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ORIGINAL PAPER

# Salt-tolerant *Acidihalobacter* and *Acidithiobacillus* species from Vulcano (Italy) and Milos (Greece)

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## Abstract

Ferrous iron- and sulfur-oxidizing *Acidihalobacter* species and similar so far unclassified bacteria have been isolated from the islands of Vulcano (Italy) and Milos (Greece), specifically from where seawater was acidified at sulfide-rich geothermal sites. *Acidithiobacillus* species which tolerated concentrations of chloride that inhibit most *Acidithiobacillus* spp. were also isolated from sites on both islands: these were *At. thiooxidans* strains and an unclassified species, *Acidithiobacillus* sp. strain V1. The potential of salt-tolerant acidophiles for industrial application in promoting copper extraction from mineral sulfides where chloride is naturally present at concentrations which would inhibit most acidophiles, or where seawater rather than fresh water is available, appears to be limited by the sensitivity of ferrous-iron oxidizing *Acidihalobacter* spp. to copper. However, tolerance of copper and chloride shown by *At. thiooxidans* strain A7 suggests it could oxidize sulfur and benefit acid leaching if ferric iron or copper was provided as the primary oxidant of sulfide ores.

**Keywords** *Acidihalobacter* · *Acidithiobacillus* · Salt-tolerance · Copper toxicity

## Introduction

There have been extensive surveys of microbial populations close to shallow, hydrothermal vents of the islands of Milos (Greece) and Vulcano (Italy) (Maugeri et al. 2010; Giovannelli et al. 2013; Antranikian et al. 2017) but the saline and strongly acidic niches where some thermal, sulfide-rich areas are adjacent to acidified seawater have been less studied. This relatively rare combination of factors for extreme environments appears to have selected for few iron- and sulfur-oxidizing bacteria that grow well under such conditions, notably species of *Acidihalobacter* and *Acidithiobacillus* (Huber and Stetter 1989; Simmons and Norris 2002; Norris et al. 2010).

*Thiobacillus prosperus* and an unnamed species (VC15) from Vulcano were the first moderately halotolerant, mineral sulfide-oxidizing acidophiles to be described (Huber and Stetter 1989). Several potentially new species of bacteria related to these were indicated by 16S rRNA gene sequences found in DNA extracted from littoral, acidic sediments of Vulcano and Milos (Norris et al. 2010). Isolates from Vulcano which were the source of two of these RNA gene sequences were referred to as *T. prosperus*-like strain V6 and *Acidihalobacter ferrooxidans* strain V8 (Norris and Simmons 2004). The original isolate of

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*T. prosperus* (Huber and Stetter 1989) was classified as *Acidihalobacter prosperus* (Pablo Cárdenas et al. 2015) and *T. prosperus* strain V6 as *Acidihalobacter aeolianus* (Khaleque et al. 2019). *Ah. ferrooxidans* strain V8 was classified as *Ah. ferrooxydans* (Khaleque et al. 2019). Another closely related isolate (*Ah. prosperus* F5) was obtained from acidic, saline drains in Australia (Khaleque et al. 2017).

Two different *Acidithiobacillus* spp. were represented in a 16S rRNA gene clone bank derived from DNA extracted from an acidic pool which was adjacent to the bay of the Porto di Levante, Vulcano (Simmons and Norris 2002). The source species of these cloned rRNA gene sequences were isolated from sulfur-oxidizing enrichment cultures: the salt-tolerant isolate V1 appeared related to *At. caldus*; isolate V2 appeared to be a salt-tolerant isolate of *At. thiooxidans*. Isolate V1 was maintained at about 50% of the population in mixed culture with *Ah. ferrooooxydans* (strain V8) with pyrite as a sole substrate in serial batch cultures in the presence of 3% (w/v) NaCl (Norris and Simmons 2004). Another *At. thiooxidans*-like isolate (V2A) was also present in less abundance after several months further serial culturing with pyrite in the presence of 6% w/v salt, while *Ah. ferrooxydans* remained dominant in the mixed culture.

The possibility of using iron- and sulfur-oxidizing *Acidihalobacter* spp. for extraction of metals from mineral sulfide ores in the presence of salt concentrations that inhibit more familiar mineral sulfide-oxidizing bacteria was noted when *Ah. prosperus* was first described with resistance to high concentrations of nickel, cobalt and zinc in solution, but a relative sensitivity to copper was also noted (Huber and Stetter 1989). The issue of metal tolerance of these bacteria, therefore, remains a consideration in their potential for industrial use because their activity could be of most relevance to copper extraction in ore heap leaching where saline water is present, particularly in Chile where seawater use in some (non-biological) mineral processing operations is expected to increase or require desalination (Cisternas and Gálvez 2018).

Draft genomes of two salt-tolerant *Acidithiobacillus* isolates (*At. thiooxidans* CLST, Quatrini et al. 2017; *Acidithiobacillus* sp. SH, Kamimura et al. 2018) and four *Acidihalobacter* isolates (National Center for Biotechnology Information Genome Database, USA, http://ncbi.nlm.nih.gov/: accession numbers given in Fig. 1) are available, but descriptions of the distribution and growth of these and similar isolates are few. The presence of *Acidithiobacillus* and *Acidihalobacter* species in samples from Milos and

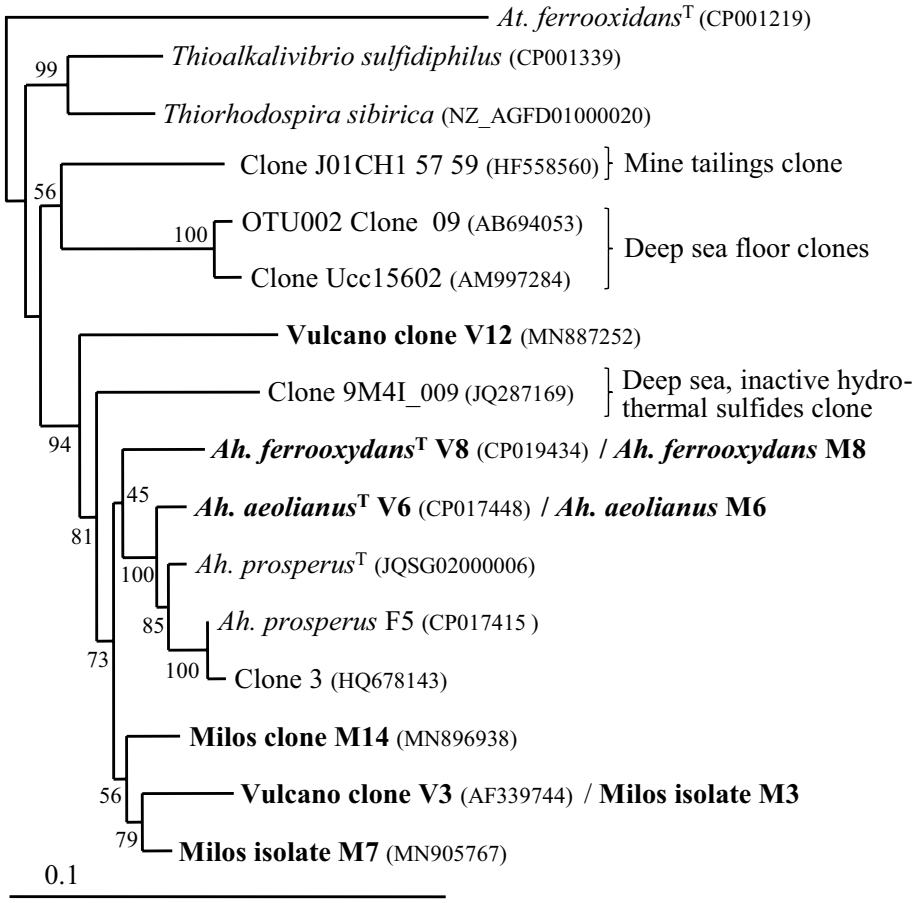


**Fig. 1** Maximum likelihood phylogenetic tree of 16S rRNA gene sequences (1473 aligned nucleotides) with *Acidithiobacillus ferrooxidans* out-group. Bootstrap values are for 100 replications and scale bar for nucleotide changes per site

Vulcano are reviewed here with additional focus on some characteristics of their growth in pure and mixed cultures and on their tolerance of copper.

## Methods

### Sample sites and organism isolation

After storage at −80 °C, a sample from on-shore at Baia di Levante, Vulcano Island was re-examined with DNA extraction and analysis as described previously (Simmons and Norris 2002). The sample comprised seawater-saturated sand and gravel and was at 45 °C and pH 3.5 on collection. Samples from acidic, geothermal sediments from the island of Milos in the Aegean Sea were previously treated and examined similarly (Norris et al. 2010) and additionally more recent samples from the same Milos sites were collected for further analysis. Samples were from the surface of sediments in two pools of acidic water which were ten metres apart at the base of cliffs about 50 metres west of the main beach of Palaeochori Bay, Milos. The temperature was about 60 °C a few centimetres below the sediment surface and hotter with increased depth. Both pools were subjected to flushing from fluctuating sea levels but had the following characteristics at the time of sampling. The first pool (sample site 12) was one metre in front of the cliff face, with water 25 cm deep at pH 2.1 and 35 °C. The second pool (site 14) was at the base of the cliff face and two samples were taken; sample 14c was 40 °C and pH 2.2, sample 14d from under the overhanging cliff face was 60 °C and pH 2.6. Ferrous iron in solution was not detectable in sample 12 but was present at 45 and 30 mg $l^{-1}$ in samples 14c and 14d respectively. DNA was also extracted from a sample of acidic soil (50–60 °C at 2 cm depth) from a geothermal site on the clifftop near Palaeochori Bay.

Enrichment cultures were established from the Milos saline sediment samples in mineral salts media (Simmons and Norris 2002) containing 2.5 or 5% w/v NaCl and powdered elemental sulfur (5 g $l^{-1}$) or ferrous iron (50 mM). In some cases, ferrous iron medium was supplemented with yeast extract (0.01% w/v) or tetrathionate (0.5 mM $K_2S_4O_6$). Enrichment cultures were also established at 30 and 48 °C with sediment from Milos sample 14c with pyrite as substrate and subjected to DNA extraction and analysis. Media (at pH 2) solidified with Phytagel at a final concentration 0.4% w/v were supplemented with tetrathionate (1 mM) or ferrous iron (25 mM) to allow isolation of single colonies of sulfur- or ferrous iron-oxidizing acidophiles from the cultures.

### Growth conditions and assays

For growth on ferrous iron, the medium contained $MgSO_4.7H_2O$ (0.4 g $l^{-1}$), $(NH_4)_2SO_4$ (0.2 g $l^{-1}$), $K_2HPO_4$ (0.1 g $l^{-1}$) and $FeSO_4.7H_2O$ (13.9 g $l^{-1}$; equivalent to 50 mM $Fe^{2+}$). For growth on sulfur and tetrathionate, the medium contained $MgSO_4.7H_2O$ (0.5 g $l^{-1}$), $(NH_4)_2SO_4$ (0.4 g $l^{-1}$), $K_2HPO_4$ (0.2 g $l^{-1}$), $FeSO_4•7H_2O$ (10 mg $l^{-1}$) and either elemental sulfur powder (5 g $l^{-1}$) or $K_2S_4O_6$ (2.5 or 5 mM). Shaken flask cultures (150 rpm) were used to test the effect of supplements (yeast extract or tetrathionate) with inocula from cultures previously grown under identical conditions to those of test media (with or without supplements as appropriate). Inocula were similarly prepared to assess the effect of copper and chloride in combination. For determination of optimum temperatures, stirred cultures were grown in water-jacketed vessels and gassed with air or 1% v/v carbon dioxide in air. Ferrous iron oxidation was determined by titration of residual ferrous iron with ceric sulfate and with phenanthroline/ferrous sulfate as an indicator. Cell numbers and biomass (total particle volume) were determined by orifice electrical flow impedance using a CellFacts Particle Size Analyser (CellFacts Instruments Ltd., Coventry, UK). Optical density measurements were taken at 440 nm. When elemental sulfur was the substrate, biomass measurements were of planktonic cells with no attempt to remove any cells attached to solid sulfur.

Primers were designed for PCR amplification of partial 16S rRNA genes of species and novel bacteria that belong or could belong to the *Acidihalobacter* or *Acidithiobacillus* genera and further primers, used in combination with universal forward or reverse primers, were designed to be specific for individual *Acidihalobacter* species for the re-examination of the sample from Vulcano (Supplementary Table S1). For examination of enrichment cultures, the specificity of PCR was improved with forward and reverse primers designed to amplify 16S rRNA sequences of individual species (Supplementary Table S2) and the specificities demonstrated (Supplementary Fig. S1) using DNA from isolated bacteria or cloned sequences of uncultured bacteria (TOPO TA Cloning Kit, Invitrogen). GenBank Accession Numbers for 16S rRNA gene sequences of clones and isolates obtained during this work are given in Figs. 1 and 4. Phylogenetic trees were constructed using PHYLIP programmes (Felsenstein 2006).

## Results and discussion

### Diversity and distribution of *Acidihalobacter* and related bacteria

A phylogenetic tree of *Acidihalobacter* spp. and related bacteria derived from 16S rRNA genes is presented (Fig. 1)

with revision from a previous version (Norris et al. 2010) to take account of more recent bacterial isolations and nomenclature changes following formal classification of some species. Their closest relatives were previously indicated as halophiles of the *Ectothiorhodospiraceae* (Goebel et al. 2000 and Khaleque et al. 2019). However, 16S rRNA gene sequences of some uncultured bacteria from the deep-sea floor and, exceptionally, from mine tailings, suggest closer relatives (Fig. 1). Three isolates obtained from Milos sediment enrichment cultures via ferrous iron-supplemented Phytagel-solidified plates, isolates M6, M8 and M3, shared 16S rRNA gene sequences with *Ah. aeolianus*$^T$, *Ah. ferrooxydans*$^T$ and Vulcano clone V3 respectively (Fig. 1).

Specific PCR primers were used to target 16S rRNA gene sequences of bacteria belonging or potentially belonging to the *Acidihalobacter* or *Acidithiobacillus* genera in DNA extracted from Vulcano and Milos acidic littoral sediments and enrichment cultures (Table 1) to assess the presence of particular bacteria in samples with greater sensitivity than was possible with universal primers and the small clone banks of previous work. The presence of *Ah. aeolianus* and *Ah. ferrooxydans* was detected in Vulcano and Milos samples. The 16S rRNA gene of isolate M7 was amplified from DNA extracted directly from two Milos sites and from a pyrite-enrichment culture (Table 1). This thermotolerant or moderately thermophilic isolate was isolated previously from a Milos enrichment culture (Norris et al. 2010). The clone M14 sequence was found in DNA extracted from samples of acidic, warm sediments at the base of Milos sea cliffs but was not detected in the Vulcano sample.

Additional PCR primers were designed for improved specificity and to account for sequences reported since the initial design of primers (Supplementary Table S2). For example, the original forward primer intended to be specific for *Ah. aeolianus* was also a match for isolate F5 from Australian saline water. Primers specific for the cloned M14 sequence were used to look for enrichment of the source bacterium under a range of growth conditions (temperatures from 30 to 52 °C, a range of substrates, salt concentrations from 2 to 6% w/v, with or without supplements of yeast extract and/or glycine betaine and/or sucrose). Weak PCR products of the M14 sequence were obtained in initial enrichment cultures at lower temperatures (up to 42 °C) but a strong product was only seen from cultures that were supplemented with sucrose at 35 °C with 2 and 3% w/v NaCl. The sequence was not detectable beyond a third serial culture. *Ah. ferrooxydans* appeared to displace other iron-oxidizing *Acidihalobacter* species in pyrite enrichment cultures at 30-35 °C (Fig. S1). The bacteria obtained from various enrichment cultures as single colonies on solid media were isolates of *Ah. aeolianus*, *Ah. ferrooxydans* and isolates M3 and M7, so the source bacterium of sequence M14 remains to be isolated, along with that of cloned sequence V12.

## Growth of *Acidihalobacter* spp

Autotrophic growth of *A. aeolianus*$^T$ was more rapid than that of *Ah. ferrooxydans*$^T$ in medium supplemented with tetrathionate and the rate of ferrous iron oxidation by *Ah. ferrooxydans*$^T$ declined with substrate remaining unoxidized making a slow growth rate difficult to measure (Table 2). The most rapid growth-associated ferrous iron oxidation by *Ah. ferrooxydans*$^T$ occurred in the presence of yeast extract. Elsewhere, the growth characteristics of *Ah. ferrooxydans*$^T$ have been found as similar to those of *Ah. aeolianus*$^T$ apart from the former's greater salt tolerance (Khaleque et al. 2019). There are some differences in reported optimum and maximum NaCl concentrations for growth of *Ah. aeolianus*$^T$ and *Ah. ferrooxydans*$^T$ with ferrous iron as energy source, perhaps reflecting different laboratory procedures or inocula preparations. The optimum NaCl concentrations for both species have been reported as 0.17–0.26 M (Simmons 2001, shown as Supplementary Fig. S2) and as 0.43 M (Khaleque et al. 2019). The maximum NaCl concentrations allowing the growth of *Ah. aeolianus*$^T$ and *Ah. ferrooxydans*$^T$ have

**Table 1** The presence (+) or absence (−) of specific 16S rRNA genes in DNA extracted from Vulcano (V) and Milos (M) sites (see "Methods") determined by PCR with primers designed for individual *Acidihalobacter* spp. or related bacteria

| Primer source | Sample sites | | | | |
|---|---|---|---|---|---|
| | V | M12 | M14c | M14d | M14[a] |
| *Ah. aeolianus* | + | − | + | − | + |
| *Ah. ferrooxydans* | + | + | + | + | + |
| Milos isolate M3 | + | − | + | + | + |
| Milos isolate M7 | − | − | − | + | + |
| Milos clone M14 | − | + | + | + | + |
| Vulcano clone V12 | + | − | − | − | − |

[a]Pyrite enrichment cultures of Milos site 14 sediment

**Table 2** Effect of tetrathionate (1 mM) and yeast extract (0.01% w/v) on growth *Acidihalobacter* species at 35 °C with ferrous iron (50 mM) as substrate in medium containing 2% w/v NaCl

| Medium supplements | *Ah. aeolianus*$^T$ | *Ah. ferrooxydans*$^T$ |
|---|---|---|
| Ferrous iron (Fe) | No growth | No growth |
| Fe + tetrathionate | 5.7 | 15–40[a] |
| Fe + tetrathionate and yeast extract | 5.7 | 5.0 |
| Fe + yeast extract | no growth | 5.3 |

Doubling times (hours) are shown for ferrous iron oxidation over the period between oxidation of 10 and 20 of the provided 50 mM ferrous iron

[a]Variable rates of poor growth

been observed as 1.0 M and 1.4 M, respectively (Simmons 2001) and as 1.3 M and 0.86 M, respectively (Khaleque et al. 2019). The apparent capacity of *Ah. ferrooxydans*$^T$ to oxidize pyrite more efficiently than *Ah. aeolianus*$^T$ at high chloride concentrations (Norris and Simmons 2004) was confirmed with optimum growth of *Ah. ferrooxydans*$^T$ on pyrite with 0.85 M NaCl compared to 0.25 M NaCl for *Ah. aeolianus*$^T$ (Khaleque et al. 2019).

Although *Ah. ferrooxydans*$^T$ grew poorly in pure culture in the absence of yeast extract compared to *Ah. aeolianus*$^T$ (Table 2), it appeared to be the *Acidihalobacter* species preferentially selected by serial culture with pyrite as a substrate in the absence of yeast extract in a mixed culture which also contained the sulfur-oxidizing *At. thiooxidans* strain V2A (Norris and Simmons 2004). *Ah. ferrooxydans* could benefit from cross-feeding of a growth factor from *At. thiooxidans* or other bacteria in a mixed culture. Growth of *Ah. ferrooxydans* (strain M8) in pure culture was enhanced by a yeast extract concentration low enough to suggest it was principally supplying a growth factor rather than organic carbon for growth (Fig. 2). In the absence of yeast extract, autotrophic growth-associated ferrous iron oxidation was enhanced in mixed culture with the sulfur-oxidizing *At. thiooxidans* strain A7 from Milos (Fig. 3), indicating the possible use of a growth factor provided by the latter. Ferrous iron oxidation in the culture containing only strain A7, which does not oxidize ferrous iron, was equivalent to oxidation in a sterile control.

*Acidihalobacter*-like moderately thermophilic isolates M3 and M7 appear to share the requirement for reduced sulfur during growth on ferrous iron as described for *Ah. aeolianus*$^T$ (Le C Nicolle et al. 2009) and several archaeal and moderately thermophilic bacterial acidophiles that oxidize ferrous iron, including *Acidimicrobium ferrooxidans*, some but not all *Sulfobacillus* sp. (Norris and Barr 1985) and *Acidithiomicrobium* sp. (Davis-Belmar and Norris 2009). Both isolates, which remain to be classified, grow autotrophically with optimum temperatures about 47 °C on ferrous iron, sulfur and tetrathionate as well as mineral sulfides (Le C Nicolle 2007).

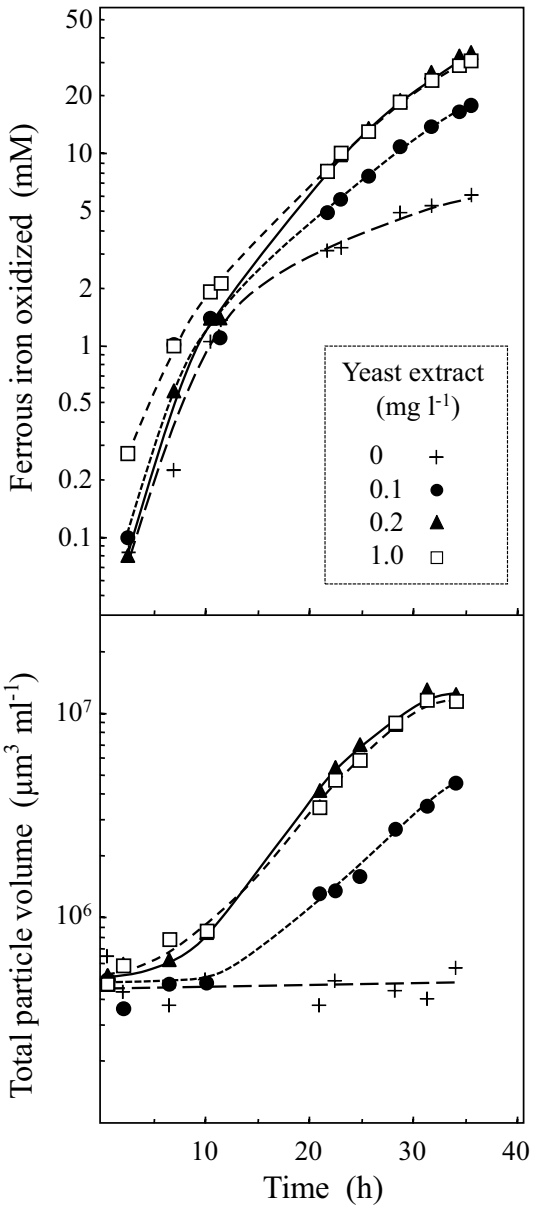


**Fig. 2** The effect of yeast extract concentration on growth (total particle volume) and associated oxidation of ferrous iron (50 mM) by *Ah. ferrooxydans*$^T$ at 30 °C

## Diversity and distribution of salt-tolerant *Acidithiobacillus*-like isolates

Comparisons of 16S rRNA sequences of *Acidithiobacillus* sp. have shown distinct clusters of strains of *At. thiooxidans* and *At. caldus* (Ni et al. 2008; Nuñez et al. 2017). The Vulcano isolate V1 falls into a group defined as *Acidithiobacillus* Group 6 (Ni et al. 2008) and *At. caldus* Subclade 1C (Nuñez et al. 2017) (Fig. 4). A similar isolate but with an identical sequence to that of *At. thiooxidans* AAU in the same cluster was also obtained from a sulfur-enrichment culture of a Milos sediment sample. Differences in tree topologies obtained with distance matrix and maximum likelihood programmes were limited to the relationship of *At. sulphuriphilus* and clone M66 to other groups (not shown). The M66 sequence was cloned from DNA extracted from the Milos cliff-top, acidic soil sample and could represent a novel species of *Acidithiobacillus*. The sequence of Vulcano isolate V2A is identical to that of isolate A7 from a sulfur-enrichment culture of Milos site 14c. These isolates cluster with the type strains of *At. thiooxidans* and *At. albertensis*: these type strains have one base difference in their 16S rRNA gene sequences and genome analysis has

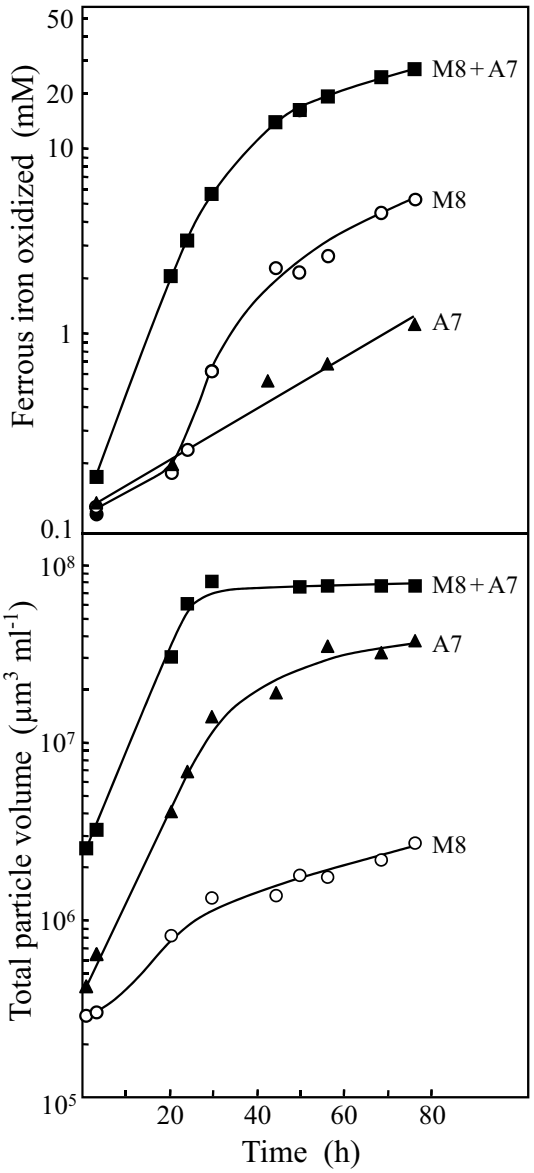


**Fig. 3** Growth and ferrous iron oxidation by *Ah. ferrooxydans* strain M8 and *At. thiooxidans* strain A7 in pure cultures and in combination in a medium containing 50 mM ferrous iron, 1 mM tetrathionate and 2% w/v NaCl at 30 °C

suggested the validity of *At. albertensis* as an independent species should be reconsidered (Castro et al. 2017). The 16S rRNA gene sequences of isolate V2 and the marine *Acidithiobacillus* sp. strain SH from Japan (Kamimura et al. 2003, 2018) are identical and have diverged slightly more from that of *At. thiooxidans*$^{T}$ than have the sequences of isolates V2A and A7, but still share over 99.6% identity with that of the type strain.

The salt-tolerant *Acidithiobacillus* sp. from Vulcano, Milos and Japan were isolated from saline environments, but apparently similar isolates have been obtained from environments for which a high salt concentration was not reported. For example, an isolate with a 16S rRNA gene sequence identical to that of isolate V1 was obtained from a copper ore heap leach site in the USA (Norris, data not shown) and several other similar but not identical sequences have been found in samples from various other sites, including metal-contaminated soils (Table S3). Conversely, a small clone bank (20 clones) of 16S rRNA genes constructed following PCR amplification using *Acidithiobacillus* genus-specific primers with DNA extracted from Milos sample site 14c comprised sequences matching the *At. caldus* type strain (Norris et al. 2010) and the probable source strain that was subsequently isolated from this site showed a similar lack of salt tolerance to that of the type strain (data not shown). *At. caldus* with limited salt tolerance was also isolated from the Vulcano saline samples that were the source of the salt-tolerant isolate V1 (Simmons and Norris 2002).

## Growth and salt tolerance of *Acidithiobacillus* spp.-related isolates

Isolate V1, *At. thiooxidans* V2 and V2A from Vulcano and *At. thiooxidans* A7 from Milos were found to be salt-tolerant mesophiles which did not oxidize ferrous iron and did not require salt for growth with sulfur, thiosulfate or tetrathionate. The growth rate of isolate V1 at its optimum temperature of about 36 °C (Supplementary Fig. S3) did not change with an enhanced concentration of $CO_2$, but the biomass yield appeared to increase (Fig. 5). Growth with 10 mM tetrathionate was much slower in the absence of pre-adaptation to the higher substrate concentration (not shown).

The tolerance of isolate V1 to salt increased through serial cultures with incremental increases in salt concentration with growth not inhibited by 0.85 M NaCl after several serial cultures at this salt concentration (Fig. 6). It was successfully sub-cultured with up to 1.5 M NaCl (9% w/v) (data not shown). In comparison, *At. caldus* was limited to growth with 0.5 M salt after adaptation. *At. caldus* is far more sensitive to sodium chloride than to sodium sulfate (Watling et al. 2012) whereas inhibition of isolate V1 occurred at similar molarities of sodium ions from chloride or sulfate salts and at similar molarities of chloride from sodium or magnesium chlorides. This indicated a response to ionic strength of the medium rather than specific chloride inhibition at least up to 0.8 M NaCl (Fig. 7). The reduction in growth rates of *Acidithiobacillus*-like isolate V1 by sodium sulfate in the absence of chloride was similar to that seen with *Ah. aeolianus* (Davis-Belmar et al. 2008).

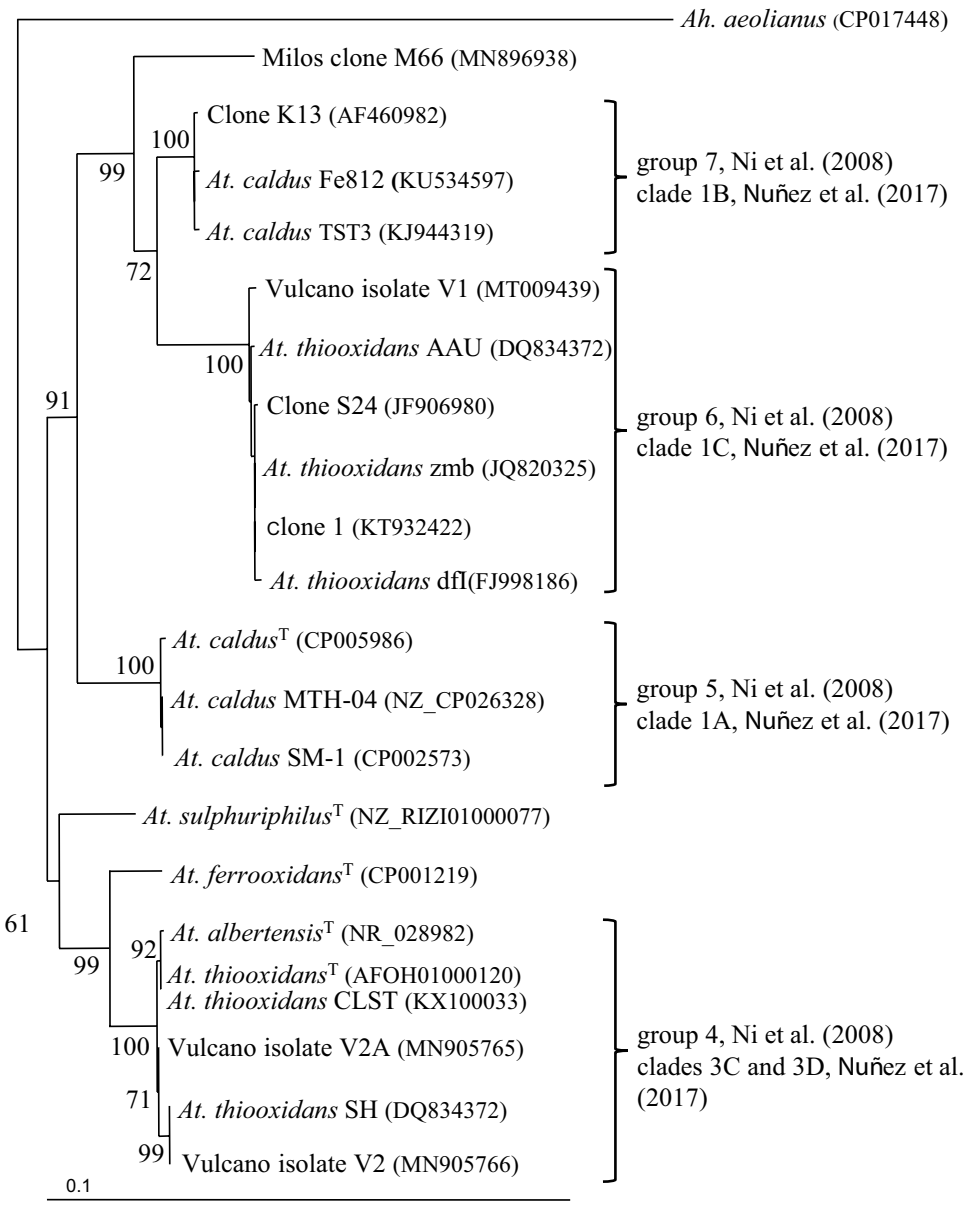


**Fig. 4** Distance phylogenetic tree of 16S rRNA gene sequences (1463 aligned nucleotides) with *Ah. aeolianus*$^{T}$ outgroup. Bootstrap values are for 100 replications and scale bar for nucleotide changes per site

## Effect of salt on copper tolerance of *Acidihalobacter* and *Acidithiobacillus* spp.: relevance to ore bioleaching

Although *Ah. prosperus* has been described as relatively sensitive to copper in contrast to its tolerance of several other metals (Huber and Stetter 1989), mesophilic *Acidihalobacter* spp. maintained ferrous iron oxidation during copper ore column leaching (Davis-Belmar et al. 2008), although this was more difficult to achieve with thermotolerant or moderately thermophilic *Acidihalobacter* spp. (Norris et al. 2010). The potential industrial application of *Acidihalobacter* spp. was also suggested with a demonstration of chalcopyrite bioleaching (Khaleque et al. 2017). However, the copper concentrations in solution were low (< 1 g $l^{-1}$) in these examples. A mixed enrichment culture from Vulcano was maintained on pyrite in presence of 3% w/v NaCl $l^{-1}$ and 3 g Cu $l^{-1}$ with monthly sub-culturing for 2 years but growth was very poor, with some inhibition by copper at 1 g $l^{-1}$ (data not shown). In contrast, nickel leaching from a pentlandite concentrate proceeded with microbial oxidation of ferrous iron and sulfur in the presence of 3% w/v salt and 5 g nickel $l^{-1}$ in mixed cultures of salt-tolerant *Acidithiobacillus* spp. and mesophilic *Acidihalobacter* spp. at 36 °C and thermotolerant *Acidihalobacter*-like species at 45 °C (Norris, data not shown; Le C Nicolle 2007). During nickel leaching, the dominant bacteria in the cultures assessed by 16S rRNA gene sequences in clone banks of extracted DNA were isolate V1 and *Ah. ferrooxydans* at 36 °C and *At. caldus* and isolate M7 at 45 °C (data not shown). However, nickel

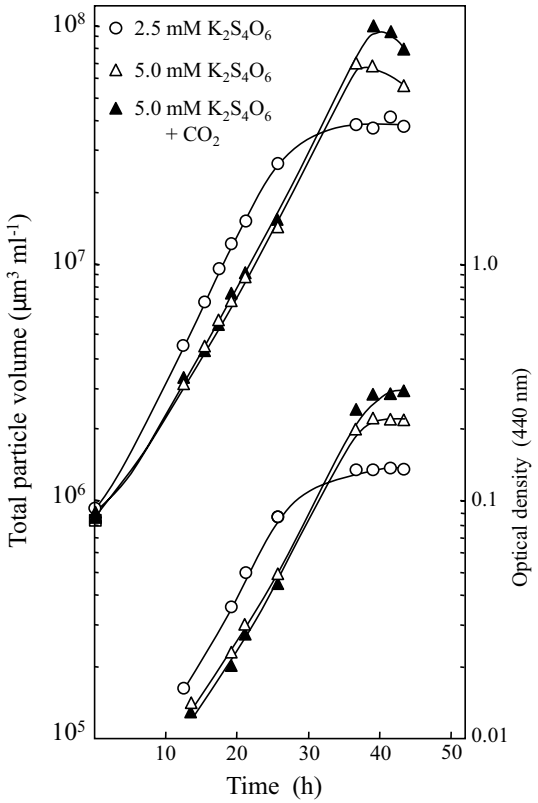


**Fig. 5** Biomass (total particle volume) and optical density during growth of *Acidithiobacillus* sp. V1 on tetrathionate at 36 °C

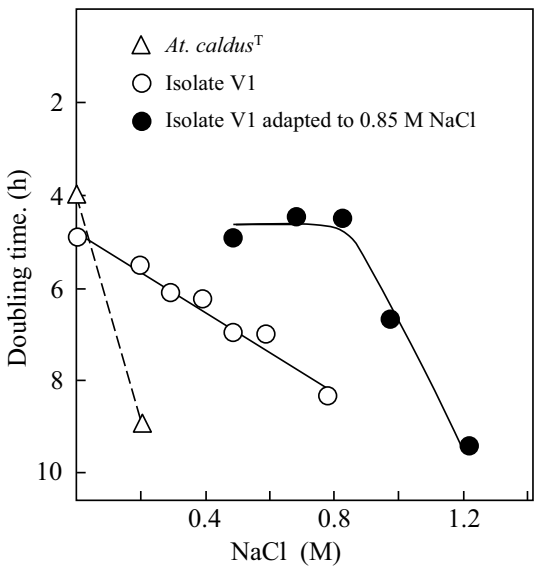


**Fig. 6** Effect of NaCl on the growth of *Acidithiobacillus* sp. V1 on tetrathionate (5 mM) derived from biomass (total particle volume) measurements

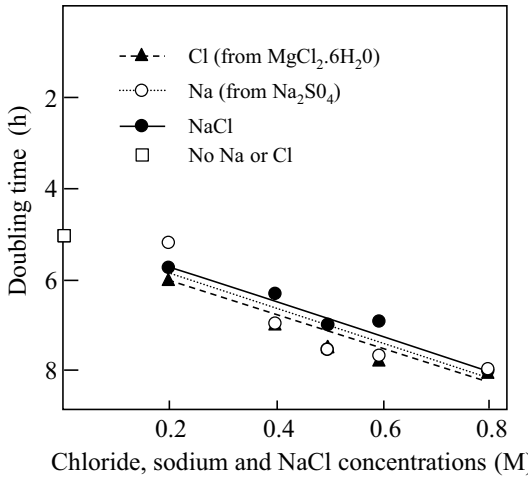


**Fig. 7** Inhibition by salts of *Acidithiobacillus* sp. V1 growth on tetrathionate (5 mM) at 36 °C

leaching was less rapid and extensive than in mixed cultures in the absence of salt at 36 °C (with *At. ferrooxidans*, *Leptospirillum ferriphilum* and *At. caldus*) and at 45 °C (with *At. caldus*, *Acidithiomicrobium* sp. and *Sulfobacillus thermosulfidooxidans*) (data not shown).

The response of sulfur-oxidizing *Acidithiobacillus* spp. and related isolates to copper in the presence of salt was examined initially in the presence of a salt concentration (10 g NaCl $l^{-1}$) that allowed the growth of *At. thiooxidans*$^T$ and *At. caldus*$^T$ (which show relatively low tolerance of salt) as well as the salt-tolerant isolates V1, V2a and A7. Inhibition was observed with copper at 0.5 g $l^{-1}$ with *At. thiooxidans*$^T$, *At. caldus*$^T$ and isolate V1 and precipitation of black copper sulfide was seen with *At. thiooxidans*, as reported for *At. thiooxidans* CLST (Quatrini et al. 2017). This sensitivity to copper contrasts with tolerance in the absence of salt, with *At. caldus,* for example, growing in the presence of at least 10 g copper $l^{-1}$ (Norris et al. 2017). Isolates V5 and A7 showed greater tolerance of copper with no inhibition at 1 g $l^{-1}$ in the presence of chloride. Growth of *At. thiooxidans* A7 was investigated further after prolonged adaptation of the culture (over 1 year) to increasing amounts of copper and salt (Fig. 8). Substantial inhibition of the growth rate occurred at the highest concentrations of salt and copper but some acid generation was maintained, indicated by the final pH values of 1.3-1.4 compared to pH 2.5 immediately after inoculation. Inhibition by the combination of copper and salt was most severe at the highest salt concentration rather than the highest copper concentration (Supplementary Table S4). The presence of anionic copper chloride complexes has been suggested to account for higher toxicity compared to when only one or the other of these elements was present during the growth of *At. thiooxidans* CLST and other acidophiles (Falagán and Johnson 2018). There could be some metabolic

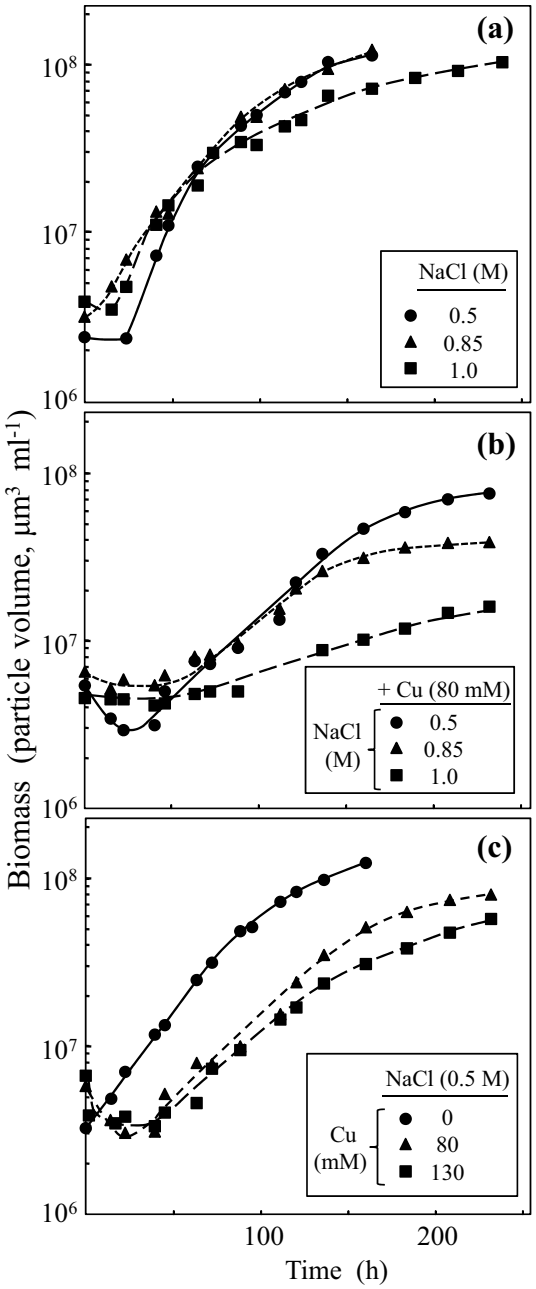


**Fig. 8** Effect of chloride (**a**) and various concentrations of chloride and copper (**b**) and **c** on growth on elemental sulfur (5 g $l^{-1}$) of *At. thiooxidans* A7 at 30 °C in shaken flasks

costs in mitigating apparently sub-inhibitory concentrations of copper and chloride when present alone that are additive and revealed as inhibition when both are present. However, the permeability of acidophiles to anions in response to their positive internal membrane potential (Alexander et al. 1987) would in this case (with presumed anionic copper chloride) result in some acidification of the cytoplasm and entry of a toxic metal which would otherwise have probably not been accumulated to the same extent.

Improved extraction of copper from copper sulfide concentrates can occur with chloride solutions (compared to sulfate solutions) and several hydrometallurgical processes have been developed to exploit this (Watling 2014). The concentration of copper in solution that would be reached in concentrate leaching at a typical, industrial process concentrate pulp density would preclude any of the isolates described here and any other known acidophilic bacteria contributing to a chloride-enhanced bioreactor process. Lower concentrations of copper are typical for heap bioleaching of low-grade ores but successful leaching of a chalcocite ore in laboratory ore columns by *Leptospirillum ferrooxidans* and *At. thiooxidans* was limited by chloride at 6 g $l^{-1}$ (Davis-Belmar et al. 2014). The growth of *At. thiooxidans* A7 at much higher chloride and copper concentrations suggests that inhibition of the iron-oxidizing bacteria would be the limiting factor in the application of mixed culture bioleaching. The activity of *Acidihalobacter* spp. under such conditions in a heap have not been demonstrated and their potential application might be restricted to the regeneration of ferric iron reduced to ferrous iron during sulfide ore oxidation, after copper recovery from the circulating solution.

In summary, some *Acidihalobacter* species have been shown as common to geographically separated sites of similar chemistry while a wider distribution of others has yet to be established. In contrast to *Acidihalobacter* spp., at least some salt-tolerant *Acidithiobacillus* spp., including isolate V1 which probably represents a novel species, do not require salt for growth and their salt tolerance appears related to osmotic pressure tolerance rather than a specific response to chloride toxicity. Salt-tolerant *At. thiooxidans* A7 could oxidize sulfur and promote acidification in ore heaps where ferric iron or copper was used as an oxidant and seawater was an alternative to freshwater or in operations where an even higher concentration of chloride was naturally present.

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