Solid State Phenomena
ISSN: 1662-9779, Vol. 262, pp 339-343
doi:10.4028/www.scientific.net/SSP.262.339

Submitted: 2017-03-31
Revised: 2017-05-26
Accepted: 2017-05-30
Online: 2017-08-21

16S rRNA and Multilocus Phylogenetic Analysis of the Iron Oxidizing Acidophiles of the *Acidiferrobacteraceae* Family

Issotta Francisco[1,a], Covarrubias Paulo C.[1,2,b], Moya-Beltrán Ana[1,c], Bellenberg Sören[3,d], Thyssen Christian[3,e], Sand Wolfgang [4,5,f], Nuñez Harold[1,g], Mena Cristóbal[6,h], Holmes David S.[1,7,i], Quatrini Raquel[1,j*] and Vera Mario[6,k*]

[1]Fundación Ciencia & Vida, Santiago, Chile

[2]uBiome, Inc., San Francisco, California, USA

[3]Biofilm Centre, Universität Duisburg-Essen, Essen, Germany

[4]TU Bergakademie Freiberg, Freiberg, Germany

[5]Donghua University, Songjiang, Shanghai, China

[6]Pontificia Universidad Católica de Chile, Santiago, Chile

[7]Universidad Andrés Bello, Santiago, Chile

[a]fissotta@gmail.com, [b]paulo.covarrubias@gmail.com, [c]ana.moyabeltran@gmail.com, [d]soeren.bellenberg@uni-due.de, [e]christian.thyssen@uni-due.de, [f]wolfgang.sand@uni-due.de, [g]hnunez@ cienciavida.org, [h]clmena@uc.cl, [i]dsholmes2000@yahoo.com, [j]rquatrini@cienciavida.org, [k]mariovera@uc.cl

Keywords: *Acidiferrobacter thiooxydans*^{T}. *Acidiferrobacter* SP-III/3, comparative genomics, phylogenetic analysis

Abstract. The family *Acidiferrobacteraceae* (order *Acidiferrobacterales*) currently contains three genera of chemolithoautotrophs: *Sulfuricaulis* (2016), *Sulfurifustis* (2015) and *Acidiferrobacter* (2011). While the two former are neutrophilic sulfur oxidizers isolated from lake sediments in Japan, the latter is an extremely acidophilic, moderately osmophilic, thermotolerant iron/sulfur oxidizer known to occur in macroscopic streamers in Rio Tinto, Spain and in acid waters worldwide. The type strains of both *Sulfuricaulis limnicola* (HA5^{T}) and *Sulfurifustis variabilis* (skN76^{T}) have been sequenced, and the draft genome of the ZJ isolate of *Acidiferrobacter thiooxydans* (MDCF01) has recently been deposited in public databases. Despite this fact, little evidence on the genomic diversity and evolution of this group has been presented so far. Using comparative genomic analyses and phylogenetic reconstruction strategies, we explored the evolutionary information contained in the available genome sequences to shed light on the taxonomic status of a novel isolate of the genus *Acidiferrobacter* (SP-III/3; DSM 27195).

Introduction

The family *Acidiferrobacteraceae* of the order *Acidiferrobacterales* accommodates three genera of chemolithoautotrophs; *Sulfuricaulis* [1], *Sulfurifustis* [2] and *Acidiferrobacter* [3]. While the two former genera are neutrophilic sulfur oxidizers isolated from lake sediments in Japan [1, 2], the later is an extreme acidophilic, moderately osmophilic, thermotolerant iron oxidizer known to occur in macroscopic streamers from Rio Tinto in Spain and mine waters worldwide [3].

Acidiferrobacter thiooxydans m-1^{T} (DSM 2392), is the type strain of the single species that conforms the genus [3], and corresponds to an anomalous strain assigned to *Acidithiobacillus ferrooxidans* that troubled phylogenetic reconstruction of the acidithiobacilli for a long time. Differentiating characteristics included a much higher chromosomal G+C content (8-10 mol% higher) and the inability to oxidize thiosulfate and elemental sulfur [3, 4]. In 2011, Hallberg and colleagues [3] published a comprehensive description of the physiological and phylogenetic

characteristics of strain m-1 and demonstrated that it grew autotrophically by ferrous iron oxidation and by oxidation of elemental sulfur, sulfide and tetrathionate, using either oxygen or ferric iron as terminal electron acceptor.

Strains and sequence clones assigned to the genus have since been identified in different acidic environments around the world, including mine waters, mine tailings, mineral concentrates, volcanic ashes and macroscopic streamers [3, 5-8]. One such isolate is *Acidiferrrobacter* sp. SP-III/3 (DSM 27195), obtained from an acid mine drainage in Cartagena (Murcia, Spain) [6]. This isolate differs from *A. thiooxydans* m-1^T in in cell morphology and optimal temperature for growth, being that of strain SP-III/3 lower (30ºC). These features suggest that taxonomic assignment of the isolate SP-III/3 should be investigated in greater detail. Using comparative genomic analyses and phylogenetic reconstruction strategies, herein we explore the evolutionary information contained in available genomic sequences of the *Acidiferrobacteraceae* family to shed light on the taxonomic status of the *Acidiferrobacter* sp. SP-III/3 isolate.

Materials and Methods

The small ribosomal RNA subunit (16S rRNA) gene sequences of *A. thiooxydans* m-1 and the *Acidiferrobacter* sp. SP-III/3 isolate were amplified by PCR using universal primers 8F and 1391R and subsequently sequenced (Macrogen Inc., Korea). Additional 16S rRNA sequences used in this study were obtained from public databases (https://www.ncbi.nlm.nih.gov/nuccore). Downstream phylogenetic analysis was carried out using 25 16S rRNA gene sequences assigned to the genus *Acidiferrobacter, Sulfuricaulis* and *Sulfurifustis* by given taxonomy or sequence identity.

Small subunit ribosomal RNA gene sequences of *Acidiferrobacter* strains and phylogenetically related sequence clones were aligned with the MAFFT software (v7.229) using the L-INS-I method. The resulting alignments were trimmed and masked (>50%) manually with the MEGA7 software (v7.0). Phylogenetic trees were generated by Bayesian posterior probabilities using MrBayes [9] and handled with DendroPy for subtree pruning and regrafting [10]. The phylogenetic tree was assessed using 1,000,000 generations and sample trees were saved each 100 generations. Tree analysis was performed as in Nuñez et al. [11]

Publically available genomes pertaining to the *Acidiferrobacteraceae* family were recovered from the NCBI genome repository (https://www.ncbi.nlm.nih.gov/genome/). Genes encoding proteins to perform a Multi Locus Sequence Analysis (MLSA) were selected as described by Nuñez et al. [12]. Internal gene fragments for each marker were amplified by PCR using genomic DNA obtained from *A. thiooxyidans* m-1^T and *Acidiferrobacter* SP-III/3 strains. The resulting amplicons were sequenced (Macrogen Inc., Korea), aligned and manually curated, when appropriate. Concatenation of the MLSA markers was done with MEGA 7 (v7.0). The phylogenetic trees were constructed and analysed as described above.

Results and Discussion

16S rRNA Gene-Based Phylogeny. To assess the phylogenetic relationships between members of the *Acidiferrobacteraceae* family, available 16S rRNA genes belonging to strains and sequence clones of the *Acidiferrobacter, Sulfuricaulis* and *Sulfurifustis* species were retrieved from public genome and gene sequence databases. The goal was to assign taxonomy to strain SP-III/3, constructing a comprehensive phylogenetic tree for the group. A total of 25 sequences were used in the phylogenentic reconstruction. After filtering for redundancy, sequence length, eliminating ambiguous characters and positions with >50% gaps in the alignment (masked), a tree was calculated using Bayesian posterior probabilities. *Chromatium okenii* DSM 169^T was included as outgroup in order to root the tree. The group of selected and curated sequences encompassed 1,292 bp of the full 16S rRNA gene sequence, containing 272 variable sites and 133 parsimony informative sites, (96.5% pairwise identity). The Bayesian phylogenetic tree built for this dataset is shown in Figure 1.

The tree obtained showed a clear separation of all sequenced representatives in a number of distinctive clades (A – I). Even when the sequence divergence at the 16S rRNA gene level between *Acidiferrobacter* SP-III/3 and *A. thiooxydans* type strain (m-1^T) was as low as 0.1%, and fell below the cutoff used to delineate species (1.3%, [13]) *Acidiferrobacter* SP-III/3 (clade H) branched apart from *A. thiooxydans* strains m-1^T and ZJ (clade I) in the tree constructed. The average distance between isolates from the same clade of the tree (clade H) and *A. thiooxydans* m-1^T (clade I) remained low (0.3%), and below the cutoff value. Interestingly, one branch (clade G) composed as of yet of uncultured clones (from a volcanic ash deposit located in Japan [7]) showed divergences at the 16S rRNA level of 1.4% against both the m-1^T/ZJ and the SP-III/3 branches, indicating the presence of a new group within the genus. This novel branch remains to be characterized.

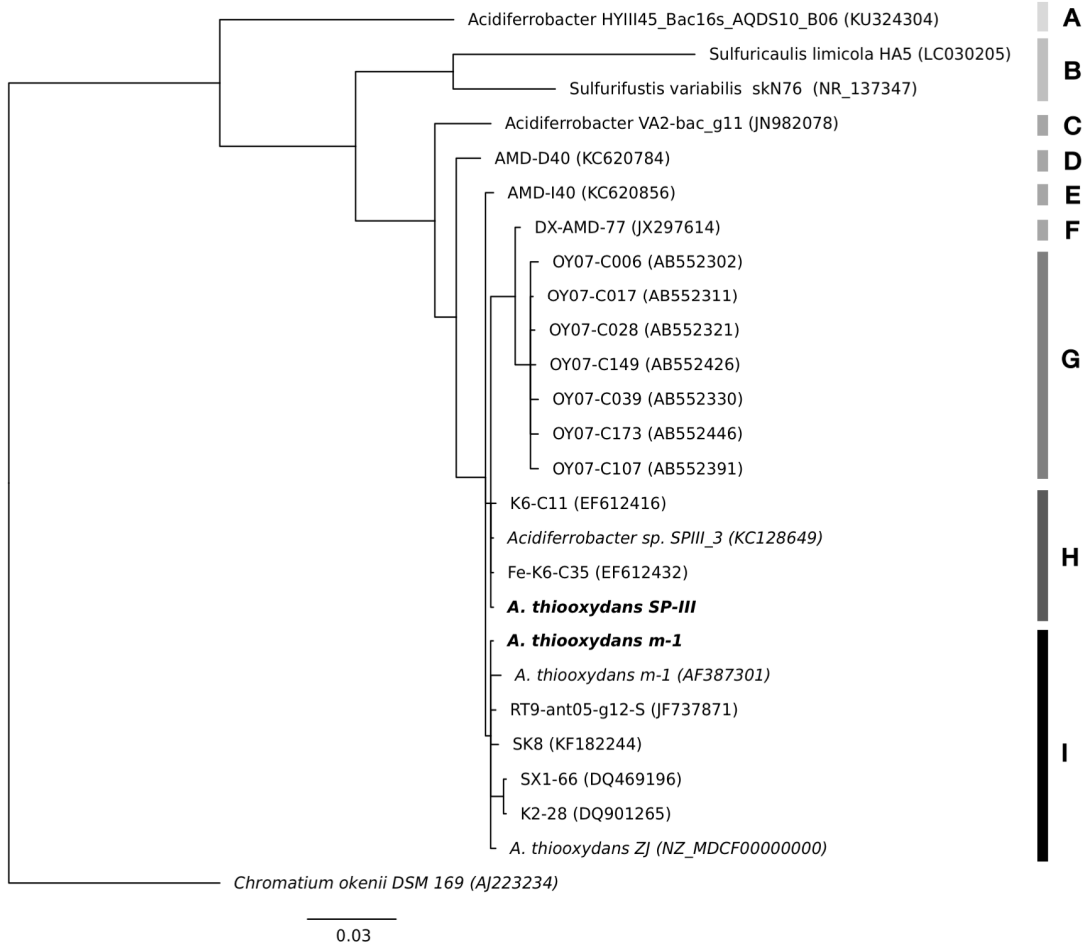

Fig. 1. *Acidiferrobacter* species phylogenetic tree built using Bayesian inference and 16S rRNA gene sequences of 25 strains and/or sequence clones. Proposed clades are as follows: Clade A – I.

MLSA-Marker Based Phylogeny. MLSA was used next to better define the phylogenetic structure of the *Acidiferrobacteraceae* family, at a higher resolution level. Informative markers were selected using a previously developed scheme for identification of genes suitable for MLSA [12] and the genomes of *A. thiooxydans* ZJ (MDCF01), *Sulfuricaulis limnicola* HA5^T (LC030205) and *Sulfurifustis variabilis* skN76^T (NR137347). Seven candidate protein encoding genes (*rpoB, hbzC1, mfd, ileS, dnaE, carB* and *clpB*) that met the amplicon size requirements of the pipeline were selected for further phylogenetic analysis. Internal gene fragments of the seven markers were amplified from genomic DNA obtained from *Acidiferrobacter m-1* and SP-III/3 strains by PCR, using a high fidelity polymerase and sequenced (Macrogen Inc., Korea).

Bayesian phylogenetic trees were constructed using a sequence concatenate of all seven markers (Figure 2). The concatenate comprised 21,844 nucleotides and consisted of 8444 variable sites. The seven protein-coding genes loci showed a mean nucleotide sequence diversity of 16.5%, in contrast with that using the 16S rRNA gene alone, which yielded only 10.8% polymorphic sites. Parsimony

informative sites, i.e. positions in the sequence set under comparison that contain at least two types of nucleotides in at least two different sequences, varied form a maximum of 85% (*rpoB*) to a minimum of 73.9% (*ileS*).

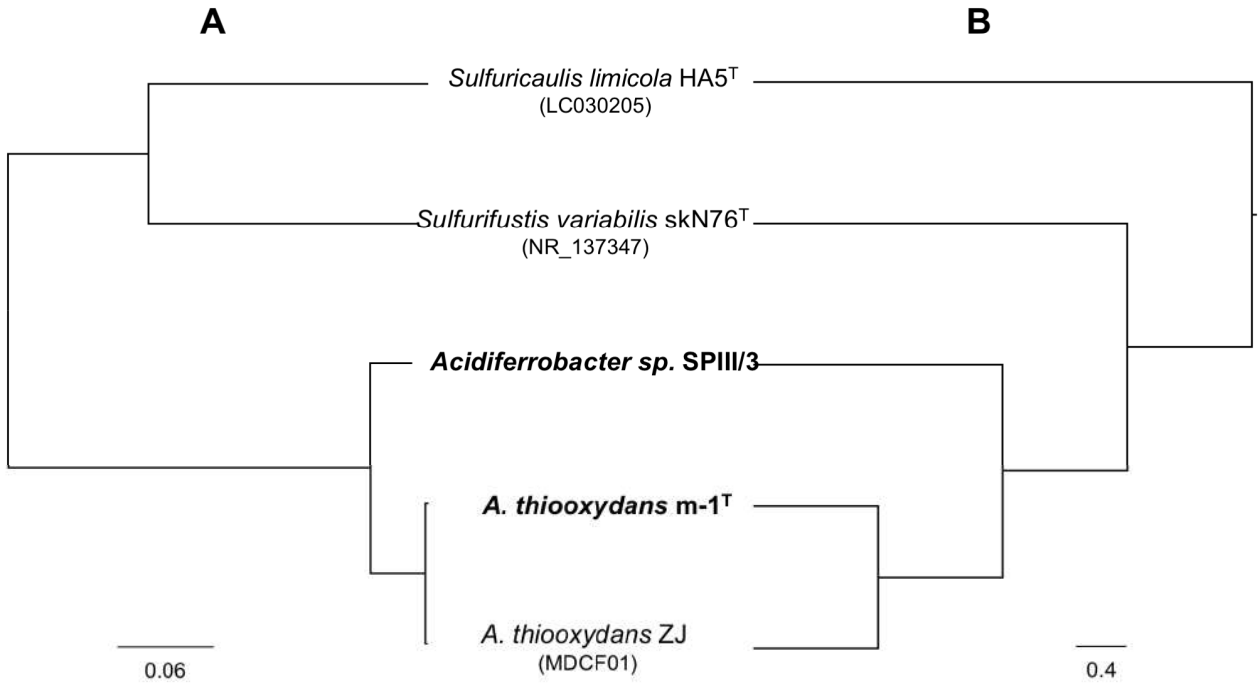

Fig. 2. *Acidiferrobacteraceae* species bayesian tree inferred from the concatenate of seven selected MLSA markers (A) versus the 16S rRNA bayesian tree derived for the same microorganisms (B). The *rpoB, hbzC1, mfd, ileS, dnaE, carB* and *clpB* gene sequences (21,844bp) from 5 representative strains of the family were concatenated and analysed using MEGA7 (v7.0). The bar represents expected nucleotide substitutions per site.

Topology of the concatenate-based tree was congruent with the topology of single-gene trees generated (data not shown), suggesting that none of the markers utilized was the object of active gene flow. Phylogenetic analysis of the concatenate produced 3 separate clades supported by bootstrap values of > 87% (Figure 2). Despite some disagreement in the topology of the MLSA-based tree and the 16S rRNA gene-based tree, the analysis supports assignment of *Acidiferrobacter* sp. SP-III/3 to a new species different from *A. thiooxydans* (represented by strain m-1^T). MLSA concatenate divergence values between both the m-1^T and SP-III/3 strains (5.1%) and the ZJ and SP-III/3 strains (5.2%) were larger than the threshold for species delineation in other microbial groups (3% [14]), supporting this assertion. In agreement with the phylogenetic reconstruction evidence, the DNA-DNA hybridization value determined experimentally for the m-1^T and SP-III/3 strains (DSMZ, Germany) was of 62%, below the well-recognized cut off of 70% [13], further validating the need for a proper taxonomic revision of the SP-III/3 isolate.

Conclusions

All together the data derived from the 16S rRNA and MLSA-concatenate phylogenetic reconstruction as well as the DNA-DNA hybridization analysis presented here, supports the assignment of strain SP-III/3 to a new species within the *Acidiferrobacter*, different from *A. thiooxydans* (m-1^T). Another clade emerging from the 16S rRNA-based phylogeny meets the levels of 16S rRNA sequence divergence to be recognized as a new species. However, no representative isolate has been described so far. Genomic sequencing of the SP-III/3 strain is deemed necessary to enable a more comprehensive comparative genomic study that will not only help refine the taxonomic structure of the *Acidiferrobacter,* but also facilitate the identification of further differentiating characteristics.

Acknowledgements

FONDECYT 1140048, 1161007, Basal CCTE PFB16, CONICYT and UNAB Scholarships.

References

[1] H. Kojima, W. Tomohiro, M. Fukui, *Sulfuricaulis limicola* gen. nov., sp. nov., a sulfur oxidizer isolated from a lake, Int. J. Syst. Evol. Microbiol. 66 (2016) 266–270.
[2] H. Kojima, A. Shinohara, M. Fukui, *Sulfurifustis variabilis* gen. nov., sp. nov., a sulfur oxidizer isolated from a lake, and proposal of *Acidiferrobacteraceae* fam. nov. and *Acidiferrobacterales* ord. nov., Int. J. Syst. Evol. Microbiol. 65 (2015) 3709–3713.
[3] K.B. Hallberg, S. Hedrich, D.B. Johnson, *Acidiferrobacter thiooxydans*, gen. nov. sp. nov.; an acidophilic, thermo-tolerant, facultatively anaerobic iron- and sulfur oxidizer of the family *Ectothiorhodospiraceae*, Extremophiles. 15 (2011) 271–279.
[4] A.P. Jr Harrison, Genomic and physiological diversity amongst strains of *Thiobacillus ferrooxidans*, and genomic comparison with *Thiobacillus thiooxidans*, Arch. Microbiol. 131 (1982) 68–76.
[5] Z. He, X.H. Xie, Z.G. He, S.M. Xiao, J.S. Liu, Microbial diversity of mine water at Zhong Tiaoshan copper mine, China. J. Basic. Microbiol. 47 (2007) 485–495.
[6] D. Mitchell, K. Harneit, G. Meyer, W. Sand, E. Stackebrandt, Systematic analysis of our culture collection for ‘‘genospecies’’ of *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*, in: V.S.T. Ciminelli, O. Garcia (Eds.), Biohydrometallurgy: fundamentals, technology and sustainable development. Elsevier, Amsterdam, 2004, pp. 1369–1378.
[7] R. Fujimura, Y. Sato, T. Nishizawa, K. Nanba, K. Oshima, M. Hattori, T. Kamijo, H. Ohta, Analysis of early bacterial communities on volcanic deposits on the island of Miyake (Miyake-jima), Japan: a 6-year study at a fixed site, Microbes Environ. 27 (2012) 19-29.
[8] A. Garcia-Moyano, E. Gonzalez-Toril, A. Aguilera, R. Amils, Prokaryotic community composition and ecology of floating macroscopic filaments from an extreme acidic environment, Rio Tinto (SW, Spain), Syst. Appl. Microbiol. 30 (2007) 601–614.
[9] J.P. Huelsenbeck, F. Ronquist, MRBAYES: Bayesian inference of phylogenetic trees, Bioinformatics 17 (2001) 754–755.
[10] J. Sukumaran, M.T. Holder, DendroPy: a Python library for phylogenetic computing, Bioinformatics 26 (2010) 1569–1571.
[11] H. Nuñez, A. Moya-Beltrán, P.C. Covarrubias, F. Issotta, J.P. Cardenas, M. Gonzalez, J. Atavales, L.G. Acuña, D.B. Johnson, R. Quatrini, Molecular systematics of the genus *Acidithiobacillus*: insights into the phylogenetic structure and diversification of the taxon, Front. Microbiol. 8 (2017) 30.
[12] H. Nuñez, D. Loyola, J.P. Cárdenas, D.S. Holmes, D.B. Johnson, R. Quatrini, Multi Locus Sequence Typing scheme for *Acidithiobacillus caldus* strain evaluation and differentiation, Res. Microbiol. 165 (2014) 735–742.
[13] E. Stackebrandt, J. Ebers, Taxonomic parameters revisited: tarnished gold standards, Microbiol. Today. 33 (2006)152–155.
[14] P. Vandamme, C. Peeters, Time to revisit polyphasic taxonomy, Antonie Van Leeuwenhoek 106 (2014) 57-65.