

# Genome-based classification of two halotolerant extreme acidophiles, *Acidihalobacter prosperus* V6 (=DSM 14174 =JCM 32253) and '*Acidihalobacter ferrooxidans*' V8 (=DSM 14175 =JCM 32254) as two new species, *Acidihalobacter aeolianus* sp. nov. and *Acidihalobacter ferrooxydans* sp. nov., respectively

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## Abstract

Phylogenomic analysis of recently released high-quality draft genome sequences of the halotolerant acidophiles, *Acidihalobacter prosperus* V6 (=DSM 14174=JCM 32253) and '*Acidihalobacter ferrooxidans*' V8 (=DSM 14175=JCM 32254), was undertaken in order to clarify their taxonomic relationship. Sequence based phylogenomic approaches included 16S rRNA gene phylogeny, multi-gene phylogeny from the concatenated alignment of nine selected housekeeping genes and multiprotein phylogeny using clusters of orthologous groups of proteins from ribosomal protein families as well as those from complete sets of markers based on concatenated alignments of universal protein families. Non-sequence based approaches for species circumscription were based on analyses of average nucleotide identity, which was further reinforced by the correlation indices of tetra-nucleotide signatures as well as genome-to-genome distance (digital DNA–DNA hybridization) calculations. The different approaches undertaken in this study for species tree reconstruction resulted in a tree that was phylogenetically congruent, revealing that both micro-organisms are members of separate species of the genus *Acidihalobacter*. In accordance, it is proposed that *A. prosperus* V6<sup>T</sup> (=DSM 14174<sup>T</sup>=JCM 32253<sup>T</sup>) be formally classified as *Acidihalobacter aeolianus* sp. nov., and '*Acidihalobacter ferrooxidans*' V8<sup>T</sup> (=DSM 14175<sup>T</sup>=JCM 32254<sup>T</sup>) as *Acidihalobacter ferrooxydans* sp. nov., and that both represent the type strains of their respective species.

Only a few bacteria capable of simultaneously tolerating both low pH and high salt have been isolated [1, 2]. Therefore, the discovery and characterization of new species of halotolerant acidophiles enriches the opportunities to map the microbial diversity and shed new light on the metabolic capabilities in this mostly unexplored geochemical environment. It could also suggest novel routes by which the metabolic potential of halotolerant acidophiles could be exploited for industrial applications such as copper bioleaching from chalcopyrite [3]. This is an especially important consideration in regions with scarce water resources such as Australia and Chile where sea water could be substituted for fresh water in the bioleaching process [2].

The genus *Acidihalobacter* includes only one formally recognized species name, *Acidihalobacter prosperus* [4]. *A. prosperus* DSM 5130<sup>T</sup> is an iron- and sulfur-oxidizing, mesophilic, halotolerant acidophile that was isolated from a geothermally heated seafloor at Porto di Levante, Vulcano, Italy [5]. Based on its phenotype and a G+C content of 64 mol%, it was originally taxonomically classified as '*Thiobacillus prosperus*' by Huber and Stetter [5]. However, after the release of its draft genome sequence [6], further phylogenomic studies were undertaken and it was formally reclassified into the family *Ectothiorhodospiraceae* of the class *Gammaproteobacteria* in the new genus *Acidihalobacter*,

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**Abbreviations:** AIC, Akaike information criteria; ANI, average nucleotide identity; ANI<sub>B</sub>, average nucleotide identity based on BLAST; BIC, Bayesian information criteria; COGs, clusters of orthologous groups of proteins; dDDH, digital DNA–DNA hybridization; MLSA, multilocus sequence analysis; NIB, nucleotide identity by BLAST; Tetra, tetra-nucleotide signatures.

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*Acidihalobacter prosperus* V6: chromosome, CP017448.1; plasmid, CP017449.1. '*Acidihalobacter ferrooxidans*' V8: CP019434.1.

Four supplementary tables are available with the online version of this article.

with *A. prosperus* DSM 5130<sup>T</sup> (JCM 30709<sup>T</sup>) being named the type strain of its species [4].

More recently, two new halotolerant acidophiles, *A. prosperus* V6 and '*A. ferrooxidans*' V8, were isolated from mixed environmental cultures obtained from shallow acidic pools on the Aeolian Islands, Vulcano, Italy [7]. *A. prosperus* V6 was recognized to be similar to the type strain in its 16S rRNA gene phylogeny as well as its morphological characteristics [8, 9], but showed superior growth to the type strain on ferrous iron [5, 10]. *A. prosperus* V6 was shown to be a member of the family *Ectothiorhodospiraceae* of the class *Gammaproteobacteria* [11]. It has previously been referred to as '*Acidihalobacter aeolicus*' [12]; however, based on 99 % 16S rRNA gene sequence similarity as well as similarity in its morphological characteristics to *A. prosperus* DSM 5130<sup>T</sup> ('*Thiobacillus prosperus*' at the time), it was submitted to the DSMZ culture collection as a strain of the *A. prosperus* species and was never formally classified or named.

*A. prosperus* V6 is an aerobe, grows autotrophically and is able to oxidize ferrous iron, the sulfur sources elemental sulfur and tetrathionate and the sulfide mineral pyrite. It is unable to grow in the absence of chloride, requiring a minimum of 60 mM NaCl, is able to grow at up to 1283 mM NaCl with maximum growth at 428 mM NaCl [8, 13]. It is able to oxidize pyrite at an optimum 256 mM NaCl [13]. The temperature range for growth of *A. prosperus* V6 was 26–42 °C with an optimum of 36 °C [14]. Growth at various pH values was determined in basal salts medium [2] at 25 g l<sup>-1</sup> NaCl with 50 mM FeSO<sub>4</sub>·7H<sub>2</sub>O and 5 mM K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> [13]. *A. prosperus* V6 was able to grow between pH 1.5 and 3.0, but not at pH 1.0 and 3.5 with optimum growth observed at pH 1.8.

Similarly, '*A. ferrooxidans*' V8 was submitted to the DSMZ culture collection with its current name as it was thought to belong to a new species of the genus *Acidihalobacter* based on its 16S rRNA gene sequence and phenotypic characteristics such as slightly greater chloride ion tolerance than *A. prosperus* V6 when grown on the sulfide mineral pyrite [9, 10]. However, it was never characterized taxonomically in detail. It is aerobic and autotrophic, able to oxidize ferrous iron, elemental sulfur and tetrathionate and the sulfide mineral pyrite, and is unable to grow in the absence of chloride, having a minimum requirement of more than 60 mM NaCl when grown in the presence of soluble iron [13]. Maximum growth on ferrous iron and sulfur occurs at 428 mM NaCl and it is able to grow at up to 856 mM NaCl [13]. It also has a superior ability to oxidize pyrite in the presence of chloride compared to *A. prosperus* V6 with maximum oxidation occurring at 856 mM NaCl. '*A. ferrooxidans*' V8 is able to grow between 26 and 43 °C with an optimum of 36 °C [14]. The pH profile was determined in the same manner as *A. prosperus* V6. '*A. ferrooxidans*' V8 was able to grow between pH 1.0 and 3.0 with optimum growth observed at pH 1.8.

The high quality draft genome sequences of *A. prosperus* V6 and '*A. ferrooxidans*' V8 have recently been released

[15, 16]. Whole-genome sequencing has shown the presence of a 162 484 bp plasmid in *A. prosperus* V6 that is not present in either the type strain of *A. prosperus* or in '*A. ferrooxidans*' V8 [6, 15, 16]. We term this plasmid pABPV6. The availability of the high quality draft genomes of these strains has provided an opportunity to use phylogenomic strategies for the re-evaluation of the taxonomical positions of both species in order to properly classify them [17]. Predicted terminal oxidases from the genomes of *A. prosperus* V6 and '*A. ferrooxidans*' V8 include *aa*<sub>3</sub> (EC 1.9.3.1), *bo*<sub>3</sub> (EC 1.10.3.10), *bd*-I (EC 1.10.3.14) and fumarate reductase (quinol, EC 1.3.5.1–1.3.5.4). Predicted respiratory quinones from the genomes include ubiquinone (EC 1.14.13.-, 2.1.1.64, 2.1.1.63, 2.1.1.201, 2.1.1.222, 2.5.1.39, 2.5.1.129, 4.1.1.98).

Recent proteomic studies have found ectoine to be the major osmoprotectant in *A. prosperus* DSM 5130<sup>T</sup> [18] and *A. prosperus* V6 [19] and it is presumed it will also play a key role in osmoprotection of '*A. ferrooxidans*' V8, as preliminary analysis of the genomes of all three isolates has shown the presence of genes for the synthesis of ectoine [6, 15, 16]. However, differences in the genomes of the three isolates included the presence of a truncated SOX operon (*soxAXBYZ*) in *A. prosperus* DSM 5130<sup>T</sup> and *A. prosperus* V6 that was not found on the genome of '*A. ferrooxidans*' V8 [6, 15, 16].

The bioinformatically inferred G+C contents for the genomes of *A. prosperus* V6 and '*A. ferrooxidans*' V8 are 62.2 and 61.6 mol%, respectively [15]. These values fall within the range of 50.5–69.7 mol% DNA G+C for members of the family *Ectothiorhodospiraceae* [20]. It is also consistent with the placement of the two species in the genus *Acidihalobacter*, which is nearest to the *Ectothiorhodospira* genus, both of which show a requirement of chloride for growth [4, 5, 8, 20]. Phenotypic and genomic features of the three species of the genus *Acidihalobacter* are shown in Table 1.

In order to infer preliminary phylogenetic relationships, the 16S rRNA gene sequence similarity of *A. prosperus* V6, '*A. ferrooxidans*' V8 and *A. prosperus* DSM 5130<sup>T</sup> was compared by aligning the sequences using the default settings of the nucleotide Basic Local Alignment Search Tool (BLASTn) tool [21] and MAFFT alignment (using the L-INS-i iterative refinement) [22, 23]. Studies have suggested that 98.65 % 16S rRNA gene sequence similarity can be used as a threshold for differentiating two species [24, 25]. '*A. ferrooxidans*' V8 shared only 97 % sequence similarity [nucleotide identity by BLAST (NIB)], 16S rRNA gene sequence similarity by BLASTn) with both *A. prosperus* DSM 5130<sup>T</sup> and *A. prosperus* V6, confirming it to be a different novel species (Table 2). The 99 % sequence similarity (NIB) between *A. prosperus* V6 and *A. prosperus* DSM 5130<sup>T</sup> (Table 2), initially suggested that they might be different strains of the same species, as was previously suggested [8, 9, 11]. However, further phylogenomic analysis using other sequence and non-

**Table 1.** Comparison of genomic and phenotypic features of the three species of the genus *Acidihalobacter*

+, Present; –, absent; NA, data not available.

Feature	<i>Acidihalobacter prosperus</i>	<i>Acidihalobacter prosperus</i>	' <i>Acidihalobacter ferrooxidans</i> '
	DSM 5130 <sup>T</sup>	V6	V8
Genome size (Mbp)	3.36	3.36	3.45
G+C content (mol%)	64.5	62.2	61.6
Predicted coding DNA sequence (CDS)	3088	3194	3089
Plasmid	–	+	–
		162 484 bp (termed pABPV6)	
tRNA genes	48	46	45
Sulfur oxygenase reductase (E.C. 1.13.11.55)	+	–	+
Truncated Sox operon (soxAXBYZ)	+	+	–
Temperature range for growth (°C)	20–45 [5]	26–42 [14]	26–43 [14]
Optimum temperature for growth (°C)	33 [5]	36 [14]	36 [14]
pH range for growth	1.0–4.5 [5]	1.5–3.0	1.0–3.0
Optimum pH for growth	2.0 [5]	1.8	1.8
NaCl range for growth (mM)	70–1030 [18]	60–1283 [13]	60–856 [13]
Optimum NaCl for growth (mM) on FeSO <sub>4</sub> ·7H <sub>2</sub> O and K <sub>2</sub> S <sub>4</sub> O <sub>6</sub>	340 [5]	428 [8, 13, 14]	428 [13, 14]
NaCl <sub>opt</sub> (mM) on Fe <sub>2</sub> S	NA	256 [13]	856 [13]

sequence based techniques, as discussed below, have shown that both are members of novel species.

In addition to members of the genus *Acidihalobacter*, a total of 11 other taxonomically related genomes were selected for inclusion into a 16S rRNA gene sequence-based phylogenetic tree. *Halothiobacillus neapolitanus* ATCC 23641 was used as an outgroup. Members for inclusion were identified from the 30 closest phylogenetic neighbours, based on *ab initio* comparisons of GLIMMER3 gene candidates with a set of universal proteins and up to 200 unduplicated proteins in the SEED and Rapid Annotation of Microbial genomes using Subsystems Technology (RAST) [26, 27]. These were verified by comparison to the sequences previously used for the reclassification of the type strain of *A. prosperus* [4], as well as by comparison with nucleotide databases after running a BLASTn-based script using an

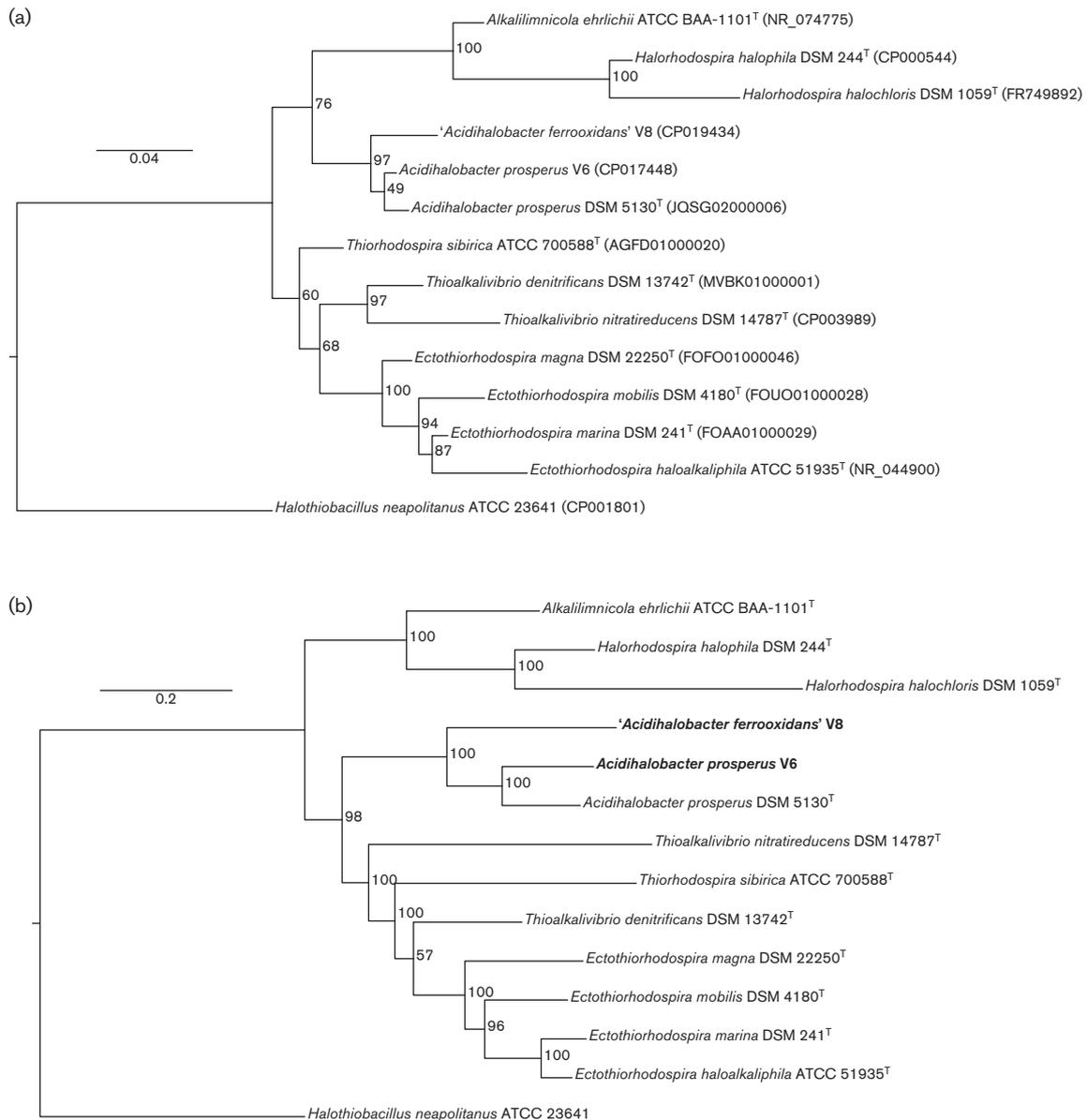
e-value threshold of 1e-5 and the databases GREENGENES [28], RDP [29] and SILVA [30].

The Tamura–Nei model [31], with a discrete gamma distribution and allowing some sites to be evolutionarily invariable (TrN+G+I), was chosen as the most appropriate model for inference of 16S rRNA gene phylogeny according to the lowest Bayesian information criteria (BIC) model selection of jModelTest [32, 33]. The phylogenetic tree was reconstructed using sequences obtained from the prokaryotic 16S rRNA gene database of NCBI and aligned in MAFFT version 7 with the L-INS-i iterative refinement [22, 23]. PhyML version 3 was used to reconstruct a maximum-likelihood tree using the bootstrap method with 1000 replicates [34, 35]. After tree reconstruction, a final of 14 members (including those of the genus *Acidihalobacter*) were selected based on the closest phylogeny as given by bootstrap values. The concise, final tree, consisting of the 14 selected members from

**Table 2.** Comparison of ANI, dDDH, TETRA and NIB of the chromosomal sequences of *A. prosperus* V6 and '*A. ferrooxidans*' V8 against the type strain, *A. prosperus* DSM 5130<sup>T</sup>, and each other

ANI, average nucleotide identity (%) as determined using ANI BLAST (%); dDDH, digital DNA–DNA hybridization (%); TETRA, nucleotide signature correlation index; NIB, 16s rRNA gene nucleotide identity by BLAST (%).

Query	<i>Acidihalobacter prosperus</i> DSM 5130 <sup>T</sup>				<i>Acidihalobacter prosperus</i> V6				' <i>Acidihalobacter ferrooxidans</i> ' V8			
	ANI	dDDH	TETRA	NIB	ANI	dDDH	TETRA	NIB	ANI	dDDH	TETRA	NIB
<i>Acidihalobacter prosperus</i> DSM 5130 <sup>T</sup>					80.60	25.20	0.9889	99	72.32	20.40	0.9151	97
<i>Acidihalobacter prosperus</i> V6	80.34	25.20	0.9889	99					72.49	21.30	0.9128	97
' <i>Acidihalobacter ferrooxidans</i> ' V8	71.93	20.40	0.9151	97	72.47	21.30	0.9128	97				



**Fig. 1.** Gene-based phylogenetic trees of 14 members of the order *Chromatiales* including the strains *Acidihalobacter prosperus* DSM 5130<sup>T</sup> (JQSG00000000.2), *A. prosperus* V6 (CP017448.1) and '*A. ferrooxidans*' V8 (CP019434.1). (a) 16S rRNA gene phylogenetic tree of 14 members of the order *Chromatiales*, including the analysed strains *Acidihalobacter prosperus* DSM 5130<sup>T</sup>, *A. prosperus* V6 and '*A. ferrooxidans*' V8. The tree was reconstructed with a discrete gamma distribution and allowing some sites to be evolutionarily invariable (TrN+G+I) [33] using PhyML 3 with log-likelihood of  $-6212.8$  [35] and the bootstrap method (with 1000 replicates) [34], the values of which were converted to statistically supported percentages. Bar, 0.04 nucleotide changes per site. (b) A multi-gene species tree was built using a concatenated alignment of nine conserved housekeeping genes (*argS*, *dnaQ*, *dnaN*, *era*, *gltA*, *gyrB*, *ppnK*, *rpoB* and *rpoD*) [40, 61, 62] from 14 members from the order *Chromatiales*. The substitution model selected for the multi-gene tree was general time-reverse with discrete gamma distribution and with some sites evolutionarily invariable (GTR+G+I) [33]. The phylogenetic tree was built using PhyML 3 using log-likelihood of  $-145524.6$  [35] and 1000 bootstraps [34] the values of which are represented as statistically supported percentage values. Bar, 0.2 nucleotide changes per site. The list of housekeeping genes used for tree elaboration is given in Table S2.

the genus *Acidihalobacter* and the order *Chromatiales* is shown in Fig. 1(a).

It has previously been shown that a better understanding of speciation events can be achieved by the use of multilocus

sequence analysis (MLSA), where the concatenation of the sequences of several protein-encoding gene fragments provides a more robust tree topology than can be obtained by 16S rRNA gene sequences alone [36–39]. In an MLSA study, housekeeping loci can be compared in order to



3 with a log-likelihood of  $-46600.9$  [35] and the bootstrap method (with 1000 replicates) [34], the values of which were converted to statistically supported percentages. Bar, 0.06 amino acid changes per site. The list of COGs used for tree elaboration is given in Table S3. (b) Phylogenomic tree of universal protein families. The tree was reconstructed using 28 out of 31 markers from universal protein families [46]. The substitution model described by Le and Gascuel [47] was utilized for multiprotein alignment, with the model utilizing four rate categories in the gamma distribution and proportion of evolutionary invariable sites (LG+G+I) [33]. The phylogenetic tree was reconstructed with PhyML 3 with a log-likelihood of  $-65827.6$  [35] and the bootstrap method (with 1000 replicates) [34], the values of which were converted to statistically supported percentages. Bar, 0.07 amino acid changes per site. The list of COGs used for tree elaboration is given in Table S4.

determine the evolutionary relationships among taxa [40]. Previously, *Salinivibrio* was determined as an ideal candidate for an MLSA study due to the low number of species available, as this allows for a more in-depth study of phylogenetic relationships [40]. As there are currently only 3 members of the genus *Acidihalobacter*, it was reasoned that an MLSA study would be appropriate for studying the phylogenetic relationship of the species of this genus. Therefore, a multi-gene species tree was built using a concatenated alignment of nine conserved housekeeping genes; *argS*, *dnaQ*, *dnaN*, *era*, *gltA*, *gyrB*, *ppnK*, *rpoB* and *rpoD* (Table S2) from 14 members from the order *Chromatiales* (Fig. 1b). The L-INS-i iterative refinement in MAFFT version 7 [22, 23] was used for the alignment of concatenated sequences; the alignments were masked to remove unreliable regions with GBLOCKS [41, 42], followed by a concatenation of all protein families. The substitution model selected for the multi-gene tree was general time-reverse with discrete gamma distribution and with some sites evolutionarily invariable (GTR+G+I), according to Bayesian and Akaike information criteria (BIC and AIC, respectively) model selection criteria, as indicated by jModelTest [32, 43]. PhyML version 3 was used to reconstruct the maximum-likelihood tree, using the bootstrap method with 1000 replicates [34, 35], as described above. The multi-gene phylogenetic tree is shown in Fig. 1(b).

Taxonomic classification of the species was further clarified through multiple locus phylogenetic analysis using two different phylogenomic approaches based on sets of clusters of orthologous groups (COGs) [44]. The complete sets of 34 and 31 COG markers (Tables S3 and S4) included in the analysis were recovered from the DOE Joint Genome Institute – Integrated Microbial Genomes and Microbiome Samples website (<https://img.jgi.doe.gov/cgi-bin/m/main.cgi>) for each micro-organism. The predicted protein-coding genes of the *Acidihalobacter* species were assigned to the COG classifications by comparison of each protein-coding sequence against the COG database by BLASTp, using a maximum e-value of  $1e-5$ . The association of each protein-coding sequence to a COG category was based on the highest hit coverage value, using an in-house Bioperl script. A multilocus phylogenomic tree was reconstructed for the 14 members of different species found to be taxonomically closest to the genus *Acidihalobacter* (Table S1, available in the online version of this article) by performing a multiple alignment of concatenated sequences of 30 COGs from 34 ribosomal protein families (Fig. 2a) that are universally

conserved in the three domains of cellular life [45]. A second multiprotein phylogenetic tree was built using complete sets of markers based on concatenated alignments of 28 out of 31 COGs retrieved from universal protein families [46] (Fig. 2b). The substitution model described by Le and Gascuel [47] was utilized for multiprotein alignment, with a discrete gamma distribution and sites evolutionarily invariable (LG+G+I), as predicted using the ProtTEST 3 tool [48, 49], according to the lowest BIC and AIC [33, 50]. The alignment of concatenated sequences, masking of unreliable regions and reconstruction of the maximum-likelihood trees was performed as described above for the multigene tree.

The gene and multiprotein phylogenomic analyses (Figs 1a, b and 2a, b) show similar relationships between the *Acidihalobacter* genomes and their positioning within and the selected members of the family *Ectothiorhodospiraceae*. While the information gained from this analysis provides important information on the evolutionary relationships of different strains, it does not show the overall similarity of the genomes. Average nucleotide identity (ANI), the correlation indices of tetra-nucleotide signatures (TETRA) and digital DNA–DNA hybridization (dDDH) calculations are sequence based techniques designed to analyse and compare interspecies boundaries between genomes [43, 51–53]. The combination of ANI results when reinforced by high TETRA correlation values can be used to help define prokaryotic species using an objective boundary [51]. Furthermore, the use of dDDH has been shown to outperform the previously used cumbersome and error prone technique of experimentally determining DNA–DNA hybridization (DDH) values and offers a more precise method for delineation of microbiological species [54]. The use of these bioinformatics approaches for clarification of taxonomical positions has previously been successfully used for *Acinetobacter* [55], *Vibrio* [56] and *Aeromonas* [53] and their advantages have been discussed extensively.

The calculation of ANI based on BLAST (ANiB) and the correlation indexes of tetra-nucleotide signatures (TETRA) were conducted using JspeciesWS (<http://jspecies.ribohost.com/jspeciesws/#Analyse>) [57], based on the recommended cut-off values for species determination ( $<95\%$  for ANiB and  $<0.989$  for TETRA) [24, 43, 51, 58]. The dDDH values were calculated using the Genome-to-Genome Distance Calculator (GGDC) web tool, (<http://ggdc.dsmz.de/distcalc2.php>), with formula 2 [52, 54] and a cut off of 70% to determine the distance between the genomes [59, 60]. The results indicate that *A. prosperus* V6 and *A. ferrooxidans* V8 belong to

different species, and provided the first evidence of *A. prosperus* V6 as a separate and novel species of the genus *Acidihalobacter*, and not a strain of *A. prosperus*. It also confirmed that '*A. ferrooxidans*' V8 is also a separate, novel species as previously suggested [9, 10]. The ANI, TETRA and dDDH values obtained from the comparison of these two species against each other and *A. prosperus* DSM 5130<sup>T</sup> are provided in Table 2.

In conclusion, results of the different sequence-based and non sequence-based phylogenomic approaches undertaken in this study show phylogenetic congruence and highlight that '*Acidihalobacter prosperus*' V6 and '*Acidihalobacter ferrooxidans*' V8 are members of separate, novel species. We, therefore, propose the reclassification *Acidihalobacter prosperus* V6 as the type strain of '*Acidihalobacter aeolianus*' sp. nov., and that of '*Acidihalobacter ferrooxidans*' V8 as the type strain of '*Acidihalobacter ferrooxydans*' sp. nov.

## DESCRIPTION OF ACIDIHALOBACTER AEOLIANUS SP. NOV.

*Acidihalobacter aeolianus* (ae.o.li.a'nus. N.L. masc. adj. *aeolianus*, referring to its isolation from the Aeolian islands, Italy).

Gram-stain-negative, motile, straight rods (1–2 µm long). Extremely acidophilic, optimum pH is 1.8 with a range of pH 1.5–3.0. Halotolerant, requires a minimum of 60 mM NaCl for growth, but can grow at up to 1283 mM NaCl with optimal growth at 428 mM NaCl. Mesophilic, optimal growth occurs at 36 °C, and capable of growth between 26 and 42 °C. Chemolithoautotrophic and aerobic. Able to utilize ferrous iron and soluble sulfur (tetrathionate) as electron donors. Grows optimally on the sulfide mineral pyrite at 256 mM NaCl. Predicted terminal oxidases from the genome include *aa<sub>3</sub>* (EC 1.9.3.1), *bo<sub>3</sub>* (EC 1.10.3.10), *bd-I* (EC 1.10.3.14) and fumarate reductase (quinol, EC 1.3.5.1–1.3.5.4). Predicted respiratory quinones from the genome include ubiquinone (EC 1.14.13.-, 2.1.1.64, 2.1.1.63, 2.1.1.201, 2.1.1.222, 2.5.1.39, 2.5.1.129, 4.1.1.98). Genome contains a truncated SOX operon (soxAXBYZ) and an operon for the biosynthesis of the osmoprotectant ectoine.

The type strain is V6<sup>T</sup> (=DSM 14174<sup>T</sup>=JCM 32253<sup>T</sup>), isolated from shallow pools of acidic, salty water in the Aeolian islands, Italy.

## DESCRIPTION OF ACIDIHALOBACTER FERROOXYDANS SP. NOV.

*Acidihalobacter ferrooxydans* (fer.ro.o'xy.dans. L. n. *ferrum*, iron; Gr. adj. *oxys*, sour or acid, used to refer to oxygen in combinations; N.L. v. *oxydare*, to turn sour, to make acid, to oxidize; N.L. part. adj. *ferrooxydans*, iron-oxidising).

Gram-stain-negative, motile rods (1 µm long), sometimes curved under stress conditions. Extremely acidophilic, optimum pH is 1.8 with a range of 1.0–3.0. Halotolerant, requires more than 60 mM NaCl for growth, grows at up to

856 mM NaCl with an optimum of 428 mM NaCl. Chemolithoautotrophic and aerobic. Uses ferrous iron and soluble sulfur (tetrathionate) as electron donors. Grows optimally on the sulfide mineral pyrite at 856 mM NaCl. Mesophilic, growth occurs in the range 26–43 °C with an optimum of 36 °C. Predicted terminal oxidases from the genome include *aa<sub>3</sub>* (EC 1.9.3.1), *bo<sub>3</sub>* (EC 1.10.3.10), *bd-I* (EC 1.10.3.14) and fumarate reductase (quinol, EC 1.3.5.1–1.3.5.4). Predicted respiratory quinones from the genome include ubiquinone (EC 1.14.13.-, 2.1.1.64, 2.1.1.63, 2.1.1.201, 2.1.1.222, 2.5.1.39, 2.5.1.129, 4.1.1.98). Genome encodes a sulfur oxygenase reductase (E.C. 1.13.11.55) and contains an operon for the biosynthesis of the osmoprotectant ectoine.

The type strain is V8<sup>T</sup> (=DSM 14175<sup>T</sup>=JCM 32254<sup>T</sup>), isolated from shallow pools of acidic, salty water in Vulcano, Italy.

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### Conflicts of interest

The authors declare that there are no conflicts of interest.

### References

- Zammit CM, Watkin ELJ. Adaptation to extreme acidity and osmotic stress. *Acidophiles: Life in Extremely Acidic Environments*. Norfolk, UK: Caister Academic Press; 2016. pp. 49–62.
- Zammit CM, Mutch LA, Watling HR, Watkin ELJ. The characterization of salt tolerance in biomining microorganisms and the search for novel salt tolerant strains. *Adv Mat Res* 2009;71-73:283–286.
- Watling HR, Collinson DM, Corbett MK, Shiers DW, Kaksonen AH et al. Saline-water bioleaching of chalcopyrite with thermophilic, iron(II)- and sulfur-oxidizing microorganisms. *Res Microbiol* 2016; 167:546–554.
- Pablo Cárdenas J, Ortiz R, Norris PR, Watkin E, Holmes DS. Reclassification of '*Thiobacillus prosperus*' Huber and Stetter 1989 as *Acidihalobacter prosperus* gen. nov., sp. nov., a member of the family *Ectothiorhodospiraceae*. *Int J Syst Evol Microbiol* 2015;65: 3641–3644.
- Huber H, Stetter KO. *Thiobacillus prosperus* sp. nov., represents a new group of halotolerant metal-mobilizing bacteria isolated from a marine geothermal field. *Arch Microbiol* 1989;151:479–485.
- Ossandon FJ, Cardenas JP, Corbett M, Quatrini R, Holmes DS et al. Draft genome sequence of the iron-oxidizing, acidophilic, and halotolerant '*Thiobacillus prosperus*' type strain DSM 5130. *Genome Announc* 2014;2:e01042–01014.
- Simmons S, Norris R. Acidophiles of saline water at thermal vents of Vulcano, Italy. *Extremophiles* 2002;6:201–207.
- Davis-Belmar CS, Nicolle JLC, Norris PR. Ferrous iron oxidation and leaching of copper ore with halotolerant bacteria in ore columns. *Hydrometallurgy* 2008;94:144–147.
- Norris PR, Davis-Belmar CS, Nicolle JLC, Calvo-Bado LA, Angelatou V. Pyrite oxidation and copper sulfide ore leaching by halotolerant, thermotolerant bacteria. *Hydrometallurgy* 2010;104: 432–436.
- Norris P, Simmons S. Pyrite oxidation by halotolerant, acidophilic bacteria. *Biohydrometallurgy: a Sustainable Technology in Evolution*, Part II National Technical University of Athens. 2004. pp. 1347–1351.
- Hallberg KB, Hedrich S, Johnson DB. *Acidiferrobacter thiooxydans*, gen. nov. sp. nov., an acidophilic, thermo-tolerant, facultatively

- anaerobic iron- and sulfur-oxidizer of the family *Ectothiorhodospiraceae*. *Extremophiles* 2011;15:271–279.
12. Kelly D, Wood A, Stackebrandt E. *Thiobacillus Beijerinck* 1904b, 597 AL. *Bergey's Manual of Systematic Bacteriology*; 2005. pp. 764–769.
  13. Khaleque HN, Kaksonen AH, Boxall NJ, Watkin ELJ. Chloride ion tolerance and pyrite bioleaching capabilities of pure and mixed halotolerant, acidophilic iron- and sulfur-oxidizing cultures. *Miner Eng* 2018;120:87–93.
  14. Simmons S. The microbial ecology of acidic environments. *PhD thesis*, University of Warwick.
  15. Khaleque HN, Ramsay JP, Murphy RJT, Kaksonen AH, Boxall NJ et al. Draft genome sequence of the acidophilic, halotolerant, and iron/sulfur-oxidizing *Acidihalobacter prosperus* DSM 14174 (Strain V6). *Genome Announc* 2017;5:e01469–01416.
  16. Khaleque HN, Ramsay JP, Murphy RJT, Kaksonen AH, Boxall NJ et al. Draft genome sequence of *Acidihalobacter ferrooxidans* DSM 14175 (Strain V8), a new iron- and sulfur-oxidizing, halotolerant, acidophilic species. *Genome Announc* 2017;5:e00413–00417.
  17. Delsuc F, Brinkmann H, Philippe H. Phylogenomics and the reconstruction of the tree of life. *Nat Rev Genet* 2005;6:361–375.
  18. Dopson M, Holmes DS, Lazcano M, McCredden TJ, Bryan CG et al. Multiple osmotic stress responses in *Acidihalobacter prosperus* result in tolerance to chloride ions. *Front Microbiol* 2016;7.
  19. Khaleque HN, Shafique R, Kaksonen AH, Boxall NJ, Watkin ELJ. Quantitative proteomics using SWATH-MS identifies mechanisms of chloride tolerance in the halophilic acidophile *Acidihalobacter prosperus* DSM 14174. *Res Microbiol* 2018;169:638–648.
  20. Imhoff JF. Family II. Ectothiorhodospiraceae Imhoff 1984b, 339VP. *Bergey's Manual of Systematic Bacteriology: Volume 2: The Proteobacteria, Part B: The Gammaproteobacteria, Part B: The Gammaproteobacteria*, vol. 2; 2007. pp. 41.
  21. Madden T. The BLAST sequence analysis tool. 2013. Mar 15. In: *The NCBI Handbook [Internet]*, 2nd ed. Bethesda (MD): National Center for Biotechnology Information (US); 2013.
  22. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 2002;30:3059–3066.
  23. Katoh K, Standley DM. MAFFT: iterative refinement and additional methods. *Methods Mol Biol* 2014;1079:131–146.
  24. Kim M, Oh HS, Park SC, Chun J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* 2014;64:346–351.
  25. Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W et al. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat Rev Microbiol* 2014;12:635–645.
  26. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ et al. The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res* 2014;42:D206–D214.
  27. Delcher AL, Bratke KA, Powers EC, Salzberg SL. Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* 2007;23:673–679.
  28. Desantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006;72:5069–5072.
  29. Cole JR, Wang Q, Cardenas E, Fish J, Chai B et al. The Ribosomal database project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* 2009;37:D141–D145.
  30. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 2007;35:7188–7196.
  31. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 1993;10:512–526.
  32. Posada D. jModelTest: phylogenetic model averaging. *Mol Biol Evol* 2008;25:1253–1256.
  33. Schwarz G. Estimating the dimension of a model. *Ann Stat* 1978;6:461–464.
  34. Brown JK. Bootstrap hypothesis tests for evolutionary trees and other dendrograms. *Proc Natl Acad Sci USA* 1994;91:12293–12297.
  35. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W et al. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 2010;59:307–321.
  36. Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S et al. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl Environ Microbiol* 2005;71:5107–5115.
  37. Thompson FL, Gomez-Gil B, Vasconcelos AT, Sawabe T. Multilocus sequence analysis reveals that *Vibrio harveyi* and *V. campbellii* are distinct species. *Appl Environ Microbiol* 2007;73:4279–4285.
  38. Pascual J, Maclán MC, Arahál DR, Garay E, Pujalte MJ. Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, recA, pyrH, rpoD, gyrB, rctB and toxR genes. *Int J Syst Microbiol* 2010;60:154–165.
  39. Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AR et al. Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonus* sp. nov. *Front Microbiol* 2013;4:414.
  40. López-Hermoso C, de La Haba RR, Sánchez-Porro C, Papke RT, Ventosa A. Assessment of multiLocus sequence analysis as a valuable tool for the classification of the genus *Salinivibrio*. *Front Microbiol* 2017;8:1107.
  41. Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 2000;17:540–552.
  42. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst Biol* 2007;56:564–577.
  43. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P et al. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81–91.
  44. Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B et al. The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* 2003;4:41.
  45. Yutin N, Puigbò P, Koonin EV, Wolf YI. Phylogenomics of prokaryotic ribosomal proteins. *PLoS One* 2012;7:e36972.
  46. Ciccarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B et al. Toward automatic reconstruction of a highly resolved tree of life. *Science* 2006;311:1283–1287.
  47. Le SQ, Gascuel O. An improved general amino acid replacement matrix. *Mol Biol Evol* 2008;25:1307–1320.
  48. Darriba D, Taboada GL, Doallo R, Posada D. ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics* 2011;27:1164–1165.
  49. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 2003;52:696–704.
  50. Akaike H. A new look at the statistical model identification. In: Parzen E, Tanabe K and Kitagawa G (editors). *Selected Papers of Hirotugu Akaike*. New York, NY: Springer New York; 1998. pp. 215–222.
  51. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci USA* 2009;106:19126–19131.

52. Auch AF, von Jan M, Klenk HP, Göker M. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Stand Genomic Sci* 2010;2:117–134.
53. Colston SM, Fullmer MS, Beka L, Lamy B, Gogarten JP *et al.* Bioinformatic genome comparisons for taxonomic and phylogenetic assignments using *Aeromonas* as a test case. *mBio* 2014;5:e02136.
54. Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.
55. Chan JZ, Halachev MR, Loman NJ, Constantinidou C, Pallen MJ. Defining bacterial species in the genomic era: insights from the genus *Acinetobacter*. *BMC Microbiol* 2012;12:302.
56. Thompson CC, Vicente AC, Souza RC, Vasconcelos AT, Vesth T *et al.* Genomic taxonomy of *Vibrios*. *BMC Evol Biol* 2009;9:258.
57. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2016;32:929–931.
58. Teeling H, Meyerdieks A, Bauer M, Amann R, Glöckner FO. Application of tetranucleotide frequencies for the assignment of genomic fragments. *Environ Microbiol* 2004;6:938–947.
59. Tindall BJ, Rosselló-Móra R, Busse HJ, Ludwig W, Kämpfer P. Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* 2010;60:249–266.
60. Wayne LG, Moore WEC, Stackebrandt E, Kandler O, Colwell RR *et al.* Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int J Syst Evol Microbiol* 1987;37:463–464.
61. Gomila M, Peña A, Mulet M, Lalucat J, García-Valdés E. Phylogenomics and systematics in *Pseudomonas*. *Front Microbiol* 2015;6:214.
62. Yonezuka K, Shimodaira J, Tabata M, Ohji S, Hosoyama A *et al.* Phylogenetic analysis reveals the taxonomically diverse distribution of the *Pseudomonas putida* group. *J Gen Appl Microbiol* 2017;63:1–10.

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