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A New *Thioalkalivibrio* sp. Strain Isolated from Petroleum-Contaminated Brackish Estuary Sediments: A New Candidate for Bio-Based Application for Sulfide Oxidation in Halo-Alkaline Conditions

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Abstract: A new halo-alkaline sulfur-oxidising bacterial strain was isolated from brackish estuary sediments contaminated by total petroleum hydrocarbon. The isolate was classified as a new strain of *Thioalkalivibrio sulfidiphilus* sp., showing a higher capability of adaptation to pH and a higher optimal sodium concentration for growth, when compared to *Thioalkalivibrio sulfidiphilus* sp. HL-EbGr7, type strain of the species. The strain was capable to grow in saline concentrations up to 1.5 M Na^+ and pH up to 10. The genome of the new isolate was sequenced and annotated. The comparison with the genome of *Thioalkalivibrio sulfidiphilus* sp. HL-EbGr7 showed a duplication of an operon encoding for a putative primary sodium extruding pump and the presence of a sodium/proton antiporter with optimal efficiency at halo-alkaline conditions. The new strain was able to oxidize sulfide at halo-alkaline conditions at the rate of 1 mmol/mg-N/h, suitable for industrial applications dedicated to the recovery of alkaline scrubber for H_2S emission absorption and abatement.

Keywords: *Thioalkalivibrio sulfidiphilus* sp.; sulfide oxidation; sodium extruding pump; sodium/proton antiporter; Dissimilatory Sulfite Reductase complex; Hetero Disulfide Reductase complex; alkaline sodium/proton antiporter

1. Introduction

In strongly antropized environments, contaminated matrices should be evaluated also as source of microorganisms of interest capable of strategies of adaptation to the environment that might be similar to the one that the "properly said" extremophiles adopt to survive to extreme conditions. Historical contaminations are associated to changes in the chemical and/or physical characteristics of most of the ecological niches afflicted by the contamination, with possible alteration of the natural pH, depletion of waters, and other diverse effects. For these reasons, contaminated matrices can be a source of microbial candidates with interesting features that might find applications in the abatement of contaminants. In fact, contaminated matrices have already been a source of isolation of interesting new microbial strains, showing metabolic features exploitable for diverse biotechnological applications [1–4].

In this study, a new strain of the haloalkaliphilic genus *Thioalkalivibrio* was isolated from brackish estuary sediments, contaminated by petroleum-derived hydrocarbons. Most of the strains of the genus *Thioalkalivibrio* have been extensively and nearly exclusively described as colonising the microflora of soda lakes [5,6], characterized by double extreme conditions, high pH and salinity. However, the presence of the genus *Thioalkalivibrio* in brackish sediments has also been recorded by the screening of clone libraries from metagenomic extracts or deep sequencing of metagenomes from different sampling sites [7–11]. In this case, the information was limited to the genomic data acquisition. From these environments, any strains were actually isolated, cultivated and characterized.

The *Thioalkalivibro* genus grows chemolithoautotrophically using hydrogen sulfide (H_2S) as the electron donor. The genus is of interest for the bio-based industrial sector and several *Thioalkalivibrio* sp. strains found application in bio-based processes for the oxidation of H_2S emissions and the recycling of adsorbing alkaline scrubbers, characterized by a pH up to 10 and high sodium concentration [12–14]. H_2S is a highly toxic gas, produced as a waste product in several industrial processes, such as leather tanning and paper manufacturing, energy production by petroleum refining, biogas. and geothermal sources [15]. Classic physical–chemical treatments for H_2S removal, like the Claus–Scott and the Stretford processes, are demanding in terms of energy consumption and/or chemicals [15].

The scope of this work was the description of a new bacterial strain that is capable of oxidizing H_2S in halo-alkaline conditions (pH = 10 and sodium concentration between 0.6 and 1 M), finding applications in the development of processes dedicated to the control of H_2S emissions. The new strain, isolated from brakish and neutral sediments, might update the description of the genus *Thioalkalivibrio*, up to now described as colonising dual extreme environments with both extremely high pH and Na^+ concentration, and might also update the microbial resources recoverable from the environment for the abatement of H_2S. The genome of the strain was sequenced and the genes, responsible for H_2S oxidation and homeostasis in halo-alkaline growing conditions, were annotated.

2. Materials and Methods

2.1. Total Petroleum Hydrocarbon Quantification in Sediments

Sediment samples were dried for 2 h at 105 ± 1 °C in an oven. A total of 10 g (dry weight) were put in 125 mL glass-stoppered flasks and extracted in 40 mL acetone/n-heptane (1:1), containing 15 µL/L dodecane and 15 mg/L tetracontane as internal standards. The samples were extracted by shaking on a rotary shaker at 200 rpm for one hour, at room temperature. Extracts were decanted by gravity and the supernatants were hand shaken in a separating funnel containing 100 mL of water to remove acetone. The step was repeated twice. The residual organic layer (containing n-heptane, internal standards, and total petroleum hydrocarbons, TPHs) was cleaned up by purification on pre-washed Florisil® columns, mesh 60, and dewatered with anhydrous sodium sulfate. A volume of 1 µL of extract was injected in a gas chromatograph (GC; Trace 1300, Thermo Fisher, Milano, Italy), equipped with a flame ionization detector (FID) and a 12 m, DB5.625 column with 0.25 mm inner diameter (J&W Scientific, Folsom, CA, USA). The GC running conditions were as follows: the oven temperature was programmed as 5 min at 50 °C, ramp to 300 °C at 10 °C/min, 300 °C for 12 min; the FID was maintained at 325 °C. Chromatograms were recorded with Chrom-card data system (Thermo Fisher, Milano, Italy). External calibration was performed using mineral oil A + mineral oil B standards (Merck, Milano, Italy), in n-heptane, containing 15 µL/L dodecane and 15 mg/L tetracontane, at concentrations ranging from 100 to 10,000 mg/L. Area integration was performed on blank subtracted spectra, from dodecane retention time to tetracontane retention time, according to UNI EN 16703.

2.2. Isolation of Thioalkalivibrio sp. 10fs10

The sediments, contaminated by total petroleum hydrocarbons (3576 mg/Kg dw/dw), were characterized by a neutral pH (pH = 7.2), chlorine and sodium concentrations of 0.45 and 0.51 M respectively. The enrichment of halo-alkaliphilic bacterial strains was performed in the DSMZ

(Deutsche Sammlung von Mikroorganismen und Zellkulturen) n° 925 added with a trace element solution (2 mL/L), $MgCl_2$ (0.5 mM), KNO_3 (5 mM), and $Na_2S_2O_3$ (40 mM) by incubating 1 g of sediments in 250 mL of the 1:2 diluted DSMZ n° 925 medium for one month at 28 ± 1 °C on an orbital shaker at 250 rpm in the dark. A sub-culturing passage was performed inoculating the bacterial suspension into full strength DSMZ n° 925 (5% v/v) and, after two months of incubation, serial dilutions of the enrichment suspension were plated on agarized DSMZ n° 925 medium. A single bacterial morphotype at visual inspection was identified. A total of ten different colonies with the same morphotype were analysed by amplified ribosomal DNA restriction analysis (ARDRA) [16] and repetitive sequence-based PCR (REP–PCR) [17,18]. The ARDRA analysis was performed digesting the amplification products with Sau 3A, Alu I e Hae III restriction enzymes. All analyses were performed at least twice for each isolate. A single operational taxonomic unit (OTU), 10fs10, was recovered.

2.3. Thioalkalivibrio sp. 10fs10 Genome Sequencing

Thioalkalivibrio sp. 10fs10 genomic DNA was extracted by GenElute™ Bacterial Genomic DNA, following producer's instructions. DNA quantification was performed using Qubit® 3.0 (Invitrogen, Thermo Fisher Life Technologies, Milan, Italy) following producer's instructions. *Thioalkalivibrio* sp. 10fs10 genome was sequenced in St. Petersburg state University, St. Petersburg (Russia) (for more details see Supplementary Materials and Methods). Genome assembly and annotation project are available at IMG, under IMG submission ID 183849.

2.4. Taxonomic Characterisation of the Thioalkalivibrio sp. 10fs10

The full-length gene coding for the 16S rRNA of *Thioalkalivibrio* sp. 10fs10 isolate was extracted from the draft genome. The sequences of the genes coding for the 16S rRNA of 77 different *Thioalkalivibrio* sp. strains were downloaded from Silva SSU database and from Joint Genome Institute (JGI, Berkeley, CA, USA) using IMG. All sequences were aligned using MUltiple Sequence Comparison by Long-Expectation (MUSCLE) algorithm [19] and trimmed to match the length of the shortest deposited sequence (1374 nucleotides). Maximum likelihood tree was computed using the Tamura-Nei DNA evolutionary model [20] (for more details see Supplementary Materials and Methods). The accuracy of the calculated tree was assessed by bootstrapping 1000 replicates.

2.5. Maximum Likelihood Tree Based on Multi-Locus Sequence (MLS) Analysis

Maximum Likelihood MLS tree was computed using concatenated amino acid sequences of ClpA-DnaJ-GyrA-RpoH-RpoS-SecF proteins, codified by respective housekeeping genes obtained from genomic annotation of *Thioalkalivibrio* sp. 10fs10 and from available complete genomes of other *Thioalkalivibrio* spp. on IMG The corresponding sequences of amino acids were aligned with MUSCLE algorithm [19] and concatenated in the reported order. Maximum likelihood tree was computed using a method based on the Whelan and Goldman + Freq. model [21] (for more details see Supplementary Materials and Methods).

2.6. Classification of Thioalkalivibrio sp. 10fs10 by ANI-BBH, dDDH, and Genome Taxonomy Database

Average Nucleotide Identity was performed using Genome comparison tools available on IMG/M platform. The Average nucleotide identity (ANI) was based on Bidirectional Best Hits (BBH) with a CDS identity cut off value of 70% [22]. Digital DNA–DNA Hybridisation (dDDH) was performed using Genome to Genome Distance Calculator (GGDC) v. 2.0 [23] available on http://ggdc.dsmz.de, using an algorithm based on "distance 2" calculation. This tool tolerates up to 80% genome deletion to achieve a resolution up to strain level [24]. MSA analysis was performed by Genome Taxonomy Database Toolkit (GTDB-Tk) tool starting from *Thioalkalivibrio* sp. 10fs10 draft genome. All 120 reference housekeeping genes used by Genome Taxonomy Database (https://gtdb.ecogenomic.org/) classification were identified, translated into amino acid sequences, concatenated, aligned and masked to reduce the full amino acid sequence length from 41,155 to 5035 aa. A total of 30 reduced and concatenated amino

acid sequences out of 17,413 entries of the Genome Taxonomy Database (release 86) were selected: 24 available strains previously classified as belonging to the genus *Thioalkalivibrio* and 6 out-groups identical to those used to compute the Phylogenetic and ML trees. Those sequences, plus queries 10fs10 and ALJ17 sequences were used to construct a Maximum likelihood tree. Maximum likelihood tree was computed using a method based on the Whelan and Goldman + Freq. model [21] (for more details see Supplementary Materials and methods).

2.7. Thioalkalivibrio sp. 10fs10 Growth in Halo-Alkaline Conditions

Na^+ dependent growth tests were performed in DSMZ n° 925 medium added with the previously described trace element solution and increasing Na^+ concentration amended as NaCl, from 642 mM, up to 1500 mM. The medium was inoculated with a bacterial inoculum deriving from the growth in DSMZ n° 925 of a single colony picked from an agarized plate, and performed at an optical density at 600 nm (SpectroStar Nano, BMG Labtech, Ortenberg, Germany) of 0.05 units of absorbance and incubated at 28 ± 1 °C on an orbital shaker at 250 rpm in the dark for 7 days. The pH dependent growth tests were performed as previously described at decreasing pH values starting from 10.2, pH value of the DSMZ n° 925 medium, down to 7.3. The pH was corrected by sulfuric acid. Bacterial growth in the different growth conditions was periodically quantified measuring the spectrophotometric absorbance of the bacterial suspension at 600 nm.

2.8. Thioalkalivibrio sp. 10fs10 Hydrogen Sulfide Oxidation Kinetics

The hydrogen sulfide oxidation kinetics were performed in 2- and 5-L Infors-HT Labfors 5 reactors, Redox and pH probes were calibrated with reference solutions before autoclaving, while the oxygen electrode was calibrated in complete growth medium and at stable operative conditions (temperature, stirring and gas flows) at 0% saturation by flushing nitrogen and 100% by flushing air at 0.25 or 0.09 NL/min and 1 bar relative pressure. The bacterial biomass for the oxidation test was grown in the 2-L reactor in DSMZ n° 925 medium with a starting thiosulfate concentration of 40 mM at pH 10, temperature constantly held at 28 °C, constant stirrer speed at 480 rpm, and air flow set to 0.25 NL/min at constant inlet pressure of 1 bar in the 2-L reactor. After two weeks of pre-growth on thiosulfate, the whole reactor volume was pelleted in a Beckman coulter centrifuge equipped with a jla8.1000 rotor, at 14,000 g at room temperature for 20 min. The pellet was immediately re-suspended in 20 mL of DMSZ n° 925 medium and used to inoculate the 5-L reactor for sulfide oxidation tests in the same experimental conditions described before, with the exception of the amendment of sodium sulfide as electron donor instead of thiosulfate. Sulfide was amended by adding solid disodium salt in the reaction vessel. Sulfide residual concentration was estimated by Trüpet-Schlegel spectrophotometric method. The inflection of the recoded redox potential curve indicated the drop of sulfide concentration to negligible residual levels, and was selected as a fast method to estimate hydrogen sulfide consumption rate. Increasing amounts of sodium sulfide to the 5-L reactor were performed sequentially: the same amount of the sodium salt was added three consecutive times. Each amendment was performed after complete oxidation of the previous addition. Sets of three additions were performed with an increasing final hydrogen sulfide concentration, ranging from 1 to 2.5 mM HS. Bacterial growth was quantified by the Bradford assay.

3. Results

3.1. Thioalkalivibrio sp. 10fs10 Strain Isolation and Phylogenetic Characterisation

The opportunity of the isolation of the new strain was envisaged by analysing results of a shot-gun sequencing of the metagenome of the sediments of interest, which showed a non-negligible relative abundance of the genus *Thioalkalivibrio* (5‰). The enrichment ended up with the isolation of a single strain that, by the sequencing of the partial gene coding for the 16S rRNA showed 100% of homology

with the full length *Thioalkalivibrio sulfidiphilus* HL-EbGr7 16S rRNA gene (GenBank Accession number NC_011901).

In Figure 1, the 16S rRNA gene phylogenetic tree and the amino-acidic based MLS tree are shown. The clade containing *Thioalkalivibrio* sp. 10fs10 was unmodified in both phylogenetic and MLS tree topologies and indicated that the strain was classified as a *Tv. sulfidiphilus* species.

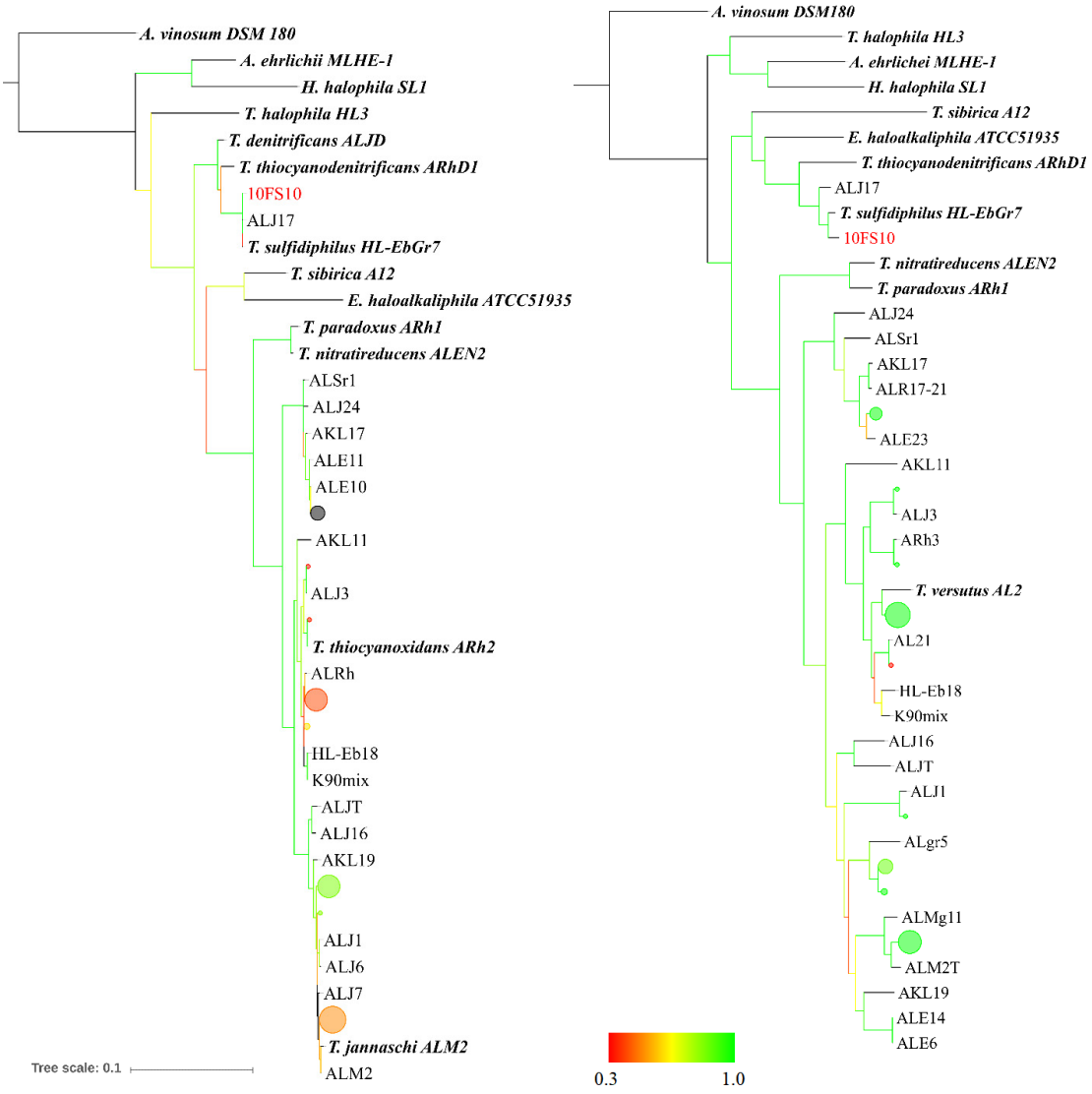

Figure 1. Phylogenetic and multi-locus sequence (MLS) analyses of *Thioalkalivibrio* spp. Tree on the left: 16S rRNA phylogenetic tree; tree on the right: MLS maximum likelihood tree, based on concatenated amino acid sequences of Clp-DnaJ-GyrA-RpoH-RpoS-SecF. Type strains are in bold. Circles represent collapsed leaves (branch length < 0.001 for Phylogenetic tree and <0.05 for MLS tree): their dimensions are proportional to the number of leaves collapsed. Colour scheme of nodes represents bootstrapping values, as reported in the legend.

The ML phylogenetic tree based on Genome Taxonomy Database entries and classification classified the 10fs10 as a new strain belonging to the genus *Thioalkalivibrio A sulfidiphilus*, whose species type-strain is the HL-EbGr7 (Figure 2).

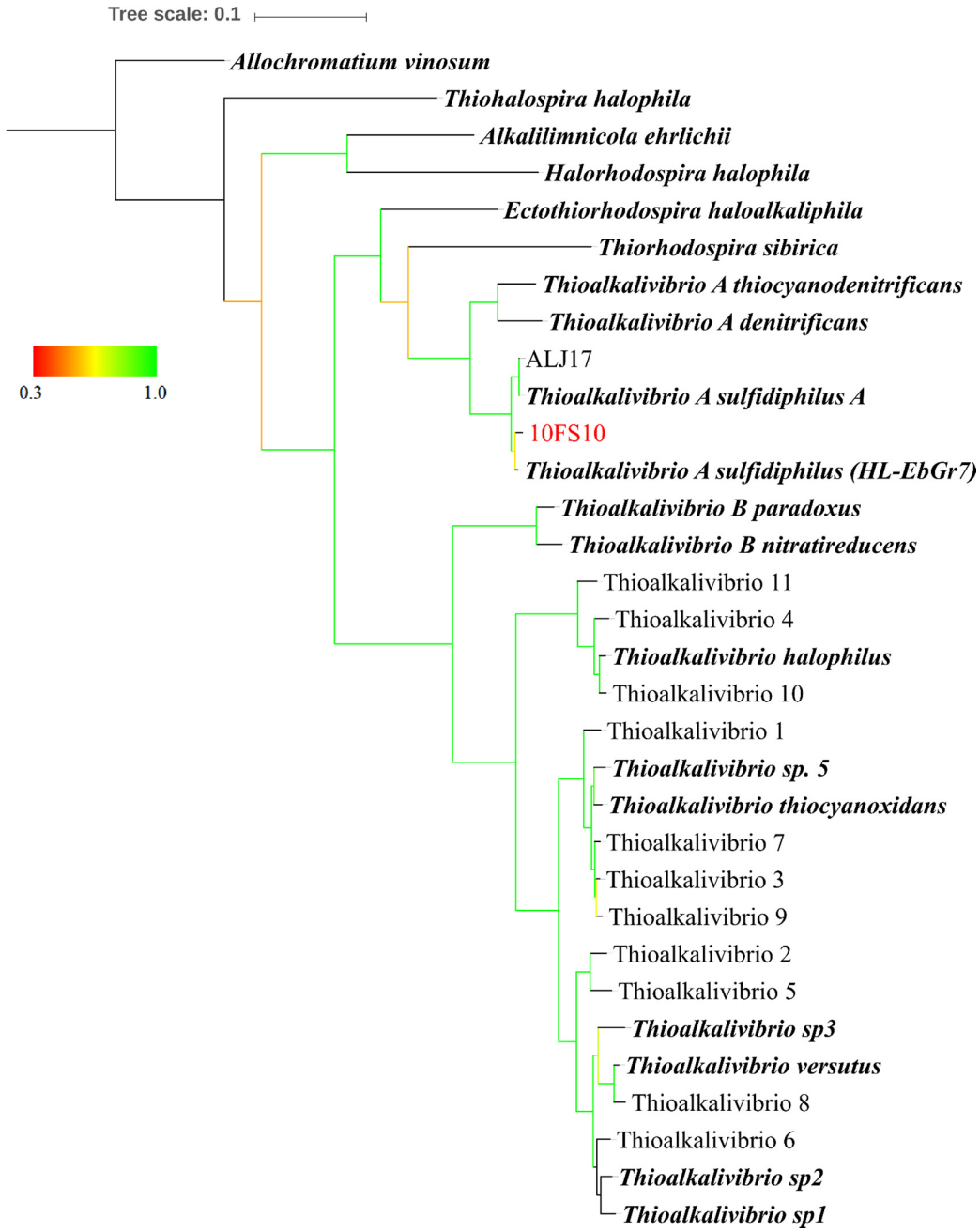

Figure 2. Maximum Likelihood tree performed using masked sequences of 120 housekeeping genes, as suggested by Genome Taxonomy Database criterion of classification, using the Genome Taxonomy Database Toolkit (GTDB-Tk) tool.

The ANI-BBH analysis of the genomes of the two already genome sequenced strains of the *Tv. sulfidiphilus* species (HL-EbGr7 and ALJ17) and the 10fs10 isolate, confirms that *Thioalkalivibrio* sp. 10fs10 shares the same genus of both HL-EbGr7 and ALJ17 strains, but not necessarily the same species (Table 1). Data were partially confirmed by the high taxonomic resolution achieved by dDDH calculation (Table 1), indicating that 10fs10 is a different strain from both *Tv. sulfidiphilus* HL-EbGr7 and ALJ17, but also suggested that 10fs10 belongs to a different species with reference to *Tv. sulfidiphilus* HL-EbGr7, even though with a borderline value (dDDH value 56.8–62.4; $P_{dDDH>70\%}$ 51.07%).

Table 1. Average nucleotide identity (ANI) and digital DNA hybridisation (dDDH) calculated for *Thioalkalivibrio* sp. 10fs10 and other *Tv. sulfidiphilus* strains with pairwise ANI–Bidirectional Best Hits (BBH) tool available in IMG and Genome to Genome Distance Calculator (GGDC) v. 2.0 respectively. AF represents the aligned fraction expressed in percentage, ANI 1->2 and ANI 2->1 and related AF values indicate ANI and AF calculation swapping query and reference genomes. $P_{dDDH>70\%}$ and $P_{dDDH>79\%}$ represent false negative probability related to dDDH calculation. ANI-BBH values comprised between 94 and 96 indicate same genus, while dDDH values comprised between 70% and 79% indicate same species.

Genomes	**ANI-BBH**				**dDDH**		
Reference genome	ANI 1->2	ANI 2->1	AF 1->2	AF 2->1	Estimated DDH value (%)	$P_{dDDH>70\%}$	$P_{dDDH>79\%}$
Tv. sulfidiphilus HL-EbGR7	95.52	95.52	78.39	80.31	56.8–62.4	51.07%	12.52%
Tv. sulfidiphilus ALJ17	94.33	94.33	82.68	73.67	49.1–54.4	24.30%	5.08%

3.2. Genome Properties

Statistics of genome assembly and annotation are reported in Table 2. The draft genome of the isolate 10fs10 consists of 17 contigs greater than 500 kbp, contigs which less than 500 bp in length were discarded. The total length of contigs was 3.42 Mbp. A length of 3.16 Mbp characterizes one of that contigs, which cover more than 92% of the entire genome. The G + C percentage determined from the genome sequence is 64.95%. A total of 3243 coding DNA sequences (CDS) were predicted, of which 3175 were annotated as protein coding sequences, while 68 were annotated as RNA genes. A single copy of the genes coding for ribosomal RNA subunits 5S, 16S, and 23S was retrieved. More than 69% of CDS were assigned to cluster of orthologous-group classification and functional categories (COG).

Table 2. *Thioalkalivibrio* sp. 10fs10 genome assembly and annotation statistics.

Genome Properties	**Value**
Total scaffolds	17
Length	3.42 Mbp
G + C content	64.95%
Total DNA coding region	3243
Protein coding region	3175
rRNA genes	3 (5S rRNA, 16S rRNA, 23s rRNA)
tRNA	52
tmRNA	1
Protein coding region with Pfam	2966
Protein coding region with COG	2241
Protein coding region with KEGG	1656
Transmembrane protein coding gene	781
CRISPR repeats	2

3.3. Halo-Alkaline Condition of Growth

The growth of *Thioalkalivibrio* sp. 10fs10 in presence of thiosulfate and increasing Na^+ concentration was measured. Results obtained (Figure 3) showed that 10fs10 was capable to grow at a wide interval of Na^+ concentrations with an optimal growth at 750 mM total Na^+ content. The 10fs10 isolate can grow up to 1.5 M Na^+ concentration.

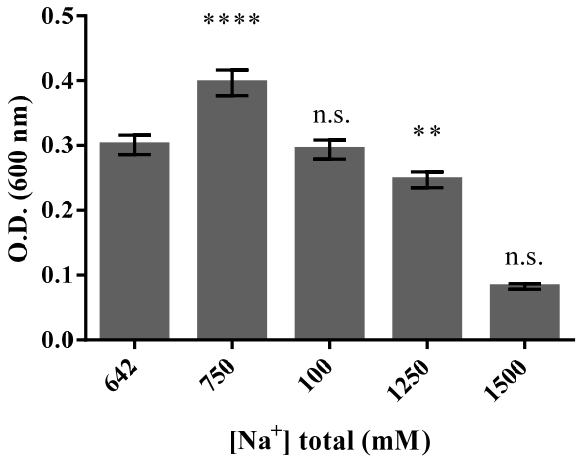

Figure 3. *Thioalkalivibrio* sp. isolate 10fs10 growth after 7 days of incubation at increasing sodium concentrations: maintenance medium (DSMZ 925 with 40 mM thiosulfate and nitrate 5 mM, pH10) was added with NaCl at the total sodium concentration reported. Error bars represent SD of 6 replicates. Asterisks indicate significance level at 99% (**), and 99.99% (****). n.s—statistically not significant.

The growth of *Thioalkalivibrio* sp. 10fs10 at different pH is reported in Figure 4. The best growth performances were achieved in the pH range of 10.2–9.02, however the strain can growth also at neutral pH.

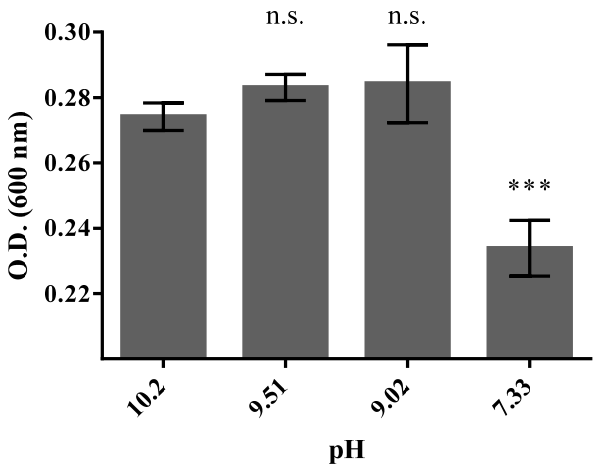

Figure 4. *Thioalkalivibrio* sp. 10fs10 growth after 7 days at different pH: maintenance medium (DSMZ 925 with 40 mM thiosulfate and nitrate 5 mM) was adjusted at reported pH by addition of sulfuric acid. Error bars represent SD of 6 replicates. Asterisks indicate significance level at 99.9% (***). n.s—statistically not significant.

3.4. Thioalkalivibrio sp. 10fs10 Capacity of Hydrogen Sulfide Oxidation in Halo-Alkaline Growth Conditions

Bench scale fed-batch experiments of sulfide oxidation in alkaline conditions (pH 10) were performed to estimate the hydrogen sulfide oxidation rate of the 10fs10 isolate. The Haldane inhibition model was adopted to interpolate the specific data points of hydrogen sulfide oxidation rate with reference to the initial sulfide concentration added ($(HS^-)_{init}$) and to evaluate the kinetics parameters maximum specific oxidation rate (V_{max}), Michaelis–Menten constant (K_M) and Haldane inhibition constant (Ki). Results obtained showed that *Thioalkalivibrio* sp. 10fs10 oxidized hydrogen sulfide to an optimal concentration of 4 mM with an air flow of 0.25 NL/min, as shown in Figure 5 and Table 3.

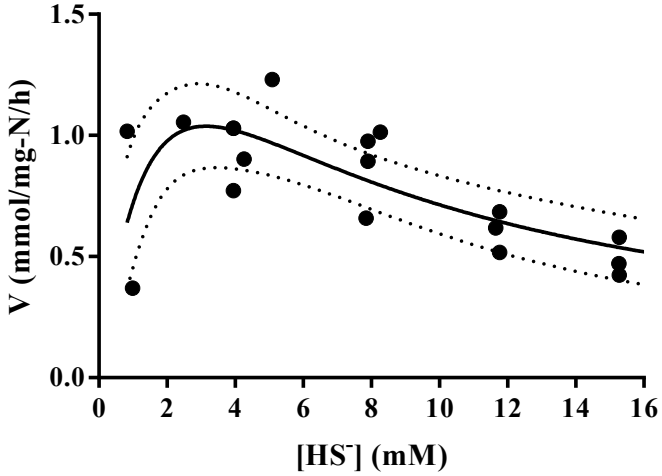

Figure 5. Oxidation performances of *Thioalkalivibrio sp.* isolate 10fs10 in fed batch reactor for a range of sulfide initial concentrations 1–15 mM with an air flushing at 0.25 NL/min: graph reports specific oxidation rate of sulfide (i.e., normalized for biomass concentration) against initial sulfide concentration. Continuous line represents Haldane inhibition model applied to substrate. Dashed lines represent 95% confidence interval of the interpolation.

Table 3. Haldane kinetic parameters for sulfide oxidation tests at diverse air flows.

Air Flow	0.25 NL/min	0.09 NL/min
Vmax (mmol/mg-N/h)	2.648	0.745
K_M (mM)	2.438	4.547
K_i (mM)	4.053	3.491

Reduced air flow (0.09 NL/min of air) was tested to check the variation in specific oxidation rate in growth conditions described as favouring the production of elemental sulfur [13]. Results are shown in Figure 6 and Table 3.

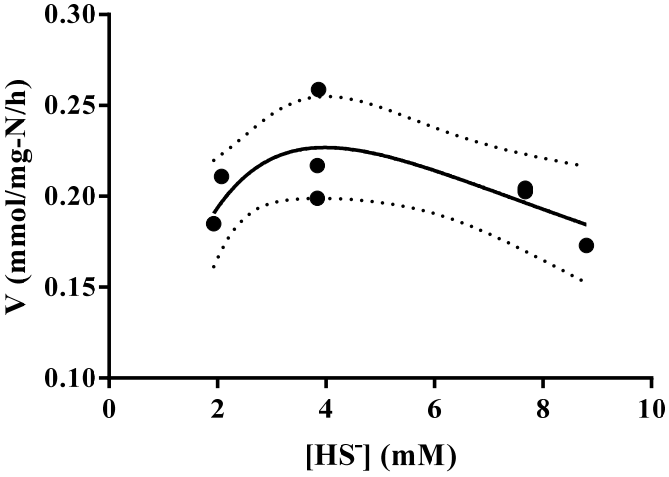

Figure 6. Oxidation performances of *Thioalkalivibrio* sp. isolate 10fs10 in fed batch reactor for a range of sulfide initial concentrations 1 mM to 10 mM with an air flushing at 0.09 NL/min: graph reports specific oxidation rate of sulfide (i.e., normalized for biomass concentration) against initial sulfide concentration. Continuous line represents Haldane inhibition model applied to substrate. Dashed lines represent 95% confidence interval of the interpolation.

Inhibition constants were similar in high and low airflow tests, showing that *Thioalkalivibrio* sp. 10fs10 efficiently oxidizes sulfur also in microoxic conditions. Affinity for substrate increases with

oxygen flow, in fact the K_M decreases from 4.55 to 2.44 mM when air flow is increased from 0.09 to 0.25 NL/min. Lower sulfide affinity in poorly oxygenated medium is associated to a reduced specific oxidation rate and it is reasonable to assess that elemental sulfur, instead of sulfate, is produced as suggested by the whitish colour of the growth medium (data not shown).

In Table 4, the comparison of two oxidation processes of the same starting sulfide and biomass content, performed at high and low oxygen flows is reported: the pH drop, due to the oxidation of elemental sulfur to sulfate with the generation of two acidic equivalents, is significant in high oxygen flow conditions.

Table 4. Comparison of low air flow and high air flow oxidations for the same starting hydrogen sulfide and similar biomass concentration.

Parameters	Low Air Flow (0.018 NL/min)	High Air Flow (0.25 NL/min)
(HS^-) initial (mM)	5.21	6
V_{ox} spec (mmol/(mg-N·h))	0.057	0.318
Biomass (mg-N/L)	7.31	6.51
ΔpH	Negligible	−0.1
Elemental sulfur	Yes	no

3.5. Thioalkalivibrio sp. 10fs10 Genome Sequencing and Annotation

Table 5 reports the list of the genes and complete operons implied in sulfur oxidation that could be retrieved in *Thioalkalivibrio* sp. 10fs10 genome. The Kelly–Friedrich pathway for sulfur oxidation [25,26] relies on a periplasmatic complex composed by four proteins SoxXA, SoxB, SoxYX, and SoxCD that catalyse thiosulfate disproportion to sulfate and elemental sulfur, and sulfide oxidation to sulfate. In microorganisms able to store sulfur in elemental form, the hemomolibdoprotein SoxCD is absent. *Thioalkalivibrio* sp. 10fs10 has one copy of *sox*B, three copies of *sox*A and *sox*X, and two copies of *sox*Y and *sox*Z. Genes coding for SoxCD are absent.

Table 5. Comparison of gene copy numbers related to genes implied in sulfur metabolism between *Tv.* 10fs10, *Tv. sulfidiphilus* Hl-EbGr7^T, and *Tv. sulfidiphilus* ALJ17.

Gene	10fs10	HL-EbGr7	ALJ17
*sox*A	3	4	4
*sox*X	3	4	4
*sox*Y	2	1	not found
*sox*Z	2	1	1
*sox*B	1	1	1
*sox*C	0	0	0
*sox*D	0	0	0
*fcc*A	1	3	2
*fcc*B	1	3	2
*dsr*A	1	1	1
*dsr*B	1	1	1
*dsr*C	1	1	1
*apr*A	1	1	1
*apr*B	1	1	1
sat	2	1	1
*hdr*A	1	1	1
*hdr*B	2	1	1
*hdr*C	2	1	1
*sor*A	1	1	1
sor B	1	1	1

A putative second mechanism for sulfide oxidation to elemental sulfur, up to now described only in vitro, is performed by the Flavocytochrome-C dependent oxidase complex (FccAB) [27]. *Thioalkalivibrio* sp. 10fs10 has one copy of *fcc*AB.

The cytoplasmatic Dissimilatory Sulfite Reductase complex, which, in sulfur oxidizers, runs in reverse (rDSR), is involved in further oxidation of elemental sulfur to sulfite [28]. The mechanism of oxidation involves an intracellular cascade of carrier proteins (Rhd, TusA, DsrEFH, DsrC, and the membrane bound DsrE2A) [29] transporting sulfur moiety to the active site of the cytosolic enzyme Sulfite Reductase [30,31], codified by the *dsr*AABB operon coding for the rDSR complex. The membrane-bound DsrMKJOP electron-transporting complex [32,33] might be involved in the transport of electrons to the iron-sulfur flavoprotein DsrL, with the concomitant reduction of NAD^+ [34]. *Thioalkalivibrio* sp. 10fs10 has a single copy of all the genes implied in transcription of the aforementioned proteins. A second pathway for elemental sulfur oxidation retrieved in 10fs10 was the Hetero Disulfide Reductase complex (HDR) based on a gene operon, *hdr*ABB_1CC_1, recently described by [35] for its involvement in the sulfur oxidation pathway.

Further oxidation of sulfite to sulfate within the genus *Thioalkalivibrio* can be performed by two pathways: a direct oxidation, and an indirect oxidation pathway, carried out with the intermediate Adenosine 5′-Phosphosulfate (APS). Direct oxidation is performed by the heterodimeric sulfite oxidase enzyme SorAB, consisting of a subunit containing a molybdopyranopterin cofactor (SorA) and cytochrome c 552 type with a single heme prosthetic group (SorB) [36]. The indirect pathway is performed by the AprABM complex, docked to the cytoplasmic membrane by the electron-channeling subunit AprM that transfers electrons in the quinone pool [37]. This complex performs the first sulfurylation step of adenosine monophosphate by the docked heterodimeric enzyme APS reductase (AprBA) [38]. Subsequent phosphorylation of APS is performed by ATP sulfurylase (Sat), with the concomitant production of ATP [39]. *Thioalkalivibrio* sp. 10fs10 has genes for both the direct and indirect sulfite oxidation to sulfate.

The results of the genomic annotation of *Thioalkalivibrio* sp. 10fs10, referred to the genes and complete operons codifying for proteins and enzymes implied in osmotic stress and high pH adaptation are listed in Supplementary Table S1.

In *Thioalkalivibrio* sp. 10fs10, the genes coding for the primary osmoprotectant glycine/betaine synthesis by glycine poly-methylation have been retrieved. The genes involved in the synthesis of secondary osmoprotectants, sucrose, and sucrose phosphate, have been also recovered.

The genes involved in the synthesis of the highly unsaturated, non-polar membrane lipid squalene, by non-mevalonate pathway, putatively involved in the decrease of the membrane permeability to the migration of ions into the cell [39], have been retrieved, as well as the genes for the synthesis of cardiolipin. Cardiolipin synthesis is supposed to improve the efficiency of proton capture through the membrane [40], to stabilize the respiratory complex in alkaline conditions [41], and to improve the Cytochrome-C scavenging effect of reactive oxygen species [42].

About primary and secondary ion pumps for osmoregulation, the genes encoding for sodium/proton translocation machinery have been retrieved. More in details, *Thioalkalivibrio* sp. 10fs10 harbours one *nuo*ABCDEFGHIJKLMN complete operon for proton extruding machinery, one Na^+/H^+ multi-subunit antiporter coded by the *mrp* operon (*mrp*ABCDEFG) and two *rnf* ABCDGE operons coding for a primary sodium or proton extruding pump (NADH or ferredoxin dependent). Moreover, the *nha*D gene codifying for a single unit sodium/proton antiporter, described for its efficiency at alkaline pH (pH > 8) [43] was also retrieved.

4. Discussion

The shot-gun sequencing of the metagenome of the contaminated sediments here in study, showed a non-negligible relative abundance of the genus *Thioalkalivibrio,* suggesting an ecological role of the latter in the sediments. *Thioalkalivibrio* in brackish environments was already reported as the result of the screening of clone libraries from metagenomic extracts or deep sequencing of metagenomes.

However, any ecological role has been already suggested in environments very diverse from those associated to halo-alkaline soda lakes, characterized by very high pH and salinity. All the already described strains are involved in the sulfur cycle in those extreme environments, but the sediments here analysed were characterized by a neutral pH and a sodium concentration referable to a brackish environment. The further characteristic of the sediments was eventually a significative contamination by petroleum derived hydrocarbons. The quantification of the nutrient concentrations in the sediments indicated a high sulfur concentration, referable to the anoxic nature of the sediments. In the context of the contamination, the presence of plastic metabolic traits of sulfur oxidizing or reducing bacteria might be expected and actually the genus *Thioalkalivibrio* might have shown a cosmopolitan attitude and eventually a higher flexibility in the adaptation to different growth conditions and propagation than the already described.

The here isolated new strain of the genus *Thioalkalivibrio* was phylogenetically compared to strains of the same genus, isolated from different soda lakes and treatment plants for H_2S containing gas. As reported by [44], the ALJ and ARh strains were isolated from soda lakes in Kenya. The HL-EbGr7 and HL-Eb18 were isolated from full scale sulfide removing bioreactors of Eerbeek (Netherlands). The ALR and ALBR strains were isolated from sulfide oxidising bioreactors inoculated with sediments of Mongolian soda lakes. The ALE strains were isolated from Wadi Natrum Lake (Egypt), the ALM strain was isolated from Mongolian soda lakes, and the AKL strain and K90mix were isolated from a soda lake in Russia.

Both phylogenetic and MLS tree topologies suggested that the 10fs10 belongs to the *Tv. sulfidiphilus* species. The ANI-BBH analysis confirmed that 10fs10 belongs to the genus *Thioalkalivibrio* and the high taxonomic resolution achieved by dDDH calculation, indicated that 10fs10 is a new strain in the genus when compared to already isolated and sequenced strains HL-EbGr7 and ALJ17 of the *sulfidifilus* species. On Genome Taxonomy Database [45] several bacteria and archaea were reclassified on the base of genomic comparison and results here obtained confirmed the classification of 10fs10 as a new strain belonging to the genus *sulfidiphilus.*

In relation to the putative participation of the strain to the sulfur cycle in the sediments, the 10fs10 genome annotation suggested a metabolic plasticity of the strain with reference to the capability to grow in environments characterized by changes in the redox potential. *Thioalkalivibrio* sp. 10fs10 efficiently oxidizes sulfur in microoxic conditions or high redox potential. Affinity for reduced sulfur increases with the increase in the redox potential, showing that *Thioalkalivibrio* sp. 10fs10 adopts a more efficient sulfur oxidation metabolism when oxygen availability is higher. In fact, the 10fs10 genome annotation showed that the strain is capable to catalyse thiosulfate and sulfide oxidation to elemental sulfur in the periplasm, to store elemental sulfur in extracellular compartments and to oxidize these latter to sulfite by both the rDSR and the HDR complexes. Actually, all sequenced genomes of the *Thioalkalivibrio* spp. harbour the HDR complex [46]. However, the coexistence of the HDR and rDSR complexes was, up to now, restricted to *Thioalkalivibrio sulfidiphilus* HL-EbGr7, *Thioalkalivibrio sulfidiphilus* ALJ17, *Thioalkalivibrio nitratireducens* ALJD, *Thioalkalivibrio* AKL19, and *Thioalkalivibrio* ALE19. Moreover, the 10fs10 genome annotation indicated that the strain oxidizes sulfite to sulfate by both direct (SorAB) and indirect (Apr/Sat) oxidations. In this context, it is worth mentioning that, as suggested also by other authors [47], the rDSR/SorAB pathway might be related to sulfur oxidation carried out at high redox potentials, while the HDR/Apr/Sat pathway might be related to low redox potentials. Moreover, the coexistence of two distinct pathways for sulfur accumulation in its elemental form, starting from both highly reduced sulfide or poorly reduced thiosulfate, suggests that *Thioalkalivibrio* sp. 10fs10 is suited to face shortage of reduced sulfur forms.

The plasticity in the sulfur metabolism associated to the above described genome organisation, might be the signature for a high plasticity and adaptability of the genus *Thioalkalivibrio* to the environment, higher than the already described, and the 10fs10 strain might be one representative of the not yet described adaptability. In fact, as here described, the 10fs10 strain showed a higher flexibility with reference to halo-alkaline conditions of growth, when compared to the type strain of

the species, the HL-EbGr7. The 10fs10 genome annotation showed a duplication of the *rnf* ABCDGE operon coding for a putative primary sodium or proton extruding pump, retrieved in single copy in HL-EbGr7. Actually, by inspection of the halo-alkaliphilic *Thioalkalivibrio* sp. genomes deposited on IMG (study name: Genome sequencing of 100 strains of the halo-alkaliphilic chemolithoautotrophic sulfur-oxidizing bacterium *Thioalkalivibrio)*, the duplication of the Rnf complex can be found in only *Thioalkalivibrio paradoxus* Arh1 and *Thioalkalivibrio versutus* AL2. The duplication of *rnf* gene cluster is a quite rare feature [48] retrieved in few microorganisms characterized by high metabolic plasticity [49] and nitrogen fixation capabilities [50]. A clear participation of the cluster to the adaptation to the halo-alkaline conditions of growth is not already demonstrated, however, an Rnf complex variant in the alkalitolerant *Acetobacterium woodi* was shown to have an essential role in homeostasis [51,52].

On the other hand, the 10fs10 genome annotation indicated that the strain shows very similar features to the *Thioalkalivibrio* genus in terms of genetic arrangement for adaptation to pH variation and osmotic stress, harbouring genes for the synthesis of osmolyte such as glycine/betaine and sucrose [53] and for highly unsaturated, non-polar lipids like squalene, synthetized for reinforcing the structure of the cell membrane to withstand osmotic pressure [47,53,54]. Moreover, in 10fs10 and in the *Thioalkalivibrio* genus, the genes for the cardiolipin synthesis have been retrieved. Cardiolipin is supposed to improve efficiency of proton capture through the membrane [40], to stabilize the respiratory complex in alkaline conditions [41] and to improve the Cytochrome-C scavenging effect of reactive oxygen species [42]. On the other hand, it is worth mentioning that 10fs10 harbours the *nha*D gene, coding for a single unit sodium/proton antiporter, characterized by a high efficiency at alkaline pH that has never been retrieved in any of the genomes of the strains already sequenced in the genus *Thioalkalivibrio*.

5. Conclusions

A new isolate of the *Thioalkalivibrio sulfidiphilus* sp. that, with reference the type strain of the species, the HL-EbGr7, shows a higher capacity to adapt to pH variation of the growth environment, was isolated from brackish sediments contaminated by petroleum derived hydrocarbons. Even though *Thioalkalivibrio* sp. 10fs10 shows very similar features to *Tv sulfidiphilus* HL-EbGr7 in terms of genetic arrangement for sulfur oxidation and accumulation, the higher flexibility of the new strain with reference to halo-alkaline condition of growth might be related to a different organization of the genome. In fact, the duplication of an operon encoding for a putative primary sodium or proton extruding pump was retrieved in 10fs10 and not in HL-EbGr7. Moreover, the presence of a sodium/proton antiporters, with alkaline-adapted capabilities was recorder in 10fs10 and, up to now, not retrieved in the *Thioalkalivibrio* genus. The metabolic flexibility of the bacterial species in relation to the redox potential and the capability to utilize reduced sulfur as electron source was successfully exploited in fed batch and continuous reactor scale processes, where the maximal specific oxidation rate for reduced sulfur obtained for 10fs10 in low air flow tests, reached values very similar to the one observed in previously described operational conditions [11]. At increasing air flows, *Thioalkalivibrio* sp. 10fs10, increased the specific oxidation rate of about five times, resulting to be an interesting candidate for the development of bio-based processes dedicated to the control of H_2S emissions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/5/1385/s1, Table S1: Genes involved in bacterial homeostasis in saline and alkaline environments annotated in *Thioalkalivibrio* sp. 10fs10 genome. IMG-ER and Rast identifiers are reported.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be interpreted as a potential conflict of interest.

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