic Acid						■
44	Tween 80		■	■			■	124	D,L-Citramalic Acid	■	■				■
45	a-Hydroxyglutaric Acid-g-Lactone		■	■			■	125	D-Glucosamine				■	■	■
46	Adonitol	■	■		■	■	■	126	4-Hydroxybenzoic Acid			■			■
47	Maltotriose				■		■	127	b-Hydroxybutyric Acid		■				■
48	2'-Deoxyadenosine				■			128	g-Hydroxybutyric Acid		■				■
49	Adenosine	■	■		■		■	129	Itaconic Acid			■			■
50	m-Inositol		■		■	■	■	130	D-Lactic Acid Methyl Ester	■	■				■
51	Fumaric Acid		■		■	■	■	131	Malonic Acid		■				■
52	Bromosuccinic Acid		■	■			■	132	Melibionic Acid		■	■			■
53	Mucic Acid		■	■	■		■	133	Oxalomalic Acid	■	■	■			■

54	Glycolic Acid	134	Quinic Acid
55	D-Cellobiose	135	D-Ribono-1,4-Lactone
56	Inosine	136	Sebacic Acid
57	Gly-Glu	137	Sorbic Acid
58	L-Serine	138	Succinamic Acid
59	L-Alanine	139	D-Tartaric Acid
60	Ala-Gly	140	L-Tartaric Acid
61	N-Acetyl-D-Mannosamine	141	Acetamide
62	Mono-Methylsuccinate	142	L-Alaninamide
63	Methylpyruvate	143	N-Acetyl-L-Glutamic Acid
64	D-Malic Acid	144	L-Arginine
65	L-Malic Acid	145	L-Histidine
66	Gly-Pro	146	L-Homoserine
67	p-Hydroxyphenyl Acetic Acid	147	Hydroxy-L-Proline
68	m-Hydroxyphenyl Acetic Acid	148	L-Isoleucine
69	Tyramine	149	L-Leucine
70	D-Psicose	150	L-Lysine
71	Glucuronamide	151	L-Methionine
72	Pyruvic Acid	152	L-Ornithine
73	L-Galactonic Acid-g-Lactone	153	L-Pyroglutamic Acid
74	D-Galacturonic Acid	154	L-Valine
75	Chondroitin Sulfate C	155	D,L-Carnitine
76	a-Cyclodextrin	156	Sec-Butylamine
77	b-Cyclodextrin	157	D,L-Octopamine
78	g-Cyclodextrin	158	Putrescine
79	Dextrin	159	2,3-Butanediol
80	Gelatin	160	2,3-Butanone
		161	3-Hydroxy 2-Butanone

Metal reduction by wild-type lysogenic and phage-induced bacteria: Cells of the wild-type *D. vulgaris* Hildenborough strain reduced 10 mM Fe(III) to 8.5 mM Fe(II) within 24 h (85% reduction) with lactate as the electron donor, while its UV-exposed counterpart showed only a 60% reduction under similar conditions. The non-induced strain D/LZK1/GE16 achieved a 62% reduction but the induced cells could only reduce 26% of the initial Fe(III) added. Strains D/GE19 and D/LZK1/GE20/1 transformed 80% of the initial Fe(III) to Fe(II), while their phage-induced counterparts could only reduce 56% and 50% of initial Fe(III), respectively (Figure 8; a, b, c, d). As a control, we examined the iron reduction capability of the non-lysogenic strains *D. vulgaris* DvH/GE15 and D/PA35E4/GE7 (previously UV-exposed, but no prophage released). Untreated strain DvH/GE15 reduced 80% of Fe (III) to Fe(II) in 24 h with lactate as the electron donor (Figure 8; e). The cells of strain D/PA35E4/GE7 equally transformed 10 mM Fe(III) to 8 mM Fe(II) in 24 h before and after UV irradiation (Figure 8; f). No reduction of Fe(III) was observed in parallel incubations of these bacterial strains without an electron donor (Figure 8; e, f), proving that the reduction of Fe(III) was due to the SRB activity.

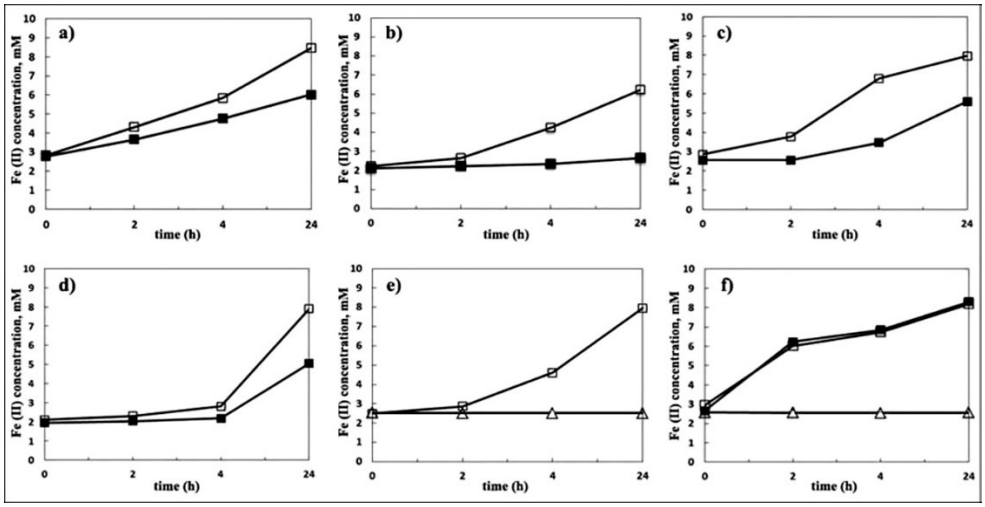


Figure 8. Iron reduction by wild-type and UV-irradiated *Desulfovibrio* bacterial cells
a) DvHildenborough; b) D/LZK1/GE16, c) D/GE19, d) D/LZK1/GE20/1, e) DvH/GE15,
f) D/PA35E4/GE7.
Note: □ – *wild-type cells,* ■ **-** *UV-induced cells,* Δ **-** *incubation without electron donor (e, f).*

Overall, these results indicated that temperate phages support high rate of iron transformation in lysogenic bacterial hosts.

To determine the role of temperate phages in hexavalent chromium [Cr(VI)] reduction, 3 lysogenic SRB strains (DvHildenborough, D/GE19, and D/LZK1/GE20/1) were tested. The ability of Cr(VI) reduction by the wild-lysogenic and UV-treated (non-lysogenic) strains differed: the lysogenic strain *D. vulgaris* Hildenborough reduced 63.4% of the Cr(VI) supplied as potassium chromate within 24 h with lactate as the electron donor, while its UV-exposed counterpart only achieved a 24% reduction. Wild-type of D/GE19 transformed 68.2% of the initial Cr(VI), but the phage-induced counterpart reduced only 38.5%. Similarly, native cells of strain D/LZK1/GE20/1 reduced 67.2% of the initial chromium (VI) compared to the UV-exposed counterpart (Figure 9). It appears that temperate phages support Cr(VI) reduction in SRB, as chromate transformation in induced (non-lysogenic) bacterial cells was relatively low in comparison with the cells of these strains before phage induction.

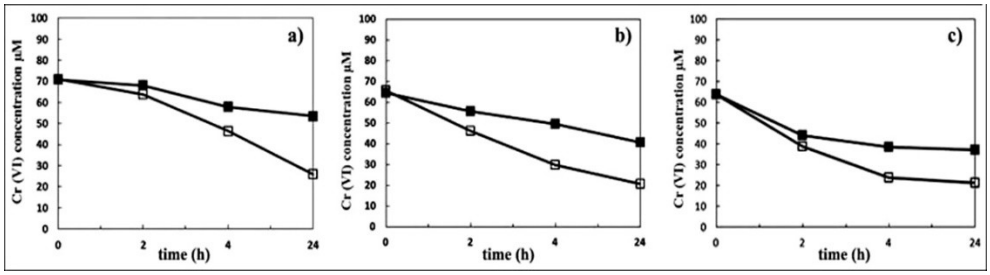


Figure 9. Chromium reduction by wild-type and UV-induced *Desulfovibrio* bacterial cells
a) DvHildenborough, b) D/GE19, c) D/LZK1/GE20/1.
Note: □ - *wild cells;* ■ - *UV-induced cells.*

DISCUSSION

The connection between activity of environmental microorganisms and cases of anaerobic iron corrosion was recognized almost a century ago [16]. In contrast, these same microorganisms have particularly important applications in biotechnology, bioremediation of toxic heavy metals, wastewater hydrogen sulfide decontamination, and in preventing bio-corrosion [30]. A number of publications have focused on their involvement in bio-corrosion of ferrous metal installations in various industries [15, 31]. More recent studies have documented the potential of SRB to bioremediate toxic heavy metals (*e.g.,* chromium and mercury), even radionuclides, (*e.g.,* uranium) [20, 45]. Despite ongoing investigations to understand the genetic bases of metal transformation by this group of bacteria, still not much is known about their sulfate reduction and electron transport.

Bacterial viruses play a key role in the environment. Diverse bacteria and their viruses co-exist in nutrient limited water and groundwater environments [17]. Viruses depend upon the metabolism of their host and thus, influence active microbial communities. Temperate phages have the same lytic capacity as virulent phages but in addition, they can choose a non-lytic lifestyle [21]. Prophages are integrated into the chromosome of their host until conditions favor their reactivation. Bacteriophages incorporated into the host bacterial genome play important role in regulating growth, diversity, abundance, and the overall function of a microbial community in various environments. They also act as a catalyst of geochemical nutrient cycles. The maintenance of lysogeny depends on a fine balance between phage and host. Production of lysogenic phages depends on the number of cells that contain prophages and the presence of an inducing agent [44], [50].

Lysogeny is thought to be the preferred viral life cycle when growth conditions for the host are unfavorable for rapid growth, while the phage can survive times of low host abundance as prophage. Also, bacterial viruses together with other mobile genetic elements are the most prominent facilitators of horizontal gene transfer in bacterial populations.

The Black Sea ecology, the oil terminal in Batumi and the marine loading complex, which currently processes about 5.4 million tons of crude oil and petroleum products annually, explains the presence of anaerobic, sulfate-reducing bacteria in the world's largest meromictic basin; water from the depths of the sea does not readily mixes with the upper layers. As a result, over 90% of the depth is depleted in dissolved oxygen. In the upper layers, the salinity is about 17 PSU due to the constant influx of fresh river water. Deeper waters and sediments contain mainly biotic hydrogen sulfide. Thus, it was not surprising that sulfate-reducing bacteria were identified in this region, and all (7) environmental sources, from where SRB strains are isolated were located in the Western part of Georgia, near the Black Sea cost. The samples were taken from various sites near oil refineries (river water, sea water, sludge, dry corrosion sites).

Ten identified bacterial strains were confirmed as *Desulfovibio* spp. by sequencing the 16S rRNA-coding genes. Identification of genetically closely related strains, isolated from different sources may, in part, be explained by a possible transmission of isolates from natural spring waters to the rivers and finally, to the sea water, since these samples were collected in western part of Georgia mainly near estuaries flowing into the Black Sea.

Together with the newly isolated environmental strains, two well-characterized SRB strains were used in our research, including *Desulfovibrio vulgaris* Hildenborough, the first SRB for which the whole genome was sequenced [23]. In the 3,570,858 bp chromosome there were two copies of nearly identical mu-like bacteriophages annotated, one lambdoid bacteriophage and remnants of another bacteriophage genome. Phages acquire new genetic information for transfer by recombination with superinfecting phages, resident prophages, or other mobile genetic elements directly from the bacterial chromosome. The newly acquired genes are then passed to the bacterial host. Novel pathways by which prophages can influence their hosts can be determined while studying the lysogenic strain. We identified three environmental strains as lysogenic and induced *Siphoviridae* morphotype temperate phages from these strains. Metabolism of carbon sources and transformation of metals (ferrum and chromium) in lysogenic and prophage-induced strains were substantially impacted by phages integrated in host bacterial chromosome. Substantial differences in the metabolism of phage-induced and untreated bacterial strains became evident, indicating that temperate phages incorporated in the genome of host bacteria may alter significant functions in bacterial activity. Data obtained in this study indicated significant contribution of phages in acquisition of genetic traits by sulfate-reducing bacteria.

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