Isolation and characterization of a novel acidophilic zero-valent sulfur- and ferric iron-respiring Firmicute

Holanda, Roseanne; Johnson, D. Barrie

Research in Microbiology

DOI:
10.1016/j.resmic.2020.07.003

Published: 18/08/2020

Peer reviewed version

Journal Pre-proof

Isolation and characterization of a novel acidophilic zero-valent sulfur- and ferric ironrespiring *Firmicute*

Roseanne Holanda, D. Barrie Johnson

PII: S0923-2508(20)30077-2

DOI: https://doi.org/10.1016/j.resmic.2020.07.003 Reference: RESMIC 3774

To appear in: *Research in Microbiology*

Received Date: 9 April 2020

Revised Date: 17 June 2020

Accepted Date: 17 July 2020

Please cite this article as: R. Holanda, D.B. Johnson, Isolation and characterization of a novel acidophilic zero-valent sulfur- and ferric iron-respiring *Firmicute*, *Research in Microbiologoy*, https://doi.org/10.1016/j.resmic.2020.07.003.

Isolation and characterization of a novel acidophilic zero-valent sulfur- and ferric iron-respiring *Firmicute*.

Roseanne Holanda[§]*, D. Barrie Johnson

School of Natural Sciences, Bangor University, UK

Present address: SENAI Innovation Institute for Mineral Technologies, National Service for Industrial Training, Belém/PA, 66035-405, Brazil.

rosebarata@gmail.com (correspondence and reprints)

correspondence: rosebarata@gmail.com; Tel: +55 091 99354 3850

§

d.b.johnson@bangor.ac.uk

*

Abstract:

A novel, obligately anaerobic, acidophilic bacterium (strain I2511), isolated from sediment in an abandoned copper mine, was shown to couple the oxidation of organic electron donors to the reduction of both zero-valent sulfur and ferric iron in acidic media. The isolate was an obligate heterotroph that used a variety of organic compounds as electron donors and required yeast extract for growth. Alternative electron acceptors (sulfate, tetrathionate, thiosulfate and nitrate) were not used by the novel isolate. The strain grew as motile, endospore-forming rods, and was mesophilic and moderately acidophilic, with a growth rate of 0.01 h^{-1} at optimum pH (3.7) and temperature (35°C). Analysis of its 16S rRNA gene sequence placed strain I2511 within the phylum *Firmicutes*, distantly related to validated species. Phylogenetic analysis and physiological traits indicate that the novel strain represents a species of a candidate novel genus. Strain I2511 was included in a microbial consortium in a low pH "hybrid" sulfidogenic bioreactor designed to remove chalcophilic metals from metal-contaminated liquors and was present in >50 % relative abundance when bioreactor was operated at pH ~ 2.0. Results indicate that the novel isolate could be applied in biotechnologies to treat acidic and neutral pH, metal-rich effluents.

Keywords: Acidophile, Iron reduction, *Firmicutes*, Obligate anaerobe, Sulfidogenesis, Sulfur reduction

1. Introduction

Elemental (zero-valent) sulfur (ZVS) and several sulfur oxyanions can be used as terminal electron acceptors by some prokaryotes, generating hydrogen sulfide. While there are a number of reports describing microbially–catalyzed sulfidogenesis in very acidic natural

and engineered eco-systems (reviewed in [1]), there have been relatively few reports of pure cultures of bacteria that are capable of generating H_2S at extremely low pH (< 3). *Thermodesulfobium narugense*, isolated from a hot spring in Japan, was the first acidtolerant sulfate-reducing bacterium to be validated [2]; it grows between pH 4 and 6.5 and is a moderate thermophile. *Desulfosporosinus* (*D.*) *acidiphilus* and *Desulfosporosinus acididurans* are moderately acidophilic sulfate-reducing bacteria (aSRB) that are able to grow at lower pH (3.6 and above) and can also reduce ZVS [3 - 5]. *D. acididurans* was reported to represent ~ 6 % of the bacterial population in a sulfidogenic bioreactor maintained at pH 2.4, which was dominated by currently non-validated SRB [6]. A novel *Deltaproteobacteria*, *Desulfurella* (*Ds.*) *amilsii*, was shown to reduce ZVS at low pH, growing between pH 3 and 7 and optimally at pH 6 - 6.5 [7]. More recently, the aSRB *Thermodesulfobium acidiphilum* [8] and *Thermodesulfobium* sp. strain 3baa [9] have been described as thermophiles which can grow at pH range of 3.7 - 6.5 and 2.6 – 6.6, respectively. Currently, there are no reports of ZVS-reducing bacteria that grow optimally at pH < 4. However, dissimilatory reduction of ZVS at extremely low pH has been reported for some, mostly hyperthermophilic, archaea, such as *Acidianus* spp., *Stygiolobus azoricus* and *Sulfurisphaera ohwakuensis*. Species of the genus *Acidianus* (e.g. *A. brierleyi, A. infernus, A. ambivalens* and *A. sulfidivorans*), isolated from solfatara environments and acidic thermal springs, are able to grow at pH as low as 1, and the minimum growth pH reported for *A. sulfidivorans* is 0.35 [10].

Many early attempts to isolate sulfidogens in low pH were unsuccessful because organic acids (e.g. lactate) were used as the main carbon/energy source in enrichment cultures [1]. At pH below their pK_a values, organic acids exist mainly in non-dissociated forms. These tend to be lipophilic, and can diffuse into the cells lowering intracellular pH, ultimately causing cell death. Non-ionic substrates such as glycerol, hydrogen and methanol are more suitable electron donors for sulfidogenesis at low pH [5, 6]. Another challenge with cultivating sulfidogens in acidic media, also related to the toxicity of organic acids, is that some species are known to be “incomplete substrate oxidizers,” and produce and excrete small molecular

weight organic acids when metabolizing larger molecular weight substrates, again inducing toxicity (e.g. *D. acididurans* produces acetic acid in stoichiometric equivalents to glycerol oxidized).

Depending on pH, hydrogen sulfide can react with chalcophilic metals, such as copper and zinc, precipitating them as sulfide phases. This is the principle behind sulfidogenic bioreactors, which have been widely studied to remove and recover metals from mine-impacted effluents [6, 11]. Industrial-scale bioreactors currently used for this purpose utilize species of neutrophilic sulfidogens that tend to be highly sensitive to both acidity and soluble transition metals that have to be shielded from direct contact with contaminated mine waters [12].

The present study reports the characteristics of a novel obligately anaerobic, acidophilic bacterium (*Firmicute* strain I2511) which was found to couple the oxidation of organic electron donors to the reduction of ZVS and ferric iron. The novel sulfidogen was included in a microbial consortium in a low pH "hybrid" sulfidogenic bioreactor (HSB), designed to target the removal of chalcophilic metals from metal-contaminated liquors.

2. Materials and methods

2.1. Enrichment, isolation and cultivation of Firmicute I2511

Black sediments collected from an acidic stream draining an abandoned copper mine, Mynydd Parys, North Wales, UK (53° 22' 59.9988" N; 4° 20' 60" W) that had a distinct hydrogen sulfide odor, were used to enrich acidophilic ZVS-reducers. Ten grams of sediment was added to 100 mL of liquid medium containing 5 mM glycerol, 0.01% w/v, yeast extract; chloride salts medium (g L^{-1}: 0.36 NH_4Cl, 0.05 KCl, 0.04 $MgCl_2.6H_2O$, 0.006 Na_2HPO_4, 0.05 KH_2PO_4, 0.014 $Ca(NO_3)_2.4H_2O$) and 1%, w/v sterile hydrophilic ZVS. To prepare hydrophilic ZVS, sulfur powder (Sigma-Aldrich, UK), which is hydrophobic, was added (10%, w/v) to a liquid culture inoculated with the sulfur-oxidizing acidophilic bacterium *Acidithiobacillus (At.) thiooxidans*, and incubated at 30ºC, shaken at 50 rpm, for 7 days, a technique widely reported

to “wet” ZVS, causing it to become hydrophilic. The ZVS was then allowed to settle, removed from the culture, and sterilized at 110°C for 60 min.

The enrichment culture was placed in sealed jars under anaerobic atmosphere (Oxoid™ AnaeroJar™, Thermo Fisher Scientific; USA) and incubated at 30ºC, shaken at 50 rpm. Hydrogen sulfide production was confirmed by the appearance of CuS precipitates inside a “copper trap”, which consisted of an open universal bottle containing 10 mL of 20 mM $CuSO_4$ which was placed inside the sealed jar (Supplementary Fig.1). Following this, the enrichment cultures were streaked onto aSRB plates [13] and incubated under anaerobic atmospheres at 30ºC. Single colonies were differentiated by their morphologies and purified by repeated re-streaking onto fresh solid media. From these, isolates were cultivated in 5 mM glycerol, 0.01% (w/v) yeast extract, chloride basal salts, trace elements [13] and 1% (w/v) sterile ZVS pH 3, incubated anaerobically at 30 ºC and 50 rpm.

2.2. Growth Characteristics of isolate I2511

2.2.1. Standard cultivation conditions

Several attempts to cultivate isolate I2511 in aSRB liquid medium [13] in the absence of ZVS, did not result in increased cell numbers or H_2S production. Therefore, it was assumed that the isolate was not able to catalyze the dissimilatory reduction of sulfate. Hence, unless otherwise indicated, the chloride-based basal salts used in the enrichment cultures medium was subsequently replaced by sulfate salts (acidophile basal salts (ABS); [13]). The “standard medium” used to cultivate isolate I2511 contained 5 mM glycerol, 1% (w/v) ZVS, 0.01% (w/v) yeast extract, ABS and trace elements [13], pH adjusted to 3.0 with sulfuric acid. ZVS-containing and ZVS-free liquid media were heat-sterilized at 110 ºC for 60 min, and 120 ºC for 30 min, respectively. The standard incubation conditions used were anaerobic (generated in sealable jars containing Oxoid™ AnaeroJar™ sachets, Thermo Fisher Scientific; USA), 30 °C, shaken at 50 rpm. Growth was monitored by enumerating cells using a Helber counting

chamber marked with Thoma ruling (Hawksley, UK) and viewed with a Leitz Labolux phase-contrast microscope at a magnification of 400x.

2.2.2. Growth under aerobic and micro-aerobic conditions

Growth under aerobic conditions was tested in liquid and on solid media and for micro-aerobic conditions on solid media only. Micro-aerobic atmosphere was generated in sealable jars containing Campy*Gen*™ CN25 sachets (Thermo Fisher Scientific; USA). The selected solid media used (all overlay) were: YE3o and FeSo [14]. Liquid media tested were: (a) ZVS (1%, w/v), 5 mM glycerol and yeast extract (0.01%, w/v), pH 3.0 and (b) 10 mM Fe^{2+} and yeast extract (0.02%, w/v), pH 2.0. Solid and liquid media were inoculated with culture grown in liquid medium containing ZVS (1%, w/v), 5 mM glycerol and yeast extract (0.01%, w/v) pH 3.5, incubated anaerobically. To evaluate growth in liquid media, planktonic bacterial cells were counted and pH measured after 10 and 20 days.

2.2.3. Carbon metabolism

To investigate whether isolate I2511 required yeast extract for growth, isolate I2511 was grown in standard liquid medium with or without yeast extract. Planktonic cells were enumerated after 11 and 18 days. The effect of different concentrations (0.005 or 0.05%, w/v) of yeast extract or peptone on cell yields was tested in replicate liquid cultures. Two other liquid culture variants were set up: (a) 0.05% (w/v) yeast extract or peptone but no ZVS; (b) standard medium with 0.005% (w/v) yeast extract. Planktonic cells were enumerated after 18 days.

The ability of isolate I2511 to grow on a range of defined small molecular weight organic compounds was tested in replicate liquid cultures containing ABS and trace elements, 1% (w/v) ZVS, 0.01% (w/v) yeast extract pH 3.0 supplemented with the following compounds: glucose, fructose,1,3-propanediol, glycerol, lactic acid and citric acid (all at 5 mM); ethanol and methanol (both at 10 mM). In parallel, control cultures were set up containing ZVS and 0.01% (w/v) yeast extract with no additional organic electron donor. To assess growth by fermentation, replicate cultures were supplemented with glucose, glycerol, L-malate or

fumarate (all at 5 mM) in the absence of ZVS at pH 4. Growth was assessed from counts of planktonic cells after incubating for 12 - 18 days.

2.2.4. The use of alternative electron acceptors by isolate I2511

Dissimilatory reduction of ferric iron was tested by growing strain I2511 in liquid medium containing 5 mM glycerol and 0.005% (w/v) yeast extract and ~15 mM $Fe_2(SO_4)$. The pH was adjusted to 2.6 with sterile 1 M NaOH, which caused partial precipitation of the ferric iron. Cultures were incubated in sealed jars under anaerobic atmospheres. Replicate cultures were inoculated with an active culture of strain I2511, grown previously in the presence of ferric iron. Replicated non-inoculated controls were set up in parallel. Ferrous iron concentrations and culture pH were determined after 17, 25 and 30 days.

To determine whether strain I2511 could catalyze the dissimilatory reduction of tetrathionate, thiosulfate, cysteine or nitrate, replicate cultures were incubated anaerobically at 30ºC in aSRB medium supplemented with either 5 mM potassium tetrathionate, 10 mM sodium thiosulfate, 0.02% (w/v) L-cysteine hydrochloride monohydrate or 10 mM sodium nitrate. Positive (ZVS-containing) and negative (no electron acceptor) controls were set up in parallel. Growth was evaluated by enumerating planktonic cells.

2.2.5. Effect of pH and temperature on the growth rate of isolate I2511

Isolate I2511 was grown in a pH- and temperature-controlled bioreactor (FerMac 310/60 unit, Electrolab Biotech, UK) fitted with a 2.2 L glass vessel and stirred at 150 rpm. The standard liquid medium was slightly modified by including less yeast extract (0.005%, w/v). A continuous stream of oxygen-free nitrogen (OFN, at $\sim$ 200 mL min^{-1}) was used to maintain anaerobic conditions and to deliver H_2S generated inside the bioreactor vessel to an off-line glass vessel that contained 500 mL of 20 mM $CuSO_4$.To determine the effect of pH on the growth rate of isolate I2511, this was varied between 2.8 and 4.5 and the bioreactor maintained at 30 ºC. To determine the effect of temperature on the growth rate of isolate I2511, this was

varied between 23°C and 35°C and the bioreactor maintained at pH 3.7. Samples from the bioreactor vessel and the off-line vessel were removed regularly to determine concentrations of glycerol, acetic acid and copper. Semi-logarithmic plots of glycerol oxidized against time were used to determine growth rates. Rates of H_2S production were determined by changes in concentrations of copper in the off-line vessel. Additional tests were carried out to assess the minimum pH (at 30ºC) and maximum temperature (at pH 3.7) at which isolate I2511 could grow. Growth was evaluated by counting planktonic bacterial cells after 11 and 18 days of incubation.

2.2.6. Dissimilatory reduction of zero-valent sulfur on solid medium

Reduction of ZVS on solid media was assessed by streaking liquid culture of strain I2511 onto an overlay ZVS (S^0o) plates, developed jointly with Ivan Ñancucheo (Facultad de Ingeniería y Tecnología, Universidad San Sebastian, Chile). The S^0o plate contained 5 mM glycerol, 0.01% (w/v) yeast extract, chloride basal salts, 0.5 % (w/v) agarose, adjusted to pH 3.0 with hydrochloric acid, to which, after setting, 100 µL of a suspension of ZVS (40%, m/v) was spread on the top. Inoculated plates were placed in sealed jars, incubated under an anaerobic atmosphere, together with a universal bottle containing 20 mM copper sulfate to indicate production of H_2S, incubated at 30 ºC.

2.2.7. Tolerance to copper, sodium chloride and acetic acid

Replicate cultures of I2511 in standard medium (pH 2.5) were supplemented with copper sulfate (at 0.1, 1.0, 5.0 and 10 mM) to assess tolerance to Cu^{2+}. Copper-free control cultures were set up in parallel. Growth was assessed by enumerating planktonic cells and from formation of CuS (as a result of production of H_2S). A similar approach was used to determine the tolerance to sodium chloride (50, 100 and 500 mM). To investigate tolerance of strain I2511 to acetic acid, replicate cultures containing 0.5, 1.0 and 3.0 mM acetic acid were set up in standard medium at pH 2.5, together with acetic acid-free controls.

2.3. Relative abundance of isolate I2511 in a low pH sulfidogenic bioreactor

Isolate I2511 was included in a microbial consortium in a low pH "hybrid" sulfidogenic bioreactor (HSB). The design of the HSB, and its effectiveness in removing contaminant transition metals from neutral pH mine waters, are detailed in [15]. The HSB contained both ZVS and sulfate as potential electron acceptors, and glycerol and yeast extract were provided as electron donors/carbon sources. The bioreactor (an upflow biofilm bed sulfidogenic bioreactor of the 2.2 L working volume) was operated at 30 °C, agitated at 50 rpm in continuous flow mode with a feed solution containing 2 mM glycerol, 0.01% (w/v) yeast extract, ABS with pH adjusted to either 2.0 or 2.5 with sulfuric acid. The composition of microbial populations in the HSB, both planktonic and sulfur-attached cells, were analyzed. Samples were taken from the surface liquor of the bioreactor and from the ZVS layer, and treated as previously described [15]. DNA from the biomass was extracted using PowerSoil UltraClean microbial DNA isolation Kits (QIAGEN, Denmark), following manufacturer's instructions. Bacterial and archaeal 16S rRNA genes were amplified and analyzed by terminal restriction enzyme fragment length polymorphism (T-RFLP; [16]), a semi-quantitative approach used to calculate relative abundances in microbial communities.

2.4. Biomolecular Analysis

Genomic DNA was extracted from liquid cultures using modified CTAB/high-salt extraction, followed by alcohol precipitation [17]. The 16S rRNA gene of strain I2511 was amplified using 27F (5′–3′ AGAGTT TGATCM TGGCTCAG) and 1387R (5′–3′ GGGCGGWGTGTACAAGGC) primers and PCR products were sequenced by Sanger method (Macrogen Inc., South Korea) and analyzed by T-RFLP [16]. The draft genome sequence of strain I2511 (GenBank accession number QXHL00000000) indicated only one 16S rRNA gene copy to be present, enabling more accurate calculation of relative abundances using T-RFLP analysis.

2.5. Phylogenetic Analysis

The 16S rRNA gene sequence of strain I2511 was deposited in the GenBank database and compared with those of related species (retrieved from EZBioCloud;[18]).

Multiple sequence alignment was performed using SINA [19]. The phylogenetic analysis was performed by MEGAX with the Maximum Likelihood method implemented [20]. Selection of best-fitting substitution models was performed with the Bayesian Information Criterion using bootstrap analysis using 1000 replicates.

Analytical methods

The pH of liquid cultures were measured using a pHase combination glass electrode coupled to an Accumet 50 pH meter. Concentrations of glycerol, sulfate, and acetic acid were determined by ion chromatography [6]. Concentrations of ferrous iron determined using the Ferrozine assay [22]. Concentrations of soluble copper were measured using a

Isolation and characteristics of isolate I2511

After 12 days of anaerobic incubation, hydrogen sulfide was detected as being

Model Selection implemented in iQtree [21]. Tree topology reliabilities were confirmed by

2.6.

colorimetric assay [23].

3. Results

3.1.

241

242 generated by liquid enrichment cultures of the black sediments in ZVS-glycerol medium.
243 Single colonies from aSRB plates streak-inoculated from the enrichment cultures were put
244 into standard ZVS medium, and one of these (I2511) was able to generate H_2S. T-RFLP
245 analysis confirmed purity of the culture. Cells were motile rods (3 - 5 µm long and ~0.4 µm
246 wide) that formed oval endospores located at the cell termini. Isolate I2511 produced very
247 small off-white colonies (≤ 1 mm diameter) on aSRB solid medium but these were not
248 encrusted with ZnS, a feature indicative of sulfate-reducers [13]. Strain I2511 also produced
249 small white colonies on S^0o plates, and generated H_2S (confirmed by formation of CuS in a
250 universal bottle containing $CuSO_4$). ZVS particles disappeared where colonies grew,
251 indicating they were being used to generate H_2S (Supplementary Fig. 2). No growth of

isolate was observed under aerobic or micro-aerobic conditions, confirming that it is an obligate anaerobe.

3.2. Phylogenetic and genomic analysis

The partial 16S rRNA gene sequence of isolate I2511 (1,388 bp) was deposited in GenBank (accession number KY576736). Analysis of its 16S rRNA gene sequence showed that isolate I2511 was a member of the phylum *Firmicutes* (order Bacillales, family *Alicyclobacillaceae*) and its closest validated relatives were *Alicyclobacillus contaminans* 3A191^T and *Alicyclobacillus tolerans* K1^T, which shared 92.9% and 93.1% sequence similarity to isolate I2511, respectively. Isolate IR2 and clone G13 were more closely related bacteria, both sharing 96% identity of their 16S rRNA genes to strain I2511. Strain IR2 was isolated from a sulfidogenic bioreactor inoculated with enrichment culture containing biomass of an acidic stream draining a copper mine [6]. The clone G13 was obtained from glucose enrichment culture inoculated with acidic mine tailings containing high concentrations of methylmercury [24]. Phylogenetic analysis of the 16S rRNA gene of strain I2511 showed that the isolate clustered with strain IR2 and clone G13 in a separate clade from that represented by the closest validated *Alicyclobacillus* species. The phylogenetic relationship of I2511 with the validated species of the genus *Alicyclobacillus* and closely related bacteria is shown in Fig. 1.

3.3. Carbon metabolism

There were no increase in cell numbers of isolate I2511 in media where glycerol was the only carbon source provided. However, the isolate grew in glycerol medium that also contained yeast extract, and in medium containing only either yeast extract or peptone as carbon source/electron donor, both of which were also coupled to the reduction of ZVS, generating H_2S. Isolate I2511 grew on a range of organic compounds (Supplementary Fig. 3), all of which were coupled to the dissimilatory reduction of ZVS. Lactic acid was not utilized,

and cell numbers were less than in control cultures. The isolate was not able to grow by fermentation of yeast extract or of any of the defined organic substrates tested.

3.4. Alternative electron acceptors

Cultures of I2511 containing glycerol and ferric iron (mostly present as a solid phase) but no ZVS showed increases in both pH and Fe^{2+} concentrations after 17 days of incubation, but beyond this cell number increases were only marginal, and yeast extract (0.005%, v/v) added at day 25, did not induce further iron reduction (Fig. 2). Minor changes in pH and Fe^{2+} concentrations were measured in non-inoculated cultures during the same time. Concentrations of glycerol oxidized and acetic acid produced after 25 days were 0.54 mM and 0.51 mM, respectively.

In contrast, media containing either tetrathionate or thiosulfate as potential alternative electron acceptors to ZVS had similar cell counts to the negative (ZVS-free) control cultures. Similarly, cell numbers did not increase and pH did not change in cultures amended with cysteine. Cell numbers in nitrate-amended cultures were slightly more than in controls but far fewer than those in ZVS-containing cultures. In a repeat of this experiment, no increases in cell numbers were observed. From these experiments, it was concluded that isolate could use ferric iron as an alternative electron acceptor to ZVS, but not tetrathionate, thiosulfate, nitrate or cysteine.

3.5. Effect of pH and temperature on growth rates

Growth experiments showed that hydrogen sulfide production was correlated with both glycerol oxidation (r = 0.93) and planktonic cell numbers (r =0.91) (Fig. 3). Concentrations of acetic acid increased with time, and the ratio of acetic acid produced to glycerol oxidized was ~ 0.4 (Fig. 4*).* When incubated at 30ºC, isolate I2511 had an optimum growth at pH ~ 3.7 with a culture doubling time of ~ 62 h (Fig. 5). Under optimum pH (3.7) and 35ºC, its culture doubling time was 54 h, corresponding to a μ_{max} of 0.01 h^{-1}. Isolate I2511 did not grow (or generate H_2S) at temperatures ≥ 38°C, and below 23 °C growth was very slow (doubling time ~ 80 h; at pH 3.7 and 23 °C). No growth or H_2S production was observed at pH 2.5 and 30ºC in bioreactor

cultures. However, tests carried out in universal bottles, where pH was not controlled, suggested that the isolate was able to generate H_2S in media poised initially at 1.8 (Supplementary Fig 4).

3.6. Tolerance to copper, sodium chloride and acetic acid

Isolate I2511 was highly sensitive to both copper (II) ions and salt (NaCl), with growth being inhibited by the lowest concentrations of these (0.1 and 50 mM, respectively) tested. All cultures supplemented with acetic acid had lower cell counts than the control culture at same pH after 10 days of incubation, though by day 15 cultures containing 0.5 and 1.0 mM acetic acid had similar planktonic cell counts to the control cultures at the same pH (Supplementary Fig. 5). Growth of strain I2511 was completely inhibited by 3 mM acetic acid. Culture pH at day 15 for all tests (including control) were similar at ~ pH 2.6.

3.7. Relative abundance of isolate I2511 in a low pH sulfidogenic bioreactor

While the HSB operated over a wide pH range [15] there was particular interest in the microbial community composition when it was operated at extremely low pH. Prior to the start of experiment, the bioreactor was allowed to adapt to the operating conditions for 20 days for the pH 2.5 feed liquor, and for 16 days for the pH 2.0 feed liquor. With pH 2.5 feed liquor, the pH within the vessel was 2.6, and when using pH 2.0 feed liquor the bioreactor pH ranged from 1.98 - 2.05. Hydrogen sulfide was generated throughout both experiments, but no significant differences in rates were found when using pH 2.0 and pH 2.5 feed liquors (ttest: $t(11) = 0.30$, $p = 0.77$; Supplementary Fig. 6). Changes in rates of sulfate reduction and H_2S production using pH 2.5 and pH 2.0 feed liquors are shown in Supplementary Fig. 7.

Some of the sulfate present in the pH 2.5 influent liquor was reduced (these accounted for 77 ± 8% of the H_2S produced), but there was no detectable net sulfate reduction when the bioreactor pH equilibrated at ~ 2.0.

The microbial communities that were attached to ZVS particles in the HSB were similar to those of planktonic bacteria at both pH values, though there were major differences in the dominant bacteria present (Fig. 6). At pH 2.6, the sulfate-reducing bacteria *Peptococcaceae*

CEB3 [1] and *D. acididurans* were found to be the dominant bacteria, but at pH ~2.0 neither of these was detected, and the most abundant bacterium was strain I2511 (51% relative abundance in the liquid phase and 55% of the attached community), and the facultative anaerobe *At. ferrooxidans* and a putative fermentative *Clostridium* sp. were also detected.

4. Discussion

This study has shown that a novel mesophilic acidophilic isolate of the phylum *Firmicutes,* strain I2511, was able to generate hydrogen sulfide at low pH, *via* the dissimilatory reduction of ZVS. This is only the second non sulfate-reducing acidophile to be demonstrated to have this trait, and the data show that I2511 is far more tolerant of extreme acidity than *Ds. amilsii* [7]. The isolate was an obligately anaerobe that (like other *Firmicutes*) formed endospores, and was also an obligate heterotroph. The only other electron acceptor that I2511 used (of those tested) was ferric iron. This possibly accounts for its initial growth on solid media that contained iron but not ZVS (at the pH of the aSRB plates there would be some anticipated oxidation of Fe^{2+} to Fe^{3+} during preparation and storage). Rigorous testing confirmed that I2511 was not able to use sulfate, tetrathionate, thiosulfate or nitrate as a terminal electron acceptor, or grow *via* fermentation. Isolate I2511 was able to use a variety of organic compounds but required yeast extract for growth. It oxidized glycerol incompletely, generating acetic acid, but not in stoichiometric amounts. The isolate was mesophilic, with no growth observed at a maximum temperature 38°C. Its pH optimum was 3.7, categorizing it as a moderate, rather than an extreme acidophile [25]. Although it did not grow in the fixed pH bioreactor at pH 2.5, data from the HSB (and to a lesser extent from the batch culture experiment in universal bottles) suggested that it may be capable of growth-decoupled sulfidogenesis at pH values less than 2. This apparent discrepancy might be explained by carryover of acetic acid in the bioreactor when setting up a new growth test (generally ~ 80% of the bioreactor was drained and replaced with fresh medium) which at the HSB in the pH 2.0

feed liquor test, acetic acid concentrations were low (≤ 0,31 mM). Generating H_2S at low pH is an unusual and relatively rare trait (for acidophilic bacteria, though not for acidophilic archaea) that could be useful in the development of sulfidogenic biotechnologies used to treat acidic, metal-rich effluents.

Phylogenetic analysis of the 16S rRNA gene of strain I2511 showed that the isolate clustered in a separate clade from that represented by the closest validated *Alicyclobacillus* species. Some characteristics of strain I2511 (i.e. a mesophilic obligate anaerobe, which catalyzes the dissimilatory reduction of both ZVS and ferric iron and has relatively low tolerance to copper and sodium chloride) are distinct from its closest known relatives *Alb. contaminans*^{T} and *Alb. tolerans*^{T}. For example, *Alb. contaminans*^{T} is a moderately thermophilic strict aerobe, and tolerates up to ~350 mM NaCl. *Alb. tolerans*^{T} is a moderately thermophilic facultative anaerobe and grows as a heterotroph and also autotrophically using Fe^{2+} and ZVS as electron donors. The acidophilic genera of the family *Alicyclobacillaceae* (*Alicyclobacillus, Sulfobacillus* and "*Acidibacillus"*) share several characteristics. Many species are strict aerobes, others are facultative anaerobes and use either molecular oxygen or ferric iron as electron acceptors, many are thermo-tolerant or moderately thermophilic, several are obligate heterotrophs, some are facultative autotrophs, some species can use ferrous iron or reduced sulfur as electron donors, and display elevated tolerance to transition metals. However, none of the species described to date can grow anaerobically by sulfur respiration, which clearly delineates isolate I2511 (which is a strict anaerobe) from other species of the family *Alicyclobacillaceae.*

While more research is required in order to validate isolate I2511, the data suggest that it is the first cultivated species of a novel genus within the phylum *Firmicutes*. Questions about how it interacts with other microorganisms in the wider environment need also to be addressed, along with more work on examining its potential for bioremediation of polluted environments.

385

386 **Conflict of interest**

The authors confirm that there are no conflicts of interest.

Acknowledgements

Roseanne Holanda is grateful to the National Council of Technological and Scientific Development (Brazil) for provision of a research studentship.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://xxxx

References

[1] Johnson DB, Sánchez-Andrea I. Dissimilatory reduction of sulfate and zero-valent sulfur at low pH and its significance for bioremediation and metal recovery. Adv Microb Physiol 2019;75:205–231.

[2] Mori K, Kim H, Kanagawa T, Hanada S. A novel lineage of sulfate-reducing microorganisms: *Thermodesulfobiaceae* fam. nov., *Thermodesulfobium narugense*, gen. nov., sp. nov., a new thermophilic isolate from a hot spring. Extremophiles 2003;7:283–290.

[3] Kimura S, Hallberg KB, Johnson DB. Sulfidogenesis in low pH (3.8 – 4.2) media by a mixed population of acidophilic bacteria. Biodegradation 2006;17:57–65.

[4] Alazard D, Joseph M, Battaglia-Brunet F, Cayol JL, Ollivier B. *Desulfosporosinus acidiphilus* sp. nov.: a moderately acidophilic sulfate-reducing bacterium isolated from acid mining drainage sediments. Extremophiles 2010;14:305–312.

[5] Sánchez-Andrea I, Stams AJ, Hedrich S, Ňancucheo I, Johnson DB. *Desulfosporosinus acididurans* sp. nov.: an acidophilic sulfate-reducing bacterium isolated from acidic sediments. Extremophiles 2015;19:39–47.

[6] Ňancucheo I, Johnson DB. Selective removal of transition metals from acidic mine waters by novel consortia of acidophilic sulfidogenic bacteria. Microb Biotechnol 2012; 5:34–44.

[7] Florentino AP, Brienza C, Stams AJ, Sánchez-Andrea I. *Desulfurella amilsii* sp. nov., a novel acidotolerant sulfur-respiring bacterium isolated from acidic river sediments. Int J Syst Evol Microbiol 2016; 66:1249–1253.

[8] Frolov EN, Kublanov IV, Toshchakov SV, Samarov NI, Novikov AA, Lebedinsky AV, et al. *Thermodesulfobium acidiphilum* sp. nov., a thermoacidophilic, sulfate-reducing, chemoautotrophic bacterium from a thermal site. Int J Syst Evol Microbiol 2017; 67:1482–1485.

[9] Rüffel V, Maar M, Dammbrück MN, Hauröder B, Neu TR, Meier J. *Thermodesulfobium* sp. strain 3baa, an acidophilic sulfate reducing bacterium forming biofilms triggered by mineral precipitation. Environ Microbiol 2018;20:3717–3731.

[10] Golyshina OV, Ferrer M, Golyshin PN, Diversity and physiologies of acidophilic archaea, in Quatrini R, Johnson DB (Eds), Acidophiles: Life in Extremely Acidic Environments, Caister Academic Press, Norfolk, 2016, pp 93–106.

[11] Santos AL, Johnson DB. Design and application of a low pH upflow biofilm sulfidogenic bioreactor for recovering transition metals from synthetic waste water at a Brazilian copper mine. Front Microbiol 2018;9:2051.

[12] Bratty M, Lawrence R, Kratochvil D, Marchant B. Applications of biological H_2S production from elemental sulfur in the treatment of heavy metal pollution including acid

436 rock drainage, in Proceedings of the 7th International Symposium of Acid Rock Drainage
437 (ICARD), St. Louis, MO, 2006, pp 271–281.

438 [13] Ňancucheo I, Rowe OF, Hedrich S, Johnson DB. Solid and liquid media for isolating and
439 cultivating acidophilic and acid-tolerant sulfate-reducing bacteria. FEMS Microbiol Lett
440 2016;363:1-6.

441 [14] Johnson DB, Hallberg KB. Techniques for detecting and identifying acidophilic
442 mineraloxidising microorganisms. In: Rawlings DE, Johnson DB, editors. Biomining.
443 Heidelberg: Springer-Verlag; 2007, p 237-62.

444 [15] Holanda R, Johnson DB. Removal of zinc from circum-neutral pH mine-impacted waters
445 using a novel “hybrid” low pH sulfidogenic bioreactor. Front Environ Sci 2020; 8:22.

446 [16] Kay C, Rowe O, Rocchetti L, Coupland K, Hallberg KB, Johnson, DB. Evolution of
447 microbial “streamer” growths in an acidic, metal-contaminated stream draining an
448 abandoned underground copper mine. Life 2013;3:189–210.

449 [17] Wilson K. Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol 2001;2:2-4.

450 [18] Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, Seo H, et al. Introducing EzBioCloud: a
451 taxonomically united database of 16S rRNA gene sequences and whole-genome
452 assemblies. Int J Syst Evol Microbiol 2017;67:1613–1617.

453 [19] Pruesse E, Peplies J, Glöckner FO. SINA: accurate high throughput multiple sequence
454 alignment of ribosomal RNA genes. Bioinformatics 2012;28:1823–1829.

455 [20] Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary
456 Genetics Analysis across computing platforms. Mol Biol Evol 2018;35:1547-1549.

457 [21] Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ. W-IQ-TREE: a fast online
458 phylogenetic tool for maximum likelihood analysis. Nucl Acids Res 2016;44(W1): W232-

459 W235.

461 [22] Stookey L. Ferrozine e a new spectrophotometric reagent for iron. Anal Chem 462 1970;42:779-81.

463 [23] Anwar MA, Iqbal M, Qamar MA, Rehman M, Khalid AM. Technical communication: 464 determination of cuprous ions in bacterial leachates and for environmental monitoring.

465 World J Microbiol Biotechnol 2000;16(2):135-8.

466 [24] Winch, S., Mills, H.J., Kostka, J.E., Fortin, D. and Lean, D.R.S. Identification of sulfate467 reducing bacteria in methylmercury-contaminated mine tailings by analysis of

rRNA genes. FEMS Microbiol Ecol 2009*;* 68:94–107.

[25] Johnson DB, Quatrini R. Acidophile Microbiology in Space and Time. Curr Issues Mol

Figure 1. Phylogenetic analysis by the Maximum Likelihood method showing the relationship of isolate I2511 16S rRNA gene sequences to closely related bacteria and validated species SSU

468

469

470 Biol 2020;39:63-76.

471

472

Legends to figures

of the genus *Alicyclobacillus*. The support in bootstrap analysis (1000 replicates) with values ≥ 50% are indicated by "●". The bar represents 0.05 substitutions per site. The 16S rRNA gene sequence of *Sulfobacillus thermosulfidooxidans*^{T} (NR040945) was used as the outgroup.

Figure 2. Reductive dissolution of solid phase ferric iron by isolate I2511 (solid lines) and non-inoculated cultures (broken lines) after 17, 25 and 30 days of incubation at 30°C. Key: (▲) ferrous iron concentrations (mM) and (■) pH. Bars show mean values and error bars indicate data ranges (n=2).

485 Figure 3. Relationship between hydrogen sulfide production and glycerol oxidation (▲; r =
486 0.93) and hydrogen sulfide production and planktonic cell numbers (●; r = 0.91) when isolate
487 I2511 was grown in a bioreactor at pH 3.2 and 30°C.

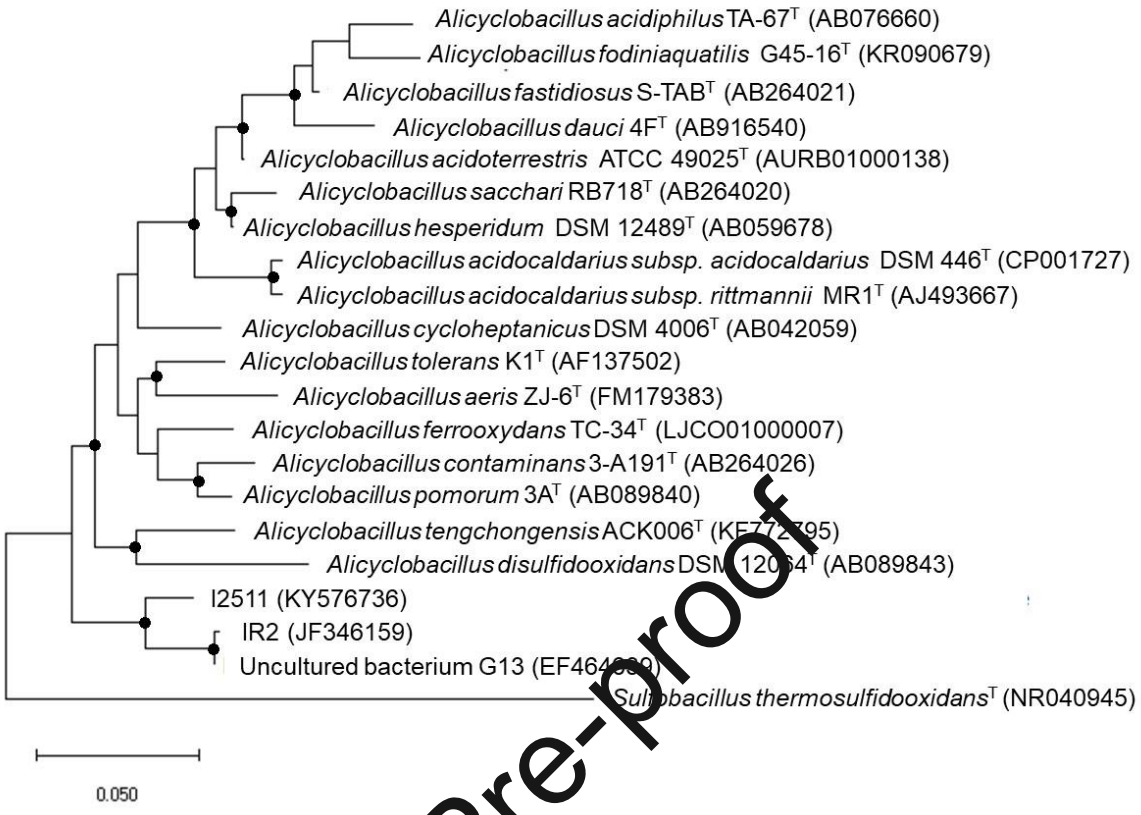
488 Figure 4. Cumulated amounts of hydrogen sulfide produced (■), glycerol oxidised (▲) and
489 acetic acid produced (●) of isolate at pH 2.8 and 30°C.

490 Figure 5. Effect of pH on the culture doubling times (t_d) of isolate I2511 at fixed temperature
491 (30°C).

Figure 6. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during experiments operating the HSB at low pH in continuous flow mode. “Sulfur phase” corresponds to DNA extracted from sulfur-attached bacterial communities. “Liquid phase” corresponds to DNA extracted from planktonic cells.

492

496



gene sequences to

validated species of the genus *Alicyclobacillus*. The

analysis (1000 replicates) with values ≥ 50% are indicated by "●". The bar

represents 0.05 substitutions per site. The 16S rRNA gene sequence of

Figure 1. Phylogenetic analysis by the Maximum Likelihood method showing the relationship of isolate I2511 16S rRNA closely related bacteria

and support in bootstrap

Sulfobacillus thermosulfidooxidans^{T} (NR040945) was used as the outgroup.

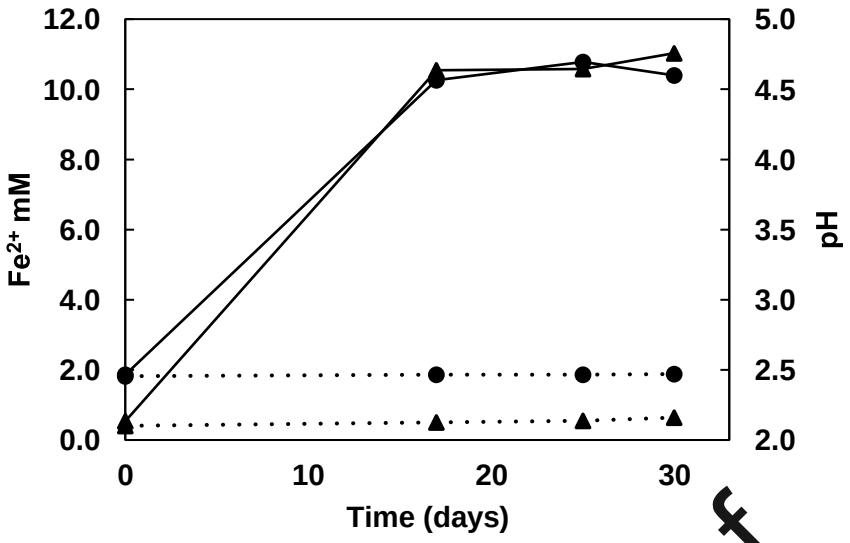


Figure 2. Reductive dissolution of solid phase ferric iron by isolate I2511 (solid lines) and non -inoculated cultures (broken lines) after 17, 25 and 30 days of incubation at 30°C. Key: (▲) ferrous iron concentrations (mM) and (●) pH. Bars show mean values and error bars indicate data ranges (n=2).

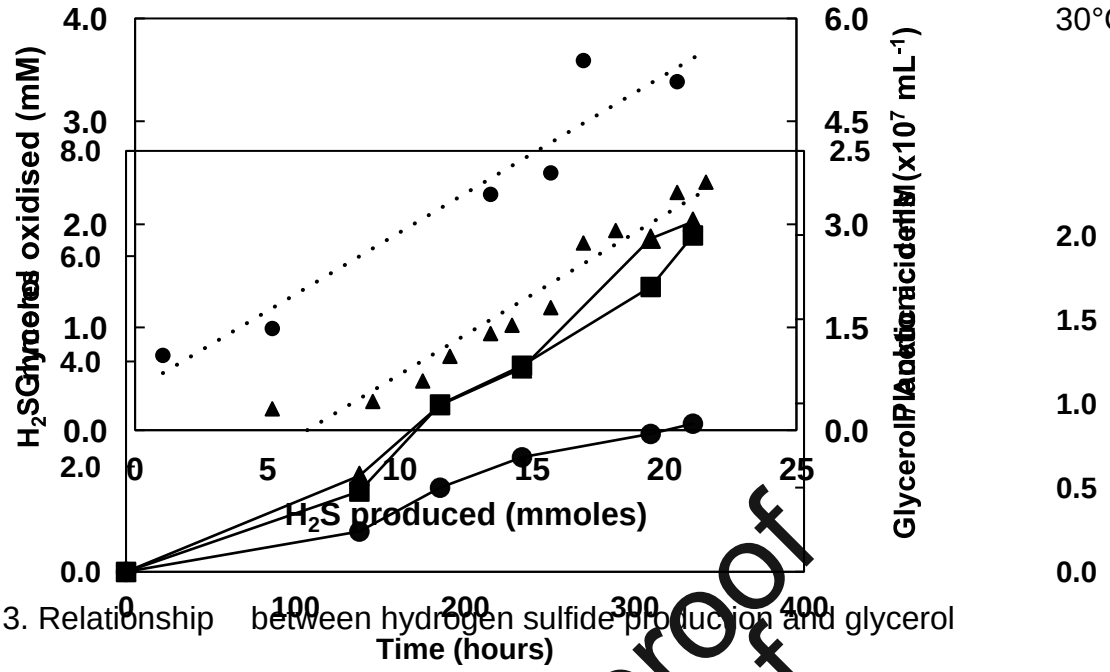


Figure 3. Relationship between hydrogen sulfide production and glycerol oxidation (▲; r = 0.93) and hydrogen sulfide production and planktonic cell numbers (●; r = 0.91) when isolate I2511 was grown in a bioreactor at pH 3.2 and 30°C.

Figure 4. Cumulated amounts of hydrogen sulfide produced (■), glycerol oxidised (▲) and acetic acid produced (●) of isolate at pH 2.8 and 30°C.

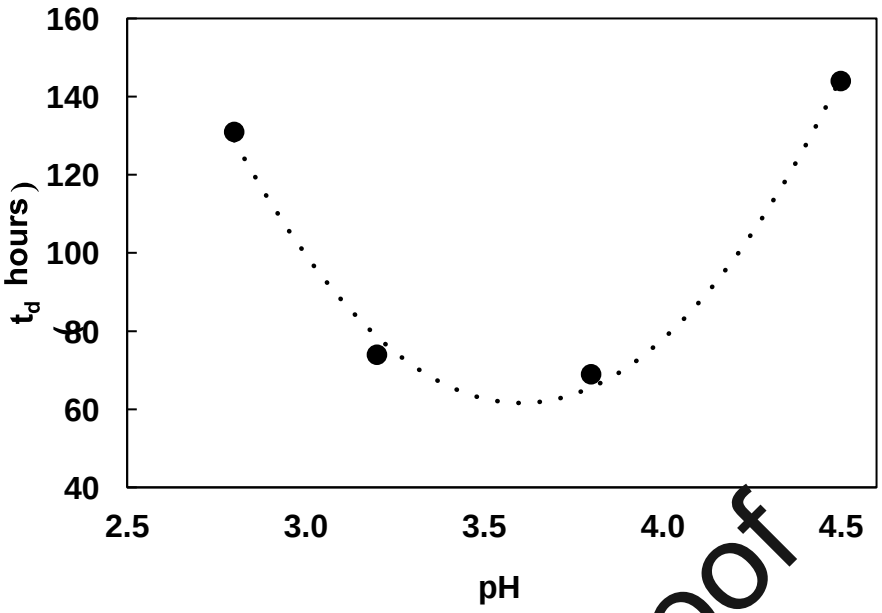


Figure 5. Effect of pH on the culture doubling times (t_d) of isolate I2511 at fixed temperature (30°C).

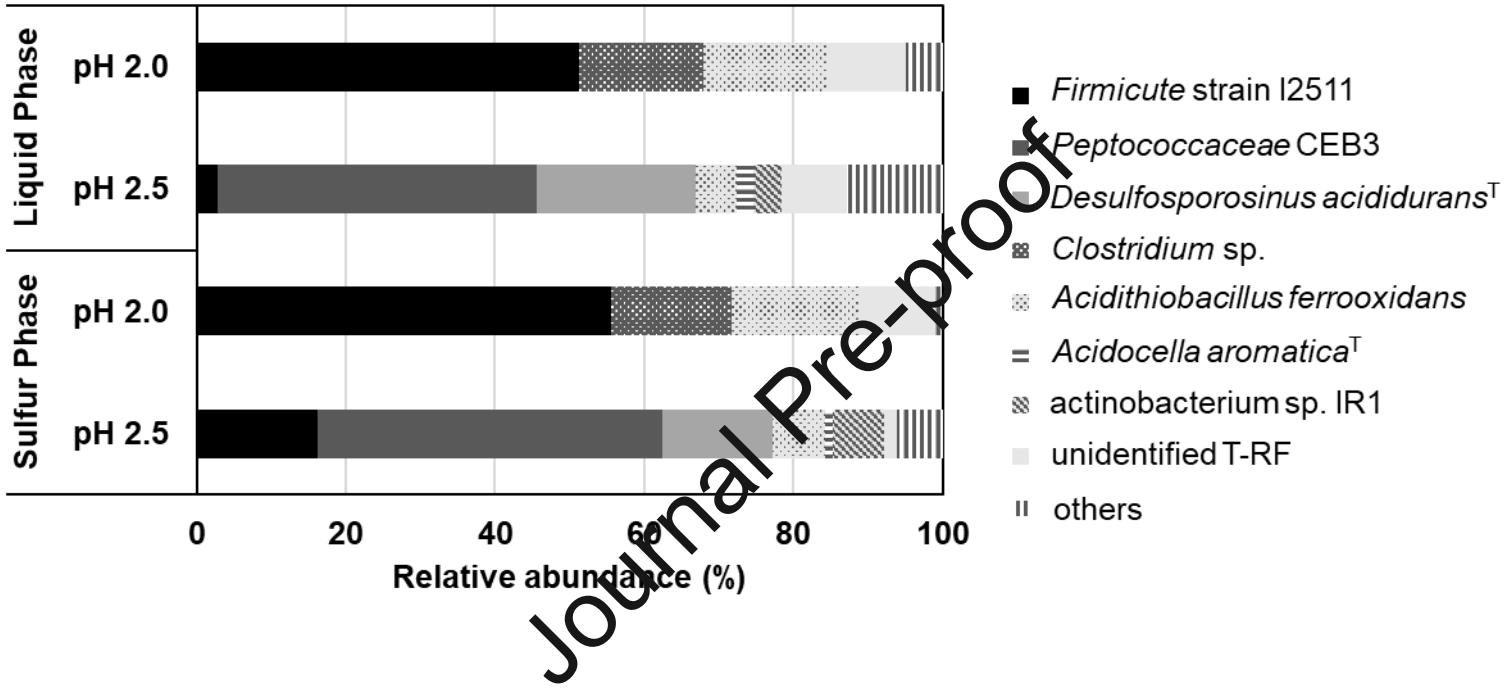


Figure 6. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during experiments operating the HSB at low pH in continuous flow mode. Sulfur phase correspond to DNA extracted from sulfurattached bacterial communities. Liquid phase correspond to DNA extracted from planktonic cells.