School of Biomedical Sciences

Acidihalobacter: Novel Halotolerant Iron- and Sulfur-oxidizing Acidophiles with Potential for Saline Water Bioleaching

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: Ismail Nalveeran Khalegue.

Date: 10/09/2017.

Dedication

To my beloved parents and my wonderful husband.

Acknowledgements

First of all, I would like to thank my supervisor and mentor, Professor Elizabeth Watkin. Throughout this journey, you have supported me, motivated me, guided me and been there to celebrate my victories and help me through the tough times. Working with you has been one of the greatest experiences in my life and I will be forever grateful for all that you have done for me. From the bottom of my heart, I thank you.

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List of Publications

This thesis is assembled by publications (either published, accepted, submitted or prepared for submission) which form the individual chapters of the thesis.

The publications are listed as follows:

Chapter 2

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. Minerals Engineering (accepted for publication in Minerals Engineering).

Chapter 3

Khaleque HN, Ramsay JP, Murphy RJ, Kaksonen AH, Boxall NJ, Watkin EL. Draft Genome Sequence of the Acidophilic, Halotolerant, and Iron/Sulfur-Oxidizing Acidihalobacter prosperus DSM 14174 (Strain V6). Genome Announcements. 2017 Jan 19;5(3):e01469-16.

Khaleque HN, Ramsay JP, Murphy RJ, Kaksonen AH, Boxall NJ, Watkin EL. Draft Genome Sequence of Acidihalobacter ferrooxidans DSM 14175 (Strain V8), a New Iron-and Sulfur-Oxidizing, Halotolerant, Acidophilic Species. Genome Announcements. 2017 May 25;5(21):e00413-17.

Khaleque HN, Corbett MK, Ramsay JP, Kaksonen AH, Boxall NJ, Watkin ELJ. Complete genome sequence of *Acidihalobacter prosperus* strain F5, an extremely acidophilic, iron- and sulfur-oxidizing halophile with potential industrial applicability in saline water bioleaching of chalcopyrite. Journal of Biotechnology, Elsevier, 2017, 262, pp 56-59.

Chapter 4

Khaleque HN, González C, Kaksonen AH, Boxall NJ, Holmes DS, Watkin ELJ. Genome-based reclassification and renaming of two extremely acidophilic, iron- and sulfur-oxidizing halophiles *Acidihalobacter prosperus* strain V6 and *'Acidihalobacter ferrooxidans'* strain V8 as two new species, *Acidihalobacter aeolianus* sp. nov., and *Acidihalobacter vulcanensis* sp. nov., respectively. International Journal of Systematic and Evolutionary Microbiology (under revision for publication).

Khaleque HN, González C, Kaksonen AH, Boxall NJ, Holmes DS, Watkin ELJ
Genome-based reclassification of the extremely acidophilic, iron- and sulfur-oxidizing halophile *Acidihalobacter prosperus* strain F5 as a new species, *Acidihalobacter yilgarnensis* sp. nov. International Journal of Systematic and Evolutionary Microbiology (prepared for submission).

Chapter 5

Khaleque HN, Kaksonen AH, Boxall NJ, Watkin ELJ. Metabolic capabilities and adaptive mechanisms of the members of the *Acidihalobacter* genus: A comparison of four *Acidihalobacter* genomes (prepared for submission).

Chapter 6

Khaleque HN, Kaksonen AH, Boxall NJ, Watkin ELJ. Identifying the mechanisms of osmotic stress tolerance in the halotolerant, extreme acidophile, '*Acidihalobacter aeolianus'* DSM 14174^{T} (prepared for submission to Research in Microbiology).

Statement of Contribution by Others

I hereby declare that the work presented in this thesis was primarily designed, experimentally executed, interpreted, and written by the first author of the individual manuscripts (Himel Nahreen Khaleque). Contributions by colleagues are described in the following. The signed statement by co-authors are in Appendix 2.

Chapter 2

ELJW, AHK and NJB provided significant contributions to the conception and design of this study. They also provided intellectual input on the interpretation of the results and critical revision of the manuscript.

Chapter 3

JPR assisted with genome assembly and annotations. RJM performed the Miseq sequencing of the genomic DNA for '*Ac. ferrooxidans*' DSM 14175 (strain V8) and *Ac. prosperus* strain F5. ELJW, JPR, AHK and NJB provided intellectual input on the interpretation of the results and critical revision of the manuscripts. MKC provided data for the bioleaching studies on *Ac. prosperus* strain F5.

Chapter 4

DSH and ELJW provided significant contributions to the conception and design of this study. CG helped in bioinformatics analyses and generation of images. ELJW, DSH, AHK and NJB provided critical revision of the manuscripts.

Chapter 5

ELJW provided significant contributions to the conception and design of this study. ELJW, AHK and NJB provided intellectual input on the interpretation of the results and critical revision of the manuscript.

Chapter 6

ELJW provided significant contributions to the conception and design of this study. ELJW, AHK and NJB provided intellectual input on the interpretation of the results and critical revision of the manuscript.

Abstract

In this study, the tolerance of seven environmental acidophilic cultures to increasing chloride ion concentrations (2-45 g/L chloride ion) was tested. From these, the four mesophilic cultures that showed highest chloride ion tolerances were further screened for their ability to oxidize 1% w/v pyrite at 9, 15 and 30 g/L chloride ion. The results showed that cultures V6 and V8 (pure cultures of *Acidihalobacter prosperus* DSM 14174 strain V6 and *'Ac. ferrooxidans'* DSM 14175 strain V8, respectively) showed the maximum ability to tolerate high concentrations of chloride ion (up to 45 g/L) as well as the ability to oxidize pyrite at up to 30 g/L chloride ion. Furthermore, the mixed culture 14C, which was dominated by *Acidihalobacter* spp. (93% relative abundance) was also able to tolerate higher chloride concentrations than the other mixed cultures. Another iron-and sulfur-oxidizing *Ac. prosperus* isolate, *Ac. prosperus* strain F5, was included into the study due to its ability to tolerate and oxidize pyrite, chalcopyrite and pentlandite at up to 45 g/L chloride ion.

The genomes of *Ac. prosperus* DSM 14174, '*Ac. ferrooxidans'* DSM 14175 and *Ac. prosperus* strain F5 were sequenced using PacBio long read sequencing technology, which allowed for the public release of the high quality genomes at the National Centre for Biotechnology Information (NCBI). Phylogenomic studies of the newly sequenced genomes was undertaken using both sequence and non-sequence based approaches. The results of the analyses revealed that the isolates belonged to separate species of the *Acidihalobacter* genus. Accordingly, the isolates were reclassified as '*Ac. aeolianus'* DSM 14174^T (previously *Ac. prosperus* DSM 14174), *'Ac. vulcanensis'* DSM 14175^T (previously *'Ac. ferrooxidans'* DSM 14175) and *'Ac. yilgarnensis'* strain F5^T (previously *Ac. prosperus* strain F5), with each representing type strains of their species.

The genomes of all the members of the *Acidihalobacter* genus (including *Ac. prosperus* DSM 5130^T) were compared to increase the understanding on their mechanisms of nutrient assimilation, energy acquisition and tolerance to acid, osmotic, metal and oxidative stresses. The results suggested that all members of the *Acidihalobacter* species are able to utilize a variety of substrates including carbon dioxide, bicarbonate, ammonium, urea, sulfate and

phosphate for their metabolic requirements. *Ac. prosperus* DSM 5130^T, '*Ac. aeolianus'* DSM 14174^T, and *'Ac. yilgarnensis*' strain F5 share mechanisms of iron and sulfur oxidation through the use of proteins encoded in the classical *rus* operon for iron oxidation and the SOX subunits for sulfur oxidation. The iron and sulfur oxidation pathways for '*Ac. vulcanensis'* DSM 14175 differed in the repertoire of genes for iron and sulfur oxidation and require further analysis for an understanding of energy acquisition pathways for this species. However, all members showed genes encoding proteins involved in survival of the extreme stresses present in bioleaching environments, namely, acid, metal and oxidative stress. The *Acidihalobacter* species have the unique ability to tolerate high chloride concentrations in conjunction with the other stresses, making them unique in comparison to other bioleaching microorganisms which tend to be sensitive to high chloride levels. Therefore, the genomes were searched for genes involved in the biosynthesis of osmoprotectants and the regulation of chloride ion entry into the cell. The results revealed pathways for the synthesis of ectoine, periplasmic glucans, osmotic shock proteins and amino acids related to osmoprotection (glutamate, glutamine, lysine, and proline). It also revealed the presence of transporters for the uptake of glycine betaine and taurine. Furthermore, the presence of multiple chloride transporters were found on the genome which may have a role in efflux of chloride ions to prevent their damaging effect on the reversed transmembrane potential maintained by acidophiles.

Proteomic analysis of '*Ac. aeolianus'* at high and low chloride concentrations was performed using Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) analysis. Analysis of the differential response at high (30 g/L chloride) and low (5 g/L) chloride concentrations led to the generation of a proposed model for simultaneous tolerance to low pH and high chloride concentrations by '*Ac. aeolianus'* DSM 14174^T. The model suggested that at elevated chloride concentrations, '*Ac. aeolianus'* DSM 14174^T used multiple strategies for defence against osmotic, acid and metal stresses. This included the increased production of osmoprotectants (ectoine, glutamate, glutamine, lysine and proline), increased abundance of proteins with roles in membrane biosynthesis and DNA repair and the increased efflux of metal ions through an increased number of metal ion transporters. The most significant protein that increased in abundance by 422.9 fold at high chloride concentrations was the enzyme, ectoine synthase. This suggested that ectoine accumulation

was instrumental in the protection of '*Ac. aeolianus'* DSM 14174^T under osmotic stress caused by high chloride concentrations. The reduction of energy acquisition through iron and sulfur oxidation at high chloride levels, as evidence by a large decrease in the fold change of proteins such as the cytochrome C oxidase, rusticyanin, SOX subunits and Blr3520 homolog, was compensated for by the increase in proteins linked to glycogen metabolism. Furthermore, the decrease in uptake of ammonia and phosphates, the reduced biosynthesis of new flagella as well as the decrease in the oxidative stress response suggested mechanisms of conserving energy at high chloride concentrations.

The characterization of novel members of the *Acidihalobacter* genus in this study provided key insights into their metabolic processes. It also allowed for a deeper understanding of the mechanisms of stress tolerance, especially to low pH and high chloride levels. Therefore, this study highlights the potential applicability of the use of members of the *Acidihalobacter* genus as an alternative to other commonly used bioleaching microorganisms that cannot tolerate acid and chloride stress simultaneously.

1 Chapter 1

1.1 Literature Review

1.2 Biomining

The term 'biomining' is generally used to describe the processes of 'bioleaching' and 'biooxidation', where the former refers to the biological oxidation and complexation processes that result in the release of soluble metal from insoluble ore, and the latter entails the biooxidation and removal of interfering sulfides to expose the metals in ores for further recovery through chemical extraction (1).

The decreasing quality of primary ores that can be mined using existing technologies remains one of the biggest challenges faced by the mining industry (2). As higher grade ores become depleted, the focus is moving towards the utilisation of lower grade ores and those with higher mineralogical complexity (2). The use of traditionally applied extraction technologies may not be economically feasible for these lower grade and complex ores (3). In some instances, biomining can provide an alternative that is more economically feasible for the extraction of metals from these ores (4). Furthermore, the requirement for more environmentally friendly techniques with reduced carbon emissions to the atmosphere also makes biomining a more environmentally benign technology for the mineral processing (5). Biomining has been used to process a wide range of sulfide ores to recover commodities such as copper, zinc, cobalt, nickel, silver, gold and uranium (6).

1.3 The oxidation of metal sulfides by microorganisms

The biologically mediated oxidation of metal sulfides can proceed via two alternative pathways, namely the thiosulfate pathway and the polysulfide pathway. The oxidation pathway is generally determined by the acid solubility of the mineral (7-9). In the model proposed by Schippers and Sand (8), acid insoluble metal sulfides such as pyrite, molybdenum disulfide and tungsten disulfide, are oxidized via the thiosulfate pathway, whereas acid soluble metal sulfides including chalcopyrite, pentlandite and chalcocite, are oxidized via the polysulfide pathway. In the thiosulfate pathway, ferric iron acts as the leaching agent, and is subsequently reduced to ferrous iron which is biologically oxidised back to ferric iron. In the polysulfide pathway, both protons (H^+) and ferric iron attack the ore, thereby solubilizing the

metals and liberating sulfur compounds, such as hydrogen sulfide. The hydrogen sulfide is then oxidized to various polysulfides by sulfur oxidizing microorganisms to generate sulfate and protons, which are cycled back into the pathway

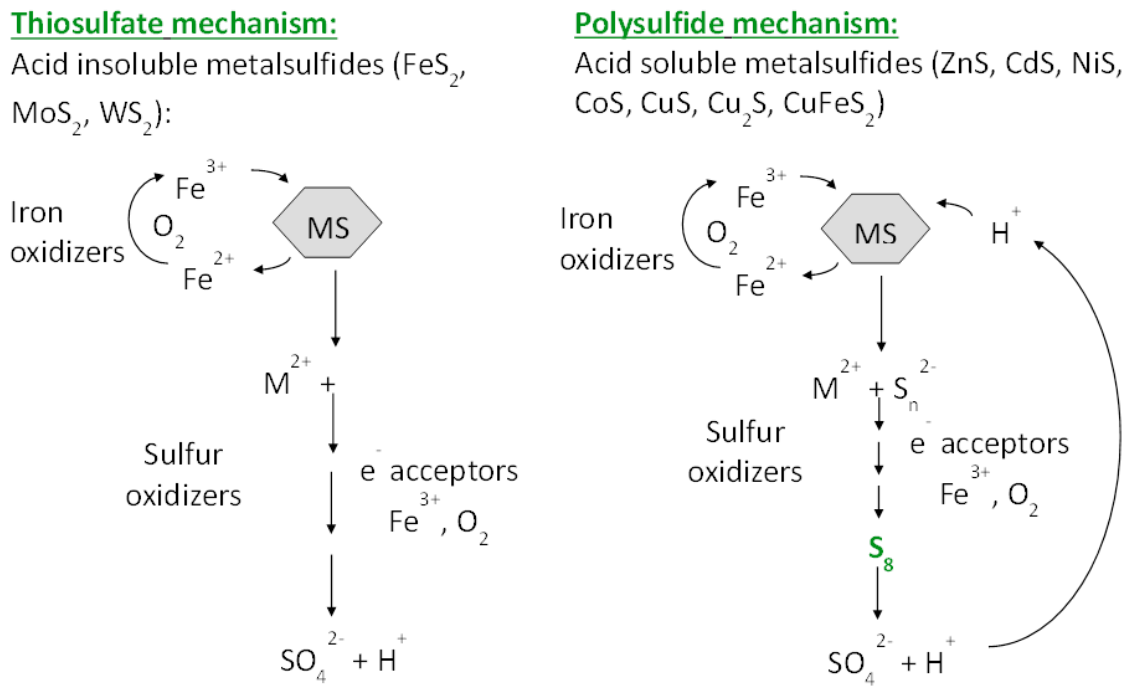

Figure 1. Bioleaching mechanisms through the thiosulfate and polysulfide mechanisms (8).

1.4 General features of biomining microorganisms

Biomining microorganisms share several physiological features. Although some biomining microorganisms are heterotrophs and use organic carbon sources, many are autotrophs, and able to fix carbon dioxide from the atmosphere for growth, though the efficiency with which they do so varies from species to species (10). Many bioleaching microorganisms are also chemolithotrophs, and hence able to oxidize inorganic electron donors, such as ferrous iron and/or reduced inorganic sulfur compounds (RISC) for their energy metabolism (11). Microorganisms that can catalyze the leaching of metal sulfides display a wide range of temperature requirements. Most commonly described biomining bacteria are generally mesophilic (20 – 45 °C) or moderately thermophilic (40 –55 °C), whereas common biomining archaea include mesophiles, moderate thermophiles and thermophiles (55 –80 °C) (12). The temperatures to which microorganisms are exposed in biomining operations depend on the engineering applications. For example, stirred tank reactors have more stable temperatures and may have heating or cooling to achieve optimal temperature range for microorganisms. However, in dump and heap leaching processes, the temperature may vary in different parts

of the dump or heap, and also over time as the biooxidation generates heat and the temperature can only be partially influenced by adjusting air and solution flow rates and applying covers to retain heat (1, 2).

Biomining microorganisms are acidophilic, with most growing below pH 3 (13). In order to survive low pH, they show diverse mechanisms of pH homeostasis (13, 14), including i) the maintenance of a reverse transmembrane potential to keep their intracellular environment more positive than their extracellular environment thus inhibiting the influx of protons, ii) maintenance of cell membranes that are highly impermeable to protons, iii) the use of energy dependent ion channels to pump protons out of the cells, and iv) various buffering mechanisms involving enzymes and proteins to sequester protons (13, 14). These mechanisms ensure that their intracellular pH remains near neutral, even when the pH of the external environment is extremely acidic (13, 14).

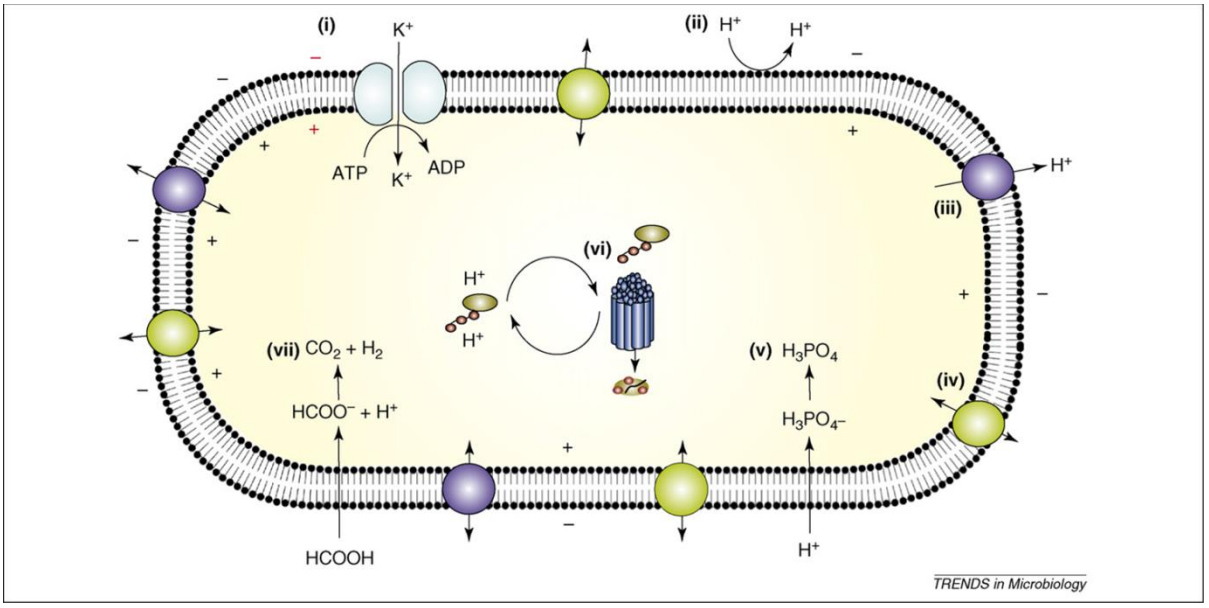

Figure 2. Mechanisms of pH homeostasis in acidophiles (13). (i) Generation of a reversed transmembrane potential through the active influx of K^+; ii) Highly impermeable cell membranes retard the influx of protons into the cell; (iii) Active proton export by transporters; (iv) Secondary transporters to reduce the energy demands associated with pumping necessary solutes and nutrients into the cell; (v) Enzymes and/or chemicals capable of binding and sequestering protons; (vi) DNA and protein repair systems; (vii) Degradation of organic acids that function as uncouplers.

Finally, biomining microorganisms must have good metal tolerance in order to survive the high concentrations of metal ions that accumulate in bioleach liquors during the biomining

process (15, 16). Metal resistance mechanisms can be based on 1) efflux of metals from the cell by proteins; 2) intra/extracellular binding of metal reducing its toxicity; 3) exclusion of metals via a permeability barrier, i.e. selective transport proteins that exclude the metal; 4) alteration of cellular components to lower their sensitivity metals; and 5) conversion of metals to less toxic form, e.g. the reduction of Hg^{2+} to Hg^{0} which volatilizes out of the cells (16).

1.5 General metabolic pathways for energy production, nutrient assimilation and stress tolerance in biomining microorganisms

As described above, iron- and sulfur-oxidizing microorganisms inhabit an extreme environmental niche characterized by low pH and high metal contents. They have developed a suite of unique metabolic features for energy production, nutrient assimilation and stress tolerance. Here, some of the key pathways used by acidophilic microorganisms are discussed.

1.5.1 Iron oxidation

Ferrous iron uptake and oxidation strategies have been studied for a range of biomining microorganisms, including members of the *Acidithiobacillus*, *Leptospirillum*, *Sulfolobus* and *Ferroplasma* spp. as well as *Metallosphaera sedula* and *Acidihalobacter prosperus* strain V6 (17-22). A comparison of known Fe(II) oxidation pathways in acidophilic microorganisms by Bonnefoy and Holmes (17) has suggested that electrons in bacterial strains are primarily removed from their ferrous iron substrates using an outer membrane cytochrome C. The study suggested that oxidation of soluble ferrous sulfate substrates thereby helps in avoiding the production of damaging free radicals and the formation of ferric oxyhydroxides within the cells. *At. ferrooxidans* contains a *cyc*2 gene encoding a high molecular cytochrome C protein which is hypothesized to serve the function of electron transfer. However genes with high identity to *cyc*2 have not been found to be present in the cytochrome c oxidase operons of other acidophiles such as *Ac. prosperus* or *Leptospirillum* spp. The study also found strong dissimilarity in the components of the ferrous iron oxidation system in acidophilic microorganisms. Bacteria such as *At. ferrooxidans* and *Ac. prosperus* have been shown to encode genes for rusticyanin in their ferrous iron oxidation

system (19-22). In addition, the terminal oxidase can be either of the aa3 type (as seen in *At. ferrooxidans* and *Ac. prosperus*) or the cbb3 type (as seen in *Leptospirillum* spp) (17, 18, 20, 23) . On the other hand, the archaeal *Ferroplasma* spp. also has a cbb3 terminal oxidase and uses an alternative copper protein called sulfocyanin, which is thought to have a similar role to rusticyanin. In other archaea such as the *Sulfolobus* spp. and *M. sedula* however, the role of terminal oxidase may be accomplished by Fe-S proteins (17).

As reviewed by Bonnefoy and Holmes (17), despite the difference in terminal oxidase, bioleaching microorganisms were found to share the feature of having a terminal oxidase that is located in the inner membrane and that uses the 'downhill' pathway where electrons derived from the oxidation of Fe(II) are used to remove protons that enter the cell through an adenosine triphosphate (ATP) synthase. They achieve this by either extruding protons or by the reduction of oxygen, leading to partial consumption of protons in the process. This, thereby, is thought to help in the maintenance a neutral intracellular environment. Another shared feature is the ability of all the microorganisms to use the bc1 complex for an 'uphill' reaction, where proton motive force is used against a thermodynamically favorable gradient, in order to reduce nicotinamide adenine dinucleotide (NAD+).

1.5.2 Sulfur oxidation

The sulfur oxidation systems in biomining microorganisms vary from species to species. The sulfur oxidation pathway of *At. ferrooxidans* has been studied in depth and has shown unique systems for which a number of novel genes for sulfur transferases, hetero disulfide reductase complexes, ATP sulfurylases and a NADH complex accessory protein have been predicted (20). The electron transport chain components for the oxidation of elemental sulfur and/or RISCs have been proposed to proceed in the following manner: Electrons generated from sulfur oxidation are transferred via the quinol pool in the inner membrane either directly or indirectly to the terminal oxidases or to the NADH complex. The proteins involved in this transfer may be the gene products of *cyd*AB, *cyo*ABCD, *pet*II, *nuo*ABCDEFGHIJKLMN operon, or the proteins cytochrome c (CycA2), or a high potential iron-sulfur protein (HiPIP) (20).

However, *At. thiooxidans* ATCC 19377 and *At. caldus* ATCC 51756 have shown to have the genes *sox*ABXYZ, *hyp* and *res*BC which belong to the SOX sulfur oxidase system, which are not present in the genome of *At. ferrooxidans* (20, 24-27). In the pathways described for *At.*

ferrooxidans, it appears that sulfur is mobilized by a thiol bearing outer membrane protein which transports it to the periplasmic space where it then undergoes a number of steps until it reaches a reduced form (20). However, the SOX pathways described for *At. thiooxidans* ATCC 19377 and *At. caldus* 51756 allow for the microbial oxidation of sulfur, sulfide, thiosulfate and sulfite to sulfate (28, 29). Moreover, these microorganism appear to have a truncated version of the SOX oxidation system, where the genes *sox*CD encoding sulfate thiohydrolase (which interacts with the periplasmic sulfur oxidation proteins coded for by *sox*YZ) are not present (24, 25, 29). Other genes which have proposed functions in sulfur oxidation include those for the sulfide quinone oxidoreductase (*sqr*), thiosulfate: quinone oxidoreductase (*tqo*) and tetrathionate hydrolase (*tet*H). The *sqr* gene product is thought to be involved in the oxidation of sulfide and sulfite (30, 31). TQO converts thiosulfate to tetrathionate which can then be further used as a substrate for the formation of thiosulfate, sulfate and sulfur by the TetH. The hydrolysis of tetrathionate results in thiosulfate, pentathionate and finally sulfate (20, 29).

Bacterial and archaeal sulfur oxidizing pathways have also been found to be significantly different from one another (24). In archaea, such as *M. sedula* sulfur oxygenase catalyzes the oxidation of sulfur(20).

1.5.3 Inorganic sulfur assimilation

Sulfur, usually in the form of sulfate, is taken up from the environment by a SulP sulfate permease or an ABC uptake system (CysU and CysA) (27). Sulfate is assimilated into various metabolites, including the amino acids methionine and cysteine or iron-sulfur centre (32, 33). In the assimilatory sulfur metabolism pathway, sulfate can be converted to adenylysulfate (APS) via sulfate adenylytransferase (Sat) and/or sulfate adenylytransferase subunit 1 (CysN or CysD) (34). The subsequent conversion of APS to sulfite then occurs through the products of adenylylsulfate kinase (*cys*C) and phosphoadenosine phosphosulfate reductase (*cys*H) (34). The sulfite can then be reduced to sulfide by the assimilatory sulfite reductases (CysI, CysJ and Sir) (Johnson et al. 2015). This pathway for assimilatory sulfur metabolism has been described for *At. ferrooxidans*, the genome of which encodes the *cys*JIHDNG operon (27).

1.5.4 Carbon metabolism

1.5.4.1 Carbon dioxide fixation

As reviewed by Valdés, Cárdenas (35), biomining acidophiles that are obligate autotrophs are predicted to use one of three pathways for CO_2 fixation: (a) The Calvin-Benson-Bassham cycle (CBB), characterized by the presence of the ribulose-1,5-bisphosphate carboxylase gene in aerobic autotrophic bacteria and facultative anaerobes autotrophs; (b) the reductive citric acid cycle, characterized by genes for ATP citrate lyase, pyruvate synthase and 2-oxoglutarate oxidoreductase in anaerobic and microaerophilic sulfur-oxidizing bacteria; and, (c) the hydroxypropionate cycle, characterized by acetyl CoA carboxylases and propionyl CoA carboxylases in archaea and some autotrophic bacteria (35-40). In a bioinformatics study by Valdés, Cárdenas (35), it was shown that the pathway used by bioleaching microorganisms for carbon dioxide fixation varied as temperatures increased during heap biomining. At mesophilic temperatures, bacteria dominated the consortia and fixed carbon dioxide through the CBB cycle, while at intermediate temperatures, both bacteria and archaea were present and used both the CBB cycle and the reverse citric acid cycle. At higher temperatures, the bioleaching consortia was dominated by thermophilic acidophiles that use the modified 3-hydroxypropionate pathway. The three pathways for carbon dioxide fixation used by bioleaching microorganisms is shown in Figure 3.

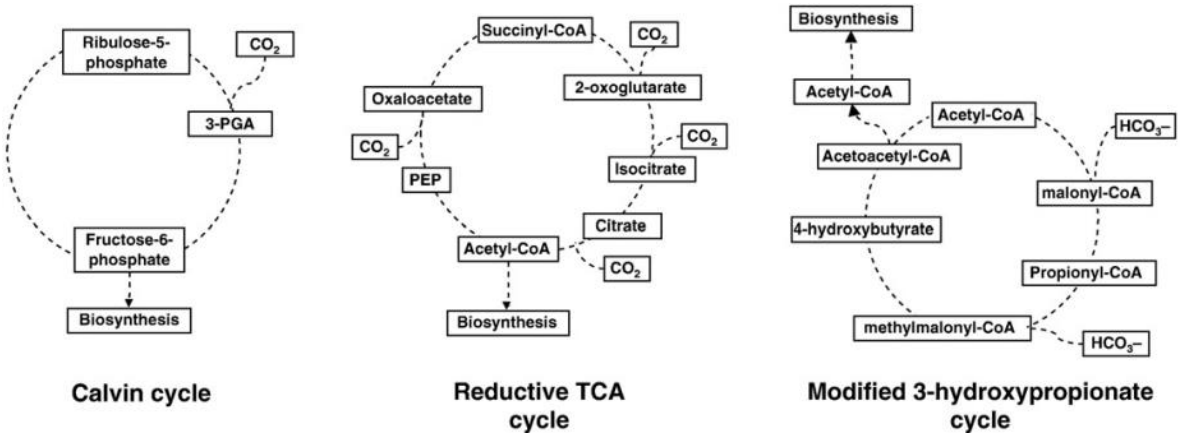

Figure 3. Carbon dioxide fixation pathways predicted in bioleaching bacteria by bioinformatics analyses (35)

1.5.4.2 The tricarboxylic acid (TCA) cycle

The absence of complete TCA cycles, notably TCA cycles lacking the irreversible enzyme oxidative alpha ketoglutarate dehydrogenase complex, has been previously suggested as a hallmark of obligate autotrophy and been termed the horseshoe TCA cycle (36, 41). It has been suggested that the absence of complete TCA cycles can be used as an indicator for obligate autotrophy in novel microbial genomes (41).

Studies on genomes of acidophiles has suggested that a horseshoe TCA cycle is found in obligate autotrophic acidophiles that use the CBB cycle for carbon dioxide fixation (41). To date, only one strain of iron oxidizing acidophiles that is known to be chemolithoautrophic has been found to harbour the complete repertoire of TCA cycle genes, namely *Ferrovum* sp. strain JA-12 (42).

On the other hand, autotrophic microorganisms and archaea that are known to lack the alpha ketoglutarate dehydrogenase complex, but use the reverse TCA cycle to fix carbon dioxide, have been found to contain genes encoding 2-oxoacid:ferredoxin oxidoreductase, which may compensate for the lack of the former gene (41).

1.5.5 Nitrogen metabolism

Nitrogen fixation is predicted to be carried out by a very limited number of biomining microorganisms, notably by *At. ferrooxidans* and members of groups I and III of the Leptospirillum genus (25, 43-48). Genome analysis of nitrogen fixation in *At. ferrooxidans* DSM 23270 and DSM 16786 have identified the key nitrogenase genes *nif*HDK next to several tandem genes important for the assembly of the nitrogenase MoFe cofactor. However, homologous genes have not been found in *At. thiooxidans* DSM 17318 or *L. ferriphilum* DSM 17947 genomes, which suggests that these microorganisms do not fix atmospheric nitrogen, but rather assimilate nitrogen through uptake of ammonia and the use of nitrate/nitrate and ferredoxin-reductases, consistent with what has also been reported for other members of *Leptospirillum* Group II and *At. thiooxidans* (25, 48-50). In addition to nitrogen fixation, biomining microorganisms assimilate nitrogen in the form of nitrate and nitrite using various mechanisms which are generally based on a transporter for nitrate and enzymes to catalyze the conversion of nitrate to nitrite and then ammonia, as described by Moir and Wood (51) and Richardson, Berks (52). In these assimilation processes, the key genes include nitrate and nitrate reductases. Furthermore, ammonia

transporters (Amt) are known to assist in the movement of ammonia across membrane and genes for ammonia permeases (AmtB) have been described in biomining microorganisms (50).

1.5.6 Phosphate utilization

Among acidophilic iron- and sulfur oxidizing microorganisms, phosphate metabolism studies have only been performed for *At. ferrooxidans*, for which a phosphate starvation response has been suggested, however, the mechanisms of phosphate metabolism, integration and regulation are still not well understood (53). Genome analysis has shown the presence of phosphate-specific transport (Pst) system previously identified in *Escherichia coli*, with two homologous genes being named *pst*S1 and *pst*S2 based on their percent identities with the former microorganism (53). PstS is the periplasmic inorganic phosphate binding protein and is one of the most-induced proteins during the inorganic phosphate starvation response in *E. coli* and many other bacteria (54). Other genes homologous to those of *E. coli* Pho regulon components have also been described in *At. ferrooxidans*, such as those encoding phosphate permease, periplasmic phosphate binding protein, polyphosphate kinase and exopolyphosphates. Furthermore, genomic analysis revealed the presence of a putative Pho regulon box in the upstream regions of the C-P lyase operon. The C-P lyase enzyme is the main bacterial enzyme involved in phosphonate (Pn) degradation in other microorganisms. This evidence strongly suggests that as in *E. coli* and other bacteria, C-P lyase expression forms part of the Pho regulon in *At. ferrooxidans*. Its presence in *At. ferrooxidans* was the first reported for phosphonate utilization in a chemolithoautotrophic microorganism. The existence of a functional C-P lyase system is a clear advantage for the survival under Pi limitation, a condition that may greatly affect the biomining of ores (53).

1.5.7 Acid stress tolerance

The general mechanisms of acid stress tolerance shown by acidophiles have been described above in Section 1.3. Therefore, in this section, the focus is on the genes responsible for the genomic determination of acid stress tolerance in these microorganisms.

According to Baker-Austin and Dopson (13), acidophile pH homeostasis requires a complex cell membrane structure. The wide repertoire of genes for cell membrane biosynthesis in *L.*

ferriphilum has been suggested to be indicative of a complex structure that probably has a role in acid tolerance in bacterial acidophiles (13, 48)

The reversed transmembrane potential of acidophiles is generated by cation transport, including those for potassium ions as well as pumps to remove protons that have entered the cytoplasm. Active proton pumping has been seen for the maintenance of a reverse transmembrane potential in *Bacillus acidocaldarius* and *Thermoplasma acidophilum* (55). Also, available acidophile genomes have shown putative proton efflux systems, such as H^+ ATPase, antiporters and symporters. Several candidate proton efflux proteins have also been identified in the sequenced genomes of *At. ferrooxidans*, *At. thiooxidans* and *At. caldus* (25). Furthermore, numerous proton-driven secondary transporters were found to be present on the genomes of *Picrophilus torridus* and *T. acidophilum* and these also probably represent an adaptation of these organisms to harvest proton motive force for metabolic purposes and survive in an extremely acidic environment (55, 56).

In addition, organic acids are known to be harmful to acidophiles because they function as uncouplers of the respiratory chain at low pH (57). Therefore, genes encoding proteins which aid in active mechanisms of organic acid degradation might be a useful method of pH homeostasis in heterotrophic acidophiles. Genes for organic acid degradation pathways, such as those encoding the enzymes propionyl-CoA synthase, acetyl-CoA synthetases and lactate-2-monooxygenase that convert lactate to pyruvate, have been found in *P. torridus* (58).

The presence of a large number of DNA and protein repair genes have been found on the genomes of several extreme acidophiles. They may have a role in repair of damaged biomolecules at low pH as a result of acid stress. The *P. torridus* genome has been shown to contain a large number of genes for DNA repair proteins (58). Also, chaperones involved in protein folding have been found to be highly expressed in *L. ferriphilum* and in *At. ferrooxidans* (49, 59). The prevalence of chaperones in this wide range of acidophiles suggests that damage to DNA and proteins is a key challenge for survival under acidic conditions.

1.5.8 Heavy metal stress tolerance

The increased solubility of metals at acidic pH leads to acidophiles being challenged by very high metal concentrations (60). Acidophilic microorganisms employ a number of strategies

and encode genes for heavy metal stress tolerance which are similar to those seen in neutrophilic microorganisms (61). Active mechanisms of metal removal include the use of efflux pumps as well as the complexation of metals into less toxic forms (60). Genome analysis of two strains of *At. ferrooxidans* (DSM 23270 and DSM 53993) has shown the presence of genomic islands that integrate metal resistance genes into the genomes of acidophiles (61, 62). Additionally, passive mechanisms for heavy metal stress tolerance in acidophiles have also been described, which includes the movement of metal cations against the chemiosmotic gradient used in the maintenance of an internal positive membrane potential as well as the decrease in concentration of free metal ions by the formation of metal sulfate complexes (60).

An additional mechanism of heavy metal tolerance, especially for copper tolerance, is the sequestration of long polymers of inorganic polyphosphate (polyp) by the enzyme polyphosphate kinase. These polymers can be liberated as activated inorganic phosphate by the exopolyphosphatase enzyme. This may enable the liberated activated inorganic phosphate to bind to heavy metals in times of heavy metal stress and metals to be eliminated through inorganic phosphate transporters (61, 62).

1.5.9 Oxidative stress tolerance

The high oxygen consumption rates of chemolithoautotrophic microorganisms is known to cause an increase in oxidative stress, which can result in damage to nucleic acid, proteins and other macromolecules by reactive oxygen species (ROS), thereby affecting cell growth and survival (63).

In a study by Cárdenas, Moya (63), comparative analysis of 44 bacterial and archaeal genomes was undertaken and revealed the presence of superoxide dismutase genes in almost all the studied genomes. However, genes for the peroxide removing enzyme, catalase were absent in the majority of the microorganisms studied. Most microorganisms also showed sparseness of the glutathione/glutaredoxin system components. It was predicted that the gene for rubrerythrin may encode a protein for the scavenging of hydrogen peroxide. Furthermore, DNA pathways for mitigation of oxidative DNA damage were found to be conserved in most studied acidophiles. It has also been suggested that the limited presence of glutathione biosynthesis and utilization genes is compensated through

the use of alternative low molecular weight thiols and/or the Co-enzyme A/ Co-enzyme A disulfide reductase system.

1.5.10 Osmotic stress tolerance

Osmotic stress is a result of the difference in concentrations of soluble extracellular and intracellular salts, resulting in an energetic stress to the microorganisms and ultimately resulting in cellular dehydration or lysis. While the mechanisms employed to tolerate osmotic stress are well studied in neutrophiles, *Ac. prosperus* is the only acidophilic species characterised to tolerate high concentrations of chloride ion (and therefore high osmotic stress), with the type strain, DSM 5130^{T}, requiring chloride ion for growth. All other studies have focussed on acidophiles that do not tolerate high levels of osmotic stress (64).

It has previously been suggested that biomining microorganisms show a number of cellular responses to osmotic stress, most of which are counterparts of osmotic stress responses in neutrophiles (64, 65). One response is the accumulation of low molecular weight compatible solutes (also known as osmoprotectants) in the cytoplasm to increase osmotic pressure and hence prevent the loss of intracellular water due to osmosis. These osmoprotectant compounds include betaine, proline, taurine, ectoine, hydroxyectoine and trehalose (66-68). Genome analysis of the type strain of the halotolerant acidophile *Ac. prosperus* DSM 5130 revealed genes involved in the biosynthesis of sucrose, glycine betaine and ectoine (69). Genes for trehalose and mannoglycerate synthesis in the *Thermus* spp. was also shown to be correlated with halotolerance of this non-acidophilic genus (70).

Halotolerant microorganisms generally show a biphasic response to osmotic stress with an initial rapid stimulation of potassium uptake and accumulation of organic anions such as amino acids to compensate for the increasing charge of potassium ions inside the cell (64). At higher levels of osmotic stress, this response is not sufficient and the production of compatible solutes is required (71). It has been shown by Parro, Moreno-Paz (66) that an increase in salinity results in the upregulation of genes for osmo-sensitive potassium channels in *L. ferrooxidans*. Thermophiles have also been shown to initially accumulate amino acids, mainly alpha- and beta-glutamate, during the initial influx of potassium ions into cells in response to low-level osmotic stress and then replace amino acids as the osmotic stress increases (71).

Proteomic studies by Zammit, Mangold (72) and Guo, Jiang (67) have also shown that there is an increased abundance in membrane biosynthesis proteins by acidophiles under chloride stress. This may help chloride challenged cells to recover from the damage to their cells due to the influx of protons. An up-regulation for proteins for amino acid biosynthesis, energy production and carbon dioxide fixation have also been seen in the salt stress response of *At. caldus*, which suggests that this biomining microorganism can undergo a metabolic shift of carbon flux under salt stress (72).

1.6 Constraints to biomining – the chloride ion problem

The presence of chloride ions in biomining systems has been identified as a limiting factor to the implementation of biomining operations (64, 72-75). This problem is seen especially in arid regions such as Western Australia and Chile, where mining is an integral part of the economy, but freshwater resources are scarce (64). The chloride can be introduced into process waters through the use of saline or brackish water and mining of ores containing chloride-rich gangue minerals, and accumulates as a result of the recirculation of leaching liquors and evaporation (64). The overall effect is that the chloride ion present in the biomining systems inhibits or delays the growth of microorganisms, and subsequently increases the time to leach ores. This decreases the profitability of biomining, and is one of the reasons that only one biomining operation is currently operational in WA (64). In Chile, the inability of biomining microorganisms to withstand the salt stress means that expensive desalination plants must be established, which increases the costs of the biomining operations (76). Also, in these regions, the presence of ores such as atacamite, which contain high levels of chloride ion, causes release of chloride into the biomining operations (77, 78).

1.7 Effect of chloride ion on acidophilic microorganisms

As discussed above, acidophiles are known to maintain a reversed transmembrane potential as one of their mechanisms of surviving in low pH environments. The effect of chloride ion on the transmembrane potential of acidophiles remains the largest cause of damage to biomining microorganisms (64). Studies have shown that chloride ion sensitivity of acidophilic microorganisms is based on its ability to cross the cell membrane and reduce internal positive charge, thereby resulting in an influx of protons and the acidification of the cytoplasm (79). While other cations and anions in brackish water, such as sulfate and

sodium ions, also have a negative impact on acidophilic microorganisms, their impact is limited to their ability to cause osmotic stress (73, 80-82).

Finally, the effect of chloride on the efficiency of biomining microorganisms may also be due to the formation of precipitates (such as jarosite) with the counter ions in salt, which may create a passivation layer that prevents oxidation of substrate by the microorganisms (64). The formation of precipitate has been shown to impact the rate of ferrous iron oxidation and reduce biomass (74).

The chloride ion tolerance of known acidophilic bioleaching microorganisms is discussed below.

1.8 Chloride ion tolerances of acidophilic bioleaching microorganisms

1.8.1 Bacteria

1.8.1.1 Acidithiobacillus

The members of the *Acidithiobacillus* genus show heterogeneity in their use of iron and/or sulfur as substrates for biomass production as well as in their physiological characteristics (83, 84). Typically, *Acidithiobacillus* spp. have very limited tolerance to chloride ions. (85-88). The growth of the most commonly studied chemolithoautotrophic iron- and sulfur-oxidizing *At. ferrooxidans* ATCC 23270 can be completely inhibited by as little as 7 g/L chloride (86, 89, 90). However, strains of *Acidithiobacillus* spp. that were identified from marine sediments (Chañaral Bay, Chile) were shown to be part of a mixed culture that was able to oxidize pyrite in the presence of 35 g/L chloride (91). A salt tolerant marine strain of sulfur-oxidizing, *At. thiooxidans* (stain SH), with the ability to grow in conditions with an optimum pH of 4.0 and optimal salt concentration requirement of 12.3 g/L chloride has been described. (92).

1.8.1.2 Acidiphilium

The majority of the members of the *Acidiphilium* genus are strictly aerobic, chemoorganoheterotrophic bacteria. *Acidiphilium* spp. vary in their pH optima between 1.5

and 5.9 (93). *Acidiphilium* spp. have been identified in acidic saline groundwater and in acidic saline drains in Western Australia (94, 95). An isolate of *A. cryptum* has shown the ability to withstand up to 42.08 g/L chloride, but showed reduction in cell numbers at 17.5 g/L chloride ion (96).

1.8.1.3 Leptospirillum

Leptospirillum spp. are iron-oxidizing, chemolithoautotrophic acidophiles that normally show limited chloride ion tolerance. Batch experiments on *L. ferriphilum* have shown that growth was completely inhibited by 7 g/L chloride. However, in a chemostat culture containing a mixed population of microorganisms, the cell numbers of *L. ferriphilum* decreased three-fold after 21 days exposure to just 1.75 g/L chloride (74, 97). Other studies have demonstrated that *L. ferriphilum* can grow at up to 10.5 g/L chloride, but when in mixed culture with other microorganisms, is outcompeted by other microorganism upon the addition of NaCl to the media (98-100). Another study showed that a "*L. ferrooxidans*"-like (strain LA) bacterium isolated from a copper heap biomining operation in Chile was able to oxidize ferrous iron when grown at 10.5 g/L chloride (88).

1.8.1.4 Sulfobacillus

Sulfobacillus spp. can grow either autotrophically or heterotrophically and are able to oxidize both ferrous and RISCs (101) (101, 102). The species show variability in their salt tolerances. *S. thermosulfidooxidans* type strain DSM 9293 has been shown to tolerate up to 10.5 g/L chloride in pure and mixed culture whereas another closely related strain of the species was partially inhibited by as little as 1.05 g/L chloride (98, 103). Australian-native isolates of *Sulfobacillus* spp. have been shown to be inhibited by 3.16 g/L chloride, whereas *Sulfobacillus* spp. were identified from Chañaral Bay in Chile and shown to grow at 35 g/L chloride (91, 104). *Sulfobacillus* sp. strain TPY, originally isolated from hydrothermal vents in the Pacific Ocean, was shown to tolerate 10.5 g/L chloride during the biomining of chalcopyrite (at pH 1.8) (105, 106)

1.8.1.5 Acidiferrobacter thiooxydans

Acidiferrobacter thiooxydans is an acidophilic thermo-tolerant, iron and sulfur oxidizing chemolithoautotrophic bacterium, which is phylogenetically closer to alkaliphilic *Ectothiorhodospira* spp. than other acidophilic biomining bacteria (107). *Ac. thiooxydans* is

both an osmophile (with optimal growth observed at an external osmotic potential of 5 bar) as well as a halotolerant (growing better at 3 g/L chloride than in the absence of salt). However, it was only found to grow at up to 5.96 g/L chloride ion (108).

1.8.1.6 Halothiobacillus neapolitanus and H. kellyi

Halothiobacillus spp. are obligatory chemolithoautotrophic bacteria which oxidize RISCs (109). They are halotolerant and halophilic microorganisms that grow optimally between 30-40°C and pH 6.5 to 8.0, however they are also able to grow at lower pH, such as during their oxidation of RISCs when the pH of the medium drops to 2.8-3.3 (109). One strain, *H. kellyi*, which was isolated from a shallow water hydrothermal vent in the Aegean Sea, has an optimum pH for growth of pH 6.5, with growth observed down to a pH of 3.5 (110). Members of *Halothiobacillus* spp. with a pH ranging from pH 2 to 4 and growing at 35 g/L chloride were identified from samples obtained from Chañaral Bay, Chile (91).

1.8.2 Archaea

1.8.2.1 Acidianus

Acidianus spp. are facultative aerobes that can both oxidise and reduce sulfur, depending on the availability of O_2, and are also known to grow autotrophically. During aerobic growth, elemental sulfur (S°)or ferrous oxidation occurs and anaerobically ferric or S° are reduced with H_2 or H_2S as electron donor (111). *Acidianus* spp. show growth over a relatively large pH range of 0.3 to 6.0 and temperature (45 to 90 °C) range (111-115). *Acidianus* spp. have been shown to grow at 25.5 g/L chloride (114, 116). However, some strains show a lower tolerance to Cl^-, such as *Ac. infernus* and *Ac. sulfidivorans*, which show optimum growth at 1.1 and 3.2 g/L chloride, respectively. (111, 114).

1.8.2.2 Sulfolobus

Sulfolobus spp. are generally sulfur oxidizers that have been isolated from a variety of geothermal sites and shown to grow at high temperatures, with their optimum growth at ~75 °C (117-123). *Sulfolobus* spp.

1.8.2.3 Thermoplasma

Thermoplasma acidophilum and *T. volcanium* were isolated from solfatara fields in the Azores, Iceland, Italy and USA, and are thermophilic (optimum temperature 55 and 60 °C, respectively), sulfur oxidizing acidophiles (optimum pH 1.0 and 2.0, respectively) (116). In a mixed culture of microorganisms related to *S. metallicus* and *T. acidophilum*, oxidation was completely inhibited at 5.96 g/L chloride (126).

1.8.2.4 Ferroplasma

Ferroplasma spp. are mesophilic or moderately thermophilic iron-oxidizing acidophiles and grow heterotrophically in pure culture (127-131). *Ferroplasma* spp. also grow at extremes of pH (pH 1.4 or less) (131-134). The type strain *F. acidiphilum* DSM 12658 has been shown to grow at up to 11.9 g/L chloride at pH 1.8 (98).

1.8.2.5 Metallosphaera

Metallosphaera spp. have been isolated from a range of biomining environments, such as a German uranium mine, a solfataric field in Italy and a hot spring in Japan (112, 121, 135, 136). *Metallosphaera* spp are facultative autotrophs or mixotrophs and oxidise iron or reduced sulfur compounds (12). *Metallosphaera cuprina* has been reported to grow in the presence of up to 5.96 g/L chloride (137).

1.9 The search for halotolerant acidophilic biomining microorganisms

The inability of most iron and sulfur oxidizing acidophiles to tolerate chloride ion has led to the search for halotolerant acidophiles to use in biomining processes (64, 65). This would allow for the use of seawater at mines, thereby reducing the costs associated with desalination. However, environmental requirements for the isolation of halophilic and acidophilic microorganism are only available at a few locations on Earth that provide low pH and high saline environments simultaneously (64).

Some acidic saline environments from which halotolerant acidophiles can be bioprospected are man-made, such as biomining environments. Others have developed naturally, such as salt lakes or volcanoes near seawater (64). Salt lakes are formed when salts are carried into acidic systems through the influx of seawater (138, 139). Evaporation of marine waters can

also result in the formation of deposits (139). In southern Western Australia and in some areas of South Australia, the Yilgarn shows the presence of many salt lakes (140, 141). These locations provide ideal conditions for sourcing acidophilic halophiles, however few microbiological studies have been carried out at such locations (64). Halotolerant acidophiles have, however, been sourced from volcanoes near seawater (89, 103). The mixture of iron-rich silicate materials in magma together with seawater forms shallow acidic pools that provide a good source for these microorganisms (89). Furthermore, bioleaching environments that stem from tailings dams, acid mine drainage and heaps in bioleaching operations, also provide a rich environment for the sourcing of halotolerant acidophiles (64).

As a result of the limited locations from which halotolerant acidophiles can be sourced, few microorganisms have been characterised that tolerate salt and acid stress simultaneously. The first halotolerant, metal-mobilizing acidophile to be isolated and have its genome sequenced was the *Acidihalobacter prosperus* type strain (previously '*Thiobacillus prosperus*') (69, 89). *Ac. prosperus* DSM 5130^T is an iron- and sulfur-oxidizing chemolithoautotrophic acidophile that requires a minimum of 1.42 g/L chloride ion for growth (Davis-Belmar, Nicolle, and Norris 2008). Two other isolates of the *Acidihalobacter* genus, *Ac. prosperus* strain V6 and '*Ac. ferrooxidans'* strain V8 have since been isolated from similar environments (103, 142). Strain V6 was found to be similar to the type strain but showed a growth rate similar to *At. ferrooxidans* on ferrous iron (142). '*Ac. ferrooxidans'* strain V8 was found to dominate a mixed culture growing on pyrite under salt stress (142). Another "*Ac. prosperus*"-like isolate (strain F5) was isolated from a saline drain in Western Australia was shown to grow at up to 17.5 chloride and to dominate a mixed culture of biomining microorganisms at up to 12.3 g/L chloride (72, 94). These four novel members of the *Acidihalobacter* genus represent halotolerant acidophiles that have potential in saline water bioleaching.

1.10 Importance of OMICS in the study of halotolerant acidophiles

Bioinformatic analysis of genomes can be a powerful tool to gain insights into the genetic and metabolic potential of bioleaching acidophiles, some of which tend to be recalcitrant to genetic manipulation (143). The genome of the salt sensitive *At. ferrooxidans* was one of the first biomining microorganisms to be studied extensively, and has formed the basis for the prediction of metabolic pathways in other acidophiles (20, 26, 35, 41, 143, 144). While *At. ferrooxidans* has become the best studied model for a biomining microorganism, many other permanent draft genomes of other biomining microorganism have since been made available, extending the prediction of genetic and metabolic potential of biomining microorganisms (143). Examples include models for energy metabolism in *At. caldus* and *At. thiooxidans* and models for the overall metabolism of *Sulfobacillus* spp. and *"Ferrovum"* spp. (25, 35, 41, 42, 67). To date, the only available genome of a halotolerant acidophile is that of *Ac. prosperus* DSM 5130 (Ossandon et al., 2014). Primary genome analysis of DSM 5130 identified the presence of genes encoding enzymes for the synthesis of the osmoprotectants, sucrose, glycine betaine and ectoine (69). However, a genome scale metabolic model is yet to be derived for this microorganism.

Although annotation of genes has come a long way, there are still a large number of genes with unknown physiological roles. Furthermore, transcriptomics based approaches suffer from the drawback of lacking the information on the state of the proteome. Proteins are, in most cases, the effectors of cellular function, therefore, one of the most informative approaches for gene-phenotypization and characterization of functional states is through the study of the proteome or its subsets. Recently, a study of differential protein expression of *Ac. prosperus* DSM 5130 at high and low salt stress was undertaken that identified the key players in its osmotic stress response (145). This has led to a better understanding of osmotic stress tolerance in this halotolerant acidophile. This demonstrates the powerful potential of using genomics reinforced with proteomics for the elucidation of mechanisms in novel halotolerant acidophiles.

1.11 Aims and significance of this research

Members of the genus, *Acidihalobacter* are a novel group of iron- and sulfur-oxidizing acidophiles that are able to tolerate high chloride concentrations and low pH simulataneously. This makes them unique amongst the acidophiles and as such worthy of study in order to characterize their metabolic potential and their exceptional ability to tolerate multiple stresses.

The aims of this study were to:

i. Establish the chloride ion tolerance of a range of acidophilic cultures enriched and/or isolated from halophilic and acidophilic environmental samples;
ii. Assess the ability of selected pure and mixed cultures of halotolerant acidophiles to bioleach pyrite under chloride ion stress;
iii. Sequence the genomes of novel isolates of halotolerant acidophiles;
iv. Phylogenetically classify novel halotolerant acidophiles;
v. Compare the genomes of the halotolerant acidophiles against other sequenced acidophile genomes for insights into metabolic pathways involved in nutrient assimilation, energy acquisition and stress tolerance: and,
vi. Study the differential proteome response to high and low chloride ion stress in the novel isolates.

The deleterious effect of chloride ions on acidophiles has been well studied and has shown that few acidophilic iron- and sulfur-oxidizing acidophiles are able to withstand high chloride ion stress. Therefore, the application of these microorganisms in bioleaching operations where high chloride levels exist is limited. The isolation and characterization of new species of halotolerant acidophiles is of key interest to the biomining industry as it would remove the need for expensive desalination processes currently used in bioleaching operations where access to fresh water is limited. It would also allow for the use of these microorganisms in regions where high chloride ions characterize the ores. This would, ultimately allow for economically feasible extraction of metals from minerals.

The first halotolerant iron- and sulfur-oxidizing acidophile to be isolated was *Acidihalobacter prosperus* DSM 5130^T (89). Since its isolation in 1989, other *Acidihalobacter*-like microorganisms have been sourced from shallow acidic pools in Italy and from acid saline

drains in Western Australia. However, further characterization and genomic studies had not been performed on these isolates. As the halotolerant, acidophilic iron- and sulfur-oxidizing members of the *Acidihalobacter* genus are each novel and unique, further characterization and classification offers insights into their abilities to tolerate acid and chloride stress simultaneously. Therefore, the key significance of this research is in the characterization of novel isolates of the *Acidihalobacter* genus with the aim of shedding new insight into their metabolic properties in order to illuminate their mechanisms of survival and growth in previously unexplored geochemical environments. This would, then, have an impact on the applicability of saline water bioleaching using these microorganisms.

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2 Chapter 2

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

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Chloride ion tolerance and pyrite bioleaching capabilities of pure and mixed halotolerant, acidophilic iron- and sulfur-oxidizing cultures

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Abstract

The search for halotolerant acidophilic microorganisms to increase the efficiency of bioleaching processes in regions of fresh water scarcity has been ongoing for the past two decades. In this study, three pure cultures (V6, V8 and M8) and four enrichment cultures (14C, L2-21, L4-9 and L6-11) from low pH, high saline environments were characterized for their ability to oxidize soluble iron and inorganic sulfur in the presence of increasing concentrations of chloride ion. The mixed cultures 14C and L2-21 contained predominantly *Acidihalobacter* and A*cidithiobacillus* spp., respectively, while L4-9 and L6-11 predominantly contained *Ferroplasma* spp. Cultures V6, V8, 14C and L2-21 were assessed for their ability to oxidize 1% pyrite at 9, 15 and 30 g/L chloride ion. Results showed that pure cultures V6 and V8 and mixed culture 14C were able to oxidize pyrite at chloride ion concentrations of 30 g/L, which is higher than the chloride concentration found in seawater (19 g/L). L2-21 was unable to oxidise pyrite, possibly due to the predominant presence of sulfur oxidizing microorganisms in the mixed culture. This illustrates the potential applicability of the cultures for saline water bioleaching.

Keywords: acidophile, bioleaching, chloride tolerance, halophile, pyrite

2.1 Introduction

Microorganisms capable of oxidizing metal sulfides are predominantly acidophilic in nature, having an optimum pH range below 3 (1). These microorganisms are useful for bioleaching applications, in which microorganisms are used to catalyze the extraction of metals from ore. Bioleaching allows the economic extraction of metals from low-grade and complex ores, the processing of which would not be feasible using traditional mining methods (2, 3). It also provides the benefits of having relatively low energy demand and atmospheric emissions, making it a more environmentally benign alternative compared to more traditional extraction methods such as roasting and smelting or leaching with strong inorganic acids (4).

The use of acidophilic microorganisms has already been successfully applied to a number of sulfide ores for the extraction of base metals such as copper, nickel, cobalt and zinc (5). These microorganisms have also been used for the biooxidation of refractory gold minerals (6, 7). It has been estimated that the utilizable copper, zinc and nickel ore reserves in the world may only last another twenty to forty years (8). As higher grade ores become less available, the mineral industry is faced with the challenge of finding low carbon footprint technologies, such as bioleaching, that may improve the economic viability of the mining sector (9). However, the applicability of typical bioleaching microorganisms is restricted in areas like parts of Western Australia and Chile where chloride content of soils and source waters is extremely high (>100 g/L) and access to fresh water is scarce, often leading to the use of seawater or brackish or brine ground and surface waters at some mines (5, 10-12). This has led to a strong interest in the search for bacterial cultures that are able to actively bioleach in seawater media (5).

The ability of bioleaching microorganisms to tolerate chloride ion varies between domain, genus and species, but most of these microorganisms cannot tolerate the levels of chloride ion present in seawater and can be inhibited by concentrations as low as 6.6 g/L (11-14). For example, *Acidithiobacillus ferrooxidans* is inhibited by 4.2 g/L chloride and *Leptospirillum* (*L.*) *ferriphilum* by 12.3 g/L chloride (10, 12, 15). *Sulfobacillus thermosulfidooxidans* has been found to grow at up to 12 g/L chloride ion (12). Some archaea, such as *Sulfolobus* spp., are inhibited by 18 g/L chloride (16, 17).

Chloride ion stress is thought to affect the viability of acidophiles used in bioleaching by affecting the mechanisms they use to tolerate low pH. Cell membranes are permeable to

chloride and when chloride enters the cell, the negative charge of the ion leads to the collapse of the inside positive membrane potential that is normally maintained by the acidophilic cell (13, 18). This causes protons to simultaneously enter the cell disrupting to the internal pH (19). The cytoplasm becomes acidified, reducing metabolic activity and ultimately causes cell death (20). Another effect of chloride ions may be on the osmotic potential of the cells. Ojumu, Petersen (21) showed that energy stress is created by the osmotic gradient formed between the interior and external environment due to an increased ionic strength, decreasing the growth and bioleaching efficiency of *L. ferriphilum*.

While low pH environments and those high in osmotic stress can be found in a diverse range of locations, environments where both stresses co-exist are rare. There are a few places where the geological conditions provide both high salinity and low pH, such as acidic lakes and drains and volcanoes near seawater, hence only a limited number of microorganisms capable of simultaneously tolerating both stresses have been isolated to date. In this study, iron oxidation and the bioleaching capabilities of a number of halotolerant acidophiles, both pure isolates and mixed cultures, were investigated. The ability of these acidophilic environmental cultures for pyrite leaching under high chloride ion stress was evaluated to explore the potential for using these microorganisms in bioleaching operations in arid regions where access to freshwater is limited.

2.2 Materials and Methods

2.2.1 Environmental cultures and growth conditions

The cultures used in this study (Table 1) were obtained from culture collections at CSIRO Land and Water (L2-21, L4-9, L6-11) and the University of Exeter (V6, V8, M8, 14C). The mixed cultures from CSIRO Land and Water were enriched from acidic, saline soils and surface waters from the Western Australian Wheat belt and Southwest region. The pure and mixed cultures from the University of Exeter were obtained from shallow acidic pools at the Aeolian Islands, Vulcano, Italy (V6, V8 and 14C) and Milos, Greece (M8). The growth conditions for the maintenance of the cultures are shown in Table 1.

Table 1. Growth conditions of the pure and mixed environmental cultures used in the study.

Type of culture	Maintenance chloride ion	Source of chloride ion	Growth temperature

		(g/L)		(˚C)
V6 *Acidihalobacter prosperus* DSM 14174	Pure	12	Sodium chloride	30
V8 *Acidihalobacter ferrooxidans* DSM 14175	Pure	12	Sodium chloride	30
M8 *Acidihalobacter ferrooxidans*	Pure	12	Sodium chloride	30
14C	Mixed	30	Sodium chloride	30
L2-21	Mixed	21	Sea salts	30
L4-9	Mixed	9	Sea salts	45
L6-11	Mixed	11	Sea salts	45

2.2.2 Chloride tolerance and bioleaching tests

The tolerance of the environmental cultures to chloride ion was determined in triplicate in 50 mL of the following media: V6, V8, M8 and 14C in basal salts (in g/L Milli-Q™ H_2O: 0.4, $(NH_4)_2SO_4$, 0.5, $MgSO_4.7H_2O$ and 0.2, K_2HPO_4 acidified to pH 2.0 with concentrated H_2SO_4) supplemented with 13.9 g/L $FeSO_4.7H_2O$, 1.51 g/L $K_2S_4O_6$ and 1 mL/L of filter sterilized (0.2 µm cellulose acetate filter, Millipore) trace element solution (mg/L Milli-Q™ H_2O; $MnCl_2.2H_2O$, 62; $ZnSO_4.7H_2O$, 68; $CoCl_2.6H_2O$, 64; H_3BO_3, 30; Na_2MoO_4, 10; $CuCl_2.2H_2O$, 66; $NaVO_3$); L2-21, L4-9 and L6-11 in basal salt media consisting of 1.5 g/L $(NH_4)_2SO_4$ plus 0.01% yeast extract adjusted to pH 1.8 with concentrated H_2SO_4 and supplemented with 10 g/L $FeSO_4 \cdot 7H_2O$, 5 g/L tyndallized elemental sulfur and 1 mL/L filter sterilized trace element solution (as above). All cultures were incubated on a rotary incubator at 100 rpm at 30˚C except L4-9 and L6-11 which were incubated at 45˚C. Tolerance to chloride was tested at 2, 9, 15, 30 and 45 g/L chloride

ion, where chloride was provided as sodium chloride (V8,V6, M8 and 14C) or synthetic Sea salts (Sigma Aldrich) (L2-21, L4-9 and L6-11).

Pyrite (FeS_2) concentrates (milled to <0.75 μm) were sterilized by gamma irradiation (50 kGray). The elemental composition, as determined by inductively coupled plasma—atom emission spectroscopy (ICP-AES) after borax flux and re-dissolution in 5% (vol/vol) HNO_3 was (wt/wt) 36.6% Fe, 0.24% Cu, 0.04% Ni, and 39.8%S. Isolates V6, and V8 and mixed cultures 14C and L2-21 were incubated in 100mL of the media as described (minus the yeast extract) with 9, 15 and 30 g/L chloride ion and 1% pyrite (the sole source of iron and sulfur) and incubated on a rotary incubator at 100 rpm at 30˚C.

2.2.3 Sampling and sample processing

Samples for pH, redox potential, iron and sulfur assays for chloride ion tolerance and bioleaching tests were taken at T_0 and then every 24 h for cultures V6, V8 and 14C, and 48 h for cultures L2-21, L4-9 and L6-11. The chloride ion tolerance tests were run for 96 h for the pure cultures and 144 h for the mixed cultures. For the bioleaching tests, samples were taken every 7 days for a total duration of 28 days for all cultures tested.

The samples (2 mL) were filtered (0.2 μm cellulose acetate filter, Millipore) to remove precipitates and cells and the filtrate used for the assays. Cultures containing elemental sulfur (L2-21, L4-9 and L6-11), were centrifuged at 700 g for 3 minutes at room temperature prior to filtering the media, in order to remove excess sulfur particles. Iron oxidation was determined based on ferric and total dissolved iron concentrations using the ferric chloride assay as described by Govender, Harrison (22) against a standard curve for ferric chloride. Samples for sulfur oxidation were prepared by adding 500 μL of filtrate to 4.5 mL of 0.07M nitric acid (in Milli-Q™ H_2O). Soluble sulfur release (only for bioleaching tests) was determined using inductively coupled plasma atom emission spectrometry (ICP-AES). Solution pH of the samples was measured using Ionode IJ44A pH electrode. Redox potential (ORP) was measured using Ionode IJ64 oxidation reduction potential electrode, with results recorded in millivolts against a double junction Ag/AgCl primary reference. To account for losses due to evaporation in the experimental flasks at 45 °C, flasks were weighted before each sampling and sterile Milli-Q™ H_2O water of pH 1.8 was added.

2.2.4 Microbial community profiling

Genomic DNA was extracted from cultures as described by Zammit, Mutch (23). Diversity profiling of the total genomic DNA was performed at the Australian Genome Research Facility (Perth, Western Australia). Amplicon sequencing at a read length of paired-end 300 bp was performed using the primer pair 341F (5'- CCTAYGGGRBGCASAG-3') – 806R (5'-GGACTACNNGGGTATCTAAT-3') (24) specific for the 16S rRNA gene of bacteria and archaea. PEAR version 0.9.5 was used to assemble paired-ends reads by aligning the forward and reverse reads (25). Primers were trimmed using Seqtk version 1.0. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME) version 1.8, USEARCH version 8.0.1623 and UPARSE software (26-28). Sequences were quality filtered and full length duplicate sequences were removed and sorted by abundance using USEARCH tools. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtered using the SILVA database as a reference (29). To obtain the number of reads in each operational taxonomic unit (OTU), reads were grouped to OTUs with a minimum identity of 97%. Taxonomy was assigned with the SILVA database version 13.8 as a reference using QIIME version 1.8 (30).

2.3 Results and discussion

2.3.1 Chloride ion tolerance of the pure and mixed cultures

Growth of the pure and mixed cultures on increasing levels of chloride ion stress was assessed based on the ability of the cultures to oxidize soluble ferrous iron ($FeSO_4.7H_2O$) and reduced sulfur (either potassium tetrathionate for cultures V6, V8, M8 and 14C or elemental sulfur for cultures L2-21, L4-9 an L6-11) sources. Iron oxidation was determined based on increasing ORP (between +400 and +650 mV (vs. Ag/AgCl)) and ferric iron generation. Solution pH was used for further indication of ferrous iron oxidation as the oxidation of ferrous iron consumes protons to increase solution pH (31). Sulfur oxidation decreases pH due to the production of sulfuric acid. Therefore, sulfur oxidation was predicted when there was a decrease in solution pH (32).

Previous studies had demonstrated that growth of the pure isolates and mixed culture 14C could not be maintained in the absence of NaCl (33); P. Norris personal communication). All of the pure cultures tested for salt tolerance reached ORP above +400 mV at the chloride

ion concentrations tested (Fig 1.A, D, G). Pure cultures V6 and V8 and mixed culture 14C at 2 and 45 g/L chloride ion did not exceed an ORP of +450 mV, whereas there was a greater increase in ORP at 9, 15 and 30 g/L chloride ion (Fig 1. A, D, Fig 2. A). Ferric iron generation and pH for V6, V8 and 14C reflected these results, indicating increased iron oxidation between 9 and 30 g/L chloride ion concentrations (Fig 1. B, C, E, F. Fig 2. B, C). The ORP for M8 did not exceed +450 mV at any chloride ion concentration, with low levels of ferric iron generation and little change in pH, indicating its inability to withstand chloride ion stress (Fig 1. G, H, I). Optimal iron oxidation was seen at 15 g/L NaCl for all of the pure isolates.

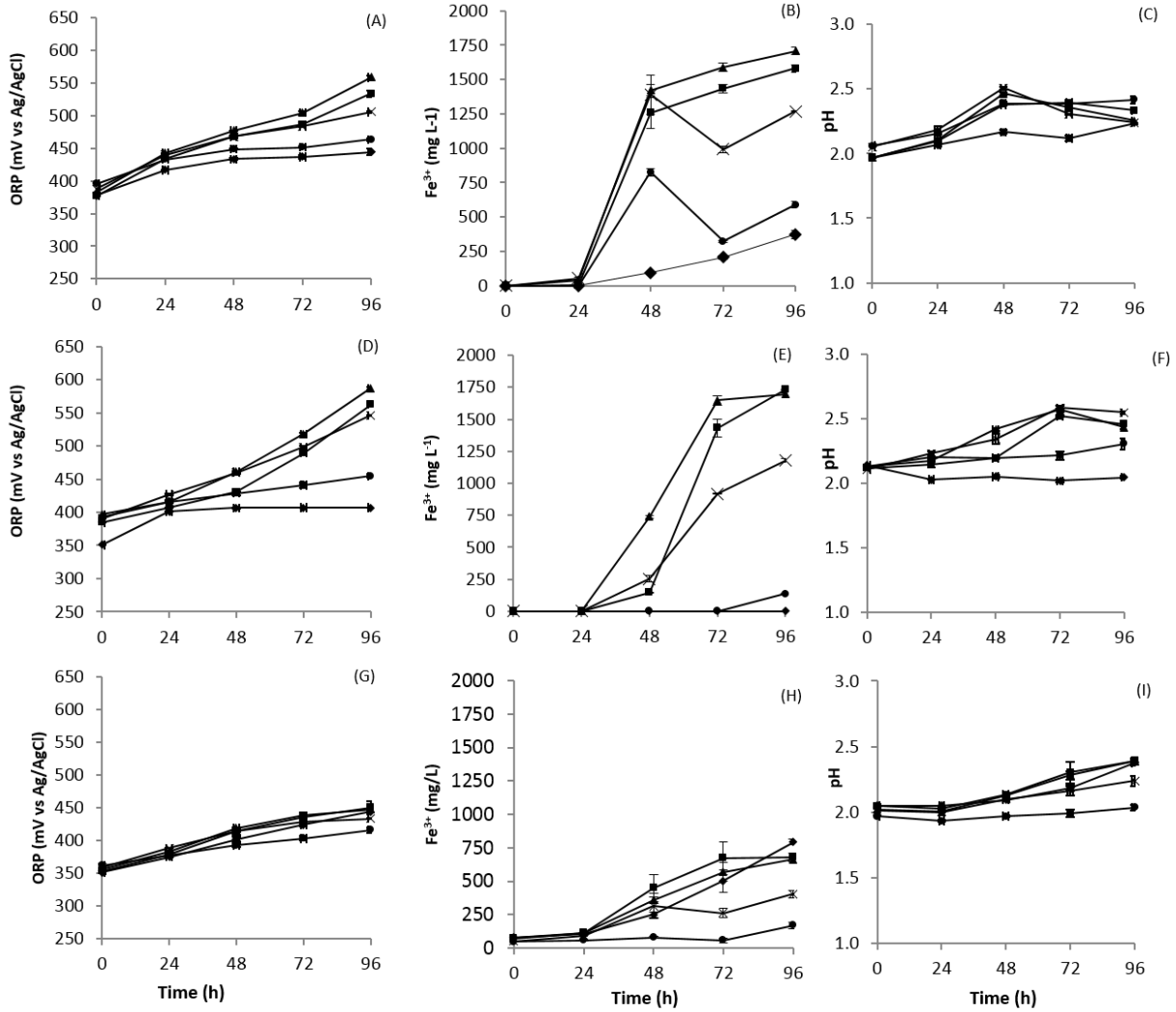

Figure 1. Redox potential (ORP), ferric iron concentration and pH during the chloride ion tolerance tests of the pure cultures V6 (A-C), V8 (D-F) and M8 (G-I). Chloride ion concentration (g/L): ◆ 2, ▪ 9, ▲ 15, x 30, • 45. Values are the average of three replicates ± standard deviation.

Mixed cultures L2-21, L4-9 and L6-11 reached a much higher maximum ORP than seen with the pure isolates or mixed culture 14C (Figure 2 D, G, J). In mixed culture L2-21 there was a decrease in ferric iron and a plateau in the ORP after 96 h at all chloride ion concentrations except 45 g/L chloride ion, suggesting the mixed population contained sulfur oxidizers (Fig 2. D, E). An increase in ORP and ferric iron concentration for L2-21 at 45 g/L chloride ion concentration was seen at 96 h, suggesting the iron oxidizers in the mixed culture had a greater chloride ion tolerance and longer lag phase than the sulfur oxidizers (Fig 2. D, E). Mixed cultures L4-9 and L6-11 performed best up to 15 g/L chloride with higher ORP and ferric iron generation than seen at 30 and 45 g/L chloride (Fig 2. G, H, J, K). Optimal performance was observed at 2 g/L chloride. Both mixed cultures had increased lag phases with higher chloride ion concentrations (Fig 2. H, K).

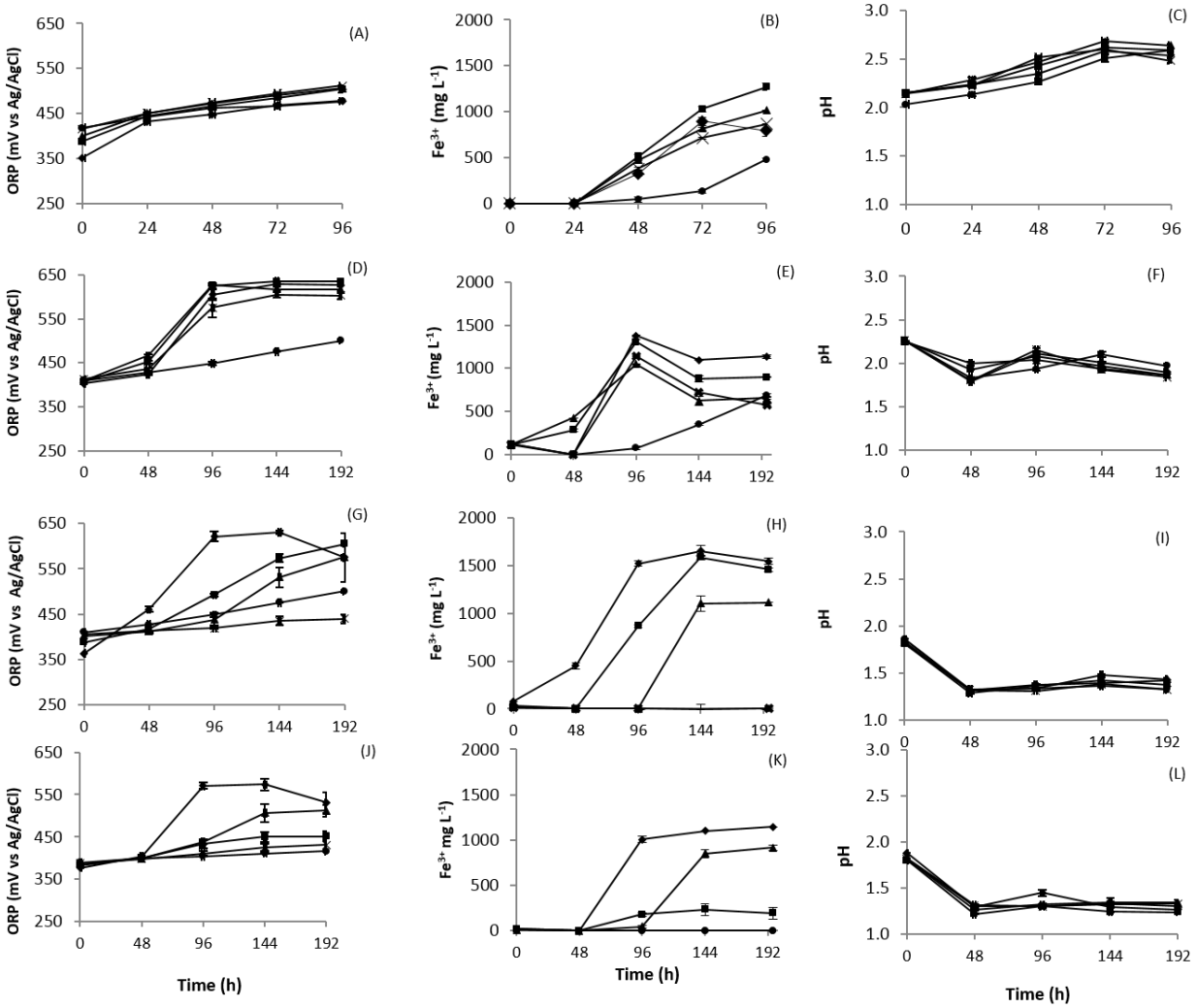

Figure 2. Redox potential (ORP), ferric iron concentration and pH during the chloride ion tolerance tests of the mixed cultures 14C (A-C), L2-21 (D-F), L4-9 (G-I) and L6-11 (J-L).

Chloride ion concentration (g/L): ◆ 2, ▪ 9, ▲ 15, x 30, • 45. Values are the average of three replicates ± standard deviation.

All the cultures used in the study were acidophilic in nature and had been routinely maintained at pH 1.8 (L2-21, L4-9, L6-11) or pH 2 (V6, V8, M8, 14C). Throughout the salt tolerance and bioleaching experiments, the cultures remained at a pH range below 3 despite iron oxidation and precipitation reactions taking place. In the chloride ion tolerance experiments with the pure cultures V6, V8 and M8 and mixed culture 14C, the initial rise in pH indicated iron oxidation (Fig 1. C, F, I. Fig 2. C). However, for mixed culture 14C, a drop in pH was seen at 96 h, possibly due to sulfur oxidizers in the mixed culture dominating the cultures under chloride ion stress (Fig 2C). For the mixed culture L2-21, the pH dropped rapidly from 2.2 to 1.8, possibly due to the initial activity of the sulfur oxidizing species in the mixed cultures. This was followed by an increase in pH at 96 h as iron oxidation began to take place (Fig 2. F). The pH then dropped after 96 h once the maximum ORP was reached, indicating sulfur oxidation was taking place. The solution pH of the mixed cultures, L4-9 and L6-11, dropped after 24 h from 1.8 to 1.3-1.5 and remained relatively stable thereafter, despite increasing ORP and ferric iron generation at 2, 9 and 15 G/L chloride ion (Fig 2. G, H, I, J, K, L). As these cultures were grown at the higher temperature of 45 ˚C is possible that this was the result of decreased solubility of ferric iron, causing it to form jarosite precipitate more easily at higher temperatures (31). A visible precipitate formed at the bottom and sides of all the flasks. Hence it is possible that the precipitate formation released protons and, therefore, decreased pH in all the cultures tested.

Previous studies of V6 and V8 had shown high chloride ion tolerance (up to 36 g/L chloride ion) by both pure cultures, with V8 showing greater salt tolerance when grown on ferrous iron substrates (34). These studies show the ability of the cultures to tolerate up to 75 g/L chloride ion. This is much higher than the previously reported salt tolerances of halotolerant *L. ferriphilum*, which are only known to tolerate up to 12 g/L chloride ion (10, 12). Furthermore, *Acidihalobacter prosperus* strains which are able to oxidize both iron- and reduced sulfur compound are known to generally exhibit greater chloride tolerance than acidophiles that solely oxidize iron (5), which may further explain the high chloride ion tolerance of the tested pure cultures.

2.3.2 Pyrite bioleaching

Based on the superior ability to oxidize ferrous iron under chloride ion stress, pyrite bioleaching was tested for pure cultures V6 and V8 and mixed cultures 14C and L2-21. Redox potential, iron oxidation and solution pH were measured, as previously done for chloride ion tolerance tests. Soluble sulfur release was also measured by ICP-AES.

All cultures with the exception of L2-21 performed better than the abiotic controls in terms of all parameters tested. In the abiotic controls, regardless of the chloride concentration, the ORP decreased from 490 mV to 430 mV and the pH decreased from 2.0 to 1.9 over 28 days. Minimal iron oxidation occurred in the abiotic controls at 15 and 30 g/L chloride with a maximum ferric concentration of 32 mg/L generated (data not shown).

For V6 and 14C, the oxidation of pyrite was highest at 9 g/L or 15 g/L chloride based on ORP and ferric iron concentrations (Fig 3. A, B, G, H). However, V8 showed very similar iron oxidation at all the chloride ion concentrations tested, whereas, for V6 and 14C there was less oxidation of the ore at 30 g/L chloride ion (Fig 3. B, E, H). After 28 days of the bioleaching experiment less soluble sulfur was released at 30 g/L chloride by V6 and 14C than at 9 and 15 g/L chloride (Fig 4). The amount of soluble sulfur released by V6 and 14C cultures was only slightly greater than the abiotic leaching of pyrite at the chloride ion concentrations tested. However, the release of soluble sulfur from pyrite by V8 was greater than the abiotic leaching tests (Fig 4). Furthermore, the drop in pH for the bioleaching tests with V6, V8 and 14C suggested the generation of sulfuric acid as a result of sulfur oxidation (Fig 3. C, F, I). On the other hand, L2-21 had very limited oxidation of the iron in pyrite. Growth of the culture may have been affected by the absence of an organic carbon source, particularly in relation to the iron oxidising activity, which was seen in the ferrous oxidation tests when yeast extract was present in the culture medium. There was a decrease in ORP and very limited generation of ferric iron at 30 g/L chloride. The pH increased at 9 and 15 g/L chloride but at 30 g/L chloride, there was an initial increase followed by a decrease in pH at 28 days (Fig 3. J, K, L). However, soluble sulfur released by L2-21 was greater in comparison to the other cultures tested, particularly at 30 g/L chloride ion (Fig 4). Chloride is an oxidant as demonstrated by the small amount of iron oxidation seen in the abiotic controls. This may have been sufficient to enable the sulfur oxidisers in the mixed culture to generate the levels of released soluble sulfur seen for the L2-21 cultures.

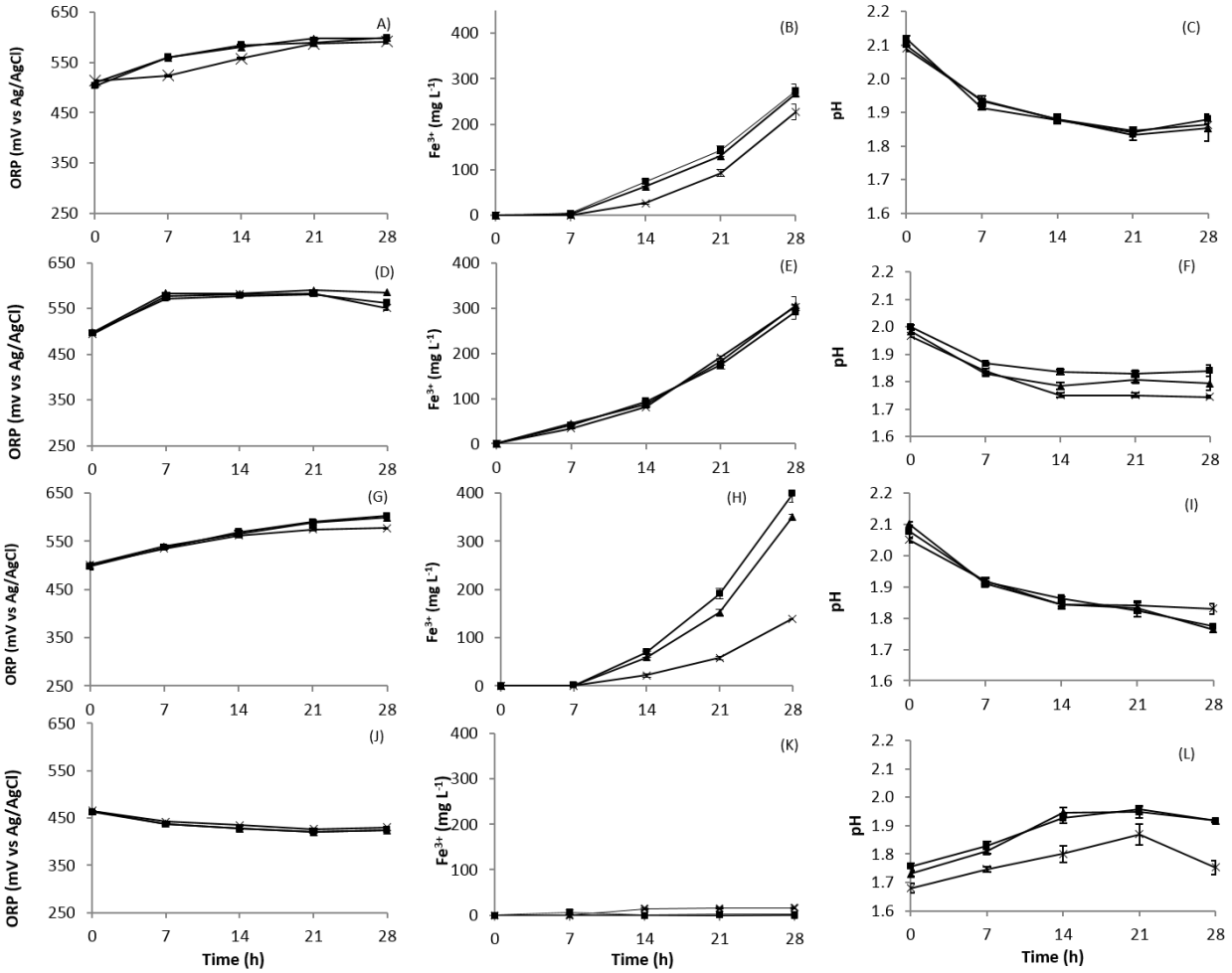

Figure 3. Pyrite bioleaching tests of V6 (A-C), V8 (D-F), 14C (G-I) and L2-21 (J-L) at chloride ion concentrations (g/L): ▪ 9, ▲ 15, x 30. Values are the average of three replicates ± standard deviation.

Previous studies have shown that V8 may have a competitive advantage over V6 in pyrite bioleaching, as determined by its ability to dominate bioreactor cultures (34). These studies confirm the ability of V8 to oxidize pyrite more efficiently than V6 under higher chloride ion stress. The pyrite bioleaching tests showed that 14C was able to oxidize pyrite, possibly due to the presence of both iron and sulfur oxidizers. However, its performance on pyrite was lower than that of the pure cultures. Mixed culture L2-21, however, seemed to be dominated by sulfur oxidizers and therefore was unable to bioleach pyrite.

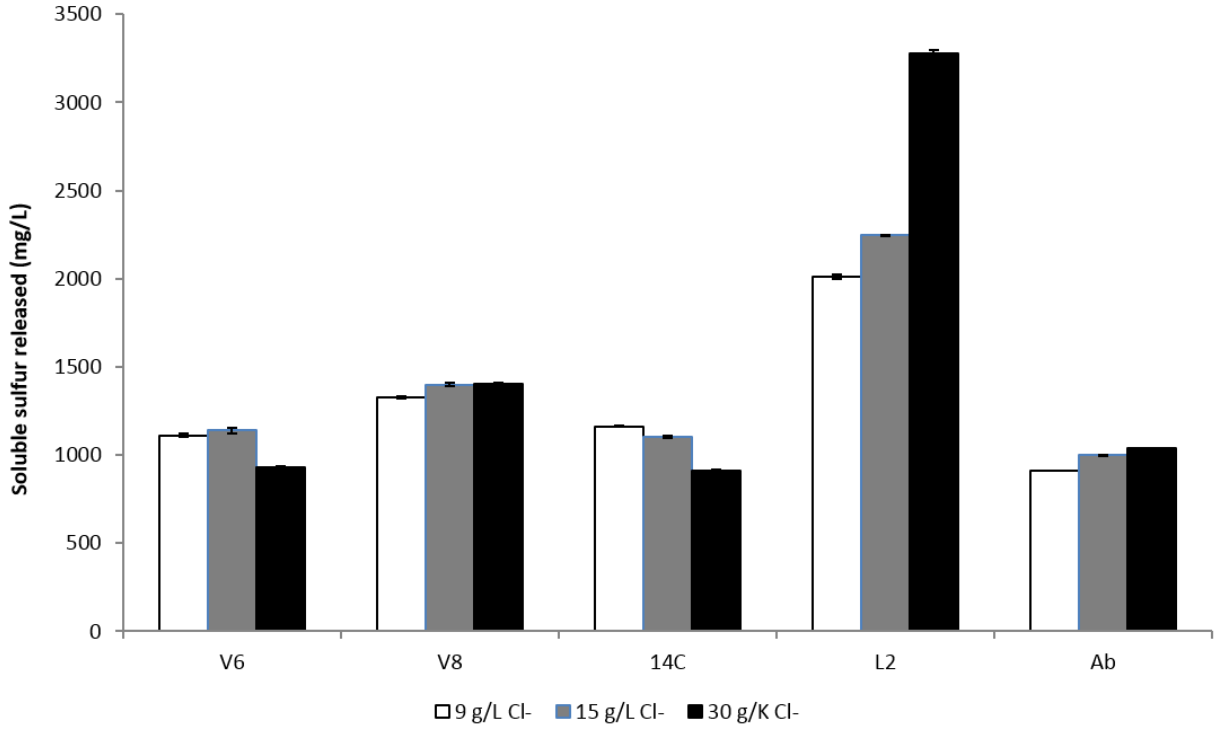

Figure 4. Soluble sulfur release from pyrite after 28 days of leaching with V6, V8, 14C, L2-21 cultures and in abiotic control (Ab). Values are the average of three replicates ± standard deviation.

2.3.3 Diversity profile of the mixed cultures

Genomic DNA extraction from acidophilic cultures grown at high salt stress have previously shown to be problematic due to low concentration of cells, high ionic strength, low pH, contamination by metals or inefficient cell lysis ((23, 35-37). The diversity profile over time of the mixed cultures 14C and L2-21 used in the bioleaching tests could not be performed due to the inability to extract high quality genomic DNA from the samples. However, the inoculant culture of 14C was dominated by *Acidihalobacter* spp. (93% relative abundance) the L2-21 inoculant by *Acidithiobacillus albertensis* (98.4% relative abundance) and the L4-9 and L6-11 inoculant by *Ferroplasma* spp. (98.9 and 99.9% relative abundance). The dominance of *A. albertensis* (a sulfur oxidizing acidophile) in culture L2-21 is likely to explain the inability to oxidize pyrite (Figure 3). This agrees with previousfindings that also showed that *A. albertensis* is unable to oxidise pyrite (38). It has been shown that some acidophiles are only able to oxidize pyrite when supplied with organic carbon such as yeast extract (31). Yeast extract was omitted in the bioleaching tests, however, as *A. albertensis* is autotrophic

this is unlikely to have had an effect (38). The presence of *Ac. prosperus* species, which oxidize both iron and sulfur, would explain the superior pyrite bioleaching capabilities by mixed culture 14C. The dominance of *Ferroplasma* spp. in L4-9 and L6-11 would explain the ability of these two cultures to successfully oxidize ferrous iron, as some *Ferroplasma* spp. are known to be effective at ferrous iron oxidation at up to 12 g/L chloride ion (10, 12).

2.4 Conclusion

This study has demonstrated the ability of pure and mixed cultures of halotolerant acidophiles to oxidize iron and sulfur at high chloride ion stress. Pure cultures V6 and V8 were able to tolerate up to 45 g/L chloride ion stress and to successfully bioleach pyrite at up to 30 g/L chloride ion, which is well above the 19 g/L chloride ion present in seawater. To date, these are the highest recorded chloride ion tolerances seen in pure cultures of halotolerant acidophiles. This highlights the potential for the use of these cultures for saline water bioleaching.

2.5 Acknowledgements

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2.6 Conflicts of Interest

The authors declare no conflict of interest.

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3 Chapter 3

Khaleque HN, Ramsay JP, Murphy RJ, Kaksonen AH, Boxall NJ, Watkin EL. Draft Genome Sequence of the Acidophilic, Halotolerant, and Iron/Sulfur-Oxidizing *Acidihalobacter prosperus* DSM 14174 (Strain V6).

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Khaleque HN, Ramsay JP, Murphy RJ, Kaksonen AH, Boxall NJ, Watkin EL. Draft Genome Sequence of *Acidihalobacter ferrooxidans* DSM 14175 (Strain V8), a New Iron-and Sulfur-Oxidizing, Halotolerant, Acidophilic Species.

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Khaleque HN, Corbett MK, Ramsay JP, Kaksonen AH, Boxall NJ, Watkin ELJ. Complete genome sequence of *Acidihalobacter prosperus* strain F5, an extremely acidophilic, iron- and sulfur-oxidizing halophile with potential industrial applicability in saline water bioleaching of chalcopyrite.

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3.1 Draft Genome Sequence of the Acidophilic, Halotolerant and Iron/Sulfur-Oxidizing Acidihalobacter prosperus DSM 14174 (strain V6)

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Abstract

The principal genomic features of *Acidihalobacter prosperus* DSM 14174 (strain V6) are presented here. This is a mesophilic, halotolerant iron/sulfur oxidising acidophile that was isolated from seawater at Vulcano, Italy. It has potential for use in biomining applications in regions where high salinity exists in the source water and ores.

Acidihalobacter prosperus (previously known as *Thiobacillus prosperus*) is a Gram-negative, halotolerant, acidophilic, mesophilic and chemolitho-autotrophic bacterium capable of oxidising both iron and reduced sulfur compounds (1). The type strain, *A. prosperus* DSM 5130, was isolated from a marine geothermal field in Italy. It requires a minimum of 0.04 M Cl^- for growth and tolerates up to 0.6 M Cl^- (1). *A. prosperus* DSM 14174 was isolated from a shallow acidic pool by the shore of Baia de Levant, Aeolion Islands of Vulcano, Italy (2). Like *A. prosperus* DSM 5130, it does not grow in the absence of salt (3). It has been used in salt-rich systems for the active biomining of metal sulfide ores (4).

Total DNA extracted from *A. prosperus* DSM 14174 was sequenced using Illumina MiSeq (204,485 paired-end 300 bp × 2 reads) and PacBio RS single-molecule real-time (SMRT) sequencing technologies (96,369 reads post-filter, 11,756 bp mean length). *De novo* hybrid assembly using SPAdes 3.9.0 (5) produced a circular 162,484-bp plasmid (~32-fold coverage) and two chromosome fragments of 2,607,071 bp and 752,753 bp (~18-fold coverage). The

two chromosome fragments were scaffolded using SSPACE-Longread version. 1.1 (6), producing a circular chromosome of 3,363,634 bp (with one gap). The genome has a G+C content of 62.2 %. The NCBI Prokaryotic Genome Annotation Pipeline version 3.3 and GeneMarkS+ were used for annotation. The genome contains 46 tRNA sequences, one rRNA operon and 3,194 protein-coding genes.

Genome analysis of *A. prosperus* DSM 14174 confirmed the presence of the previously reported *rus* operon known to be involved in iron oxidation (3). Also present were genes coding for subunits SoxAX, SoxB and SoxYZ of the sulfur oxidation system (7), as well as those for sulfur metabolism through hydrogen sulfide biosynthesis (8). Furthermore, there were genes encoding proteins involved in various catalytic reactions for oxidation/reduction of sulfur as well as transport of sulfate/sulfonate (8). Similar to the genome of the type strain *A. prosperus* DSM 5130, the genome of *A. prosperus* DSM 14174 contains a complete set of genes for carbon dioxide fixation via the Calvin-Benson-Bassham cycle as well as those for the Nif complex for nitrogen fixation, chemotaxis and formation of a polar flagellum (9).

The synthesis of compatible solutes such as ectoine, sucrose and glycine betaine assists in the survival of bacteria under high osmotic stress (10). The genome of *Ac. prosperus* DSM 14174 contains genes that encode diaminobutyrate aminotransferases, diaminobutyrate acetyltransferase, ectoine synthase and sucrose synthase. These have potential roles in ectoine and sucrose biosynthesis pathways. Genes for ABC transporters for ectoine and glycine betaine uptake were also detected.

A. prosperus DSM 14174 contains a single plasmid, pABPV6, which is unique to this strain. The plasmid pABPV6 contains an array of genes coding for replication and transfer proteins, transposases, DNA methyltransferases, recombinases, hydrolases and DNA binding proteins.

Accession number(s). The whole-genome has been deposited at DDBJ/EMBL/GenBank under the accession no. CP017448. The plasmid pABPV6 has been deposited under the accession no. CP017449. The versions described in this paper are CP017448.1 and CP017449.1 respectively.

Acknowledgements

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3.2 Draft Genome Sequence of *Acidihalobacter ferrooxidans* DSM 14175 (strain V8), a New Iron- and Sulfur-Oxidizing, Halotolerant, Acidophilic Species

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Abstract

The use of halotolerant acidophiles for bioleaching provides a biotechnical approach for extraction of metals from regions where high salinity exists in the ores and source water. Here, we describe the first draft genome of a new species of a halotolerant, iron- and sulfur-oxidizing acidophile, *Acidihalobacter ferrooxidans* DSM-14175 (strain V8).

The halotolerant acidophile, *Acidihalobacter prosperus*, is well known for its ability to oxidize iron at low pH under saline conditions (1, 2). *A. ferrooxidans* DSM 14175 (strain V8) represents a similar group of Gram-negative, mesophilic, halotolerant acidophiles that also has the ability to oxidize iron and sulfur and has a chemolithoautotrophic lifestyle. It was isolated from the same shallow acidic pool at the Aeolian Islands of Italy as *A. prosperus* DSM-14174 (strain V6) (3) and was found to dominate mixed cultures during mesophilic pyrite oxidation (4).

Total DNA was extracted from *A. ferrooxidans* DSM 14175 using the modified method of nucleic acid extraction for acidophiles, as described by Zammit et al. (5). DNA was sequenced using Illumina MiSeq (619,160 paired-end reads, 2 × 300 bp reads) and PacBio RS SMRT sequencing technologies (733,419 subreads with a mean read length of 1,602 bp). *De novo* hybrid assembly using SPAdes version 3.9.0 (6) generated 10 contigs which were then used with PacBio reads to generate a scaffold using SSPACE-Longread version 1.1 (7). The

resulting scaffold was a single circular chromosome with an approximate size of 3,448,835 bp (with 4 gaps with total approximate size of 6 kb), with approximately 13 × Illumina read depth and 355-fold PacBio read depth. The genes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline version 3.3 and GeneMarkS+. The genome has a G+C content of 61.6% and contains 45 tRNA sequences, 1 rRNA operon and 3,089 protein-coding genes.

Similar to the genomes of *A. prosperus* DSM 5130 and DSM 14174, genome analysis of *A. ferrooxidans* DSM-14175 showed the presence of the *rus* operon genes for iron oxidation (8-10).

Also found were genes for carboxysomes and carbon dioxide fixation through the Calvin-Benson-Bassham cycle (9-11) and those for nitrogen fixation through the nif complex (9, 10, 12). A complete set of genes for chemotaxis and flagellar biosynthesis, similar to those found in *A. prosperus* strains DSM 5130 and DSM 14174, were also present (9, 10). However, unlike the genomes of the *A. prosperus* strains, the genome of *A. ferrooxidans* DSM 14175 does not contain genes encoding the SoxAX, B, YZ subunits of the sulfur oxidation system (9, 10, 13); rather, it contains genes encoding sulfur oxygenase reductases, which may be responsible for sulfur metabolism in this strain (13).

The genome of *A. ferrooxidans* DSM 14175 has genes for pathways involved in tolerance to stresses such as acid and oxidative stress. Considering the ability of this strain to withstand high osmotic stress in a low pH environment, some of the most important stress tolerance genes are those encoding operons for the biosynthesis and regulation of ectoine, glycine betaine and osmoregulated periplasmic glucan, as well as for glycine betaine and choline uptake (14, 15). These proteins act as compatible solutes in acidophiles under osmotic stress and may provide assistance in survival of halotolerant acidophiles (14, 15).

Accession number(s). The whole-genome of *A. ferrooxidans* DSM 14175 (strain V8) has been deposited at DDBJ/EMBL/GenBank under the accession no. **CP019434**. The version described in this paper is the first version, **CP019434.1**.

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3.3 Complete genome sequence of Acidihalobacter prosperus strain F5, an extremely acidophilic, iron- and sulfur-oxidizing halophile with potential industrial applicability in saline water bioleaching of chalcopyrite

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Abstract

Successful process development for the bioleaching of mineral ores, particularly the refractory copper sulfide ore chalcopyrite, remains a challenge in regions where freshwater is scarce and source water contains high concentrations of chloride ion. In this study, a pure isolate of *Acidihalobacter prosperus* strain F5 was characterized for its ability to leach base metals from sulfide ores (pyrite, chalcopyrite and pentlandite) at increasing chloride ion concentrations. F5 successfully released base metals from ores including pyrite and pentlandite at up to 30 g.L^{-1} chloride ion and chalcopyrite up to 18 g.L^{-1} chloride ion. In order to understand the genetic mechanisms of tolerance to high acid, saline and heavy metal stress the genome of F5 was sequenced and analysed. As well as being the first strain of *Ac. prosperus* to be isolated from Australia it is also the first complete genome of the *Ac. prosperus* species to be sequenced. The F5 genome contains genes involved in the biosynthesis of compatible solutes and genes encoding monovalent cation/proton antiporters and heavy metal transporters which could explain its abilities to

tolerate high salinity, acidity and heavy metal stress. Genome analysis also confirmed the presence of genes involved in copper tolerance. The study demonstrates the potential biotechnological applicability of *Ac. prosperus* strain F5 for saline water bioleaching of mineral ores.

Keywords: Halophilic, acidophile, bioleaching, compatible solutes, copper tolerance

A limited number of microorganisms capable of simultaneously tolerating salt stress and low pH have been isolated, presumably as there are only a few places on Earth where geological conditions provide both conditions. Use of seawater at mines where access to freshwater is limited has led to the search of halotolerant, acidophilic, iron- and sulfur oxidizing bacterial cultures that are active in seawater concentrations of chloride ion (19.9 $g.L^{-1}$) (Watling, 2016; Zammit et al., 2012). Furthermore, many of the reported salt tolerant acidophiles do not tolerate copper and chloride simultaneously, hampering their use for biomining copper-containing ores such as chalcopyrite (Watling et al., 2016). Therefore, identifying acidophilic microorganisms that accelerate the dissolution of chalcopyrite under salt stress would result in a step change in biohydrometallurgical processing (Watling et al., 2016).

Acidihalobacter prosperus represents a group of Gram-negative, halophilic, iron- and sulfur-oxidizing, mesophilic, chemolithoautotrophic, extreme acidophiles that have potential for biomining applications in regions of high salinity and acidity (Huber and Stetter, 1989; Zammit et al., 2012). Draft genomes of two strains of *Acidihalobacter prosperus* have previously been published (Khaleque et al., 2017; Ossandon et al., 2014). Here we present the finished complete genome sequence of an *Ac. prosperus* strain, strain F5. The F5 sequence will aid in the genetic characterization of acid, salinity and heavy metal tolerance. F5 is also the first of this species to be isolated in Australia.

Ac. prosperus strain F5 was isolated from a mixed environmental culture obtained from an acidic saline drain (containing 130 $g.L^{-1}$ chloride, 1.4 $g.L^{-1}$ iron(II) at a pH of 2.1) at the Yilgarn Crater, Western Australia (Zammit et al., 2009). Enrichment and isolation of F5 was performed at the School of Biomedical Sciences, Curtin University, Perth, Australia. The pure culture was maintained at 30˚C in pH 2.5 basal salt media (0.4 $g.L^{-1}$ $(NH_4)_2SO_4$, 0.4 $g.L^{-1}$ $MgSO_4.7H_20$, and 0.4 $g.L^{-1}$ KH_2PO_4) supplemented with trace salts and soluble iron (13. 9 $g.L^{-1}$ $FeS0_4.7H_2O$) and sulfur sources (1.51 $g.L^{-1}$ $K_2S_4O_6$) (Zammit et al., 2012). Growth on

increasing levels of chloride ion (provided as NaCl) showed the ability of this isolate to tolerate up to 45 $g.L^{-1}$ chloride ion (data not shown).

Bioleaching experiments using a pure isolate of *Ac. prosperus* strain F5 were conducted over 10 days at increasing chloride ion concentrations (provided as NaCl) in basal salt media pH 2.5 with 1% weight/volume pyrite, pentlandite or chalcopyrite. The percent composition of the ores used is given in Table 1. The release of iron was measured for experiments on all three ores using the method described by Govender et al. (2012). Leachate solutions were filtered to remove any particulate matter and diluted in 1% HNO3. Copper (for chalcopyrite) and nickel (for pentlandite) release were measured by atomic absorption spectroscopy (Avanta ∑). The release of metals over 10 days of bioleaching experiments at the different chloride ion concentrations tested are shown in Figure 1.

Table 1. Composition of ore used in bioleaching tests (mass-%)

	Fe	Cu	Ni	S	Si	Ca	Na	K
Pyrite	36.6	0.239	0.044	39.8	4.67	1.04	0.686	0.442
Chalcopyrite	26.6	26.8	0.004	29.8	3.97	0.382	0.009	0.043
Pentlandite	40.7	0.73	7.01	35.4	1.74	0.169	0.042	0.025

Ac. prosperus strain F5 was able to leach base metals from the sulfide ore pyrite at up to 30 $g.L^{-1}$ chloride ion, chalcopyrite at 18 $g.L^{-1}$ chloride ion and pentlandite from 45 $g.L^{-1}$ chloride ion (Figure 1). The results suggested that *Ac. prosperus* strain F5 may be an ideal candidate for bioleaching of sulfide ores at chloride ion concentrations of sea water or above (19.9 $g.L^{-1}$).

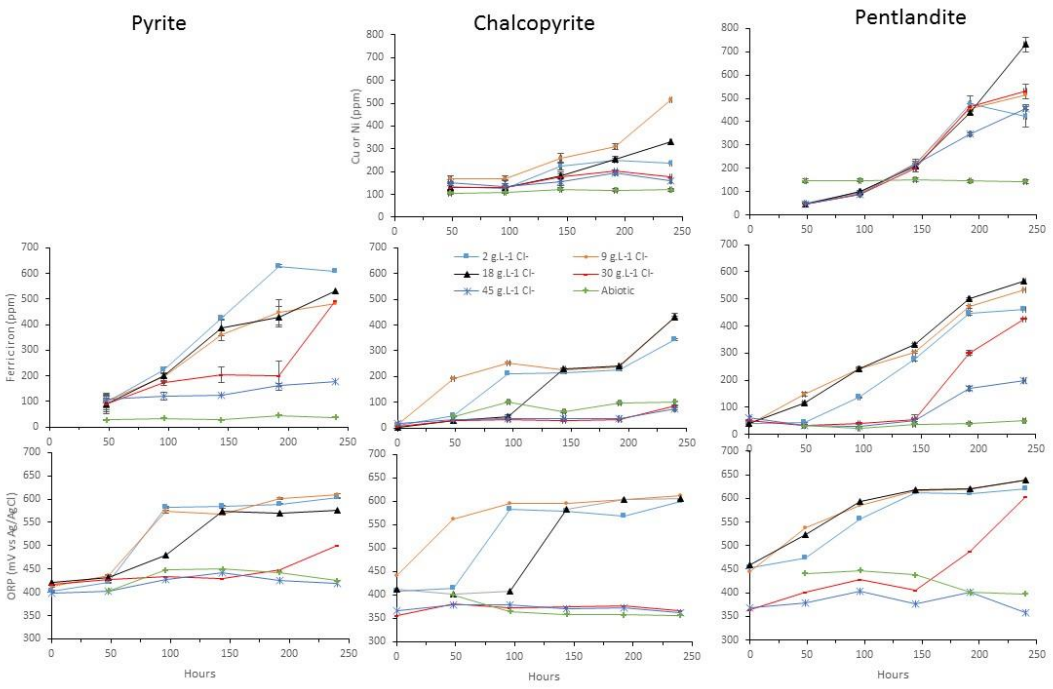

Figure 1. The effect of NaCl on the bioleaching of pyrite, chalcopyrite and pentlandite by *Acidihalobacter prosperus* strain F5 over 10 days of bioleaching. Abiotic control in the presence of 18 $g.L^{-1}$ chloride ion chosen as the optimum chloride ion concentration for *Ac. prosperus* F5 growth. Data are averages ± SE of triplicate biological replicates and three technical replicates.

Few studies have assessed the ability of pure, mesophilic halotolerant acidophiles to leach chalcopyrite. It is known that the formation of passivation layers results in the poor dissolution of chalcopyrite by mesophilic microorganisms (Hirato et al., 1987; Marhual et al., 2008; Zhou et al., 2008). Studies have been conducted with thermophilic cultures (Wang et al., 2012; Wang et al., 2014; Watling et al., 2016; Yu et al., 2014) with varied success in chalcopyrite bioleaching. However, this is the first study to demonstrate chalcopyrite bioleaching by a pure, mesophilic, halotolerant acidophile at chloride ion concentrations that have been shown to inhibit the growth of the other mesophilic acidophiles. These unique characteristics highlight the potential applicability of bioleaching the recalcitrant copper sulfide ore, chalcopyrite, using *Ac. prosperus* strain F5.

Genomic DNA was extracted from F5 using the method described by Zammit et al. (2011). F5 DNA was sequenced using PacBio RS SMRT technology which generated 92,779 post-filter reads with a mean length of 12,147 bp and ~316-fold depth of coverage. *De novo* assembly

using the long-read-only assembler canu v. 1.3 (Koren et al., 2017), produced a circular chromosome of 3,566,941 bp. Genes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline v 3.3 and GeneMarkS+. F5 contained 47 tRNA sequences, 1 rRNA operon and 3,233 protein-coding genes. The genome has a G+C content of 59.9 %. Genome features of *Ac. prosperus* strain F5 are summarised in Table 2 and Fig. 2.

Table 2. Genome features of *Acidihalobacter prosperus* strain F5.

Attribute	Value
Genome size (bp)	3,566,941
G +C content (%)	59.9
Genes (total)	3,428
CDS total	3,374
CDS coding	3,233
tRNAs	47
rRNA genes	3
ncRNAs	4
Pseudo genes (total)	141

Genome analysis of *Ac. prosperus* strain F5 confirmed the presence of genes known to be involved in iron oxidation, such as those of the previously described *rus* operon (Nicolle et al., 2009) and numerous predicted iron transport proteins (accession numbers: WP_070077550.1, WP_070077551.1, WP_070077552.1). Also present were genes encoding various sulfur oxidation, metabolism and transport pathway proteins, including those of the SoxAX, B, YZ complex (Friedrich et al., 2005) (accession numbers: WP_070078781.1, WP_083251331.1, WP_070077629.1, WP_070079247.1). Genes for proteins involved in carbon dioxide fixation (via the Calvin Benson Benham cycle) (Shively et al., 1998) and chemotaxis were found, similar to those described in the type strain *Ac. prosperus* 5130 (Ossandon et al., 2014) and the environmental isolate *Ac. prosperus* DSM 14174 (Khaleque et al., 2017) (accession numbers: JQSG00000000.2, CP017448.1).

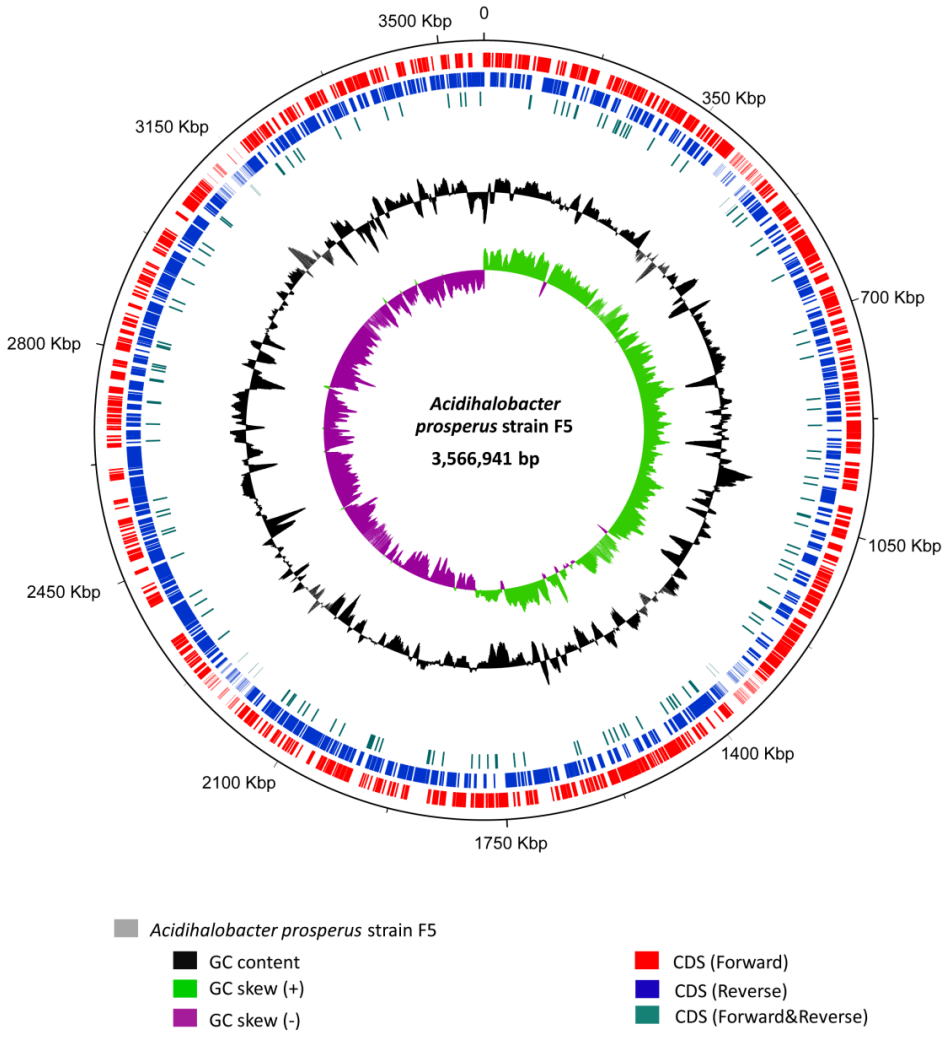

Figure 2. Circular genome plot of *Acidihalobacter prosperus* strain F5. From innermost to outermost rings: GC skew, where outwards-directed lines represent positive GC skew; GC content, where outwards-directed lines represent higher GC content; Predicted coding sequences (CDS) on both DNA strands (teal), on the reverse DNA strand (blue) and on the forward DNA strand (red).

Ac. prosperus strain F5 also carried genes encoding for the synthesis and transport of osmoprotectants including N-acetyldiaminobutyrate dehydratase, diaminobutyrate acetyltransferase, ectoine synthase and sucrose synthase (accession numbers: WP_070079295.1, WP_070079297.1, WP_070079295.1, WP_070079442.1). Genes encoding ABC transporters for ectoine and glycine betaine uptake were also present (accession numbers: WP_070078974.1, WP_083251372.1, WP_070078973.1, WP_083251085.1. These proteins have been linked to the survival of acidophiles under high osmotic pressure (Dopson et al., 2017).

Interestingly, the genome of *Ac. prosperus* strain F5 contains multiple genes encoding copper resistance proteins and a copper translocating P-type ATPase, which may be responsible for its copper tolerance and ability to leach chalcopyrite (accession numbers: WP_070078257.1, WP_070078256.1, WP_070079189.1, WP_070077568.1). Genes encoding multiple heavy metal ABC transporters for zinc, nickel, mercury and other heavy metals were also identified (accession numbers: WP_070077548.1, BI364_RS17635, WP_070078780.1, WP_070079191.1), potentially explaining the ability of F5 to leach pentlandite effectively in the presence of 45 $g.L^{-1}$ chloride ion, as shown in Fig. 1.

The information from the bioleaching tests and complete genome analysis of *Ac. prosperus* strain F5 has provided clues to understand mechanisms of salt and copper tolerance in halophilic acidophiles. The isolation of this halophilic, iron- and sulfur-oxidizing acidophile with high heavy-metal tolerance is highly significant due to its potential for use in recovery of metals from low grade ores, particularly chalcopyrite, in regions of high salinity in ores or source water.

Nucleotide sequence accession numbers. The bioproject designation for this project is PRJNA344521. The whole-genome of *Acidihalobacter prosperus* strain F5 has been deposited at DDBJ/EMBL/GenBank under the accession no. **CP017415**. The version described in this paper is CP017415.1.

Acknowledgements

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4 Chapter 4

Khaleque HN, González C, Kaksonen AH, Boxall NJ, Holmes DS, Watkin ELJ. Genome-based reclassification and renaming of two extremely acidophilic, iron- and sulfur-oxidizing halophiles *Acidihalobacter prosperus* strain V6 and *'Acidihalobacter ferrooxidans'* strain V8 as two new species, *Acidihalobacter aeolianus* sp. nov., and *Acidihalobacter vulcanensis* sp. nov., respectively.

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Genome-based reclassification of the extremely acidophilic, iron- and sulfur-oxidizing halophile *Acidihalobacter prosperus* strain F5 as a new species, *Acidihalobacter yilgarnensis* sp. nov.

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4.1 Genome-based reclassification of two extremely acidophilic, iron- and sulfur-oxidizing halophiles *Acidihalobacter prosperus* strain V6 (DSM 14174/ JCM 32253) and *'Acidihalobacter ferrooxidans*' strain V8 (DSM 14175/ JCM 32254) as two new species, *Acidihalobacter aeolianus*^{T} sp. nov., and *Acidihalobacter vulcanensis*^{T} sp. nov., respectively.

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Strain sequence accession numbers:

Acidihalobacter prosperus strain V6: chromosome - CP017448.1, plasmid - CP017449.1

Acidihalobacter ferrooxidans' strain V8: CP019434.1

Abstract

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

Only a few microorganisms 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 acidophilic halophiles 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 biol eaching process [2].

The genus *Acidihalobacter* includes only one formally recognized species, *Acidihalobacter prosperus* (DSM 5130^{T}) [4]. *A. prosperus* DSM 5130^{T} 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 *Ectothiorhodospiraceae* family of the class *Gammaproteobacteria* in the new genus of *Acidihalobacter,* with *A. prosperus* DSM 5130^T (JCM 30709) being named the type strain of its species [4].

More recently, two new halophilic acidophiles, *A. prosperus* strain V6 and '*A. ferrooxidans*' strain V8, were isolated from mixed environmental cultures obtained from shallow acidic pools at the Aeolian Islands, Vulcano, Italy [7]. *A. prosperus* strain V6 was recognized to be similar to the type strain in its 16S rRNA phylogeny as well as its morphological characteristics [8, 9] but showed superior growth to the type strain on ferrous iron [5, 10]. *A. prosperus* strain V6 grows autotrophically and is able to oxidize ferrous iron, the sulfur sources elemental sulfur and tetrathionate and the sulfide mineral pyrite. [10, 11]. It is unable to grow in the absence of chloride, requiring a minimum of 0.06 M Cl^-, is able to grow at upto 1.27 M Cl^- with maximum growth at 0.42 M Cl^- [8, 11]. *A. prosperus* strain V6 was also shown to be a member of the family *Ectothiorhodospiraceae* of the class *Gammaproteobacteria* [12]. It has previously been referred to as *'Acidihalobacter aeolicus*' [13], based on >95% 16S rRNA gene sequence similarity to *A. prosperus*^{T} ('*Thiobacillus prosperus*' at the time), but was submitted to the DSMZ culture collection as a strain of the *A. prosperus* species and was never formally classified or named.

'*A. ferrooxidans'* strain V8, on the other hand, was recognized to be 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* strain V6 when grown on the sulfide mineral pyrite [9, 10]. It is also able to autotrophically 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 0.06 M Cl^- when grown in the presence of soluble iron [11]. Maximum growth occurs at 0.42 M Cl^- and it is able to grow at 0.85 M Cl^-. It has a superior ability to oxidize pyrite in the presence of chloride compared to *A. prosperus* strain V6 with maximum oxidation occuring at 0.85 M Cl^- compared to 0.25 M Cl^- for *A. prosperus* strain V6 [11]. However, like *A. prosperus* strain V6, '*A. ferrooxidans'* strain V8 has also not been formally named or classified.

The high quality draft genome sequences of *A. prosperus* strain V6 and '*A. ferrooxidans'* strain V8 have recently been released [14, 15]. Whole-genome sequencing has shown the presence of a 162,484 bp plasmid in *A. prosperus* strain V6 that is not present in either the type strain of *A. prosperus* or in '*A. ferrooxidans*' strain V8 [6, 14, 15]. 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 [16].

The bioinformatically inferred G+C content for the genomes of *A. prosperus* strain V6 and '*A. ferrooxidans'* strain V8 are 62.2%mol and 61.6%mol, respectively [14]. These values fall within the range of 50.5–69.7% mol DNA G+C for members of the family *Ectothiorhodospiraceae* [17]. It is also consistent with the placement of the two species in the *Acidihalobacter* genus, which is nearest to the *Ectothiorhodospira* genus, both of which show a requirement of chloride for growth [4, 5, 8, 17]. Phenotypic and genomic features of the three species of the *Acidihalobacter* genus are shown in Table 1.

In order to infer phylogenetic relationships, the 16S rRNA gene sequence similarity of *A. prosperus* strain V6, '*A. ferrooxidans'* strain V8 and *A. prosperus* DSM 5130^T was compared by aligning the sequences using the default settings of the nucleotide Basic Local Alignment Search Tool (BLASTN) tool [18] and MAFFT alignment (with L-INS-i option) [19, 20]. Studies have suggested that 98.65% 16S rRNA gene sequence similarity can be used as a threshold for differentiating two species [21, 22]. '*A. ferrooxidans'* strain V8 shared only 97% sequence similarity (Nucleotide Identity by Blast (NIB), 16S rRNA gene sequence similarity by BLASTN) with both *A. prosperus*^{T} and *A. prosperus* strain V6, confirming it to be a different novel species (Table 2). The 99% sequence similarity (NIB) between *A. prosperus* strain V6 and *A. prosperus* DSM 5130^T (Table 2), initially suggested that they might be different strains of the same species, as was previously suggested [8, 9, 12] . However, further phylogenomic analysis using other sequence and non-sequence based techniques have shown that both are members of novel species.

In addition to members of the *Acidihalobacter* genus, a total of 11 other taxonomically related genomes were selected for inclusion into a 16S rRNA gene sequence-based phylogenetic tree. *Halothiobacillus neapolitanus* C2 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) [23, 24]. 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 [25], RDP [26] and SILVA [27].

The Tamura-Nei model [28] was chosen as the most appropriate model for inference of 16S rRNA phylogeny according to the lowest BIC model selection of jModelTest [29, 30]. The phylogenetic tree was reconstructed using sequences obtained from the prokaryotic 16S rRNA database of NCBI and aligned in MAFFT version 7 with the L-INS-i iterative refinement [19, 20]. Phyml version 3 was used to construct a maximum likelihood tree using the bootstrap method with 1000 replicates [31, 32]. Tree reconstruction included the use of discrete Gamma distribution and allowing some sites to be evolutionarily invariable (TrN+G+I+). After tree construction, a final of 14 members (including those of *Acidihalobacter* genus) were selected based on the closest phylogeny as given by bootstrap values. The concise, final tree, consisting of the 14 selected members from genus *Acidihalobacter* and order *Chromatiales* is shown in Figure 1.

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) [33]. The complete sets of 34 and 31 COG markers (Tables S2 and S3) 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 microorganism. 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 constructed for the 14 members of different species found to be taxonomically closest to the *Acidihalobacter* genus (Table S1) by performing a multiple alignment of concatenated sequences of 30 COGs from 34 ribosomal protein families (Figure 2a) that are universally conserved in the three domains of cellular life [34]. 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 [35] (Figure 2b). A third 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*) [36-38] from 14 members from the order *Chromatiales* (Figure 2C). For all trees, the alignment of the concatenated sequences was made using the L-INS-i Iterative refinement in MAFFT version 7 [19, 20]; the alignments were masked to remove unreliable regions with GBLOCKS [39, 40], followed by a concatenation of all protein families. The substitution model LG+G+I was used for multiprotien alignment, as predicted using ProtTEST 3 tool [41, 42], according to the lowest Bayesian and Akaike Information Criteria (BIC and AIC respectively [30, 43]). The substitution model for multi-gene tree was GTR+G+I, according to AIC and BIC model selection as indicated by jModelTest [29, 44]. Maximum likelihood trees were prepared for concatenated alignments with PhyML version 3 using 1000 replicates for the bootstrap method [31] with substitution models indicated by ProtTEST and jModelTest respectively.

A list of the NCBI accession numbers for the genomes, including their taxonomical data is provided in Table S1. The COG families used in the elaboration of the multiprotein trees are provided in Tables S2 and S3 and the housekeeping genes used in the multi-gene tree in Table S4. The resulting multiprotein phylogenomic trees and multigene phylogenetic tree are shown in Figure 2.

While the information gained from phylogenetic 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 *in silico* DNA-DNA hybridization (*is*DDH) calculations are sequence based techniques designed to analyze and compare interspecies boundaries between genomes [44-47]. The combination of ANI results when reinforced by high TETRA correlation values can be used to help define prokaryotic species using an objective boundary [45]. Furthermore, the use of *is*DDH 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 [48]. The use of these bioinformatics approaches for clarification of

taxonomical positions has previously been successfully used for *Acinetobacter* [49], *Vibrio* [50]and *Aeromonas* [47] and their advantages have been discussed extensively.

The calculation of average nucleotide identity based on BLAST (ANIb) and the correlation indexes of tetra-nucleotide signatures (Tetra) were conducted using JspeciesWS (http://jspecies.ribohost.com/jspeciesws/#Analyse) [51]. Based on the recommended cut-off values for species determination (<95% for ANIb and <0.989 for Tetra) [21, 44, 45, 52], the results provided evidence that *A. prosperus* strain V6 and '*A. ferrooxidans'* strain V8 belong to different species. This was further reinforced by the calculation of the *is*DDH values using the Genome-to-Genome Distance Calculator (GGDC) web tool, (http://ggdc.dsmz.de/distcalc2.php), with Formula 2 [46, 48]. A cut off of 70% was used to determine the distance between the genomes [53, 54]. The results provided the first evidence of *A. prosperus* strain V6 as a separate and novel species of the genus *Acidihalobacter,* and not a strain of *A. prosperus* and confirmed that *'A. ferrooxidans'* strain V8 is also a separate, novel species as previously suggested [9, 10]. The ANI, Tetra and *is*DDH values obtained from the comparison of these two species against each other and *A. prosperus* DSM 5130^T 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*' strain V6 and '*Acidihalobacter ferrooxidans*' strain V8 are members of separate, novel species. We, therefore, propose the reclassification *Acidihalobacter prosperus* strain V6 as the type strain of '*Acidihalobacter aeolianus*^{T}' sp. nov,. and that of *'Acidihalobacter ferrooxidans'* strain V8 as the type strain of '*Acidihalobacter vulcanensis*^{T}*'* sp. nov.

DESCRIPTION OF *ACIDIHALOBACTER AEOLIANUS*^{T} (STRAIN V6) SP. NOV.

Acidihalobacter aeolianus^T (ae.o.li.a'nus. N.L. masc. adj. *aeolianus*, referring to its isolation from the Aeolian islands, Italy).

Previously known as *Acidihalobacter prosperus* DSM 14174 (strain V6). An updated description of this organism is given. Cells are motile, Gram-negative rods. Extremely

acidophilic, optimum pH is 2.0. Halophilic, requires a minimum of 0.06 M Cl^- for growth but can grow at up to 1.27M Cl^-. Mesophilic, optimal growth occurs at 36°C. Chemolithoautotrophic and aerobic. Iron- and sulfur-oxidizing, grows on soluble ferrous and the soluble sulfur source tetrathionate as well as on the sulfide mineral pyrite. Found in shallow pools of acidic, salty water. The genome contains genes for the synthesis of the osmprotectants ectoine (*ect*ABC), glycine betaine (*abl*AB), proline (*pro*JA and a *pro*C homolog), and periplasmic glucans (*opg*GH) as well as transporters for glycine betaine and proline (*pro*VW, *opu*AC).

The whole-genome sequence of the 3,363,634 bp chromosome is available (GenBank accession no. CP017448.1). The genome contains a unique 162,484 bp plasmid not present in other members of the *Acidihalobacter* genus (GenBank accession no. CP017449.1). The G+C content of the DNA of the type strain genome is 62.2%mol.

The type strain is DSM 14174 (JCM 32253)

DESCRIPTION OF *ACIDIHALOBACTER VULCANENSIS*^{T} (Strain V8) SP. NOV.

Acidihalobacter vulcanensis^T (vul.can.en'sis. N.L. masc. adj. *vulcanensis*, referring to its isolation from Vulcano, Italy).

Previously known as '*Acidihalobacter ferrooxidans*' DSM 14174 (strain V8). Gram negative rods (1 µm long), sometimes curved under stressconditions. Motile. Extremely acidophilic, optimum pH is 2.0. Halophilic, requires more than 0.06M Cl^- for growth, grows at up to 0.85 M Cl^-. Oxidizes soluble ferrous and the soluble sulfur source tetrathinate. Grows on the sulfide mineral pyrite. Growth on pyrite at higher chloride concentrations is superior to that of *A. aeolianus*^{T} strain V6. Mesophilic, optimal growth occurs at 36 °C. Chemolithoautotrophic and aerobic. Found in shallow pools of acidic, salty water. The genome contains genes for the synthesis of the osmprotectants ectoine (*ect*ABC), glycine betaine (*abl*AB and *cdh*), proline (*pro*HJA), and periplasmic glucans (*opg*GH) as well as those for uptake of glycine betaine, proline and taurine (*pro*VWX, *tau*ABC).

The whole-genome sequence of the 3,448,835 bp chromosome is available (GenBank accession no. CP019434.1). The G+C content of the DNA of the type strain is 61.6%mol.

The type strain is DSM 14175 (JCM 32254)

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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

Feature	***A. prosperus*** **DSM 5130^T**	***A. prosperus*** **(Strain V6)**	***'A. ferrooxidans'*** **(Strain 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)	3,088	3,194	3,089
Plasmid	Not present	162,484bp (termed pABPV6)	Not present
16S/23S rRNA genes	1	1	1
tRNA genes	48	46	45
Predicted terminal oxidases	aa_3 bo_3 bd fumarate reductase	aa_3 bo_3 bd fumarate reductase	aa_3 bo_3 bd fumarate reductase
Predicted respiratory quinones	Ubiquinone	Ubiquinone	Ubiquinone
Iron oxidation system	Rus operon	Rus operon	Truncated Rus operon

Sulfur metabolism	SOX enzyme system, Sulfur oxygenase reductase	SOX enzyme system only	Sulfur oxygenase reductase only
T_{opt} for growth (˚C)	35 [5]	36 [55]	36 [55]
Predicted synthesized osmolytes or transporters	Ectoine, glycine betaine, proline, periplasmic glucans and transporters for glycine betaine, proline and taurine	Ectoine, glycine betaine, proline, periplasmic glucans and transporters for glycine betaine and proline	Ectoine, glycine betaine, proline, periplasmic glucans and transporters for glycine betaine, proline and taurine

Table 2. Comparison of ANI, *is*DDH, Tetra and NIB of the chromosomal sequences of *A. prosperus* strain V6 and '*A. ferrooxidans'* strain V8 against the type strain, *A. prosperus* DSM 5130^T, and each other ANI- Average nucleotide identity (%) as determined using ANI BLAST (ANIb) (%), Tetra – Tetra-nucleotide signature correlation index, GGD – Genome-to-genome distance (%), NIB – 16s rRNA Nucleotide Identity by BLAST (%)

	A. prosperus DSM 5130^T				*A. prosperus* strain V6 DSM 14174				'*A. ferrooxidans'* strain V8 DSM 14175			
Query	ANI	*is*DDH	TETRA	NIB	ANI	*is*DDH	TETRA	NIB	ANI	*is*DDH	TETRA	NIB
A. prosperus DSM 5130^T					80.60	25.20	0.9889	99	72.32	20.40	0.9151	97
A. prosperus strain V6 DSM 14174	80.34	25.20	0.9889	99					72.49	21.30	0.9128	97
'A. ferrooxidans' strain V8 DSM 14175	71.93	20.40	0.9151	97	72.47	21.30	0.9128	97				

Figure legends

Fig. 1. 16S rRNA phylogenetic tree of 14 members of order *Chromatiales*, including the analyzed strains *Acidihalobacter prosperus* DSM 5130^T, *A. prosperus* strain V6 (DSM 14174) and '*A. ferrooxidans*' strain V8 (DSM 14175). The tree was constructed using the MAFTT alignment (L-INS-i option) [19, 20] with the TrN+G+I model of jModelTest [29] according to the Bayesian Information Criterion (BIC) model [30]. The tree was built using PhyML [32] with 1000 bootstraps [31], the values of which were converted to statistically supported percentages. The scale bar represents 0.04 nucleotide changes per site.

Fig. 2. Phylogenomic trees of 14 members of order *Chromatiales* including the strains *Acidihalobacter prosperus* DSM 5130^T, *A. prosperus* DSM 14174 and '*A. ferrooxidans*' DSM 14175. (A) The tree was constructed using multi-locus concatenation of 30 out of 34 universal ribosomal proteins [34]. The concatenated proteins were aligned with MAFFT (L-INS-i option) [19, 20] and the unreliable regions were masked with GBLOCKS [39, 40]. The concatenated protein alignment was used to select the LG+G+I as the best model, according to BIC and AIC model selection [30, 43], using the ProtTEST 3 Tool [41]. The tree was built using PhyML with 1000 bootstraps [31], the values of which were converted to statistically supported percentages. The scale bar represents 0.06 amino acid changes per site. The list of COGs used for tree elaboration is given in Table S2. (B) The tree was constructed using 28 out of 31 markers from universal protein families [35]. The concatenated proteins were aligned with MAFFT (L-INS-i option) [19, 20] and the unreliable regions were masked with GBLOCKS [39, 40].

The concatenated protein alignment was used to select the LG+G+I as the best model, according to BIC and AIC model selection [30, 43], using the ProtTEST 3 Tool [41]. The scale bar represents 0.07 amino acid changes per site. The list of COGs used for tree elaboration is given in Table S3. (C) Phylogenetic tree of 9 housekeeping genes. The tree was constructed, in base to the MAFTT alignment (L-INS-i option) [19, 20], with GTR+G+I model predicted by jModelTest [29](according to BIC and AIC model selection [30, 43]). Boot strap values (1000 replicates) [31] are represented as statistically supported percentage values. The scale bar represents 0.2 nucleotide changes per site. The list of housekeeping genes used for tree elaboration is given in Table S4.

Non-standard abbreviations

COGs	clusters of orthologous groups of proteins
ANI	average nucleotide identity
ANIb	average nucleotide identity based on BLAST
Tetra	tetra-nucleotide signatures
*is*DDH	*in silico* DNA-DNA hybridization,
BLASTN	Basic Local Alignment Search Tool
MAFFT	Multiple Alignment using Fast Fourier Transform
NIB	Nucleotide Identity by Blast
RAST	Rapid Annotation of Microbial genomes using Subsystems Technology

Supplementary Files

Genome-based reclassification of two extremely acidophilic, iron- and sulfur-oxidizing halophiles *Acidihalobacter prosperus* strain V6 and *'Acidihalobacter ferrooxidans'* strain V8 as two new species, *Acidihalobacter aeolianus*^{T} sp. nov., and *Acidihalobacter vulcanensis*^{T} sp. nov., respectively.

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Table S1. List of microorganisms used in this study and their NCBI accession numbers

Organism	**NCBI Accession (FTP)**
'Acidihalobacter ferrooxidans' DSM 14175 (strain V8)	CP019434.1
Acidihalobacter prosperus DSM 5130^{T}	JQSG00000000.2
Acidihalobacter prosperus DSM 14174 (strain V6)	CP017448.1
Halothiobacillus neopolitanus C2 ATCC 23641	CP001801.1
Alkalimnicola ehrlichii ATCC BAA-1101/DSM 17681 (strain MLHE1)	CP000453.1
Ectothiorhodospira haloalkaliphila ATCC 51935	KK214995.1
Ectothiorhodospira marina DSM 241	FOAA01000040.1
Ectothiorhodospira mobilis DSM 4180	FOU001000035.1
Ectothiorhodospira magna DSM 22250 (strain B7-7)	FOFG001000058.1
Halorhodospira halochloris DSM 1059	CP007268.1
Halorhodospira halophila DSM 244	CP000544.1
Thioalkalivibrio denitrificans DSM 13742 (strain ALJD)	MVBK00000000.1
Thioalkalivibrio nitratirereducens DSM 14787	CP003989.2
Thiorhodospira sibirica ATCC 700588	AGFD01000186.1

Table S2. List of COG families used for the construction of the multiprotein concatenated ribosomal phylogenomic tree.

1. **COG0048** Ribosomal protein S12
2. **COG0049** Ribosomal protein S7
3. **COG0051** Ribosomal protein S10
4. **COG0052** Ribosomal protein S2
5. **COG0080** Ribosomal protein L11
6. **COG0081** Ribosomal protein L1
7. **COG0087** Ribosomal protein L3
8. **COG0088** Ribosomal protein L4
9. **COG0089** Ribosomal protein L23
10. **COG0090** Ribosomal protein L2
11. **COG0091** Ribosomal protein L22
12. **COG0092** Ribosomal protein S3
13. **COG0093** Ribosomal protein L14
14. **COG0094** Ribosomal protein L5
15. **COG0096** Ribosomal protein S8
16. **COG0097** Ribosomal protein L6P/L9E
17. **COG0098** Ribosomal protein S5
18. **COG0099** Ribosomal protein S13
19. **COG0100** Ribosomal protein S11
20. **COG0102** Ribosomal protein L13
21. **COG0103** Ribosomal protein S9
22. **COG0184** Ribosomal protein S15P/S13E
23. **COG0185** Ribosomal protein S19
24. **COG0186** Ribosomal protein S17
25. **COG0198** Ribosomal protein L24
26. **COG0199** Ribosomal protein S14
27. **COG0200** Ribosomal protein L15
28. **COG0244** Ribosomal protein L10
29. **COG0255** Ribosomal protein L29
30. **COG0256** Ribosomal protein L18
31. **COG0522** Ribosomal protein S4 and related proteins

32. **COG1358** Ribosomal protein HS6-type (S12/L30/L7a)
33. **COG1841** Ribosomal protein L30/L7E
34. **COG2058** Ribosomal protein L12E/L44/L45/RPP1/RPP2

Table S3. List of COG families used for the construction of the multiprotein concatenated Ciccarelli phylogenomic tree.

1. **COG0012** Ribosome-binding ATPase YchF, GTP1/OBG family
2. **COG0016** Phenylaalanyl-tRNA synthetase alpha subunit
3. **COG0048** Ribosomal protein S12
4. **COG0049** Ribosomal protein S7
5. **COG0052** Ribosomal protein S2
6. **COG0080** Ribosomal protein L11
7. **COG0081** Ribosomal protein L1
8. **COG0087** Ribosomal protein L3
9. **COG0091** Ribosomal protein L22
10. **COG0092** Ribosomal protein S3
11. **COG0093** Ribosomal protein L14
12. **COG0094** Ribosomal protein L5
13. **COG0096** Ribosomal protein S8
14. **COG0097** Ribosomal protein L6P/L9E
15. **COG0098** Ribosomal protein S5
16. **COG0099** Ribosomal protein S13
17. **COG0100** Ribosomal protein S11
18. **COG0102** Ribosomal protein L13
19. **COG0103** Ribosomal protein S9
20. **COG0172** Seryl-tRNA synthetase
21. **COG0184** Ribosomal protein S15P/S13E
22. **COG0186** Ribosomal protein S17
23. **COG0197** Ribosomal protein L16/L10AE
24. **COG0200** Ribosomal protein L15
25. **COG0201** Preprotein translocase subunit SecY
26. **COG0202** DNA-directed RNA polymerase, alpha subunit/40 kD subunit
27. **COG0256** Ribosomal protein L18
28. **COG0495** Leucyl-tRNA synthetase
29. **COG0522** Ribosomal protein S4 or related protein
30. **COG0525** Valyl-tRNA synthetase
31. **COG0533** tRNA A37 threonylcarbamoyltransferase TsaD

Supplementary Table S4. List of housekeeping genes used for the construction of the multi-gene phylogenetic tree.

1. **ArgS** Arginyl-tRNA synthetase
2. **dnaN** DNA polymerase III subunit beta
3. **dnaQ** DNA polymerase III subunit epsilon
4. **Era** GTP-binding protein Era
5. **gyrB** DNA gyrase subunit B
6. **gltA** Citrate synthase
7. **ppnK** NAD(+) kinase
8. **rpoB** RNA polymerase, beta subunit
9. **rpoD** RNA polymerase sigma factor RpoD

4.2 Genome-based reclassification of the extremely acidophilic, iron- and sulfur-oxidizing halophile *Acidihalobacter prosperus* strain F5 as a new species, *Acidihalobacter yilgarnensis* sp. nov.

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Abstract

The taxonomic position of the halotolerant acidophile *Acidihalobacter prosperus* strain F5, was clarified though phylogenomic analysis of its recently released high quality, complete genome sequence. Comparative 16S rRNA gene sequence analysis identified the strain as belonging to the species *Acidihalobacter prosperus*. However, to achieve finer phylogenetic resolution, further sequence based multiprotein phylogenetic analysis using clusters of orthologous groups (COGS) from ribosomal protein families as well as those from complete sets of markers based on concatenated alignments of universal protein families was undertaken. Furthermore, average nucleotide identity (ANI) analysis, further reinforced by the correlation indices of tetra-nucleotide signatures (Tetra), provided a non-sequence based approach for species circumscription. The different approaches undertaken in this

study resulted in a tree that is phylogentically congruent, revealing that the strain F5 was a new species within the *Acidihalobacter* genus. In accordance, it is proposed that *A. prosperus* strain F5 be formally reclassified as '*Acidihalobacter yilgarnensis*' strain F5^T sp. nov,. and that it represents the type strain of its species.

In regions like Australia and Chile, freshwater is a scarce and expensive commodity, and the use of seawater for mining in place of potable water would reduce the cost of mineral processing by bioleaching. Only a limited number of microorganisms have been isolated that are capable of tolerating the low pH and high salinity, and hence the application of seawater or brackish water in bioleaching is not currently practiced. Halotolerant acidophiles are generally isolated and enriched from environmental samples that exhibit both acidity and salinity, and only few niche environments exist across the world that can be targeted for halotoletant, acidophilic biomining microorganisms. Furthermore, most known halotolerant iron- and sulfur-oxidizing acidophiles are not able to concurrently tolerate copper, and therefore cannot be used in the bioleaching of the copper-containing ores, such as chalcopyrite (1). The discovery and characterization of new species of halotolerant acidophiles that are able to bioleach copper from chalcopyrite would be of great interest to the mining industry. Exploration of the metabolic capabilities of these unique microorganisms will further provide understanding of the mechanisms that allow these halotolerant acidophilic microorganisms to endure high osmotic stress and high chloride levels, and help to elucidate the role of chloride-tolerant iron-oxidizers in industrial bioleaching operations.

The genus *Acidihalobacter* (*A*.) currently consists of three species of iron- and sulfur-oxidizing, mesophilic, halotolerant acidophiles including *A. prosperus* strain DSM 5130^T (2), *A. prosperus* DSM 14174 strain V6 and '*A. ferrooxidans*' DSM 14175 strain V8, for which high quality draft genomes have been released (3, 4). The genome of *A. prosperus* strain F5 was also recently released (GenBank accession CP017415.1). *A. prosperus* strain F5 is a mesophilic, iron- and sulfur-oxidizing, halotolerant acidophile that has shown the ability to oxidize chalcopyrite while tolerating high acid and saline stress, making it relevant for the saline water bioleaching of copper from chalcopyrite (5). It is also the first species of the *Acidihalobacter* genus to have a complete genome sequence. The availability of the high quality genome has provided an opportunity to properly classify the species using phylogenomic strategies to re-evaluate its taxonomical position (6).

In order to infer primary phylogenetic relationships, the 16S rRNA sequence similarity of *A. prosperus* strain F5 was compared to *A. prosperus* strain DSM 5130^T , *A. prosperus* DSM 14174 strain V6 and '*A. ferrooxidans*' DSM 14175 strain V8 and by aligning the sequences using the default settings of the nucleotide Basic Local Alignment Search Tool (BLASTN) tool (7). Previous studies have suggested that 98.65 % 16S rRNA gene sequence similarity can be used as a threshold for differentiating two species (8). *A. prosperus* strain F5 was found to be 99 % similar in 16S rRNA sequence to the *A. prosperus* strain DSM 5130^T, 98% similar to *A. prosperus* DSM 14174 strain V6 and 96 % similar to '*A. ferrooxidans*' DSM 14175 strain V8.

A total of 21 genomes of taxonomically related strains, including the members of the *Acidihalobacter* genus, were selected from the prokaryotic 16S rRNA database of National Centre for Biotechnology Information (NCBI) and verified by comparison with nucleotide databases after running a BLASTN-based script using an E-value threshold of 1e^{-5} and the databases GREENGENES (9), RDP (10) and SILVA (11). These sequences were used to generate a 16S rRNA sequence-based phylogenetic tree . The Tamura-Nei model (12) was chosen as the most appropriate model for inference of 16S rRNA phylogeny based on jModelTest (13, 14). Alignment was performed in MAFFT (Version 7) with the L-INS-I iterative refinement (15, 16). A maximum likelihood tree was constructed with PhyML (Version 3) using the bootstrap method with 1,000 replicates (17). Tree reconstruction included the use of discrete Gamma distribution and allowing some sites to be evolutionarily invariable (G+I+). The concise, final tree, consisting of the 21 selected strains from the genera *Acidihalobacter* and *Ectothiorhodospira* is shown in Figure S1.

Several studies have shown that the 16S rRNA gene lacks resolution for discrimination between closely related bacterial species (Santos and Ochman, 2004; Staley, 2006; Gao et al., 2016). Therefore, taxonomic classification of *A. prosperus* strain F5 was further clarified through multiple locus phylogenetic analysis using two different phylogenomic approaches based on sets of clusters of orthologous groups (COGs) (18). For each microorganism to be included in the analysis, the complete sets of 43 COG markers (Tables S1 and S2) 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). 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}. An in-house (Center for Bioinformatics and Genome Biology,

Fundacion Ciencia y Vida and Facultad de Ciencias Biologicas, Universidad Andres Bello, Santiago, Chile) Bioperl script was used to assess the association of each protein-coding sequence to a COG category based on the highest hit coverage value.

A multilocus phylogenomic tree was constructed for the 21 strains found to be taxonomically closest to the *Acidihalobacter* genus by performing a multiple alignment of concatenated sequences of 30 COGs from 34 ribosomal protein families that are universally conserved in the three domains of cellular life (19). 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 (20). For both trees, the alignment of the concatenated sequences was made using the L-INS-I Iterative refinement in MAFFT (Version 7) (15, 16). The alignments were masked to remove unreliable regions with GBLOCKS (21, 22), followed by a concatenation of all protein families. The substitution model for concatenated alignment was predicted using ProtTEST 3 Tool (14, 23). Maximum likelihood trees were prepared for concatenated alignments with PhyML (Version 3) using bootstrap method with 1000 replicates (17). A list of the NCBI accession numbers for the genomes, including their taxonomical data, and a list of the COG families used in the elaboration of the multiprotein trees are provided in Tables S1, S2 and S3. The resulting multiprotein phylogenomic tree is shown in Figure 1.

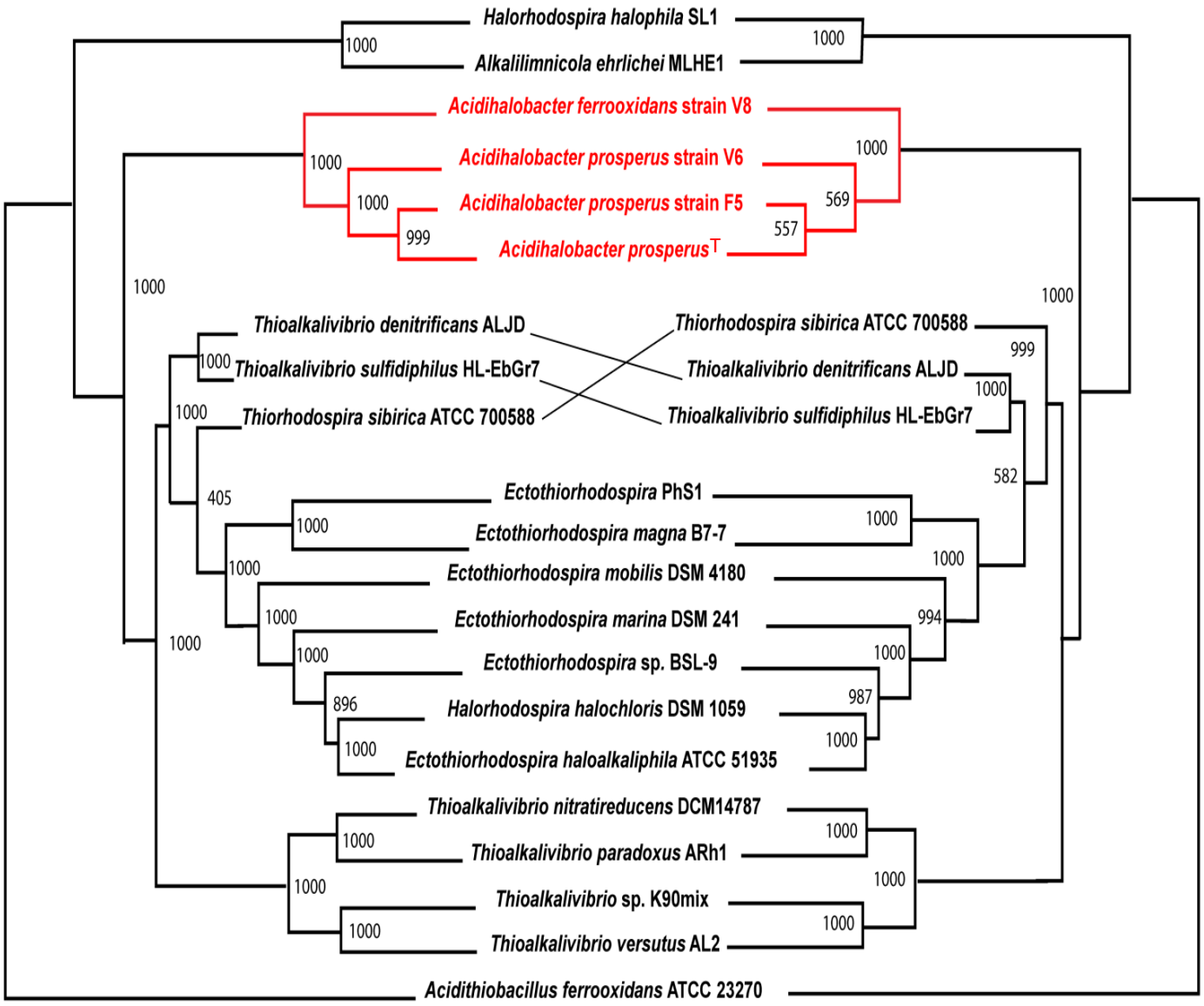

Figure. 1. Phylogenetic tree of *A. prosperus* strain F5 with selected genetic relatives based upon multi-locus concatenation of 30 out of 34 universal ribosomal proteins (19) and 28 out of 31 markers from universal protein families (20). Statistically supported bootstrap values are given at the nodes. The tree for universal ribosomal proteins with corresponding bootstrap values is shown on the left and that on the right is for conserved universal proteins. The scale bar represents 0.2 amino acid changes per site. The list of COGs used for tree elaboration are given in Tables S2 and S3.

To further reinforce the results of the sequence based phylogenomic approaches, a nonsequence based approach was undertaken in order to analyze the interspecies boundaries

between the genomes. Average nucleotide identity (ANI) and the correlation indices of tetra-nucleotide signatures (Tetra) (24) provide numerical circumscription of prokaryotic species by using an objective boundary (24). JspeciesWS (http://jspecies.ribohost.com/jspeciesws/#Analyse) was used to calculate the average nucleotide identity based on BLAST (ANIb) and the correlation indexes of tetra-nucleotide signatures (Tetra) (25). Based on the recommended cut-off values for species determination (<95 % for ANIb and <0.989 for Tetra) (8, 24, 26, 27), the results provided evidence that *A. prosperus* strain F5 is not a strain of *A. prosperus* but rather, belongs to a separate and novel species of the genus *Acidihalobacter.* A list of 16S rRNA sequence identity, ANI and Tetra values obtained from the comparison of *A. prosperus* strain F5 against other members of the *Acidihalobacter* genus is provided in Table 1. The different features of the four species of the *Acidihalobacter* genus are compared in Table 2.

Table 1. Comparison of average nucleotide identity (ANI) (%), percentage of aligned nucleotides, as determined using ANI (%) (PAN), tetra-nucleotide signature correlation index (TET) and nucleotide identity by BLAST (%) of *A. prosperus strain* F5, the type strain *A. prosperus* DSM 5130^T*, A. prosperus* strain V6, and '*A. ferrooxidans*' strain V8 and,.

	'A. prosperus' strain F5				*A. prosperus* DSM 5130^T				*'A. prosperus'* strain V6				*'A. ferrooxidans'* strain V8			
Query	ANI	PAN	TET	NIB	ANI	PAN	TET	NIB	ANI	PAN	TET	NIB	ANI	PAN	TET	NIB
A. prosperus strain F5					79.06	73.67	0.92	99.00	75.91	64.70	0.93	98.00	70.74	40.16	0.92	96.00
A. prosperus DSM 5130^T	79.22	76.57	0.92	99.00					80.45	70.75	0.99	99.00	72.16	43.14	0.92	97.00
A. prosperus strain V6	75.82	64.49	0.93	99.00	80.55	67.44	0.99	99.00					72.22	40.97	0.92	97.00
A. ferrooxidans strain V8	70.57	43.08	0.92	96.00	72.21	43.98	0.92	97.00	72.28	42.62	0.91	97.00				

Table 2. Comparison of genomic and phenotypic features of the four species of the *Acidihalobacter* genus.

Feature	*A. prosperus* (Strain F5)	*A. prosperus* DSM 5130^T	*A. prosperus* (Strain V6)	*'A. ferrooxidans'* (Strain V8)
Genome size (bp)	3566941	3359675	3363634	3448835
GC content (mol%)	59.9	64.5	62.2	61.6
Coding DNA sequence (CDS)	3,233	3,088	3,194	3,089
Plasmid	Not present	Not present	162,484 bp	Not present
tRNA genes	47	48	46	45
Optimum pH for growth	2.5	2.5	2.0	2.0
Optimum temperature for growth (˚C)	35	35	35	35

In conclusion, results of the different phylogenomic approaches undertaken in this study have shown phylogenetic congruence through both sequence based and nonsequence based techniques and have highlighted that *A. prosperus* strain F5 is a novel species. We, therefore, propose the reclassification and renaming of *A. prosperus* strain F5 to the type strain of '*Acidihalobacter yilgarnensis*' sp. nov.

DESCRIPTION OF ACIDIHALOBACTER YILGARNENSIS SP. NOV.

Acidihalobacter yilgarnensis (yil.garn.ensis. L. neut. noun. referring to its isolation from the Yilgarn Crater, Western Australia).

Previously known as *Acidihalobacter prosperus* strain F5. An updated description of this organism is given. Cells are motile, Gram negative rods. The species is extremely acidophilic, optimum pH is 2.5. Halophilic, growth occurs at up to 1.27 M chloride ion. Mesophilic, with optimal growth occuring at 35 °C. Chemolithoautotrophic growth occurs with soluble ferrous (ferrous sulfate heptahydrate) and reduced sulfur (tetrathionate) sources as well as on minerals, such as pyrite, pentlandite and chalcopyrite under aerobic conditions. The type strain was isolated from an acidic saline drain of Yilgarn Crater, Western Australia.

The whole-genome sequence of the 3,566,941 bp chromosome is available (GenBank accession no. CP017415.1). The G+C content of the DNA of the type strain genome is 59.9 mol%.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this artide.

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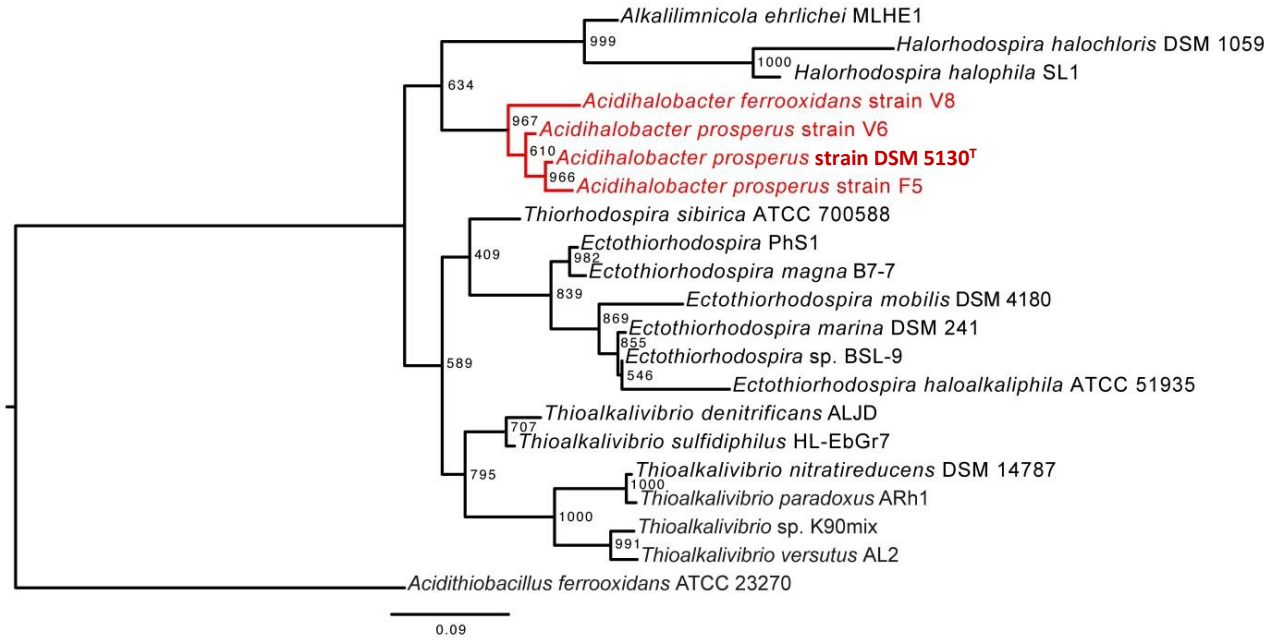
Supplementary Files

Supplementary Figure S1. 16S rRNA phylogenetic tree of the *Acidihalobacter* species together with their closest relatives.



Supplementary Figure S1. The phylogenetic position of the members of the *Acidihalobacter* genus based on their 16S rRNA genes. Statistically supported bootstrap values are labelled at the nodes. The scale bar represents 0.09 changes per site.

Supplementary Table S1. List of microorganisms used in this study and their NCBI accession numbers

Organism	NCBI Accession (FTP)
Acidihalobacter prosperus strain F5	CP017415.1
'Acidihalobacter ferrooxidans' DSM 14175 (strain V8)	CP019434.1
Acidihalobacter prosperus DSM 5130^T	JQSG00000000.2
Acidihalobacter prosperus DSM 14174 (strain V6)	CP017448.1
Acidithiobacillus ferrooxidans ATCC 23270^T	CP001219.1
Alkalimnicola ehrlichii MLHE1	CP000453.1
Ectothiorhodospira haloalkaliphila ATCC 51935	KK214995.1
Ectothiorhodospira marina DSM 241	FOAA01000040.1
Ectothiorhodospira mobilis DSM 4180	FOU001000035.1
Ectothiorhodospira PhS1	AGBG0100114.1
Ectothiorhodospira BSL 9	CP011994.1
Ectothiorhodospira magna B7-7	FOFG001000058.1
Halorhodospira halochloris DSM 1059	CP007268.1
Halorhodospira halophila SL-1	CP000544.1
Thioalkalivibrio denitrificans ALJD	MVBK00000000.1
Thioalkalivibrio nitratirereducens DSM 14787	CP003989.2
Thioalkalivibrio paradoxus ARh1	CP007029.1
Thioalkalivibrio K90mix	CP001906.1
Thioalkalivibrio sulfidiphilus HL EbGR7	CP001339.1
Thioalkalivibrio versustus AL2	MVAR01000001.1
Thiorhodospira sibirica ATCC 700588	AGFD01000186.1

Supplementary Table S2. List of clusters of orthologous group (COG) families used for the construction of the multiprotein concatenated ribosomal phylogenomic tree.

35. **COG0048** Ribosomal protein S12
36. **COG0049** Ribosomal protein S7
37. **COG0051** Ribosomal protein S10
38. **COG0052** Ribosomal protein S2
39. **COG0080** Ribosomal protein L11
40. **COG0081** Ribosomal protein L1
41. **COG0087** Ribosomal protein L3
42. **COG0088** Ribosomal protein L4
43. **COG0089** Ribosomal protein L23
44. **COG0090** Ribosomal protein L2
45. **COG0091** Ribosomal protein L22
46. **COG0092** Ribosomal protein S3
47. **COG0093** Ribosomal protein L14
48. **COG0094** Ribosomal protein L5
49. **COG0096** Ribosomal protein S8
50. **COG0097** Ribosomal protein L6P/L9E
51. **COG0098** Ribosomal protein S5
52. **COG0099** Ribosomal protein S13
53. **COG0100** Ribosomal protein S11
54. **COG0102** Ribosomal protein L13
55. **COG0103** Ribosomal protein S9
56. **COG0184** Ribosomal protein S15P/S13E
57. **COG0185** Ribosomal protein S19
58. **COG0186** Ribosomal protein S17
59. **COG0198** Ribosomal protein L24
60. **COG0199** Ribosomal protein S14
61. **COG0200** Ribosomal protein L15
62. **COG0244** Ribosomal protein L10
63. **COG0255** Ribosomal protein L29
64. **COG0256** Ribosomal protein L18

65. **COG0522** Ribosomal protein S4 and related proteins
66. **COG1358** Ribosomal protein HS6-type (S12/L30/L7a)
67. **COG1841** Ribosomal protein L30/L7E
68. **COG2058** Ribosomal protein L12E/L44/L45/RPP1/RPP2

Supplementary Table S3. List of clusters of orthologous group (COG) families used for the construction of the multiprotein concatenated Ciccarelli phylogenomic tree.

32. **COG0012** Ribosome-binding ATPase YchF, GTP1/OBG family
33. **COG0016** Phenylaalanyl-tRNA synthetase alpha subunit
34. **COG0048** Ribosomal protein S12
35. **COG0049** Ribosomal protein S7
36. **COG0052** Ribosomal protein S2
37. **COG0080** Ribosomal protein L11
38. **COG0081** Ribosomal protein L1
39. **COG0087** Ribosomal protein L3
40. **COG0091** Ribosomal protein L22
41. **COG0092** Ribosomal protein S3
42. **COG0093** Ribosomal protein L14
43. **COG0094** Ribosomal protein L5
44. **COG0096** Ribosomal protein S8
45. **COG0097** Ribosomal protein L6P/L9E
46. **COG0098** Ribosomal protein S5
47. **COG0099** Ribosomal protein S13
48. **COG0100** Ribosomal protein S11
49. **COG0102** Ribosomal protein L13
50. **COG0103** Ribosomal protein S9
51. **COG0172** Seryl-tRNA synthetase
52. **COG0184** Ribosomal protein S15P/S13E
53. **COG0186** Ribosomal protein S17
54. **COG0197** Ribosomal protein L16/L10AE
55. **COG0200** Ribosomal protein L15
56. **COG0201** Preprotein translocase subunit SecY
57. **COG0202** DNA-directed RNA polymerase, alpha subunit/40 kD subunit
58. **COG0256** Ribosomal protein L18
59. **COG0495** Leucyl-tRNA synthetase
60. **COG0522** Ribosomal protein S4 or related protein
61. **COG0525** Valyl-tRNA synthetase
62. **COG0533** tRNA A37 threonylcarbamoyltransferase TsaD

5 Chapter 5

Khaleque HN, Kaksonen AH, Boxall NJ, Watkin ELJ. Metabolic capabilities and adaptive mechanisms of the members of the *Acidihalobacter* genus: A comparison of four *Acidihalobacter* genomes.

Manuscript is prepared for submission.

*****Please note that in this chapter I have adopted the names allocated in Chapter 4 for the members of the *Acidihalobacter* genus. (*Ac. prosperus* DSM 14174 (strain V6) is *'Ac. aeolianus'* DSM 14174^{T} (strain V6); *'Ac. ferrooxidans'* DSM 14175 is *'Ac. vulcanensis'* DSM 14175^{T}; *Ac. prosperus* strain F5 is *'Ac. yilgarnensis' strain F5*^{T})

Metabolic capabilities and adaptive mechanisms of the members of the *Acidihalobacter* genus: A comparison of four *Acidihalobacter* genomes

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Abstract

Members of the genus *Acidihalobacter* are iron- and sulfur-oxidizing, halotolerant acidophiles that have the potential to bioleach ores with brackish or saline process waters. The genus consists of four species, *Ac. prosperus* DSM 5130^T, '*Ac. aeolianus*' DSM 14174 (strain V6), '*Ac. yilgarnensis*' (strain F5; culture collection identifier pending) and '*Ac. vulcanensis*' DSM 14175 (strain V8). In order to provide a comprehensive description of the metabolic capabilities and adaptive stress defence mechanisms used by the different species, an in depth analysis of their genomes was undertaken. The prediction of nutrient assimilation pathways from all four species suggested that they maintain a chemolithoautotrophic lifestyle and are able to metabolize carbon dioxide, bicarbonate, ammonium, urea, sulfate and phosphate sources for biomass production. They obtain energy from the oxidation of iron and sulfur, and show differences in their ability to use these substrates for energy acquisition. All four species show versatility in their survival mechanisms used to overcome acid, osmotic, metal and oxidative stresses. Each member of the *Acidihalobacter* genus has shown genes for unique metabolic capabilities that separate them from each other as well as from other previously studied acidophiles.

5.1 Introduction

The most extensively studied acidophiles are those that oxidize iron and/or sulfur for the catalytic dissolution of minerals in low pH environments (1). These microorganisms can be used in biomining, , i.e. for the economic extraction of metals from low grade ores, which are otherwise too expensive to process through traditional mining processes such as smelting or roasting (2). In regions like Western Australia and Chile, groundwater is often limited, and seawater may be used for bioleaching operations. However, a high concentration of chloride ion in process waters and ores inhibits the growth of conventional acidophiles, increasing the bioleaching process time. Desalination can be used to remove chloride ion but this is prohibitively expensive (3-5).

The discovery and characterization of halophilic acidophiles is important to the mining industry due to the ability of these unique microorganisms to leach base metals from high-salt ores (4, 6). Genome sequencing is a significant first step in characterising a new organism as it provides critical genetic information required to elucidate biochemical pathways underpinning its metabolic capabilities. Several acidophiles have been sequenced and comparative genomics has shed light on their metabolic processes. However, until recently, *Ac. prosperus* DSM 5130^T was the only known halotolerant acidophile with a complete genome sequence available (7). In this work, comparative genomics analysis of the *Ac. prosperus* DSM 5130^T and three new species of *Acidihalobacter* genus (‘*Ac. aeolianus’* DSM 14174 strain V6, ‘*Ac. vulcanensis’* DSM 14175 strain V8, ‘*Ac. yilgarnensis’* strain F5) was used to enhance the understanding of the metabolic features and stress tolerance mechanisms these acidophiles employ to tolerate high chloride concentrations (see Chapter 3 and 4).

5.2 Methods and Materials

5.2.1 Cultivation of the microorganisms used in the study

Growth medium for ‘*Ac. aeolianus’* DSM 14174 (strain V6) and *‘Ac. vulcanensis’* DSM 14175 (strain V8) was basal salts medium (in g/L Milli-Q H_2O: 0.4, $(NH_4)_2SO_4$; 0.5, $MgSO_4 \cdot 7H_2O$; and 0.2, K_2HPO_4 acidified to pH 2.0 with concentrated H_2SO_4) and sterilized by autoclaving. The medium was supplemented with soluble ferrous iron (13.9 g/L $FeSO_4 \cdot 7H_2O$), potassium tetrathionate (1.51 g/L $K_2S_4O_6$) and 1 mL/L of a trace element solution (mg/L Milli-Q H_2O;

$MnCl_2 \cdot 2H_2O$, 62; $ZnSO_4 \cdot 7H_2O$, 68; $CoCl_2 \cdot 6H_2O$, 64; H_3BO_3, 30; Na_2MoO_4, 10; $CuCl_2 \cdot 2H_2O$, 66; $NaVO_3$), dissolved in the basal salts medium and filter sterilized through 0.2 µm filter (Millipore). Chloride ion (15 g/L) was provided as sodium chloride. The cultures were maintained at 30 ˚C with shaking at 100 rpm.

The pure isolate of '*Ac. yilgarnensis*' strain F5 was maintained in modified basal salts medium (in g/L Milli-Q H_2O: 0.4 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4 \cdot 7H_2O$, and 0.4 g/L, KH_2PO_4; pH 2.5 with concentrated H_2SO_4) and sterilized by autoclaving. The medium was supplemented with soluble ferrous iron (13. 9 g/L $FeSO_4 \cdot 7H_2O$), potassium tetrathionate (1.51 g/L $K_2S_4O_6$) and trace element solution (as above). Chloride ion (15 g/L) was provided as sodium chloride. The culture was maintained at 30 ˚C with shaking at 100 rpm at (8).

5.2.2 Genome sequencing, assembly, annotation and comparisons

Genomic DNA was isolated using the method of DNA extraction from acidophiles as previously described by Zammit, Mutch (9). Genome sequencing and assembly were performed as previously described by Ossandon, Cárdenas (7), Khaleque, Ramsay (10) and Khaleque, Ramsay (11).

The genomes of *Ac. prosperus* DSM 5130^T, *Ac. aeolianus* DSM 14174 (strain V6), *Ac. vulcanensis* DSM 14175 (strain V8) and '*Ac. yilgarnensis*' strain F5 were downloaded from the NCBI ftp site (ftp://ftp.ncbi.nlm.nih.gov/). For the purpose of this comparison, the genomes were annotated using the Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org/) using the ClassicRAST annotation scheme (12). Comparisons were performed using the SEED and RAST servers, and Geneious v.8.1.8 bioinformatic software (13). The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for verification of metabolic pathways (http://www.genome.jp/kegg/).

5.3 Results and Discussion

5.3.1 Phylogeny and genome properties

Phylogenetic and taxonomic classification of the members of the *Acidihalobacter* spp. has been undertaken (Chapter 4). The results have shown that the members belong to different species of the *Acidihalobacter* genus. Provisional names have been given to the species, where *Ac. prosperus* DSM 14174 is now '*Ac. aeolianus*' DSM 14174 strain V6, '*Ac.*

ferrooxidans' DSM 14175 is '*Ac. vulcanensis*' DSM 14175 strain V8 and *Ac. prosperus* strain F5 is '*Ac. yilgarnensis*' strain F5 (pending allocation of DSM accession).

The salient genomic features of the genomes used in the study are presented in Table 1.

Table 1. Genomic features of the members of the *Acidihalobacter* genus

Feature	***'Ac. yilgarnensis'* strain F5**	***Ac. prosperus* strain DSM 5130^T**	***'Ac. aeolianus'* DSM 14174 strain V6**	***'Ac. vulcanensis'* DSM 14175 strain V8**
Genome size (bp)	3,566,941	3,359,675	3,363,634	3,448,835
GC content (mol%)	59.9	64.5	62.2	61.6
Coding DNA sequence (CDS)	3,233	3,088	3,194	3,089
Plasmid	Not present	Not present	162,484 bp	Not present
tRNA genes	47	48	46	45

5.3.2 Nutrient assimilation and biomass production

5.3.2.1 Carbon Metabolism

5.3.2.1.1 Carbon dioxide fixation

Members of the *Acidihalobacter* genus have previously been described as chemolithoautrophic (7, 10, 11). The genomes of *Ac. prosperus* DSM 5130^T, *Ac. aeolianus* DSM 14174 (strain V6), *Ac. vulcanensis* DSM 14175 (strain V8) and '*Ac. yilgarnensis'* strain F5 were found to contain a repertoire of genes involved in carbon dioxide fixation via the Calvin-Benson-Bassham (CBB) cycle (7, 10, 11). All four genomes contain one copy of *cbbS* and *cbbL*, encoding the small and the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), respectively. They are located in a gene cluster with

other CBB cycle enzymes such as phosophoribulokinase and transketolase. The primary product of carbon fixation via the CBB cycle is 3-phosphoglycerate, which is predicted to be further metabolise into polymer precursors for bacterial biomass through the central carbon metabolism pathways, as has been described for *Ferrovum* JA-12 (14).

The presence of genes involved in carboxysome formation has also been detected in all four species of *Acidihalobacter*. The carboxysome is a carbon concentrating mechanism made up of polyhedral protein shells (15). It is thought to be involved in improving the efficiency of carbon dioxide fixation by RuBisCO and allowing the utilisation of bicarbonate as carbon source (16, 17). According to current models bicarbonate from the cytosol of the bacterial cell enters the carboxysome by crossing the carboxysome protein shell, where it is then dehydrated by carbonic anhydrase to form CO_2. This is then utilized by RuBisCO before it can escape from the carboxysome (15). RuBisCO generally has poor catalytic activity, so the concerted action of carbonic anhydrase and RuBisCO increases the local carboxysome concentration of CO_2 in order to enhance its reaction with ribulose-1,5-bisphosphate at its active site (18). One copy each of the carboxysome shell proteins *csoS1A*, *csoS1B*, *csoS2*, *csoS3*, *csoS1D* and *csoS1D*-associated protein were found in all the strains analysed in this study. In all four genomes compared, the carboxysome genes were downstream of the *cbbS* and *cbbL* genes, as has been previously found in strains of *Thiobacillus* spp. (19). In addition, *Ac. aeolianus* strain V6, *Ac. vulcanensis* strain V8 and *Ac. yilgarnensis* strain F5 also had three copies of the carboxysome shell protein gene, *csoS1* This assembly was similar to the organization seen in the salt sensitive acidophile *Acidithiobacillus ferrooxidans* as well as the salt tolerant, sulfur oxidizing chemoautotroph *Halothiobacillus neapolitanus* (15, 20). Only one copy of the *csoS1* gene was present in *Ac. prosperus* DSM 5130. However, the organization of the genes in *Ac. prosperus* DSM 5130 was similar to the carboxysome gene cluster organization in the salt tolerant *Prochlorococcus marinus* (21). All other carboxysome gene clusters, as well as those that encode other bacterial microcompartment types, contain at least two *csoS1* (Pfam00936) homologues, although the need for these multiple paralogs is still unclear. This observation is significant because until now, *P. marinus* strain MED4 was the only other microorganism to found to show similar assembly in the *Csos1* gene, and it represents a highly efficient carbon dioxide fixing microorganism with minimal media requirements (21, 22).

A unique carboxysome shell protein *csoS1D* was also identified in all four *Acidihalobacter* genomes analysed in this study, but has not been previously detected in the genomes of characterized acidophiles. The protein has been detected in the genomes of the salt tolerant species *H. neaopolitanus* and *P. marinus* (21, 23, 24). The *csoS1D* protein has a proposed role in gated metabolite transport across the carboxysome and has a proposed role in increasing carbon dioxide fixation efficiency (ref). The presence of *csoS1D* and its associated metabolic regulator genes in all species of *Acidihalobacter* may indicate it plays a similar role in the *Acidihalobacter* species (25). As genes for carbonic anhydrase were only found on the genomes of *Ac. yilgarnensis* strain F5 and *Ac. aeolianus* strain V6 but were not present in the genomes of *Ac. vulcanensis* strain V8 and *Ac. prosperus* DSM 5130, it is likely that that the latter two species have reduced carbon dioxide fixing capabilities. It is possible that all four species increase the efficiency of their carbon dioxide fixation using the *csoS1D* protein. However, this has not been experimentally verified. The organization of the gene cluster encoding RUBISCO and carboxysome shell proteins in the members of the *Acidihalobacter* genus is shown in Figure 1.

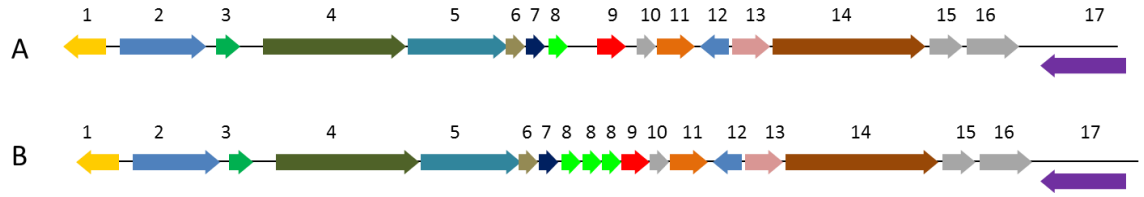


Figure 1. Organization of RUBISCO and carboxysome shell protein genes in *Ac. prosperus* DSM 5130 (A) in comparison to that of '*Ac. aeolianus*' strain V6, *'Ac. vulcanensis'* strain V8 and *'Ac. yilgarnensis'* strain F5 (B). The genes encode the following proteins: 1. RuBisCO operon transcriptional regulator CbbR; 2. RUBISCO large chain; 3. RUBISCO small chain; 4. Carboxysome shell protein CsoS2; 5. Carboxysome shell protein CsoS3; 6. Putative carboxysome peptide A; 7. Putative carboxysome peptide B; 8. Carboxysome shell protein CsoS1; 9. Bacterioferritin (cytochrome B1); 10. Hypothetical protein; 11. Chromosome (plasmid) partitioning protein ParA; 12. Hypothetical protein; 13. Rubisco activation protein CbbQ; 14. RUBISCO activation protein CbbO; 15. Carboxysome shell protein Csos1D; 16. Csos1D-associated protein; and 17. Nitrogen regulatory protein P-II.

5.3.2.1.2 The Tricarboxylic Acid (TCA) cycle

Genes for methylglyoxal metabolism, pyruvate metabolism (acetogenesis and anaplerotic reactions), interconversion between pyruvate, alanine and serine, glycolysis and gluconeogenesis, dehydrogenase complexes, the pentose phosphate pathway and glycolate - glyoxylate interconversions were identified in all four of the *Acidihalobacter* genomes analysed. Here, we limit the discussion of central carbohydrate metabolism to the tricarboxylic acid (TCA) cycle as the presence of incomplete TCA cycles in acidophiles has been reported to be a hallmark of obligate chemolithotrophy in these microorganisms (26)

The TCA cycle is a central pathway in cellular metabolism. The repertoire of TCA cycle enzymes in an organism is indicative of its lifestyle (27). Previous studies on *A. ferrooxidans* ATCC 23270 and other obligate chemolithotrophic acidophiles revealed that they have incomplete TCA cycles, often lacking the enzyme 2-oxoglutarate dehydrogenase (26). However, the genomes of the members of the *Acidihalobacter* genus were all found to encode all enzymes of the TCA cycle including the 2-oxoglutarate dehydrogenase, similar to *Ferrovum* strain JA-12 (14). This enzyme is often found in genomes of facultative heterotrophs, however all characterised members of the *Acidihalobacter* genus are chemolithoautotrophic. The complete genes of the TCA cycle have previously been found in the genomes of other neutrophilic iron oxidisers among the *Betaproteobacteria* including *Sideroxydans lithotrophicus* ES-1, *Gallionella capsiferriformans* ES-2 and *Thiobacillus denitrificans* ATCC 25259 (28, 29).

5.3.2.2 Nitrogen metabolism

5.3.2.2.1 Nitrogen Fixation

All of the species of the *Acidihalobacter* genus appear to be able to assimilate nitrogen from various sources including ammonium, nitrate and urea, but genome comparisons suggested differences in the ability of each species to fix atmospheric nitrogen. *Ac. prosperus* strain DSM 5130^{T} and '*Ac. aeolianus'* DSM 14174 strain V6 carried genes for the standard FeMO-cofactor nitrogenase complex and the nitrogenase and *nif* genes, required for fixing nitrogen for the atmosphere and were similar to those described in *A. ferrooxidans* (7, 11, 30). Genes for fixing atmospheric nitrogen were not present in '*Ac. vulcanensis'* strain V8 and *'Ac. yilgarnensis'* strain F5 and there was also substantial variation in the organisation of nitrogen assimilation and fixation genes amongst the *Acidihalobacter* genomes (10, 31). The capacity for nitrogen fixation in the acidophilic *Leptospirillum* genus has previously been

implicated as an important ecological factor because it provides the first source of fixed nitrogen in microbial communities such as in acid mine drainage and acid natural ecosystems (32). Therefore, the ability of *Acidihalobacter prosperus* DSM 5130 and *Ac. aeolianus* strain V6 to fix atmospheric nitrogen may also play an important role in providing nitrogen in their habitats.

5.3.2.2.2 Ammonium uptake and utilization

Ammonium transporters are present in the genomes of all four species of *Acidihalobacter*, as are genes coding for glutamine synthetase. Glutamine synthetase likely allows these species to incorporate transported ammonium into glutamate, making it available for production of biomass, as previously described for the iron oxidizing *Ferrovum* strain JA12 (14).

5.3.2.2.3 Nitrate reduction

Gene clusters predicted to encode transporters and reductases for nitrate and nitrite were identified in each of the members of the *Acidihalobacter* genus, indicating they can reduce nitrate to ammonia. All strains have the assimilatory nitrate reductase subunit genes and the nitrate reductase NAD(P) large and small subunit genes, which together enable the utilisation of nitrate as nitrogen source (33). Furthermore, F5 also has an ABC type nitrate/sulfonate/bicarbonate transport system, ATPase component and the respiratory nitrate reductase alpha, beta, delta and gamma subunits. This suggests that in this strain, nitrate may also serve as an alternative terminal electron acceptor (see Energy Metabolism).

5.3.2.2.4 Urea metabolism

All four species of *Acidihalobacter* were found to carry the urease alpha, beta and gamma units as well as the accessory proteins *ureDEFG* thought to be involved in the assembly of catalytically active urease (34). The urea transporter cluster *urtBCDE* was present only in '*A. vulcanensis'* strain V8. Urease hydrolyses urea to ammonia and bicarbonate in a number of organisms and may provide an alternative nitrogen source (14, 35). The presence and physiological relevance of the urease encoding gene cluster is known for the phototrophic iron oxidising *Alphaproteobacteria Rhodopseudomonas palustris* and *Rhodobacter capsulatus* (34-36). With exception of the draft genome sequences of the *"Ferrovum"*-like strain FBK7 and *Ferrovum* strain JA12, a gene cluster encoding probable urease genes has

not been detected in any other acidophilic iron oxidising *Betaproteobacteria* nor genomes of any acidophilic iron oxidising *Gammaproteobacteria* or *Acidithiobacillia* (14, 37). Carbonic anhydrases associated with carboxysomes (see carbon dioxide fixation) may also facilitate the use of bicarbonate derived from urea hydrolysis. This may suggest an additional role for carbonic anhydrases identified in *'Ac. yilgarnensis'* strain F5 and '*Ac. aeolianus'* strain V6 (38). Alternatively, urease activity may also play an important role in pH homeostasis (see below, section Strategies to adapt to acidic environments, high metal loads and oxidative stress).

5.3.2.3 Phosphate assimilation

All seven genes of the phosphate (*Pho*)-regulon as described for *Escherichia coli* by Hsieh and Wanner (39) were found in the four genomes of *Acidihalobacter* that were analysed in this study. Therefore, the strains may be able to take up inorganic phosphate via a similar mechanism to *E. coli* involving the *pstSCAB* system of transporters, which is in turn regulated by the transcription factor *phoB*, the *phoR* histidine sensor kinase and the inhibitory protein *phoU* (39). The polyphosphate kinase and the exopolyphosphatase genes within the *phoBRU* operon were present in all the *Acidihalobacter* spp. genomes and may be responsible for the synthesis and hydrolysis of polyphosphates to aid in the storage of inorganic phosphates. Furthermore, acid phosphatase genes found on the genomes of the four species may play a role in the liberation of inorganic phosphates from a range of organic molecules similar to the process previously described for *A. ferrooxidans* ATCC 23270 (Osorio et al 2008). However, the C-P lyase gene responsible for the degradation of phosphonates in *A. ferrooxidans* was not found on any of the *Acidihalobacter* spp. genomes, suggesting that they are unable to use phosphonates as a source of phosphorus under phosphate-limiting conditions (40).

The genome of *Ac. vulcanensis'* strain V8 also contained an alkaline phosphatase encoded by the *phoA* gene, downstream of a mobile genetic element which was not present in the other genomes. The *phoA* gene has previously been found to be part of a separate *phoA*-dependent pathway for phosphate utilization, which may be active in the genome of strain V8, which may indicate another means of phosphate utilization in this species.

5.3.2.4 Sulfur Assimilation

Organic sulfur assimilation in *Acidihalobacter* varies between species. Two operons, *tauABCD* and *ssuABCDE*, that allow certain bacteria to utilize aliphatic sulfonates (taurine and alkanesulfonates, respectively) as a sulfur source when faced with sulfur limitation have been previously described for *E. coli* and *Xanthomonas citri* (41-43). The *ssuABC* and *tauABC* genes code for ABC transporters for the uptake of these organosulfonates and *ssuDE* and *tauD* code for enzymes involved in their desulfonation (41). *'Ac. aeolianus'* strain V6 was found to have the genes *ssuB* and *ssuC* but not *ssuD*, suggesting their ability to take up alkanesulfonates. *'Ac. yilgarnensis'* strain F5 and *Ac. prosperus* strain DSM 5130^T carried *tauABC* genes required for taurine uptake, which are not present in the other *Acidihalobacter* genomes, but are missing the *tauD* gene for desulfonation. It is likely that taurine and alkanesulfonates are accumulated in DSM 5130, V6 and F5 but are not metabolized. The accumulation of taurine has previously been suggested as a mechanism for coping with osmotic stress in *Staphylococcus aureaus* (44) . However, the fate of alkanesulfonates in V6 and F5 is unclear. The genes for either uptake of alkanesulfonates or taurine have not been found on the genome of *'A. vulcanensis' s*train V8 or in the genome of *A. ferrooxidans* and have not been previously described in any other acidophiles at the time this paper was written.

All the *Acidihalobacter* genomes analysed in this study were found to code for a sulfate permease for the uptake of sulfate. In *A. aeolianus'* strain V6 and *'A. yilgarnensis'* strain F5, assimilation may proceed through activation of sulfate by adenosine phosphate via sulfate adenylyltransferase and subsequent reduction to sulfite by adenlylsulfate reductase, as was described for *A. ferrooxidans* (45). Finally, sulfite in F5 and V6 may then be transferred to acetyl serine generated by serine-O-acetyltransferase to form cysteine catalysed by the enzyme cysteine synthase as was described for *A. ferrooxidans* (45). However, the genomes of *Ac. prosperus* strain DSM 5130^T and *'A. vulcanensis' s*train V8 did not contain the small or large subunits of the sulfate adenylyltransferase or adenylylsulfate reductase, suggesting alternative methods of sulfate assimilation which are not at present clearly defined.

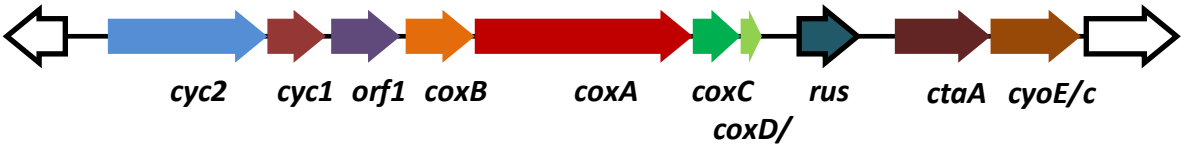
5.3.3 Energy Acquisition

5.3.3.1 Ferrous iron oxidation

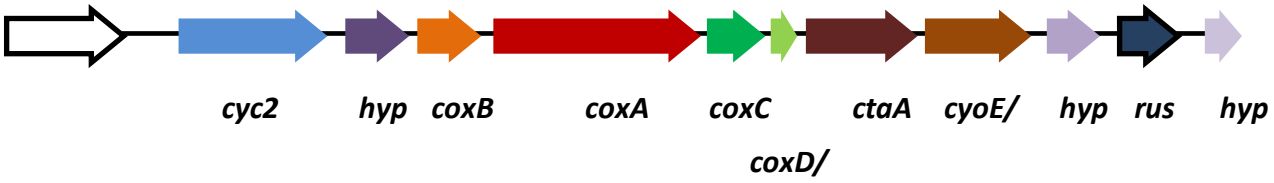
The iron oxidation pathways of *At. ferrooxidans* and *'Ac. aeolianus'* strain V6 have previously been studied and were shown to be driven by the transfer of electrons from iron to oxygen through the copper protein, rusticyanin, which is encoded as part of the *rus* operon (46-48). The *A. ferrooxidans rus* operon included the *cyc1* gene that was not found in *'Ac. aeolianus'* strain V6 (49). Comparison of the V6 *rus* operon indicates it is similar to that encoded by DSM 5130^T (50).

Two versions of rusticyanin proteins have previously been identified in *Ac. prosperus* strain DSM 5130^T, namely Form I and Form II. Form I is encoded by genes of the canonical *rus* operon and is involved in iron oxidation by the method described above. Form II rusticyanin has a presumed role in iron oxidation but is found in isolation from any other genes known to be involved in iron oxidation (50). Analysis of the genomes confirmed the presence of a similar 'classical' *rus* operon in the genomes of '*A. aeolianus'* strain V6 and *'A. yilgarnensis'* strain F5. The organization of the *rus* operons in DSM 5130^T and F5 were similar to the *rus* operon of V6, with the same sequence of genes (49). The rus Form II genes found in DSM 5130^T were also present in V6 (1 copy) and F5 (2 copies). However, the organization of the *'A. vulcanensis'* strain V8 rus operon was very different to that of the other *Acidihalobacter* species. The operon was missing genes for *cyc2*, *coxC*, *coxD* and *cyoE* (Figure 2). Furthermore, the terminal gene of the operon was annotated as the blue copper protein gene, sulfocyanin, which has previously been described for iron oxidation in the euryarchaeote *Ferroplasma* spp. (51). The *rus/sulfocyanin* operons of *At. ferrooxidans* and the *Acidihalobacter* species are shown in Figure 2.

At. ferrooxidans DSM 23270



Ac. prosperus DSM 5130, *Ac. aeolianus* DSM 14174 and *Ac. yilgarnensis* strain F5



Ac. vulcanensis DSM 14175

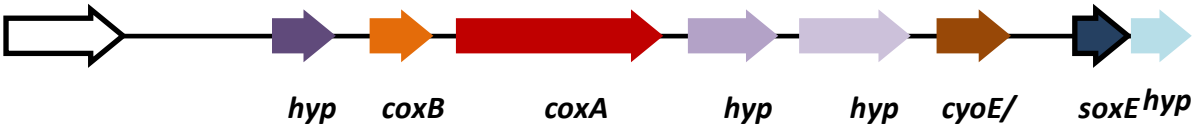


Figure 2. Organization of the rus operon in *At. ferrooxidans* ATCC 23270 compared with operons for iron oxidation in the *Acidihalobacter* spp.

5.3.3.2 Sulfur oxidation

Sulfide:quinone oxidoreductase genes were found in the genomes of *'Ac. yilgarnensis'* strain F5 and *'Ac. aeolianus'* strain V6 but not in *Ac. prosperus* strain DSM 5130^{T} or *'Ac. vulcanensis'* strain V8, suggesting that only the former strains can use hydrogen sulfide as a sulfur source. Sulfur oxidation as a method of energy utilization has been well studied in the *Acidithiobacillus* species (52-55). In these bacteria sulfide:quinone oxidoreductase (*sqr*), a periplasmic enzyme on the surface of the cytoplasmic membrane converts hydrogen sulfide to sulfur atoms (52).

Genome analysis confirmed the absence of this gene in strains DSM 5130, V6 and F5. However, the *sor* gene was found in strain V8. The presence of *sor* in *A. caldus* strain ATCC 51756 and *A. thiooxidans* strain A01 has been suggested to be as a result of horizontal gene

transfer (55, 56). However, as the genome of V8 is missing the adenylylsulfate system for conversion of sulfate to sulphite, it is possible that this is the active mechanism of sulfur assimilation in this strain. Sulfur is transferred to the cytoplasm either as sulfate in the form of thiosulfate or as polymeric sulfur by unknown mechanisms (55). Polymeric sulfur can be converted in the cytoplasm to sulfide, thiosulfate and sulfite by the enzyme sulfur oxygenase reductase (*sor*) (55, 57). Comparative studies have shown that this enzyme is not present in *At. ferrooxidans* strain ATCC 23270, *At. caldus* strain SM-1 or *At. thiooxidans* strain ATCC 19377 (54).

Furthermore, the *sox* gene system for sulfur oxidation has been found to be present in strains DSM 5130, V6 and F5 but not in V8. This system has previously been shown to be present in *A. thiooxidans* and *A. caldus* but not in *A. ferrooxidans* (54). Strains F5, V6 and DSM 5130^T have the truncated version of *sox*, without (CD)2, as also seen in *A. thiooxidans* and *A. caldus* (55, 58). However, they have the *soxYZ* (covalent sulfur binding protein and sulfur compound-chelating protein), *soxAX* (c-type cytochromes) and *soxB* (monomeric, di-manganese-containing protein which have been implicated in the oxidation of diverse sulfur compounds, including thiosulfate, sulfur, sulfite and sulfide in *A. thiooxidans* and *A. caldus* (55, 58). Furthermore, strains DSM 5130^T and F5 have *soxW* (thioredoxin) which also has been implicated in sulfur oxidation in acidophiles (59).

Periplasmic sulfur oxidation may also occur through thiosulfate:quinone oxidoreductase (*tqo*) and tetrathionate hydrolase (*tetH*), the genes of which have been found on all the genomes in this study. Enzyme *tqo* is important for the catalytic conversion of thiosulfate to tetrathionate, which can then be used as a substrate by *tetH* for the production of sulfur, sulfate and thiosulfate (55).

5.3.4 Strategies to adapt to environmental stresses

5.3.4.1 Strategies to cope with acidic environment

Acidophiles maintain a near neutral intracellular pH despite a proton gradient of up to 10,000 fold across the cytoplasmic membrane. They achieve this using a variety of mechanisms including: the maintenance of a positive membrane potential to reduce proton influx by electrostatic repulsion; using active proton pumps to export protons; altering their

cytoplasmic membrane structures; the use of enzymes such as carboxylases to consume protons; and through various cytoplasmic buffering systems (60).

The genomes of all described species of the *Acidihalobacter* genus harbour 2-3 copies of genes encoding energy dependent K^+-transporters (*trkH*, *trkA*, *kup*) that may control the flux of potassium ions into the cells in order to generate the reverse transmembrane potential. The genome of *'Ac. aeolianus' s*train V6 also contains genes for the potassium transporting ATPase chains A,B and C that are not present on the genomes of the other species. Also present were genes for multiple Na^+/H^+-antiporters for the active pumping of protons out of the cells. It is possible that the strains may also use the proton pumping activity of the respiratory chain complexes of the downhill branch to extrude protons from the cytoplasm, as has been described in other acidophiles (61). The proton influx from the ATP synthase could be compensated by the consumption of protons during the reduction of oxygen at the terminal oxidase, as described for *Ferrovum* JA-12 (14).

In the *Acidihalobacter* genomes, genes for cyclopropane fatty acyl phospholipid synthase were detected, which potentially allow the synthesis and incorporation of cyclopropane fatty acyl phospholipids into the membranes of these acidophiles. Strains DSM 5130, V6 and F5 each had one copy of the gene, but three copies were present in the genome of V8. It has been found that the cell membrane of *A. caldus* contains an increased fraction of saturated fatty acids or cyclopropane containing fatty acids (62).

The genomes of the *Acidihalobacter* spp. showed the presence of enzymes and proteins that may be involved in buffering mechanisms for the sequestration of protons, including genes for decarboxylases for arginine, glutamate and phosphatidylserine. These enzymes are involved in the generation of polyamines which are known to provide a cytoplasmic buffering capacity (60, 63). Genes for spermidine synthase, which has previously been described in acidic pH tolerance in *A. caldus* and *E. coli*, were also found on the genomes of all *Acidihalobacter* species (62, 64).

Components of the urease and polyphosphatase systems were found in the *Acidihalobacter* spp. genomes. It has been suggested that unique urease and polyphosphatase activity in the iron oxidizing acidophile *Ferrovum* sp. strain JA 12 may play a role in its adaptation to acidic environments (14). It is possible that the members of the *Acidihalobacter* genus may also be

able to benefit from the buffering capacity of polyphosphates or from the products of urea hydrolysis. Similar mechanisms for acid tolerance have previously been described for the gastric pathogens *Helicobacter pylori* and *Yersinia enterolytica* (65, 66). Furthermore, buffering could involve the use of a proton to be consumed during conversion of ammonia to ammonium, and bicarbonate could then be directed to the carboxysome for carbon dioxide fixation (14)

5.3.4.2 Strategies to cope with high metal and metalloid concentrations

Genome analysis revealed multiple genes that *Acidihalobacter* species may use to tolerate heavy metal and metalloid stresses. Among specific tolerance genes were potential arsenate detoxification genes that appear to be relatively similar to those described for *A. ferrooxidans* (67, 68). The strains possibly use an arsenate reductase to reduce arsenate to arsenite, which may then be exported by arsenite transporters (efflux pumps). The genomes also harbour a predicted mercuric reductase catalysing the reduction of Hg(II) to the volatile Hg^0, as well as genes for the transport of mercury (*merC* and *merT*), which may enable them to tolerate mercury (69).

Apart from aforementioned tolerance genes, multiple genes for putative cation/multidrug or heavy metal efflux pumps (zinc, cadmium, lead, cobalt) were detected in all the genomes of the *Acidihalobacter* species, which are possibly used to export heavy metals across the membranes as has been described previously by Dopson and Holmes (70). Furthermore, metal ion removal in these microorganisms may also be achieved through other mechanisms such as the complexation of metals with sulfate ions and the competition of metal ions and protons at low pH (14, 60, 61, 70, 71).

Several open reading frames (ORFs) have been proposed to code for putative proteins related to copper resistance in the genomes of *A. ferrooxidans* , among which genes for the copper translocating P-type ATPase for the efflux of copper have been identified to have an important role (72). All species of *Acidihalobacter* encoded multiple copper translocating P-type ATPase genes. However, the copy number of these genes varied, with 2 copies in the genome of strain V8, 4 copies in strain V6 and 5 copies in both strains DSM 5130^T and F5. Further study of these translocating ATPases is required to determine which of them have a

role in copper resistance and which are involved in transport of other heavy metals (lead, nickel, cadmium, mercury).

Also present on the *Acidihalobacter* genomes were genes for copper resistance proteins, *copC* and *copD*, which are known to be responsible for the uptake of copper, which could then be transported into the cytoplasm by the copper resistance protein *copB* (73). Strains DSM 5130 ^{T}, V6 and F5 all have the *copC* and *copD* copper uptake genes as well as the *copB* gene, whereas these genes are absent in strain V8. Furthermore, the *cusA* gene which codes for a cation efflux pump, also known to be involved in copper resistance in *A. ferrooxidans* was absent in strain V8, but present in strains F5 (6 copies), V6 (5 copies) and DSM 5130^{T} (3 copies) (72). All genomes of *Acidihalobacter* spp. had multicopper oxidase genes thought to be involved in the copper resistance of *At. thiooxidans* and *Ac. cryptum* (74, 75). Strain DSM 5130^{T} had 3 copies, strain V8 had 1 copy, strain V6 had 2 copies and strain F5 had 4 copies. Experimental data has shown the ability of strains V6 and F5 to leach chalcopyrite, demonstrating their ability to tolerate copper and suggesting their increased tolerance to copper in comparison to strains V8 and DSM 5130^{T} (31, 76). However, further work is required to determine the copper tolerance mechanisms in these acidophiles.

An additional mechanism for copper/heavy metal tolerance in the *Acidihalobacter* species may be through the poly-P dependent mechanism, as reviewed by (72). In this mechanism, acidophiles such as *A. ferrooxidans*, *A. caldus*, *A. thiooxidans* and the archaea *Sulfolobus metallicus* can synthesize and accumulate long polymers of inorganic polyphosphates (poly-P) from ATP using the enzyme polyphosphate kinase (77-81). The genes for both polyphosphate kinase and polyphosphatase were found on all the genomes of the *Acidihalobacter* species, suggesting they are all able to use this mechanism for removal of metal cations from the cytoplasm. In the presence of copper, the polyphosphatase enzyme that breaks down poly-P , is activated and this causes the release of inorganic phosphate (82). The inorganic phosphate can then bind to metal cations in the cytoplasm and be pumped out to the periplasmic space through inorganic phosphate carriers (72, 73).

5.3.4.3 Strategeies to cope with oxidative stress

The presence of high concentrations of redox-active metals such as iron in the environments of acidophilic iron oxidizers results in a high risk of oxidative stress and damage through

reactive oxygen species (ROS). Furthermore, the need of these microorganisms to maintain high oxidation rates for cellular metabolism requires them to have systems in play to protect against the direct or indirect oxidative damage caused to their DNA, proteins and membranes by ROS species such as hydrogen peroxide or super oxide radicals or from the formation of organic peroxides from alcohol groups (83).

The genome analysis of the four *Acidihalobacter* species indicated that they are able to protect themselves from oxidative damage through the presence of genes encoding for manganese-iron type superoxide dismutases. These enzymes can convert superoxide radicals into hydrogen peroxide (84). *Ac. prosperus* DSM 5130 ^{T}, *Ac. yilgarnensis* strain F5 and *Ac. aeolianus* strain V6 have genes encoding peroxiredoxins for the detoxification of hydrogen peroxide by oxidation of hydroperoxides through conserved cysteine residues. The role of peroxiredoxins has also been described in other acidophiles, such as *Sulfolobus solfataricus*, *Acidithiomicrobium* spp., *Alicyclobacillus* spp. and *Sulfobacillus* spp. (85-87). The peroxiredoxins may also play a role in the detoxification and the removal of organic peroxides, in order to protect the cells from oxidative damage. The genomes of the strains *Ac. yilgarnensis* strain F5, *Ac. prosperus* DSM 5130^{T} and *Ac. vulcanensis* strain V8 encode catalases/peroxidases which may serve the same function. Catalases have not been found in previously studied acidophilic microorganisms, and therefore, the *Acidihalobacter* species is unique in this regard (85). However, these genes were not present in the genome of strain V6, suggesting it is more sensitive to oxidative stress than the other species of *Acidihalobacter*.

5.3.4.4 Strategies to cope with high osmotic stress

Osmotic stress occurs when the soluble extracellular salts differ from the concentration within the cell that either leads to cellular dehydration or lysis (5). Genomic analysis confirmed the presence of multiple genes and potential pathways for osmotic stress tolerance in the genomes of the *Acidihalobacter* species. Accumulation of glycine, betaine, proline, taurine, ectoine, hydroxyectoine, ectoine and trehalose have previously been suggested as an adaptation in osmotically stressed acidophiles to help increase their tolerance to osmotic stress (5, 50, 88).

5.3.4.4.1 Ectoine

Genes coding for the enzymes required for ectoine biosynthesis were found in each of the *Acidihalobacter* spp. genomes. Ectoine is one of the most abundant osmolytes in nature that was first discovered in the extremely halophilic sulfobacterium *Ectothiorhodospira halochloris* (89). It has been found to improve protein folding and protect biomolecules from extremes in solutes, temperatures and chemical stresses (90). Ectoine biosynthesis proceeds through pathways involving diaminobutyrate pyruvate aminotransferase (*ectA*), L-2,4-diaminobutyric acid acetyltransferase (*ectB*) and L-ectoine synthase (*ectC*) (91) (Figure 3). The detection of these genes in the *Acidihalobacter* genomes suggested that ectoine contributed to the osmotolerance of the species in this genus (7, 10, 11, 31). Each genome also carried genes for a *marR*-like regulatory protein associated with the ectoine operon that has previously been implicated in the transcriptional regulation of the ectoine operon in the halotolerant obligate methanotroph *Methylomicrobium alcaliphilum* strain 20Z (92).

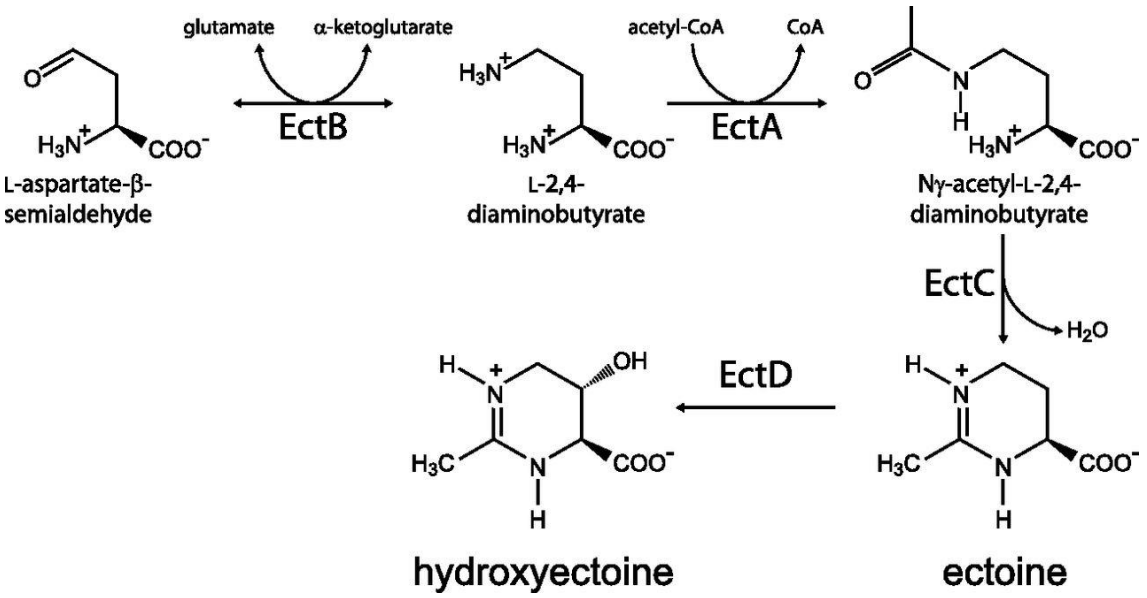


Figure 3. Ectoine biosynthesis (93)

5.3.4.4.2 Glycine betaine and proline

Glycine betaine can be synthesized by microorganisms through two independent pathways. One pathway utilizes the choline dehydrogenase gene for synthesis of glycine betaine from choline. The other pathway uses lysyl-lysine-2,3 aminomutase and a lysine acetyltransferase for synthesizing glycine betaine from glycine. The choline dehydrogenase gene was found in the genomes of *'Ac. yilgarnensis'* strain F5 and *'Ac. vulcanensis' s*train V8 but not in *'Ac. aeolianus' s*train V6 or *Ac. prosperus* strain DSM 5130^{T}. However, the lysine aminomutase

and lysine acetyltransferase genes were found on all genomes, suggesting strains V6 and DSM 5130^T can only synthesize glycine betaine through the second pathway.

Proline is the predominant compatible solute used by the moderate halophile *Halobacillus halophilus,* when faced with increasing osmotic stress (94). Proline biosynthesis from glutamate proceeds through three enzymes in this microorganism: pyrroline-5-carboxylate reductase (*proH*), glutamate 5-kinase (*proJ*) and a glutamate semialdehyde dehydrogenase (*proA*) (95). The inspection of the genomes in this study revealed the presence of these three genes in strains DSM 5130, F5 and V8. Strain V6 however lacked the *proH* gene but encoded a novel gene *yggS* in its place. *yggS* appears to be a homologue of the proline synthase (*proC*). Gene *yggS* was also present in the genomes of strains F5 and DSM 5130^T but not in the genome of strain V8. Furthermore, the strain DSM 5130 ^{T} genome contained a unique *yggA* lysine exporter gene that was not present on the other *Acidihalobacter* species genomes. These findings suggest that lysine may also play an important role in osmotic stress tolerance in the *Acidihalobacter* genus.

The transport and uptake of glycine betaine and proline can occur through the same transporters (96). All four genomes showed the presence of genes for the proline/glycine betaine ABC-transport-system permeases, *proV* and *proW*, however, only strains DSM 5130^T, V6 and F5 carried glycine betaine binding protein genes *opuAC*. Strain F5 additionally carried the permease genes, *opuAB*. Strain V8 lacked both *opuAC* and *opuAB* but carried a gene for an alternative proline/glycine betaine binding ABC transporter protein *proX*. Further work is required to understand the differences in the genes for glycine betaine uptake, however, the predicted genes imply that glycine betaine synthesis and uptake is an active osmoregulation process in all members of the *Acidihalobacter* genus.

5.3.4.4.3 Periplasmic glucan synthesis

Another mechanism of protection against osmotic stress in these microorganisms may be through the synthesis of osmoregulated periplasmic glucans, which have been reviewed for Proteobacteria by Bohin (97) and have also been described in the halophile *Halomonas elongata.* Genes *opgH* and *opgG* coding for glucosyltransferase and glucan biosynthesis precursors, respectively, that are involved in the synthesis of these glucans were found on all *Acidihalobacter* genomes. Strain DSM 5130^T also had genes for *opgD* which is another glucan biosynthesis precursor In *H. elongata*, these three enzymes potentially involved in

the biosynthesis of the core glucans were found to show differential expression under varied osmotic stress. This suggests the ability of the members of the *Acidihalobacter* genus to also synthesize osmoregulated periplasmic glucans that may have a role in osmotic stress tolerance.

5.3.4.4.4 Taurine

Taurine has previously been found to be used as an osmoprotectant in microbial communities from biofilms at the Richmond mine, Iron mountains (98). As described previously in the section on Sulfur Assimilation, the genes *tauABC* encoding proteins involved in taurine uptake were found on the genomes of strains DSM 5130^T and V8. However, both genomes lacked genes for the utilisation of taurine (*tauD*). This suggests that these microorganisms may accumulate taurine as an osmoprotectant rather than for use as a metabolite. Two taurine uptake proteins were previously found to be encoded by *Sulfobacillus* and predicted to provided osmoprotection to this species (98).

5.3.4.4.5 Possible mechanisms of dealing with high chloride ion levels

Typical acidophilic biomining strains are highly sensitive to anions and in particular chloride that have been demonstrated to inhibit ferrous iron oxidation by a *Leptospirillum ferriphilum*-dominated culture and the bioleaching ability of an undefined mixed acidophile consortium (99, 100). The greater sensitivity to the chloride anion is due to its ability to cross the cell membrane. This reduces the transmembrane potential resulting in an influx of protons and acidification of the cytoplasm (101). Other anions such as SO_2^{-4}, and cations such as Na^+, have little effect beyond their impact on osmotic potential (Blight and Ralph, 2004; Shiers et al., 2005; Davis-Belmar et al., 2008; Rea et al., 2015; Boxall et al., 2016).

Multiple genes encoding chloride ion channel proteins were present on all the genomes of the *Acidihalobacter* species. Genes for *yggT* which code for a predicted extracytoplasmic stress protein related to osmotic shock tolerance were found upstream of the chloride ion channel proteins in all the genomes. This suggests that these proteins may have a role in the expulsion of chloride ion from the cell, thereby protecting these microorganisms from the damaging effects of chloride ion. These genes have not been described for any other acidophilic iron- and sulfur-oxidizing microorganisms to date. Further investigation is required to determine the function of these genes as well as the other mechanisms the *Acidihalobacter* species may use to deal with chloride ion stress.

5.4 Conclusions

The genomic comparison of the members of the *Acidihalobacter* genus has helped to extend the current knowledge of the physiological capacities of the strains by providing a comprehensive description of the metabolic potential of these novel strains. Differences in key genes related to pathways of nutrient assimilation and energy acquisition can help to determine the roles of the *Acidihalobacter* species in the unique geochemical environments they inhabit. Furthermore, differences in their methods of tolerances to acid, osmotic and metal stress can determine their ability to be used in bioleaching processes, where these stresses tend to be high. The members of the *Acidihalobacter* genus show diversity in their methods for nutrient assimilation, energy acquisition and stress tolerance. The ability of the members of the *Acidihalobacter* genus to oxidize iron and sulfur at high acid stress and elevated chloride levels is a feature that sets them apart from other iron- and sulfur-oxidizing acidophiles. Therefore, further proteomic studies are required to shed light on the active systems used by these microorganisms under these stresses.

Nucleotide sequence accession numbers

Ac. prosperus strain DSM 5130^T (JQSG00000000.2), *Ac. prosperus* strain F5 (CP017415.1), *Ac. prosperus* strain DSM 14174 (CP017448.1), *Ac. ferrooxidans* strain DSM 14178 (CP019434.1)

5.5 Supporting Information

The full list of gene annotations showing encoded proteins can be found at the following link:

https://drive.google.com/open?id=0BwTiq6bJgkC_T1NHMGRuLUFjTEE

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5.7 Conflicts of Interest

The authors declare no conflict of interest.

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6 Chapter 6

Khaleque HN, Kaksonen AH, Boxall NJ, Watkin ELJ. Identifying the mechanisms of osmotic stress tolerance in the halotolerant, extreme acidophile, '*Acidihalobacter aeolianus'* DSM 14174^{T}.

Manuscript is prepared for submission.

*****Please note that in this chapter I have adopted the names allocated in Chapter 4 for the members of the *Acidihalobacter* genus. (*Ac. prosperus* DSM 14174 (strain V6) is *'Ac. aeolianus'* DSM 14174^{T} (strain V6); *'Ac. ferrooxidans'* DSM 14175 is *'Ac. vulcanensis'* DSM 14175^{T}; *Ac. prosperus* strain F5 is *'Ac. yilgarnensis' strain F5*^{T})

Identifying the mechanisms of osmotic stress tolerance in the halotolerant, extreme acidophile, '*Acidihalobacter aeolianus*' DSM 14174^T

Abstract

The development of microbial consortia able to withstand high chloride concentrations is of interest to the mining industry for use in regions where freshwater is scarce or where waters have high chloride ion content. The chloride ion places a stress on iron and sulfur oxidizing acidophiles, through its ability to permeate membranes, compromising pH homeostasis by leading to the collapse of the internal positive membrane potential maintained in acidophiles. '*Acidihalobacter aeolianus*' DSM 14174^T (strain V6) is an iron- and sulfur-oxidizing acidophile with biotechnological potential that has previously been determined to tolerate up to 45 g/L chloride and oxidize pyrite at up to 30 g/L chloride ion. In this study, the differential protein expression of *'Ac. aeolianus'* DSM 14174^T was studied using Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS) with the aim of understanding its mechanisms of tolerance to high chloride ion stress. The proteomic response to elevated chloride concentrations included the production of osmotic stress regulators that potentially induced the production of compatibles solutes, of which the most significant increase was in the synthesis of ectoine. Other responses directly related to the increased osmotic stress at high chloride ion levels included the increased synthesis of glutathione, changes in carbon flux, the increased production of amino acids, the efflux of metals and protons, and the increase in proteins involved in DNA repair and membrane biosynthesis. Energy generation through iron oxidation and sulfur oxidation were decreased, and energy was probably obtained from the metabolism of glycogen stores. Overall, these studies have helped to create a model of tolerance to elevated chloride levels by *'Ac. aeolianus'* DSM 14174^T that differs from the previous model developed for the *Acidihalobacter prosperus* type strain DSM 5130.

6.1 Introduction

Iron and sulfur oxidizing acidophilic microorganisms are able to catalyze the dissolution of sulfide minerals in a technique called 'bioleaching' (1). In recent years, the interest in the study of halotolerant acidophiles has increased due to their potential in saline water bioleaching in regions where high salinity characterizes the water and ores used in bioleaching operations (2, 3).

Acidophiles maintain a circumneutral intracellular pH by a variety of mechanisms, including the use of primary proton pumps to export protons during electron transport, maintenance of membranes highly resistant to the influx of protons, an inside positive membrane potential that electrostatically repulses proton influx, alterations in cytoplasmic membrane structure and reactions that consume protons, such as those using carboxylases and cytoplasmic buffering (4, 5). When high levels of extracellular chloride are present in the external environment, osmotic stress is placed on the cells. While ions such as sulfate and sodium have a damaging impact on cells at high concentrations due to their ability to cause osmotic pressure, chloride ions are able to cause damage to cells beyond their osmotic impact (2, 6-9). Chloride ion has been shown to be particularly damaging to acidophilic microorganisms due to its ability to permeate the cell membrane and reduce the transmembrane potential, ultimately leading to an influx of protons that cause acidification of the cytoplasm (6, 10, 11).

Previously, it has been suggested that responses of chloride sensitive microorganisms to increased osmotic stress include the accumulation of cytoplasmic potassium, the production of osmoprotectants, alterations in the cell membrane, increase of acidic amino acids on the surface of proteins resulting in an elevated negative potential that aids in keep proteins in solution (12-14). However, the mechanisms used by halophilic acidophiles to tolerate high chloride concentrations and low pH stress has only previously been described for the *Acidihalobacter prosperus* type strain DSM 5130^T (15). *'Ac. aeolianus'* DSM 14174^T (previously *Ac. prosperus* DSM 14174 strain V6) was isolated from hydrothermal pools at the Aeolian Islands, Vulcano, Italy (16) and its genome sequence was recently released (17). It represents a reclassified species of autotrophic, extremely acidophilic and halotolerant, iron- and sulfur-oxidizing genus, *Acidihalobacter* (see Chapter 4).

A genome comparison of *'Ac. aeolianus'* DSM 14174^T with other members of the *Acidihalobacter* genus has provided insights into novel pathways linked to nutrient assimilation, energy metabolism and stress tolerance in this microorganism (chapter 5). However, genome studies are limited to assessing the presence or absence of genes that may play a role in key pathways but cannot determine the actual mechanisms used under varying environmental conditions. Therefore, in this study, the differential response in the proteome of *'Ac. aeolianus'* DSM 14174^T at high and low chloride ion stress was investigated in order to understand the mechanisms of tolerance to osmotic and chloride ion stress by this species. Identifying differences in stress tolerance mechanisms in the different species of *Acidihalobacter* will provide for a better understanding of these isolates for use in the development of halotolerant, acidophilic consortia with promise for use in saline water bioleaching of ores.

6.2 Materials and methods

6.2.1 Strains and growth conditions

Growth medium for *'Ac. aeolianus'* DSM 14174^T was basal salts medium (in g/L Milli-Q™ H_2O: 0.4, $(NH_4)_2SO_4$, 0.5, $MgSO_4.7H_2O$ and 0.2, K_2HPO_4 acidified to pH 2.0 with concentrated H_2SO_4) sterilized by autoclaving. The medium was supplemented with 13.9 g/L $FeSO_4.7H_2O$, 1.51 g/L $K_2S_4O_6$ and 1 mL/L of a trace element solution (mg/L dH_2O; $MnCl_2.2H_2O$, 62; $ZnSO_4.7H_2O$, 68; $CoCl_2.6H_2O$, 64; H_3BO_3, 30; Na_2MoO_4, 10; $CuCl_2.2H_2O$, 66; $NaVO_3$), dissolved in the basal salts media and filter sterilized through 0.2 µm filter (Millipore). Chloride ion (5 g/L chloride ion for low chloride experiments and 30 g/L chloride ion for high chloride experiments) was provided as sodium chloride. The cultures were maintained at 30 ˚C with shaking at 120 rpm.

6.2.2 Proteomic analysis of growth of '*Ac. aeolianus*' DSM 14174^T at high or low chloride concentrations

6.2.2.1 Protein extraction

One litre cultures (triplicates) of the pure isolate of *'Ac. aeolianus'* DSM 14174^T were grown as described above. To avoid alterations within the proteome as a result of differences in growth phase, cultures were harvested at mid-exponential phase (24h for cultures at 5 g/L chloride ion and 48 h for cultures at 30 g/L chloride ion). Cells were harvested by centrifugation for 40 min at 35,000 g at 4 ^oC, and the cell pellets were washed in acidified water and re-pelleted by centrifugation at 4 ^oC for 40 min at 35,000 g. The supernatant was discarded and the cell pellets were stored at -80 ^oC in 2 mL Lobind Eppendorf tubes for protein extraction.

The cell pellets were resuspended in 1 mL lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris, 1 mM EDTA, 1 % SDS plus 1 tablet protease inhibitor cocktail for every 20 mL of buffer) and sonicated in a sonicating water bath (Diagenode Bioraptor^{TM} Plus), 20 seconds on, 20 second off, high amplitude for 40-8- cycles at 4 ^oC. Cells were checked under the microscope to ensure full cell lysis had occurred. Cellular debris was removed by centrifugation at 4,000 g for 5 min at 4 ^oC. The supernatant was removed and the total cell lysate was ultracentrifuged at 100,000 g for 1 h at 4 ^oC to pellet the membrane fraction. The supernatant was removed and stored at -80 ^oC. The pelleted membrane fraction was resuspended in 5% SDS containing protease inhibitors and heated at 75 ^oC for 15 min in a heat block then stored at -80 ^oC. Membrane fractions and total cell lysate fractions were stored separately. Both were sent to the Australian Proteomic Analysis Facility (APAF) for Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS) analysis.

6.2.2.2 Sample preparation

Individual enriched membrane fractions were precipitated with trichloroacetic acid and reconstituted in 100 mM TEAB+1% SDC) and were pooled with respective biological replicates of whole cell lysate samples (in 7 M Urea, 2 M Thiourea, 30 mM Tris-HCl, 1 mM EDTA, 1% SDS and protease inhibitors). Samples were cleaned by precipitating proteins overnight by the addition of ice cold acetone in 1:4 ratio (Sample to acetone, vol/vol). The pellet was separated by brief centrifugation (speed, time) and air dried at room

temperature. Samples were reconstituted in 200 µL of 50 mM Tris-HCl buffer (pH 8.6) containing 8 M urea. Following the dissolution, to reduce the concentration of urea to 1.6 M, samples were diluted using 50 mM Tris-HCl buffer (pH 8.6).

All the samples were subjected to buffer exchange using 3 KDa Mol wt cut-off spin column (Vivaspin, Sartorius). Briefly, the column was conditioned with 50 mM Tris-HCl buffer (pH 8.6). The sample was loaded on the spin column and centrifuged until most of the sample passed through. The flow-through was re-loaded on the spin column and centrifuged, followed by a wash using 50 mM Tris-HCl buffer (pH 8.6). The supernatant containing proteins was collected in a fresh tube. The protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Thermo-scientific, USA) as per manufacturer's instructions.

100 µg of sample was diluted using Tris-HCl buffer (pH 8.6) to make a uniform volume of 50 µL across the samples. This was followed by, reduction with dithiothreitol (final concentration of 10 mM) for 1 h at 56°C, subsequently, alkylation with iodoacetamide (final concentration of 25 mM) at room temperature for 30 min. Samples were digested with 1 µg trypsin per 50 µg of protein for 16 h at 37°C. Following the digestion, samples were acidified with formic acid (final concentration of 1% formic acid). The digested samples were dried using a vacuum centrifuge.

Before mass spectrometric analysis, the samples were reconstituted using 50 µL of loading buffer (2 % acetonitrile, 97.9 % water and 0.1 % formic acid), vortexed briefly followed by centrifugation for 10 min. 1 µl of supernatant containing 2 µg of peptides was further diluted with 8 µl of loading buffer and were subjected to Liquid Chromatography - Mass Spectrometry and Liquid Chromatography - Tandem Mass Spectrometry LC-MS/MS characterisation.

6.2.2.3 High pH (HpH) High Performance Liquid Chromatography (HPLC)

An aliquot (40 µg) of each of the pooled samples of *'Ac. aeolianus'* DSM 14174^T at 5 g/L chloride ion and at 30 g/L chloride ion (total 200 µg), cleaned with C18 Sep Pack Light Cartridges (Waters, Millford MA) and dried down using a speed-vac. Fractionation was performed by High pH Reversed Phase-High Performance Liquid Chromatography

RP-HPLC. Briefly, 5 mM ammonia solution in water (Buffer A, pH 10.5) and 5 mM ammonia solution with 90% Acetonitrile (Buffer B, pH 10.5) were used as mobile phases. The dried sample was resuspended in 5 mM ammonia solution. After sample loading and washing with 97% buffer A for 10 min, buffer B concentration was increased from 3% to 30% for 55 min and then to 70% for 10 min and to 90% for another 5 min at a flow rate of 300 µL/min. The eluent was collected every 2 min at the beginning of the gradient until 16 min and every one minute intervals for the remainder of the gradient.

The fractionated peptides were consolidated to 13 fractions and dried in vacuum centrifuge. The dried fractions were resuspended in loading buffer (2% acetonitrile, 97.9% water and 0.1% formic acid) and analysed by reversed phase nano-LC-MS/MS.

6.2.2.4 Mass spectrometry data acquisition

6.2.2.4.1 1D Information dependent acquisition

A 2 µg aliquot of peptides from each sample and HpH fractionated peptides (13 fractions) were subjected to 1D-Information Dependent Acquisition LC MS/MS (IDA-LC–MS/MS)analysis. Each sample was injected onto a reverse-phase trap for pre-concentration and desalted with 2 % acetonitrile, 97.9 % water and 0.1 % formic acid, at 5 µL/min for 5 min. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using linear solvent gradients of 5.5-33 % of solvent B (90 % acetonitrile, 9.9 % water and 0.1 % formic acid) over 60 min at a flow rate of 600 nL/min. After peptide elution, the column was cleaned with 85 % solvent B for 6 min and then equilibrated with 94.5 % solvent A for 10 min before next sample injection. The reverse phase nano-LC eluent was subject to positive ion nano-flow electrospray analysis in an IDA mode.

In the IDA mode, a time of flight (TOF)-MS survey scan was acquired (m/z 350-1500, 0.25 second), with the ten most intense multiply charged ions (2+-4+; exceeding counts per second >150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 100 milliseconds in the mass range m/z 100–1800 with rolling collision energy.

6.2.2.4.2 Data independent acquisition with Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS)

A 6 µg of samples of 'Ac. *aeolianus'* DSM 14174^T at 5 g/L chloride ion and at 30 g/L chloride ion (2 µg on column in technical replicates) samples were transferred to HPLC vials for SWATH-MS analysis. Each sample was analysed thrice (3 × 5=15 samples) using SWATH-MS. Briefly, each sample (2 µg) was injected onto a reverse-phase trap, desalted with 0.1% formic acid, 2% ACN, at 5 µL/min for 5 min. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using linear solvent gradients of 5.5-33 % of solvent B (Mobile phase A) over 60 min at a flow rate of 600 nL/min. After peptide elution, the column was cleaned with 95% solvent B for 8 min and then equilibrated with 98 % solvent A for 10 min before next sample injection. The reverse phase nano-LC eluent was subject to positive ion nano-flow electrospray analysis in a data independent acquisition mode (SWATH).

For SWATH MS, m/z window sizes were determined based on precursor m/z frequencies (m/z 350-1500) in previous IDA data (60 windows in total). In SWATH mode, first a TOF-MS survey scan was acquired (m/z 350-1500, 0.05 sec) then the 60 predefined m/z ranges were sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 0.05 sec in the mass range m/z 350-1800 with rolling collision energy optimised for lowest m/z in window +10%. To minimize bias caused by instrument condition, SWATH data were acquired in a randomized order for the samples with one blank run between every sample injection.

6.2.2.5 Data processing

6.2.2.5.1 IDA data analysis

The LC-MS/MS data of the IDA runs were searched with ProteinPilot (v5.0) (Sciex) using the Paragon™ algorithm in thorough mode. An in-house protein (APAF) database based on RAST annotations for the protein database containing 6,407 proteins was used for data search. Carbamidomethylation of Cys residues was selected as a fixed modification. An Unused Score cut-off was set to 1.3 (95 % confidence for identification). Distinct peptide summaries with relevant grouping information were exported post-search.

It should be noted that the raw IDA data files of samples *'Ac. aeolianus'* DSM 14174^T at 5 g/L chloride ion and at 30 g/L chloride ion were combined and re-search to generate a single data file (to generate ion library, Termed "Combined-IDA") and retention time of the chromatogram was aligned to that of SWATH runs using PeakView™ (v2.1). While, HpH IDA

results were searched individually and combined as described in section *SWATH quantitation*.

6.2.2.5.2 SWATH quantitation

A library was constructed by merging IDA results of all individual groups (Combined-IDA) and high pH fraction using an APAF in-house program. The combined ion library was imported into PeakViewTM (v2.1) and data were extracted using the SWATH MicroApp 2.0 (SCIEX, release 25 August 2014) with the following parameters: The top 6 most intense fragments of each peptide were extracted from the SWATH data sets (75 ppm mass tolerance, 10 min retention time window). Shared and modified peptides were excluded. After data processing, peptides (max 100 peptides per protein) with confidence 99% and FDR 1% (based on chromatographic feature after fragment extraction) were used for quantitation. Proteins with unique/non-shared peptides (termed "Non-Shared") were used for comparing protein expression within the organism.

Comparison of protein expression (SWATH protein peak areas) values was performed by APAFs in-house program. The protein peaks were normalised to the total peak area for each run and subjected to analysis of variance (ANOVA) to compare relative protein peak area between the high and low samples. Fold-change larger than 1.5 and *p*-value smaller than 0.05 were termed differentially expressed and were highlighted.

6.3 Results and Discussion

6.3.1 *'Ac. aeolianus'* DSM 14174^T differential proteomic response to the presence of high and low chloride

SWATH analysis of the total proteome, including soluble and membrane fractions, from *'Ac. aeolianus'* DSM 14174^T cultures grown in the presence of 5 and 30 g/L chloride ion identified 740 proteins which were differentially abundant (with $P < 0.05$). From this, 172 key proteins were selected based on key pathways of significance identified by genome analysis in Chapter 5. Only proteins with a fold-change larger than 1.5 and *p*-value less than 0.05 were termed differentially expressed and were selected for inclusion in the study.

6.3.1.1 Osmoprotection

The most common compatible solute to accumulate when microorganisms are faced with osmotic stress is ectoine (18). *'Ac. aeolianus'* DSM 14174^T cultures grown at 30 g/L chloride ion showed a 422.9 fold increase in abundance of the enzyme L-ectoine synthase, the key enzyme for the synthesis of ectoine (19). The increased abundance of this enzyme represented the highest fold change for proteins in the study, suggesting that this is the primary response to increased osmotic stress by *'Ac. aeolianus'* DSM 14174^T.A previous study had shown a 55.3 fold increase in abundance of an ectoine ABC transporter solute binding protein at high chloride ion concentrations in *Ac. prosperus* DSM 5130^T (15). Therefore, it is hypothesized that other members of the *Acidihalobacter* genus rely on ectoine, either through an increase in its synthesis or its uptake, as their primary protection against osmotic stress when grown at high chloride levels.

The enzyme glutathione synthetase is only found in Gram-negative bacteria and is involved in the synthesis of glutathione from gamma-glutamylcysteine and glycine (20). At high chloride ion stress in *'Ac. aeolianus'* DSM 14174^T, glutathione synthetase was found to be more abundant (7.56 fold increase). The role of glutathione for protection of bacterial cells against low pH, chlorine compounds and osmotic and oxidative stresses has been extensively reviewed by Masip et., al. 2006 (21). The combination of increased abundance of glutamate (discussed later) in conjunction with glutathione may be a key method for osmoregulation in *'Ac. aeolianus'* DSM 14174^T. Furthermore, at high chloride levels, the increased abundance of the glutathione-regulated potassium-efflux system protein, KefB, (9.2 fold increase) was seen. Activation of KefB provokes rapid potassium efflux, accompanied by acidification of the cytoplasm and influx of sodium ions (22). Accumulation of potassium ions has been suggested as the first step in response to osmotic stress tolerance in acidophiles, therefore, efflux of these ions through the KefB protein is be counterintuitive (8). However, as glutathione is known to inhibit the activation of KefB, it is hypothesized that the increase in glutathione at high chloride concentrations in *'Ac. aeolianus'* DSM 14174^T may also play a role in preventing potassium efflux, thereby allowing it to accumulate in the cell and provide protection against both low pH and high osmotic stress (23).

Amino acids can be used as osmoprotectants in osmotically stressed microorganisms (18). In many bacterial species, proline has been shown to function as a compatible solute that

accumulates within cells that are challenged by osmotic stress (24). In a proteomic study of *Acidithiobacillus caldus* SM-1 the production of enzymes involved in proline synthesis was only observed when cells were grown in the presence of 0.5 M NaCl (25). Glutamate and glutamine have both been shown to have a role in osmoprotection in *Erwinia chrysanthemi* Strain 3937 under salt stress, with glutamine being a dominant osmoprotectant in this strain (26). Glutamate synthase (1.7 fold increase) and a glutamine synthetase family protein (7.23 fold increase) were increased at high chloride levels in *'Ac. aeolianus'* DSM 14174^T, as possible osmoprotectants. As discussed previously, the joint effect of glutamate and glutathione may also play a role in osmoprotection (27). Furthermore, the abundance of the enzymes glutamate-5-kinase, that converts glutamate to proline was also increased at high chloride stress (1.7 fold), suggesting that proline is synthesized as another osmoprotectant (28). Finally, a 1.6 fold decrease was seen in the abundance of the pyrroline-5-carboxylate reductase protein, which is responsible for the breakdown of proline to pyrroline-5-carboxylate (29). This suggests that proline is accumulated rather than catabolized in *'Ac. aeolianus'* DSM 14174^T at elevated chloride concentrations. There was also a decrease of 9.48 and 2.7 fold in the glycine cleavage system H and T proteins, respectively, possibly to allow synthesis of glycine betaine in *Ac. aeolianus* DSM 14174^T at high chloride ion concentrations.

The urease alpha subunit was found to increase (7.3 fold) in *'Ac. aeolianus'* DSM 14174^T at high chloride ion levels. This increase in urease activity could serve multiple functions in this species, including assimilation of nitrogen as well as tolerance to acid stress (30). Urease converts urea to ammonia, which can then be assimilated in *'Ac. aeolianus'* DSM 14174^T for further production of glutamate and glutamine for use as osmoprotectants (31). Alternatively, the ammonia could function as a nitrogen source. At high chloride concentration, proteins involved in nitrogen assimilation through the uptake and utilization of ammonia were decreased. This included the decrease in abundance of two P-II type nitrogen regulatory proteins (9.6 and 2.2 fold, respectively) and nitrogen regulation proteins NR(I) and NtrB (2.1 and 1.9 fold, respectively). Therefore, this decrease in ammonia assimilation could be compensated by the increased abundance of urease subunits at high chloride levels. Finally, increased urease activity has been linked to increased acid tolerance in acidophiles, as has been described for the iron oxidizing acidophile, *Ferrovum* JA-12. The role of urease in *'Ac. aeolianus'* DSM 14174^T may be to assist in increasing acid tolerance at

high chloride concentrations (31, 32). *Ferrovum* spp. and *Acidihalobacter* spp. represent the only acidophiles in which the urease genes have been found, therefore ,the increased abundance of the urease protein at high chloride ion stress suggests an important role in *Acidihalobacter* (32). The differentially expressed proteins with proposed roles in osmoprotection are shown in Table 1.

Table 1. Fold change in proteins with roles in osmoprotection in '*Ac. aeolianus'* DSM 14174^T at high chloride concentrations.

Increased in abundance at high chloride concentrations	**Fold change**
L-ectoine synthase (EC 4.2.1.-)	423
Glutathione-regulated potassium-efflux system protein KefB	9.2
Glutathione synthetase (EC 6.3.2.3)	7.6
Urease alpha subunit (EC 3.5.1.5)	7.3
Glutamine synthetase family protein	7.2
Aspartokinase (EC.2.7.2.4)	1.8
Glutamate 5-kinase (EC 2.7.2.11) / RNA-binding C-terminal domain PUA	1.8
Glutamate synthase (NADPH) large chain (EC 1.4.1.13)	1.7
Decreased in abundance at high chloride concentrations	
Glycine cleavage system H protein	9.5
Glutamate decarboxylase (EC 4.1.1.15)	2.9
Pyrroline-5-carboxylate reductase (EC 1.5.1.2)	1.6
Aminomethyltransferase (glycine cleavage system T protein) (EC 2.1.2.10)	2.7

6.3.1.2 Cell shape, membrane integrity, mobility and transport

Proteins involved in cell shape determination, such as the integral membrane protein CcmA and RodA were highly abundant at high chloride levels (44.3 and 13.0 fold increase, respectively). Integral membrane protein CcmA has previously been linked to the maintenance of linearity in elongated cells while RodA has a role in cell growth and maintenance of cell shape (33, 34). Therefore, it is hypothesized that both these proteins play a role in osmoprotection by maintaining the cell membrane structure when *'Ac. aeolianus'* DSM 14174 is faced with high osmotic stress.

In *'Ac. aeolianus'* DSM 14174^T grown at high chloride stress, there was a large increase in the number of proteins related to membrane biosynthesis, cell envelope biogenesis, motility and transport of various compounds through active efflux. Cell membrane biosynthesis and cell envelope biogenesis proteins that were found to be increased in abundance at high chloride ion stress in *'Ac. aeolianus'* DSM 14174^T included the outer membrane protein, Imp (3.67 fold), which is involved in the assembly of lipopolysaccharides (LPS) in the outer membrane of *Escherichia coli* (35). Other membrane and membrane fusion proteins (unclassified) were found to be increased in expression by 8.3 and 6.7 fold, respectively. Also, a heat shock protein YciM precursor was found to be increased 1.6 fold (36). The negative impact of high chloride ion levels on membrane integrity and the need for increased biosynthesis of membrane components have previously been observed by Dew et al 2004. Proteomic studies of *At. caldus* and *Acidimicrobium ferrooxidans* (10331^T) have also shown an increase in abundance of membrane biosynthesis proteins when exposed to chloride ion (in the form of sodium chloride), therefore, it is likely that *'Ac. aeolianus'* DSM 14174^T shows a similar response (37, 38).

The flagellar synthesis regulator protein, FleN was increased 13.9 fold at high chloride concentrations in *'Ac. aeolianus'* DSM 14174^T. This protein has a proposed role in regulating flagellar number in *Pseudomonas aeruginosa* (39). There was also an increase in the flagellar motor switch protein FliG (2.8 fold increase), which has been reported to be essential for the assembly and function of the flagellar motor in *E. coli* (40). This suggests an increase in motility in response to increased chloride concentrations in *'Ac. aeolianus'* DSM 14174^T. However, the flagellar sensor histidine kinase FleS, which forms part of a two-component system with FlesR for the control of flagellar synthesis, was less abundant (4.2 fold decrease) (41). The transcriptional activation of FlesSR is controlled by the sigma-54 dependent RNA

polymerase factor, which was found to be decreased 2.4 fold at high chloride levels (42). This suggests that while motility is increased at high chloride levels, the biosynthesis of new flagella is decreased when *'Ac. aeolianus'* DSM 14174^T is under osmotic stress.

The formation of capsular polysaccharides have previously been suggested to have a role in biofilm formation in bacteria, and may have a similar role in *'Ac. aeolianus'* DSM 14174^T (43). A fatty acid synthase, WcbR, (9.4 fold increase) and glycosyltransferase biosynthesis protein WcbB (9.0 fold increase) were found to increase at high chloride concentration in *'Ac. aeolianus'* DSM 14174^T. These proteins have known roles in capsular polysaccharide biosynthesis in *Burkholderia pseudomallei* K96243 (44). Also, the probable components of the lipoprotein assembly complex lipoprotein, NlpD, which has previously been reported for its role in biofilm formation in *E. coli* (45), were found to be increased 2.8 fold at high chloride levels.

Certain porins and outer membrane precursor proteins were found to decrease at high chloride concentrations in *'Ac. aeolianus'* DSM 14174^T. The most notable decrease was in an outer membrane porin protein (37.1 fold decrease) as well as in multiple precursors for OmpA and Omp16 (2.6-4.1 fold decrease). It was previously reported that decreases in outer membrane protein assembly at high chloride levels are likely to reduce pores in the outer membrane in order to inhibit the flow of chloride into the cells, as has been reported for OmpC and OmpF in *E. coli* and OmpA in *Ac. prosperus* DSM 5130^T (15, 46).

There was also an increased abundance of transport proteins at high chloride levels, including those related to transport of metals, amino acids, lipids, and other biopolymers. Multiple multidrug transport systems were increased in abundance at high chloride concentrations, including those for an ABC multidrug transport system ATPase component (7.7 fold increase), an RNA multidrug efflux transporter with a role in acriflavin resistance (5.1 fold increase) and another ungrouped multidrug efflux transporter (5.3 fold increase). Other important transport proteins to be increased included those for a probable RND efflux membrane fusion protein (8.3 fold increase) and a type I secretion outer membrane protein, TolC precursor (2.3 fold increase) which may have a role in active transport of compounds such as antibiotics, dyes and detergents across the membrane (47). An amino acid ABC transporter protein, solute binding component was increased (6.1 fold increase), as was an amino acid ABC transporter ATP binding protein y4tH (3.8 fold increase), suggesting the movement of amino acids across the membrane, possibly for the production of

osmoprotectants (as discussed above). However, there was a decrease in certain uncharacterized ABC transporters, such as the auxiliary component YrbC (6.2 fold decrease) which is a periplasmic binding protein with a predicted function in import that actively prevents phospholipids from accumulating in the membrane (48, 49). Therefore, the decrease in this protein would allow for an increase in phospholipid accumulation in the membrane as method of protecting the cells during osmotic stress.

Metal efflux systems were also increased at high chloride concentrations, including lead, cadmium, zinc and mercury transport ATPase/copper translocating ATPase (5.1 fold increase) and a magnesium and cobalt efflux protein, CorC, (4.8 fold increase). An MFS superfamily transporter, which plays a role in the transport of small solutes across the membrane in response to chemiosmotic gradients, also showed increase in abundance at high chloride levels (5.5 fold increase) (50). This increased efflux of metals and solutes may play a role in the ability of *'Ac. aeolianus'* DSM 14174^T to tolerate high metal concentrations even when grown in high chloride concentrations under acid stress (11, 51). Tolerance to arsenic presumably increases at high chloride levels, as evidenced by increased abundance of an arsenate reductase (4.0 fold increase). However, there was a decrease in copper tolerance protein CopC (29.2 fold decrease) at high chloride levels, suggesting decreased copper tolerance at high chloride concentrations. This was countered by an increase in apolipoprotein N-acyltransferase/copper homeostasis protein CutE (4.8 fold increase), which could compensate for the decreases in the copper tolerance protein CopC at high chloride concentrations. Furthermore, the role of exopolyphosphateshave also been described for increased metal tolerance by acidophiles. Expolyphosphates were increased in *'Ac. aeolianus'* DSM 14174^T at high chloride concentrations by 1.8 fold, suggesting this may be an alternative mechanism that is activated in response to the increased sensitivity of this microorganism to heavy metal stress at high chloride concentrations.

Differentially abundant proteins with roles in cell shape, membrane formation, motility and transport across the membranes are shown in Table 2.

Table 2. Fold change in proteins that affect cell shape, membrane integrity, mobility and transport in '*Ac. aeolianus*' DSM 14174^T at high chloride concentrations.

Increased in abundance at high chloride concentrations	**Fold change**
Integral membrane protein CcmA involved in cell shape determination	44.3
Rod shape-determining protein RodA	13.0
Capsular polysaccharide biosynthesis fatty acid synthase WcbR	9.4
Capsular polysaccharide glycosyltransferase biosynthesis protein WcbB	9.0
Probable RND efflux membrane fusion protein	8.3
ABC-type multidrug transport system, ATPase component	7.7
Membrane protein	6.7
Probable amino acid ABC transporter protein, solute-binding component	6.1
HPr kinase/phosphorylase (EC 2.7.1.-) (EC 2.7.4.-)	5.9
ATP-grasp enzyme-like protein	5.7
Transporter, MFS superfamily	5.5
Multidrug-efflux transporter	5.3
RND multidrug efflux transporter; Acriflavin resistance protein	5.1
Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)	5.1
Zn-dependent protease with chaperone function	5.0
Apolipoprotein N-acyltransferase (EC 2.3.1.-) / Copper homeostasis protein CutE	4.8
Arsenate reductase (EC 1.20.4.1)	4.0

Outer membrane protein Imp, required for envelope biogenesis / Organic solvent tolerance protein precursor	3.7
Probable component of the lipoprotein assembly complex (forms a complex with YaeT, YfgL, and NlpB)	3.7
Outer membrane efflux protein	3.5
COG0488: ATPase components of ABC transporters with duplicated ATPase domains	3.0
Lipoprotein NlpD	2.8
ABC transporter, permease protein, putative	2.5
Type I secretion outer membrane protein, TolC precursor	2.3
Lipid A export ATP-binding/permease protein MsbA	2.2
Protein-export membrane protein SecD (TC 3.A.5.1.1)	2.0
Biopolymer transport protein ExbD/TolR	1.7
ABC transporter, ATP-binding protein	1.7
Heat shock (predicted periplasmic) protein YciM, precursor	1.6
Capsular polysaccharide export system inner membrane protein KpsE	1.6
Decreased in abundance at high chloride concentrations	
Outer membrane protein (porin)	37.1
HtrA protease/chaperone protein	35.8
Copper resistance protein CopC	29.2
membrane protein involved in aromatic hydrocarbon degradation	7.4
Heat shock protein 60 family co-chaperone GroES	7.0

Outer membrane lipoprotein SmpA, a component of the essential YaeT outer-membrane protein assembly complex	6.4
Uncharacterized ABC transporter, auxiliary component YrbC	6.2
Outer membrane protein (porin)	5.9
MotA/TolQ/ExbB proton channel family protein	5.0
Flagellar sensor histidine kinase FleS	4.2
Lipoprotein releasing system transmembrane protein LolC	4.1
18K peptidoglycan-associated outer membrane lipoprotein; Peptidoglycan-associated lipoprotein precursor; Outer membrane protein P6; OmpA/MotB precursor	4.1
Inner membrane protein translocase component YidC, long form	4.0
Outer membrane lipoprotein omp16 precursor	3.8
Protein export cytoplasm chaperone protein (SecB, maintains protein to be exported in unfolded state)	3.3
Outer membrane protein H precursor	3.2
MotA/TolQ/ExbB proton channel family protein	2.9
Probable Co/Zn/Cd efflux system membrane fusion protein	2.8
Outer membrane protein A precursor	2.6
RNA polymerase sigma-54 factor RpoN	2.4
Outer membrane lipoprotein LolB	2.1
Outer membrane efflux protein	2.1
TolB protein precursor, periplasmic protein involved in the tonb-independent uptake of group A colicins	2.1

Outer membrane protein NlpB, lipoprotein component of the protein assembly complex (forms a complex with YaeT, YfiO, and YfgL); Lipoprotein-34 precursor	1.9
Uncharacterized ABC transporter, periplasmic component YrbD	1.9
Outer membrane protein YfgL, lipoprotein component of the protein assembly complex (forms a complex with YaeT, YfiO, and NlpB)	1.8
MotA/TolQ/ExbB proton channel family protein	1.5

6.3.1.3 DNA repair, transcription and protein folding

High chloride ion and acid stress causes DNA damage, therefore, active repair of the cell's DNA would be necessary to cope with increased stress (4). The differential proteomic response uncovered a large number of proteins involved in DNA repair as well as RNA modifications that were increased under high chloride concentrations. These repair proteins included type III endonucleases, class V exodeoxyribonuclease chains, DNA and RNA helicases, recombination inhibitory proteins, exinuclease subunits, ligases and mismatch repair proteins, increased between 1.5-18.3 fold (Table 3). All these proteins have well characterized roles in DNA replication and repair.

The RNA polymerase sigma factor, RpoS, was found to be increased 8.65 fold at high chloride levels in *'Ac. aeolianus'* DSM 14174^T. This subunit of RNA polymerase has been found to be involved in increasing tolerance to high osmolarity, acidic pH, carbon starvation and high temperatures in *E. coli* (52). Therefore, the increased synthesis of this factor may provide tolerance to high osmotic stress and acidity in *'Ac. aeolianus'* DSM 14174^T.

The prevalence of chaperones in a wide range of acidophilic microorganisms has been suggested as a mechanism of coping with damage to their proteins under acid stress (4, 15). Chaperone with roles in protein refolding have previously been seen to be increased in *Leptospirillum* group II (53). The role of chaperones has also been discussed for *Ac. prosperus* DSM 5130^T, where it has been suggested that they play a role in the stress response to high chloride levels (15). A similar increase in protein chaperones was seen in *'Ac. aeolianus'* DSM 14174^T at 30 g/L chloride ion. This included DnaJ and DnaK molecular

chaperones (18.0 and 2.9 fold) as well as proteases with chaperone functions (4.8-5.0 fold) (Table 3).

Proteins with roles in DNA repair, transcriptional control and chaperone functions are shown in Table 3.

Table 3. Fold change in proteins involved in DNA repair, transcriptional control and protein folding upregulated in '*Ac. aeolianus*' DSM 14174^T at high chloride concentrations.

Increased in abundance at high chloride concentrations	**Fold change**
ATP-dependent DNA helicase RecG (EC 3.6.1.-)	18.3
DnaJ-class molecular chaperone CbpA	17.9
tRNA pseudouridine 13 synthase (EC 4.2.1.-)	17.1
Exodeoxyribonuclease V beta chain (EC 3.1.11.5)	10.7
RNA polymerase sigma factor RpoS	8.7
DinG family ATP-dependent helicase YoaA	8.0
Endonuclease III (EC 4.2.99.18)	5.4
G:T/U mismatch-specific uracil/thymine DNA-glycosylase	5.4
Zn-dependent protease with chaperone function	5.0
ATP-dependent DNA helicase RecQ	4.8
HtrA protease/chaperone protein	4.8
ATP-dependent RNA helicase RhlE	3.3
Chaperone protein DnaK	2.9
Transcription-repair coupling factor	2.7
LSU ribosomal protein L25p	2.5
Exodeoxyribonuclease V alpha chain (EC 3.1.11.5)	2.4

Recombination inhibitory protein MutS2	2.2
Excinuclease ABC subunit B	2.2
DNA ligase (EC 6.5.1.2)	2.1
ATP-dependent RNA helicase NGO0650	2.0
DNA repair protein RecN	2.0
Capsular polysaccharide export system inner membrane protein KpsE	1.6
Cell division protein FtsH (EC 3.4.24.-)	1.5

6.3.1.4 Carbon metabolism and nutrient assimilation

At high chloride concentrations, the abundance of two Csos1 carboxysome shell proteins decreased by 77.5 and 57.2 fold as did the ribulose 1, 5 bisphosphate carboxylase (RUBISCO) small and large chain proteins (33.1 and 23.5 fold decrease respectively). This suggests that carbon dioxide fixation was reduced at high chloride levels in *'Ac. aeolianus'* DSM 14174^{T}. However, there was an increase in some of the enzymes of the Calvin Bassham Benson (CBB) cycle, such as ribose 5-phosphate isomerase A (4.3 fold increase), Phosphoribulokinase (6.1 fold increase), NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (8.5 fold increase), triosephosphate isomerase (10.7 fold increase). At the same time, there was a decrease in other CBB cycle enzymes such as fructose 1,6 bisphosphatase type I (19.3 fold decrease) and transketolase (5.50 fold decrease), both enzymes which may have alternative roles in gluconeogenesis and the pentose phosphate pathways, respectively. Similarly, certain enzymes of the tricarboxylic acid (TCA) cycle showed an increase in abundance at high chloride levels while others showed a decrease in abundance. TCA cycle enzymes that increased in abundance included isocitrate dehydrogenase (2.6 fold increase), oxoglutarate dehydrogenase E1 component (4.0 fold increases) and fumarate hydratase class II (4.5 fold increase). A reduction in abundance was seen for the enzyme citrate synthase (2.5 fold decrease) and in dihydrolipoamide dehydrogenase and dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex (3.4 and 1.6 fold decrease, respectively). Citrate synthase converts oxaloacetate to citrate while the dihydrolipoamide dehydrogenases convert pyruvate to acetyl CoA – both key to

the TCA cycle. It is possible that the reduction in the dihydrolipoamide dehydrogenases is somewhat compensated by pyruvate dehydrogenase (1.6 fold increase), which also catalyzes the conversion of pyruvate to acetyl CoA for entry into the TCA cycle. However, it is yet to be determined how citrate is synthesized at high chloride levels in *'Ac. aeolianus'* DSM 14174^T. It is also unknown why some CBB and TCA cycle proteins were increased in abundance at high chloride concentrations despite the reduction in the proteins involved in carbon dioxide fixation. It is hypothesized that the increase in these enzymes provides components for the synthesis of capsular polysaccharides for protection of the cells at high chloride concentrations.

The phosphate ABC transporter for periplasmic phosphate binding protein PstS (3.02 fold decrease), which controls the PHO regulon for phosphate uptake, was less abundant at high chloride levels (insert Hsieh and Warner 2010 ref). Therefore, under these conditions, it is likely that the cells become phosphate-starved. This possibly explains the increased abundance of the phosphate starvation inducible protein ProH (3.0 fold) and exopolyphosphatases (1.8 fold) at high chloride concentrations – these systems are activated to release inorganic phosphates from polyphosphate. The ABC nitrate/sulfonate/bicarbonate transport system, ATPase component was increased (4.5 fold) suggesting an increase in the assimilation of nitrate, sulfonates and bicarbonates.

Fold changes for proteins all proteins involved in carbohydrate metabolism and assimilation of nutrients are shown in Table 4.

Table 4. Fold change in proteins linked to carbon metabolism and assimilation of nutrients in '*Ac. aeolianus'* DSM 14174^T at high chloride concentrations.

Increased in abundance at high chloride concentrations	**Fold change**
Triosephosphate isomerase (EC 5.3.1.1)	10.7
NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	8.5
Urease alpha subunit (EC 3.5.1.5)	7.3
Phosphoribulokinase (EC 2.7.1.19)	6.1

Fumarate hydratase class II (EC 4.2.1.2)	4.5
Ribose 5-phosphate isomerase A (EC 5.3.1.6)	4.3
2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	4.0
Isocitrate dehydrogenase (NADP) (EC 1.1.1.42)	2.6
6-phosphofructokinase (EC 2.7.1.11)	2.1
Dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61)	2.1
Fructokinase (EC 2.7.1.4)	2.1
Exopolyphosphatase	1.8
Nitrogen regulation protein NtrY (EC 2.7.3.-)	1.6
Pyruvate dehydrogenase E1 component (EC 1.2.4.1)	1.6
Aldehyde dehydrogenase (EC 1.2.1.3)	1.6
Decreased in abundance at high chloride concentrations	
Carboxysome shell protein CsoS1	77.5
Carboxysome shell protein CsoS1	57.2
Ribulose bisphosphate carboxylase small chain (EC 4.1.1.39)	33.1
Inorganic pyrophosphatase (EC 3.6.1.1)	27.8
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	23.5
Fructose-1,6-bisphosphatase, type I (EC 3.1.3.11)	19.3
Rubisco activation protein CbbQ	11.2
Nitrogen regulatory protein P-II	9.6
Ribulose-phosphate 3-epimerase (EC 5.1.3.1)	8.2

Transketolase (EC 2.2.1.1)	5.5
RuBisCO operon transcriptional regulator	3.7
Ferredoxin--NADP(+) reductase (EC 1.18.1.2)	3.6
Dihydrolipoamide dehydrogenase (EC 1.8.1.4)	3.4
Hypothetical transmembrane protein coupled to NADH-ubiquinone oxidoreductase chain 5 homolog	3.2
Phosphate ABC transporter, periplasmic phosphate-binding protein PstS (TC 3.A.1.7.1)	3.0
Citrate synthase (EC 2.3.3.1)	2.5
Nitrogen regulatory protein P-II	2.2
CsoS1D-associated protein 3	2.2
Nitrogen regulation protein NR(I)	2.1
Nitrogen regulation protein NtrB (EC 2.7.13.3)	2.0
Hypothetical transmembrane protein coupled to NADH-ubiquinone oxidoreductase chain 5 homolog	2.0
Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex (EC 1.8.1.4)	1.6
Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	1.5

6.3.1.5 Energy acquisition

At high chloride concentrations, there was a decrease in energy acquisition through iron oxidation, sulfur oxidation and the ATPase subunits. The largest decrease appeared to be in the oxidation of sulfur through the Blr3520 protein homology (4.0-40.0 fold decrease). This is interesting because sulfur oxidation in *'Ac. aeolianus'* DSM 14174^T is primarily thought to occur through the Sox system (17). The SoxA, SoxB and SoxZ subunits of the sulfur oxidation

system were also found to be reduced in abundance by 4.3, 1.7 and 26.9 fold, respectively. However, it was previously shown that SoxZ subunit was increased at high chloride concentrations (2.9 fold increase) in *Ac. prosperus* DSM 5130^T (15).

There was also a decrease in the MotA/TolQ/ExbB proton channel family protein (2.93 fold), which has been suggested to be involved in iron uptake into the cytoplasm across the outer membrane in *At. ferrooxidans* (54). This suggests that iron uptake is reduced in *'Ac. aeolianus'* DSM 14174^T at high chloride concentrations. Iron transport and oxidation through the downhill pathway showed a decrease, as shown by the decreased abundance of the proteins of the cytochrome c complex, rusticyanin and ATPases in in *'Ac. aeolianus'* DSM 14174^T at high chloride concentrations (1.6-33.7 fold decrease). There was also a decrease in Cytochrome d and O ubiquinol oxidases (1.7-2.9 fold decrease). However, the NADH-ubiquinone oxidoreductases, which are responsible for electron flow resulting in the generation of NADH from NAD+ (uphill pathway), showed an increase (1.7-9.5 fold increase). The significance of this is yet to be determined.

Enzymes involved in the metabolism of glycogen, such as 4-alpha-glucanotransferase (amylomaltase) (1.6 fold), 1,4-alpha-glucan glycogen branching enzyme, GH-13-type (3.7 fold) and glucose-1-phosphate adenylyltransferase (2.7 fold) were shown to increase at high chloride concentration. It is hypothesized that at high chloride levels *'Ac. aeolianus'* DSM 14174^T, glycogen stores are metabolized in order to produce energy to compensate for the reduction in iron and sulfur oxidation (55).

Fold changes for proteins involved in energy acquisition are shown in Table 5.

Table 5. Fold change in proteins involved in energy acquisition in *'Ac. aeolianus'* DSM 14174^T at high chloride concentrations.

Increased in abundance at high chloride concentrations	**Fold change**
NAD(FAD)-utilizing dehydrogenase, sll0175 homolog	11.3
NADH-ubiquinone oxidoreductase chain I (EC 1.6.5.3)	9.57
CoB—CoM heterodisulfide reductase subunit C (EC 1.8.98.1)	4.5
Ubiquinol-cytochrome C reductase iron-sulfur subunit (EC 1.10.2.2)	2.2

Ubiquinol—cytochrome c reductase, cytochrome B subunit (EC 1.10.2.2)	2.2
Cytochrome c-type biogenesis protein DsbD, protein-disulfide reductase (EC 1.8.1.8)	1.7
NADH-ubiquinone oxidoreductase chain E (EC 1.6.5.3)	1.7
CoB—CoM heterodisulfide reductase subunit B (EC 1.8.98.1)	1.6
Decreased in abundance at high chloride concentrations	
Blr3520 protein homolog	40.0
Cytochrome c family protein	33.7
Sulfur oxidation protein SoxZ	26.9
Blr3520 protein homolog	12.5
Blr3520 protein homolog	10.6
ATP synthase epsilon chain (EC 3.6.3.14)	9.7
Uncharacterized ABC transporter, ATP-binding protein YrbF	9.4
Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	8.7
rusticyanin related protein	6.1
ATPase associated with various cellular activities AAA_5	5.1
ATP synthase gamma chain (EC 3.6.3.14)	4.9
Transport ATP-binding protein CydD	4.6
sulfur oxidation protein SoxA	4.3
Blr3520 protein homolog	4.0
ATP synthase beta chain (EC 3.6.3.14)	3.6
Cytochrome c precursor	3.5

ATP synthase alpha chain (EC 3.6.3.14)	3.1
ATP phosphoribosyltransferase regulatory subunit (EC 2.4.2.17)	3.1
ATP synthase delta chain (EC 3.6.3.14)	2.9
Cytochrome O ubiquinol oxidase subunit III (EC 1.10.3.-)	2.9
Cytochrome c oxidase polypeptide I (EC 1.9.3.1)	2.9
Cytochrome O ubiquinol oxidase subunit II (EC 1.10.3.-)	2.6
ATP phosphoribosyltransferase (EC 2.4.2.17)	2.6
cytochrome c oxidase assembly factor	2.5
ABC transporter, ATP-binding protein	2.2
Cytochrome oxidase biogenesis protein Sco1/SenC/PrrC, putative copper metallochaperone	1.8
Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)	1.7
Sulfur oxidation protein SoxB	1.7
Bacterioferritin (cytochrome b1)	1.6

6.3.1.6 Other stress responses at high chloride concentrations

Tolerance to oxidative stress was found to decrease at high chloride ion levels in *'Ac. aeolianus'* DSM 14174^T as evidenced by the decrease in abundance of superoxide dismutase (11.6 fold decrease), thiol peroxidase (10.1 fold decrease) and rubethyrin (4.3 fold decrease) at high chloride concentrations. However, in *Ac. prosperus* DSM 5130^T at high chloride concentrations, the oxidative stress response was shown to increase (15). Also, an ATP dependent Clp protease proteolytic subunit and its ATP binding subunit were found to be decreased by 10.3 and 1.6 fold, respectively, at high chloride concentrations. Clp proteases have previously been seen to increase in *Bacillus subtilis* during high salt and oxidative stress as well as during glucose deprivation, while in *Lactococcus lactis* it is increased by changes in the pH (56, 57). Therefore, it is yet to be determined why *Ac. aeolianus* DSM 14174^T shows a

decrease in proteins involved in general stress responses commonly seen in other microorganisms. The fold changes for proteins involved in oxidative stress response and proteolysis are shown in Table 6.

Table 6. Fold change in proteins involved in oxidative stress and proteolysis in '*Ac. aeolianus'* DSM 14174T at high chloride concentrations.

Decreased in abundance at high chloride concentrations	**Fold change**
Superoxide dismutase (Fe) (EC 1.15.1.1)	11.6
ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)	10.3
Thiol peroxidase, Tpx-type (EC 1.11.1.15)	10.1
Survival protein SurA precursor (Peptidyl-prolyl cis-trans isomerase SurA) (EC 5.2.1.8)	4.35
Rubrerythrin	4.25
ATP-dependent Clp protease ATP-binding subunit ClpX	1.65

6.3.2 Model of *'Ac. aeolianus'* DSM 14174^T response to elevated chloride concentrations

High chloride ion concentrations cause both osmotic stress and an acidification of the intracellular pH in acidophilic microorganisms (Zammit and Watkin 2016). This is a result of the ability of chloride to cross the membrane, thereby causing the influx of protons, which ultimately leads to a collapse of the inside positive membrane potential maintained by acidophiles (15). The ability of *'Ac. aeolianus'* DSM 14174^T to oxidize iron and sulfur in the presence of high chloride ion concentrations as well as low pH makes it an ideal candidate for saline water bioleaching of mineral sulfides. The response of *Ac. prosperus* DSM 5130^T and *At. ferrooxidans* ATCC 23270 have previously been studied. However, this is the first study to be performed on the proteomic response of *'Ac. aeolianus'* DSM 14174^T to high chloride concentrations. Here, we propose a model of the response of *'Ac. aeolianus'* DSM

14174^T at high chloride concentrations based on differential protein expression observed at high and low chloride concentrations as determined by SWATH analysis.

The main response of *'Ac. aeolianus'* DSM 14174^T directly related to the increase in osmotic pressure caused by high chloride concentration is the increase in abundance of multiple forms of osmoprotectants. The increase in abundance of ectoine synthase by 422.9 fold is the largest increase amongst the proteins in the study, suggesting that ectoine is the primary osmoprotectant used by '*Ac. aeolianus'* DSM 14174^T at high chloride concentrations. Increase in abundance of glutathione and various amino acids (glutamate, glutamine, lysine and proline) suggest that these are also key osmoprotectants used by this species. DNA repair and transcription control as well as the biosynthesis of membrane components through the proteins that have roles in the synthesis of polysaccharides and lipopolysaccharides, are also increased at increased chloride concentrations, in order to protect the cells from the deleterious effects of increased acid stress that results from the influx of protons upon chloride entry into the cell. Protection against harmful substances such as drugs and toxic compounds is mediated by the increased efflux of these compounds across the membrane, as indicated by the increase in transport proteins for these substances at elevated chloride concentrations. Furthermore, metals are removed from the cells through the use of efflux pumps, their breakdown by reductases and through their complexation with inorganic phosphates released from the activity of exopolyphosphates.

A shift in carbon flux occurs at high chloride concentrations whereby there is a decrease in carbon dioxide fixation. However, certain enzymes involved in carbohydrate metabolism were found to increase in abundance at high chloride levels in order to provide polysaccharides for the synthesis of membrane components. Furthermore, there is a reduction in energy acquisition through iron and sulfur oxidation which is compensated for by the metabolism of glycogen to release energy as well as by conservation of energy through a reduction in the proteins involved in tolerance the assimilation of phosphates and ammonia. The enzyme activity of exopolyphosphatases and ureases can then be used to meet the cells' phosphate and ammonia requirements, respectively. The activity of the ureases may further ameliorate the increased pH stress caused by an influx of protons in response to the decreased transmembrane potential as a result of chloride entry into the cell. Overall, the response of *'Ac. aeolianus'* DSM 14174^T is manifold and has shown important differences that further solidify its differences to *Ac. prosperus* DSM 5130^T,

thereby highlighting the versatility of different members of the *Acidihalobacter* genus in their tolerances and survival at low pH and high chloride concentrations.

The proposed model for tolerance to high chloride ion levels and low pH along is shown in Figure 1.

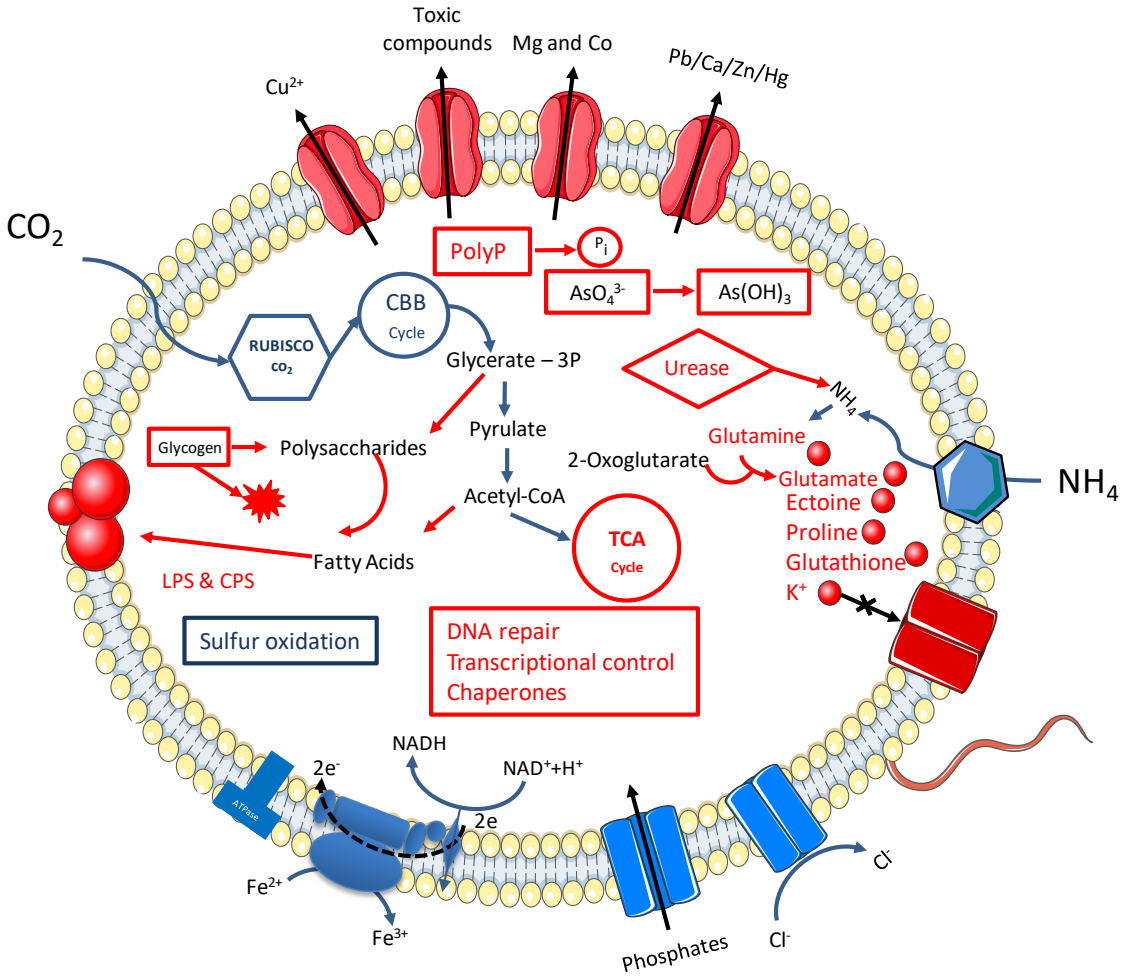

Figure 1. The multiple responses of *Ac. aeolianus* DSM 14174^T to high chloride concentrations. Red boxes, arrows and images depict proteins that increased in abundance at high chloride concentrations while those in blue depict proteins of decrease abundance at high chloride concentrations.

6.4 Conclusions

The difference in the osmotic stress response of *Ac. prosperus* DSM 5130 and *'Ac. aeolianus'* DSM 14174^T suggest that versatility exists in the mechanisms used by different species of the *Acidihalobacter* to tolerate high chloride concentrations simulatenously with low pH. Further work is required to determine the mechanisms of osmotic stress tolerance in *'Ac. vulcanensis'* DSM 14175^T and '*Ac. yilgarnensis*' strain F5 in order to gain a better understanding of the unique defining properties of all the members of the *Acidihalobacter* genus.

6.5 References

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7 Chapter 7

7.1 Discussion

The initial objective of the study was to assess the chloride ion tolerances of seven environmental cultures that had previously been sourced from acid saline environments. The cultures were obtained from culture collections at CSIRO Land and Water and the University of Exeter and contained both pure isolates and mixed enrichments of acidophilic, iron and/or sulfur-oxidizing microorganisms. Pure cultures *Acidihalobacter prosperus* DSM 14174 (strain V6) and '*Ac. ferrooxidans'* DSM 14175 (strain V8) and the mixed culture 14C were from shallow acidic pools in the Aeolian Islands, Italy. Pure culture M8 was from a similar environment in Milos, Greece. The mixed enrichment cultures, L2-21, L4-9 and L6-11, were bioprospected from acidic saline soils and surface waters from the Western Australian Wheat belt and Southwest region. When grown in basal salts media at increasing chloride ion concentrations with ferrous sulfate and tetrathionate as soluble iron and sulfur sources, respectively, it was found that pure isolates of *Ac. prosperus* DSM 14174 and '*Ac. ferrooxidans'* DSM 14175 showed the maximum ability to oxidize the soluble iron and sulfur substrates at high chloride concentrations (up to 45 g/L chloride). Analysis of the community diversity profiles revealed that the mixed cultures consisted primarily of *Acidihalobacter* spp. (14C), *Acidithiobacillus albertensis* (L2-21), *Ferroplasma* spp. (L4-9 and L6-11).

Having assessed chloride ion tolerance, a preliminary assessment of bioleaching capabilities of the four mesophilic cultures (V6, V8, 14C and L2-21) was carried out on 1% pyrite at 9, 15 and 30 g/L chloride concentrations. The results of the pyrite bioleaching studies showed that pure cultures V6 and V8 were able to oxidize pyrite more efficiently than the mixed culture 14C at high chloride levels, successfully bioleaching the mineral ore at up to 30 g/L chloride concentrations. However, mixed culture L2-21 failed to oxidize pyrite. The inability of L2-21 to release iron from pyrite was possibly due to the dominance of the sulfur oxidizing acidophile, *At. albertensis*, as had been determined during the chloride ion tolerance studies. It has previously been reported that the activity of iron oxidizers on insoluble ores is the first step in the metal release process, while the sulfur oxidizers play a role in the maintenance of the low pH of the system (1). Though the mixed culture L2-21 showed oxidation of soluble iron during the chloride tolerance tests, it is possible that the key

players in the oxidation of iron were inhibited when provided an insoluble source of iron (pyrite).

The most extensively studied iron- and sulfur oxidizing acidophile, *At. ferrooxidans*, is known to be completely inhibited by chloride concentrations as little as 7 g/L (2-4). Prior studies have shown that most other pure isolates of acidophilic microorganisms, such as those belonging to *Leptospirillum* spp., *Sulfobacillus* spp., *Sulfolobus* spp., *Thermoplasma* spp. and *Ferroplasma* spp. also do not tolerate chloride concentrations of more than 12.3 g/L chloride (5). *Ac. prosperus* DSM 5130^T is the first halotolerant acidophilic species to be described, requiring 1.4 g/L chloride ion for its growth and oxidizing ferrous iron at up to 30 g/L chloride ion (2) The pure isolates, V6 and V8, are known to be members of the *Acidihalobacter* genus. These studies have shown their ability to tolerate up to 45 g/L chloride ion on ferrous iron and the ability to oxidize pyrite at up to 30 g/L chloride ion. Therefore, due to their unique ability to oxidize iron and sulfur at high chloride concentrations simultaneously with extremely low pH, the remaining study focussed on further characterization of members of the *Acidihalobacter* genus.

The *Acidihalobacter* genus has recently been reclassified (previously '*Thiobacillus*') based on the reclassification of *Ac. prosperus* DSM 5130^T, the first and only member of this genus to be formally classified (2, 6). The V6 and V8 had previously been provisionally classified as members of the *Acidihalobacter* spp. based on their 16S rRNA sequences (7, 8). Both had been tested for pyrite oxidation in mixed culture, the results of which had determined that they are able to tolerate high levels of chloride ion, with isolate V8 dominating a mixed culture grown on pyrite (7). Isolate V6 has been named *Ac. prosperus* DSM 14174 strain V6, whereas isolate V8 had been given the provisional classification of *'Ac. ferrooxidans'* DSM 14175 strain V8. However, having tested their ability to oxidize iron and sulfur at high chloride concentrations, it was rationalized that further phylogenomic and taxonomical characterization of *Ac. prosperus* DSM 14174 and '*Ac. ferrooxidans'* DSM 14175 may lead to a better understanding of these unique microorganisms. Another isolate that was known to be an iron- and sulfur-oxidizing halotolerant acidophile of the *Acidihalobacter* genus, *Ac. prosperus* strain F5, was also included in the study due to its ability to leach the minerals pyrite, chalcopyrite and pentlandite at up to 45 g/L chloride ion stress in pure culture (9). Therefore, the genomes of *Ac. prosperus* DSM 14174, *'Ac. ferrooxidans'* DSM 14175 and *Ac. prosperus* strain F5 were sequenced using the latest in Next Generation Sequencing (NGS),

the PacBio RS SMRT System. Pacbio long-read sequencing allows for generating more comprehensive *de novo* assemblies than previous sequencing methods, providing more complete and accurate resolution of genomes (10).

A complete genome sequence for *Ac. prosperus* strain F5 was assembled from the long-read sequences obtained through Pacbio sequencing, thereby providing the first complete genome sequence of a halotolerant acidophile. *Ac. prosperus* DSM 14174 and '*Ac. ferrooxidans'* DSM 14175 were also assembled into high quality, near complete genome assemblies, however, for this purpose Illumina Mi-seq (paired end 2x300bp) sequencing results were also used to create hybrid *de novo* assemblies that were scaffolded to give the final assembly. The final genome assemblies consisted of one gap in the genome of *Ac. prosperus* DSM 14174 and six gaps in the genome of '*Ac. ferrooxidans'* DSM 14175.

The genome sequencing results provided the first evidence of differences in the genomes of the isolates, such as in the presence of a unique 162,484 bp plasmid in the genome of *Ac. prosperus* DSM 14714. This confirmed that a complete phylogenetic classification of the pure isolates of the *Acidihalobacter* spp. may lead to the discovery of further differences between the members of this genus. Therefore, in-depth phylogenomic characterization of the three genomes was undertaken. The phylogenomic study resulted in the reclassification of the isolates and provided evidence that they are novel species of the *Acidihalobacter* genus. The names proposed for the isolates are as follows: *Ac. prosperus* DSM 14174 strain V6 was reclassified as '*Ac. aeolianus'* DSM 14174^{T} strain V6; *'Ac. ferrooxidans'* DSM 14175 strain V8 as '*Ac. vulcanensis'* DSM 14175^{T}and *Ac. prosperus* strain F5 as '*Ac. yilgarnensis'* strain F5^{T} (pending allocation of a DSM number). The new names of the species will be used from this point onwards.

Having phylogenetically reclassified the members of the *Acidihalobacter* genus, the next aim of the project was to use the genome information to generate predictive models of metabolic potential and stress tolerance mechanisms of the novel members of this genus. Therefore, the genomes of all members of the *Acidihalobacter* genus were analysed for genes involved in key pathways of nutrient assimilation, energy acquisition and stress tolerance. The genomic features determined were compared between the members of the species as well as to genomes of other acidophiles and halophiles, in order to determine features unique to the members of the *Acidihalobacter* genus in their ability to assimilate

nutrients and tolerate acid, osmotic and heavy metal stress. The analysis revealed important similarities and differences, as discussed below.

Some nutrient assimilation pathways in the *Acidihalobacter* spp. appeared to be common amongst all the members, while others showed differences that suggest diversity in their metabolic potential. All members of the *Acidihalobacter* genus appear to fix carbon dioxide through the Calvin-Bassham-Benson (CBB) cycle, for which all the key genes are present on all the genomes. The genomes also contained genes encoding proteins involved in carboxysome formation, including those for the novel carboxysome shell protein Csos1D that has been suggested to have a role in metabolite transport across the carboxysome shell to increase the efficiency of carbon dioxide fixation (11-13). The gene for Csos1D has previously only been described for the halophile, *Pyrochlorus marinus*, suggesting that it is a feature of the halophilic lifestyle (11). However, there appear to be differences in the organization of carboxysome shell encoding genes as well as in the presence of the genes encoding carbonic anhydrase, suggesting that other factors may also play a role in efficiency of carbon dioxide fixation of the different species of this genus. The genomes of all species of *Acidihalobacter* were also found to contain the full set of genes for the citric acid (TCA) cycle, which has, to date, only been seen in the genome of the acidophilic, iron oxidizing '*Ferrovum*' spp. but has not been found on the genomes of other iron- and sulfur-oxidizing acidophilic microorganisms (14).

Nitrogen assimilation appears to differ in each of the species, with only *Ac. aeolianus* DSM 14175 and *Ac. prosperus* DSM 5130 carrying the required genes for fixing atmospheric nitrogen through the FeMo nitrogenase complex. However, all the members of the *Acidihalobacter* genus have genes common for nitrogen assimilation through ammonia transporters, amino acid synthesizing enzymes and nitrate/nitrite reductases. Furthermore, the genes for ureases were found on all the genomes, though only '*Ac. vulcanensis'* DSM 14175^T had a urea transporter. This suggests the ability of these microorganisms to use urea as a source of nitrogen. Ureases have also been reported to have a role in tolerance to low pH, therefore, the function of ureases in the members of the *Acidihalobacter* spp. may an additional mechanism of acid stress tolerance.

The presence of genes encoding proteins involved in the uptake of alkanesulfonates were found only in '*Ac. aeolianus'* DSM 14174^T suggests the ability of this species to use aliphatic sulfonates. Furthermore, the presence of sulfate adenylyltransferase and adenylylsulfate

reductase genes only in '*Ac. yilgarnensis'* strain F5^T and *Ac. aeolianus* DSM 14174^T may indicate their ability to utilize sulfates directly.

Potential mechanisms of energy acquisition through iron and sulfur oxidation were queried through the study of the genes involved in these pathways. *Ac. prosperus* DSM 5130^T, '*Ac. aeolianus'* DSM 14174^T and '*Ac. yilgarnensis'* strain F5^T showed similar organization of genes involved in the oxidation of ferrous iron for energy production as well as those encoding the subunits of the truncated SOX system for the oxidation of sulfur. Furthermore, the presence of a gene encoding the SoxW thioredoxin in *Ac. prosperus* DSM 5130^T and '*Ac. yilgarnensis'* strain F5^T indicate an extra gene for sulfur oxidation for these two species. However, the genome of '*Ac. vulcanensis'* DSM 14175^T showed the absence of the SOX system genes and also showed key differences in the *rus* operon for iron oxidation. Unlike the other members of the *Acidihalobacter* genus, the genome of '*Ac. vulcanensis'* DSM 14175^T contained a truncated version of the rus operon that was missing the *cyc2*, *coxC*, *coxD* and *cyoE.* Also, in the place of a gene for rusticyanin was a gene encoding the copper protein sulfocyanin

Bioleaching microorganisms thrive in habitats that are rich in acid, oxidative and metal stress and therefore require the ability of multiple mechanisms to tolerate these conditions. Genomic analysis found the presence of genes encoding ion transporters, proton pumps and multiple genes involved in membrane biosynthesis, which may play a role in acid stress tolerance in these microorganisms, as has previously been discussed (15). Genes encoding superoxide dismutases, peroxiredoxins and thiol peroxidases were found on all genomes. These may have a role in the mitigation of reactive oxygen species that cause oxidative damage to cells. A unique feature was the presence of catalases and peroxidases in the genomes of '*Ac. yilgarnensis'* strain F5^T, '*Ac. vulcanensis'* DSM 14175^T and *Ac. prosperus* DSM 5130^T. These genes have not been seen in the genomes of other studied acidophiles or in the genome of '*Ac. aeolianus'* DSM 14174^T (16). This suggests an increased ability of these *Acidihalobacter* spp. to cope with high oxidative stress. Furthermore, tolerance of heavy metal stress in these species may occur through the presence of the product of genes encoding multiple heavy metal cation efflux proteins. Also, the presence of genes for exopolyphosphates suggest that, under heavy metal stress, the members of the *Acidihalobacter* genus may use the breakdown of polyphosphates to inorganic phosphates. These inorganic phosphates can then bind to metal cations, forming complexes that can then be pumped out the cell, as has previously been described for *At. ferrooxidans* (17).

Furthermore, the differences in copy numbers of ATP copper translocating ATPases and the absence of copper uptake and cation efflux pump genes in '*Ac. vulcanensis*' DSM 14175^T, suggest possible differences in the tolerances to copper, though experimental determination is required to confirm these findings.

The key interest in this study was to understand the mechanisms used by the *Acidihalobacter* spp. to tolerate high chloride concentration, a feature that sets them apart from other previously characterized iron- and sulfur-oxidizing acidophiles. High chloride concentrations have a deleterious effect on acidophilic microorganisms through the ability to cause osmotic stress as well as the ability of the chloride ions to permeate cells and reduce the transmembrane potential maintained by these microorganisms. The genomes of the *Acidihalobacter* spp. were searched for genes which encode proteins that may have roles in osmoprotection as well as active mechanisms of chloride efflux. The genomes revealed the presence of genes involved in the biosynthesis of many metabolites which have previously been suggested roles to be involved in osmoprotection in acidophiles, such as ectoine, taurine, lysine, trehalose, glycine betaine and proline. Key differences were seen in the presence of the choline dehydrogenase gene only in '*Ac. yilgarnensis*' strain F5^T and '*Ac. vulcanensis*' DSM 14175^T, which suggest that glycine betaine can be synthesized by an additional pathway in these two species. Different transport systems were also seen for glycine betaine in all the species, suggesting differences in their ability to use this as an osmoprotectant. While glucan biosynthesis genes were present on all genomes, an extra glucan biosynthesis gene was found in the genome of '*Ac. yilgarnensis*' strain F5^T suggesting increased synthesis of glucan biosynthesis for use as an osmoprotectant in this species. Differences were also seen in the proline biosynthesis genes in all the species, including a unique lysine exporter on *Ac. prosperus* DSM 5130^T. This suggests methods of transport of proline to the cytoplasm so that it can be used as an osmoprotectant. Interesting, the presence of taurine uptake genes but the absence of taurine utilization genes in '*Ac. yilgarnensis*' strain F5^T and *Ac. prosperus* DSM 5130^T also suggest that these species accumulate taurine as an osmoprotectant rather than metabolizing it. Finally, the presence of multiple chloride ion channels proteins as well as a predicted extracytoplasmic osmotic shock tolerance protein suggests the possible efflux of chloride ion to protect the cells when faced with high chloride levels.

Having analysed the genomes of the members of the *Acidihalobacter* spp. for clues to the metabolic pathways and stress tolerance mechanisms they utilize for survival in their unique habitats, the study then aimed to validate these genome-scale models. A previous study has reported mechanisms of osmotic stress tolerance for *Ac. prosperus* DSM 5130^T in comparison to *At. ferrooxidans* ATCC 23270^T (18). However, it was hypothesized that variation may exist in the proteomic response of different species to varying levels of chloride concentrations.

Protein extractions were performed for *'Ac. aeolianus'* DSM 14174^T, *'Ac. vulcanensis'* DSM 14175^T and *'Ac. yilgarnensis'* strain F5^T at high (30 g/L chloride ion) and low (5 g/L chloride ion) concentrations. However, despite repeated attempts, proteomic analysis of *'Ac. vulcanensis'* DSM 14175 and *'Ac. yilgarnensis'* strain F5^T could not be undertaken due to the inability to extract sufficient quantities of protein for SWATH analysis. Therefore, it was only possible to study the differential proteome response of '*Ac. aeolianus'* DSM 14174^T at high and low chloride concentrations.

The response of '*Ac. aeolianus'* DSM 14174^T to high chloride concentrations in comparison with the response to low chloride concentrations was manifold. Proteogenomic studies determined that the mechanism of osmotolerance at high chloride concentrations was through the accumulation of the osmoprotectant ectoine, followed by the accumulation of glutathione and the amino acids glutamate, glutamine, lysine and proline. The increase in proteins involved in biosynthesis of membrane components, carbohydrate metabolism and DNA repair mechanisms provides the ability of '*Ac. aeolianus'* DSM 14174^T to protect itself from damage caused by acid and osmotic stress. Furthermore, the increased abundance of ureases, which are not commonly found in acidophiles, at high chloride concentrations suggests the ability of these microorganisms to utilize urea for production of amino acids as well as for defence against low pH. The increased abundance of metal cation transporters and proteins involved in metal cation binding helps to explain the ability of these microorganisms to survive high levels of metal stress even in the presence of osmotic and acid stress. While iron and sulfur oxidation decrease at high chloride concentrations, the cells have an increase in proteins involved in glycogen metabolism, which compensates for the decrease in energy production at high chloride concentrations.

Overall, the characterization of members of the *Acidihalobacter* genus has allowed for assessment of their chloride ion tolerances and bioleaching properties, which have shown

their ability to oxidize iron and sulfur at chloride ion concentrations well above the 19 g/L present in seawater and above concentrations that other described biomining microorganisms are able to tolerate. Their ability to oxidize soluble iron and sulfur sources at up to 45 g/L chloride concentrations as well as their ability to oxidize pyrite successfully at up to 30 g/L chloride ion present the highest reported tolerances for pure of acidophilic iron/sulfur oxidizing microorganisms to date. The generation of genome-scale models for these unique microorganisms has been a valuable tool for understanding the inner workings of metabolic networks and has enabled prediction of nutrient assimilation pathways as well as multiple pathways for stress tolerance that help to explain how these microorganisms are able to survive harsh environments present in bioleaching systems. Furthermore, generation of a model of the proteomic response of *'Ac. aeolianus'* DSM14174^T to high chloride concentrations has enabled verification of the mechanisms employed by this species to survive the high acid, osmotic and metal stresses that characterize the environments in which they thrive. This study has, therefore, highlighted the potential applicability of members of the *Acidihalobacter* genus in bioleaching operations where high chloride concentrations exist.

7.2 Future Considerations

It has been shown that key differences exist in the mechanisms of tolerance to high chloride concentrations by *Ac. prosperus* DSM 5130^T and *'Ac. aeolianus'* DSM 14174^T. Further work is required to understand the mechanisms of osmotic stress tolerance in the other two species of the *Acidihalobacter* genus, *'Ac. vulcanensis'* DSM 14175 and *'Ac. yilgarnensis*' strain F5. This would enable deeper understanding of the unique members of the *Acidihalobacter* genus.

Furthermore, the ability of *'Ac. yilgarnensis'* strain F5 to oxidize chalcopyrite at up to 45 g/L chloride concentrations makes it worthy of further study to determine its potential application for saline water bioleaching of chalcopyrite.

7.3 References

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8 Appendices

8.1 Appendix 1

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2. Himel Khaleque, Joshua Ramsay, Riley Murphy, Anna Kaksonen, Naomi Boxall, Elizabeth L. J. Watkin (2017). Dra

   genome sequence of the acidophilic, halotolerant, and iron/sulfur-oxidizing *Acidihalobacter prosperus* DSM 14174 (strain V6). Genome Announc. January 2017 5:Doi:10.1128/Genomea.01469-16

Many thanks

Professor Elizabeth Watkin

Director of Research Training | School of Biomedical Sciences

Faculty of Health Sciences

Curtin University

8.2 Appendix 2

The following pages contain the written statements of the co-authors of published or submitted manuscripts forming the chapters of this thesis.

To Whom It May Concern,

I, Himel Nahreen Khaleque, as the first author of the publication entitled ""**Chloride ion tolerance and pyrite bioleaching capabilities of pure and mixed halotolerant, acidophilic iron- and sulfur-oxidizing cultures**"", declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.

Himel Nahreen Khaleque.

First author signature

I, as a Co-author, endorse that this level of contribution by the first author indicated above is appropriate.

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To Whom It May Concern,

I, Himel Nahreen Khaleque, as the first author of the publication entitled **"Draft Genome Sequence of the Acidophilic, Halotolerant, and Iron/Sulfur-Oxidizing Acidihalobacter prosperus DSM 14174 (Strain V6)"**, declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.

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To Whom It May Concern,

I, Himel Nahreen Khaleque, as the first author of the publication entitled **"Draft Genome Sequence of Acidihalobacter ferrooxidans DSM 14175 (Strain V8), a New Iron- and Sulfur-Oxidizing, Halotolerant, Acidophilic Species"**, declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.

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To Whom It May Concern,

I, Himel Nahreen Khaleque, as the first author of the publication entitled **"Complete genome sequence of Acidihalobacter prosperus strain F5, an extremely acidophilic, iron- and sulfur-oxidizing halophile with potential industrial applicability in saline water bioleaching of chalcopyrite"**, declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.

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To Whom It May Concern,

I, Himel Nahreen Khaleque, as the first author of the publication entitled **"Genome-based reclassification of two extremely acidophilic, iron- and sulfur-oxidizing halophiles *'Acidihalobacter prosperus'* strain V6 and *'Acidihalobacter ferrooxidans'* strain V8 as two new species, *Acidihalobacter aeoliarum* sp. nov., and *Acidihalobacter vulcanensis* sp. nov., respectively"**, declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.

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8.3 Appendix 3

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PROKARYOTES

Draft Genome Sequence of the Acidophilic, Halotolerant, and Iron/Sulfur-Oxidizing *Acidihalobacter prosperus* DSM 14174 (Strain V6)

Himel Nahreen Khaleque,[a] Joshua P. Ramsay,[a] Riley J. T. Murphy,[a] Anna H. Kaksonen,[b] Naomi J. Boxall,[b] Elizabeth L. J. Watkin[a]

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ABSTRACT The principal genomic features of *Acidihalobacter prosperus* DSM 14174 (strain V6) are presented here. This is a mesophilic, halotolerant, and iron/sulfur-oxidizing acidophile that was isolated from seawater at Vulcano, Italy. It has potential for use in biomining applications in regions where high salinity exists in the source water and ores.

Received 2 November 2016 **Accepted** 14 November 2016 **Published** 19 January 2017

Citation Khaleque HN, Ramsay JP, Murphy RJT, Kaksonen AH, Boxall NJ, Watkin ELJ. 2017. Draft genome sequence of the acidophilic, halotolerant, and iron/sulfur-oxidizing *Acidihalobacter prosperus* DSM 14174 (strain V6). Genome Announc 5:e01469-16. https://doi.org/10.1128/genomeA.01469-16.

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Acidihalobacter prosperus (previously known as *Thiobacillus prosperus*) is a Gram-negative, halotolerant, acidophilic, mesophilic, and chemolitho-autotrophic bacterium capable of oxidizing both iron and reduced sulfur compounds (1). The type strain, *A. prosperus* DSM 5130, was isolated from a marine geothermal field in Italy. It requires a minimum of 0.04 M Cl^- for growth and tolerates up to 0.6 M Cl^- (1). *A. prosperus* DSM 14174 was isolated from a shallow acidic pool by the shore of Baia de Levant, Aelion Islands of Vulcano, Italy (2). Like *A. prosperus* DSM 5130, it does not grow in the absence of salt (3). It has been used in salt-rich systems for the active biomining of metal sulfide ores (4).

Total DNA extracted from *A. prosperus* DSM 14174 was sequenced using Illumina MiSeq (204,485 paired-end 300 bp × 2 reads) and PacBio RS single-molecule real-time (SMRT) sequencing technologies (96,369 reads postfilter, 11,756 bp mean length). *De novo* hybrid assembly using SPAdes 3.9.0 (5) produced a circular 162,484-bp plasmid (~32-fold coverage) and two chromosome fragments of 2,607,071 bp and 752,753 bp (~18-fold coverage). The two chromosome fragments were scaffolded using SSPACE-LongRead version 1.1 (6), producing a circular chromosome of 3,363,634 bp (with one gap). The genome has a G+C content of 62.2%. The NCBI Prokaryotic Genome Annotation Pipeline version 3.3 and GeneMarkS+ were used for annotation. The genome contains 46 tRNA sequences, one rRNA operon, and 3,194 protein-coding genes.

Genome analysis of *A. prosperus* DSM 14174 confirmed the presence of the previously reported *rus* operon known to be involved in iron oxidation (3). Also present were genes coding for subunits SoxAX, SoxB, and SoxYZ of the sulfur oxidation system (7), as well as those for sulfur metabolism through hydrogen sulfide biosynthesis (8). Furthermore, there were genes encoding proteins involved in various catalytic reactions for oxidation/reduction of sulfur as well as the transport of sulfate/sulfonate (8). Similar to the genome of the type strain *A. prosperus* DSM 5130, the genome of *A. prosperus* DSM 14174 contains a complete set of genes for carbon dioxide fixation via the Calvin-Benson-Bassham cycle, as well as those for the Nif complex for nitrogen fixation, chemotaxis, and formation of a polar flagellum (9).

The synthesis of compatible solutes, such as ectoine, sucrose, and glycine betaine, assists in the survival of bacteria under high osmotic stress (10). The genome of *A. prosperus* DSM 14174 contains genes that encode diaminobutyrate aminotransferases, diaminobutyrate acetyltransferase, ectoine synthase, and sucrose synthase. These have potential roles in ectoine and sucrose biosynthesis pathways. Genes for ABC transporters for ectoine and glycine betaine uptake were also detected.

A. prosperus DSM 14174 contains a single plasmid, pABPV6, which is unique to this strain. The plasmid pABPV6 contains an array of genes coding for replication and transfer proteins, transposases, DNA methyltransferases, recombinases, hydrolases, and DNA binding proteins.

Accession number(s). The whole-genome sequence has been deposited at DDBJ/EMBL/GenBank under the GenBank accession no. CP017448. The plasmid pABPV6 has been deposited under the GenBank accession no. CP017449. The versions described in this paper are CP017448.1 and CP017449.1, respectively.

ACKNOWLEDGMENTS

This study was funded by the Curtin Strategic International Research Scholarship and CSIRO Mineral Resources Postgraduate Scholarship.

We thank Paul Norris, University of Exeter, for his generous donation of the pure culture of *Acidihalobacter prosperus* DSM 14174 (strain V6).

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Draft Genome Sequence of *Acidihalobacter ferrooxidans* DSM 14175 (Strain V8), a New Iron- and Sulfur-Oxidizing, Halotolerant, Acidophilic Species

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ABSTRACT The use of halotolerant acidophiles for bioleaching provides a biotechnical approach for the extraction of metals from regions where high salinity exists in the ores and source water. Here, we describe the first draft genome of a new species of a halotolerant and iron- and sulfur-oxidizing acidophile, *Acidihalobacter ferrooxidans* DSM 14175 (strain V8).

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The halotolerant acidophile *Acidihalobacter prosperus* is well known for its ability to oxidize iron at low pH under saline conditions (1, 2). *A. ferrooxidans* DSM 14175 (strain V8) represents a similar group of Gram-negative, mesophilic, halotolerant acidophiles that also has the ability to oxidize iron and sulfur and has a chemolithoautotrophic lifestyle. It was isolated from the same shallow acidic pool at the Aeolian Islands of Italy as *A. prosperus* DSM 14174 (strain V6) (3) and was found to dominate mixed cultures during mesophilic pyrite oxidation (4).

Total DNA was extracted from *A. ferrooxidans* DSM 14175 using the modified method of nucleic acid extraction for acidophiles, as described by Zammit et al. (5). DNA was sequenced using Illumina MiSeq (619,160 paired-end reads, 2 × 300-bp reads) and PacBio RS SMRT sequencing technologies (733,419 subreads with a mean read length of 1,602 bp). *De novo* hybrid assembly using SPAdes version 3.9.0 (6) generated 10 contigs, which were then used with PacBio reads to generate a scaffold using SSPACE-LongRead version 1.1 (7). The resulting scaffold was a single circular chromosome with an approximate size of 3,448,835 bp (with 4 gaps with a total approximate size of 6 kb) with approximately 13× Illumina read depth and 355× PacBio read depth. The genes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline version 3.3 and GeneMarkS+. The genome has a G+C content of 61.6% and contains 45 tRNA sequences, 1 rRNA operon, and 3,089 protein-coding genes.

Similar to the genomes of *A. prosperus* DSM 5130 and DSM 14174, genome analysis of *A. ferrooxidans* DSM 14175 showed the presence of the *rus* operon genes for iron oxidation (8–10).

Also found were genes for carboxysomes and carbon dioxide fixation through the Calvin-Benson-Bassham cycle (9–11) and those for nitrogen fixation through the Nif complex (9, 10, 12). A complete set of genes for chemotaxis and flagellar biosynthesis, similar to those found in *A. prosperus* strains DSM 5130 and DSM 14174, were also present (9, 10). However, unlike the genomes of the *A. prosperus* strains, the genome of *A. ferrooxidans* DSM 14175 does not contain genes encoding the SoxAX, B, and YZ subunits of the sulfur oxidation system (9, 10, 13); rather, it contains genes encoding

sulfur oxygenase reductases, which may be responsible for sulfur metabolism in this strain (13).

The genome of *A. ferrooxidans* DSM 14175 has genes for pathways involved in tolerance to stresses such as acid and oxidative stress. Considering the ability of this strain to withstand high osmotic stress in a low-pH environment, some of the most important stress-tolerance genes are those encoding operons for the biosynthesis and regulation of ectoine, glycine betaine, and osmoregulated periplasmic glucan, as well as for glycine betaine and choline uptake (14, 15). These proteins act as compatible solutes in acidophiles under osmotic stress and may provide assistance in the survival of halotolerant acidophiles (14, 15).

Accession number(s). The whole genome of *A. ferrooxidans* DSM 14175 (strain V8) has been deposited at DDBJ/EMBL/GenBank under the accession number CP019434. The version described in this paper is the first version, CP019434.1.

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Short genome communications

Complete genome sequence of *Acidihalobacter prosperus* strain F5, an extremely acidophilic, iron- and sulfur-oxidizing halophile with potential industrial applicability in saline water bioleaching of chalcopyrite

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Copper tolerance

ABSTRACT

Successful process development for the bioleaching of mineral ores, particularly the refractory copper sulfide ore chalcopyrite, remains a challenge in regions where freshwater is scarce and source water contains high concentrations of chloride ion. In this study, a pure isolate of *Acidihalobacter prosperus* strain F5 was characterized for its ability to leach base metals from sulfide ores (pyrite, chalcopyrite and pentlandite) at increasing chloride ion concentrations. F5 successfully released base metals from ores including pyrite and pentlandite at up to 30 g L^{-1} chloride ion and chalcopyrite up to 18 g L^{-1} chloride ion. In order to understand the genetic mechanisms of tolerance to high acid, saline and heavy metal stress the genome of F5 was sequenced and analysed. As well as being the first strain of *Ac. prosperus* to be isolated from Australia it is also the first complete genome of the *Ac. prosperus* species to be sequenced. The F5 genome contains genes involved in the biosynthesis of compatible solutes and genes encoding monovalent cation/proton antiporters and heavy metal transporters which could explain its abilities to tolerate high salinity, acidity and heavy metal stress. Genome analysis also confirmed the presence of genes involved in copper tolerance. The study demonstrates the potential biotechnological applicability of *Ac. prosperus* strain F5 for saline water bioleaching of mineral ores.

A limited number of microorganisms capable of simultaneously tolerating salt stress and low pH have been isolated, presumably as there are only a few places on Earth where geological conditions provide both conditions. Use of seawater at mines where access to freshwater is limited has led to the search of halotolerant, acidophilic, iron- and sulfur oxidizing bacterial cultures that are active in seawater concentrations of chloride ion (19.9 g L^{-1}) (Watling, 2016; Zammit et al., 2012). Furthermore, many of the reported salt tolerant acidophiles do not tolerate copper and chloride simultaneously, hampering their use for biomining copper-containing ores such as chalcopyrite (Watling et al., 2016). Therefore, identifying acidophilic microorganisms that accelerate the dissolution of chalcopyrite under salt stress would result in a step change in biohydrometallurgical processing (Watling et al., 2016).

Acidihalobacter prosperus represents a group of Gram-negative, halophilic, iron- and sulfur-oxidizing, mesophilic, chemolithoautotrophic, extreme acidophiles that have potential for biomining applications in regions of high salinity and acidity (Huber and Stetter, 1989; Zammit et al., 2012). Draft genomes of two strains of *Acidihalobacter prosperus* have previously been published (Khaleque et al., 2017; Ossandon et al., 2014). Here we present the finished complete genome sequence of an *Ac. prosperus* strain, strain F5. The F5 sequence will aid in the genetic characterization of acid, salinity and heavy metal tolerance. F5 is also the first of this species to be isolated in Australia.

Ac. prosperus strain F5 was isolated from a mixed environmental culture obtained from an acidic saline drain (containing 130 g L^{-1} chloride, 1.4 g L^{-1} iron(II) at a pH of 2.1) at the Yilgarn Crater, Western Australia (Zammit et al., 2009). Enrichment and isolation of F5 was performed at the School of Biomedical Sciences, Curtin University, Perth, Australia. The pure culture was maintained at 30° C in pH 2.5 basal salt media (0.4 g L^{-1} $(NH_4)_2SO_4$, 0.4 g L^{-1} $MgSO_4{\cdot}7H_20$, and 0.4 g L^{-1} KH_2PO_4) supplemented with trace salts and soluble iron (13.9 g L^{-1} $FeSO_4{\cdot}7H_2O$) and sulfur sources (1.51 g L^{-1} $K_2S_4O_6$) (Zammit et al., 2012). Growth on increasing levels of chloride ion (provided as NaCl) showed the ability of this isolate to tolerate up to 45 g L^{-1} chloride ion (data not shown).

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Table 1
Composition of ore used in bioleaching tests (mass-%).

	Fe	Cu	Ni	S	Si	Ca	Na	K
Pyrite	36.6	0.239	0.044	39.8	4.67	1.04	0.686	0.442
Chalcopyrite	26.6	26.8	0.004	29.8	3.97	0.382	0.009	0.043
Pentlandite	40.7	0.73	7.01	35.4	1.74	0.169	0.042	0.025

Bioleaching experiments using a pure isolate of *Ac. prosperus* strain F5 were conducted over 10 days at increasing chloride ion concentrations (provided as NaCl) in basal salt media pH 2.5 with 1% weight/volume pyrite, pentlandite or chalcopyrite. The percent composition of the ores used is given in Table 1. The release of iron was measured for experiments on all three ores using the method described by Govender et al. (2012). Leachate solutions were filtered to remove any particulate matter and diluted in 1% HNO3. Copper (for chalcopyrite) and nickel (for pentlandite) release were measured by atomic absorption spectroscopy (Avanta Σ). The release of metals over 10 days of bioleaching experiments at the different chloride ion concentrations tested are shown in Fig. 1.

Ac. prosperus strain F5 was able to leach base metals from the sulfide ore pyrite at up to 30 g L^{-1} chloride ion, chalcopyrite at 18 g L^{-1} chloride ion and pentlandite from 45 g L^{-1} chloride ion (Fig. 1). The results suggested that *Ac. prosperus* strain F5 may be an ideal candidate for bioleaching of sulfide ores at chloride ion concentrations of sea water or above (19.9 g L^{-1}).

Few studies have assessed the ability of pure, mesophilic halotolerant acidophiles to leach chalcopyrite. It is known that the formation of passivation layers results in the poor dissolution of chalcopyrite by mesophilic microorganisms (Hirato et al., 1987; Marhual et al., 2008; Zhou et al., 2008). Studies have been conducted with thermophilic cultures (Wang et al., 2012; Wang et al., 2014; Watling et al., 2016; Yu et al., 2014) with varied success in chalcopyrite bioleaching. However, this is the first study to demonstrate chalcopyrite bioleaching by a pure, mesophilic, halotolerant acidophile at chloride ion concentrations that have been shown to inhibit the growth the other mesophilic acidophiles. These unique characteristics highlight the potential applicability of bioleaching the recalcitrant copper sulfide ore, chalcopyrite, using *Ac. prosperus* strain F5.

Table 2
Genome features of *Acidihalobacter prosperus* strain F5.

Attribute	Value
Genome size (bp)	3,566,941
G +C content (%)	59.9
Genes (total)	3428
CDS total	3374
CDS coding	3233
tRNAs	47
rRNA genes	3
ncRNAs	4
Pseudo genes (total)	141

Genomic DNA of was extracted from F5 using the method described by Zammit et al. (2011). F5 DNA was sequenced using PacBio RS SMRT technology which generated 92,779 post-filter reads with a mean length of 12,147 bp and ~316-fold depth of coverage. *De novo* assembly using the long-read-only assembler canu v. 1.3 (Koren et al., 2017), produced a circular chromosome of 3,566,941 bp. Genes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline v 3.3 and GeneMarkS+. F5 contained 47 tRNA sequences, 1 rRNA operon and 3233 protein-coding genes. The genome has a G + C content of 59.9%. Genome features of *Ac. prosperus* strain F5 are summarised in Table 2 and Fig. 2.

Genome analysis of *Ac. prosperus* strain F5 confirmed the presence of genes known to be involved in iron oxidation, such as those of the previously described *rus* operon (Nicolle et al., 2009) and numerous

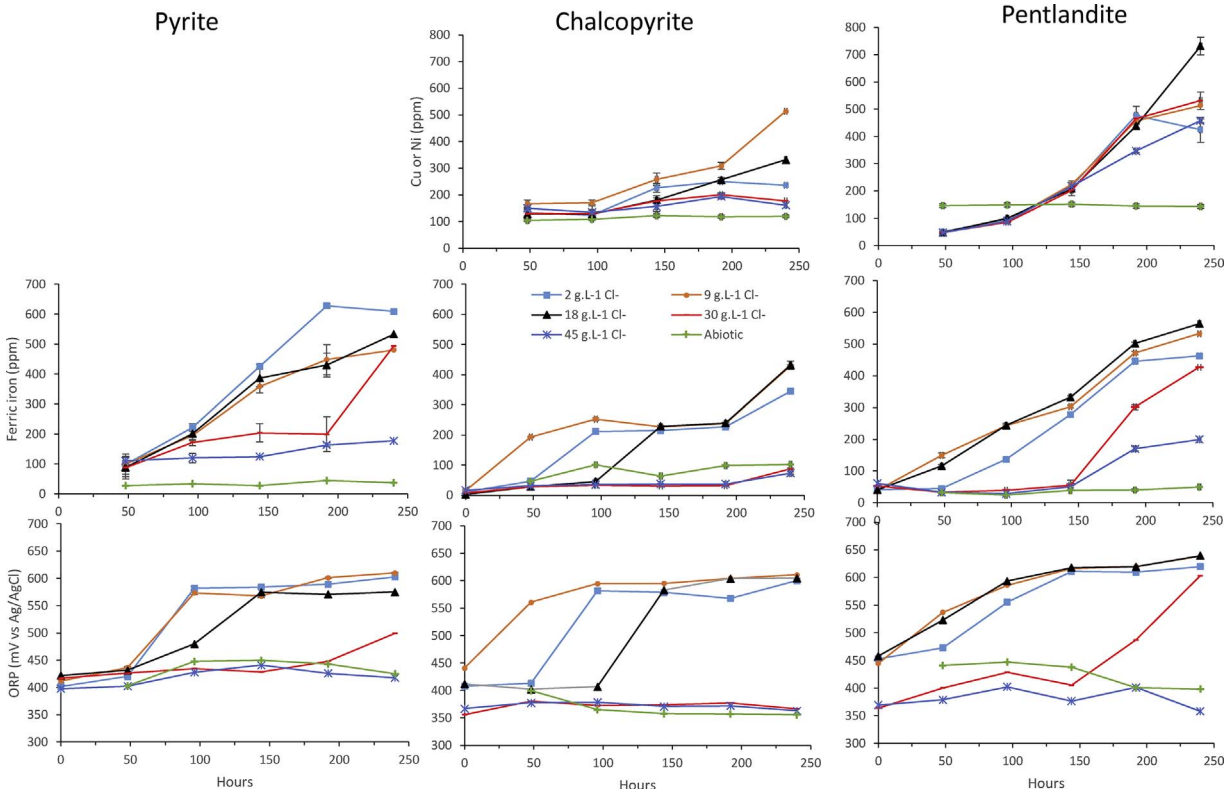

Fig. 1. The effect of NaCl on the bioleaching of pyrite, chalcopyrite and pentlandite by *Acidihalobacter prosperus* strain F5 over 10 days of bioleaching. Abiotic control in the presence of 18 g L^{-1} chloride ion chosen as the optimum chloride ion concentration for *Ac. prosperus* F5 growth. Data are averages +/− SE of triplicate biological replicates and three technical replicates.

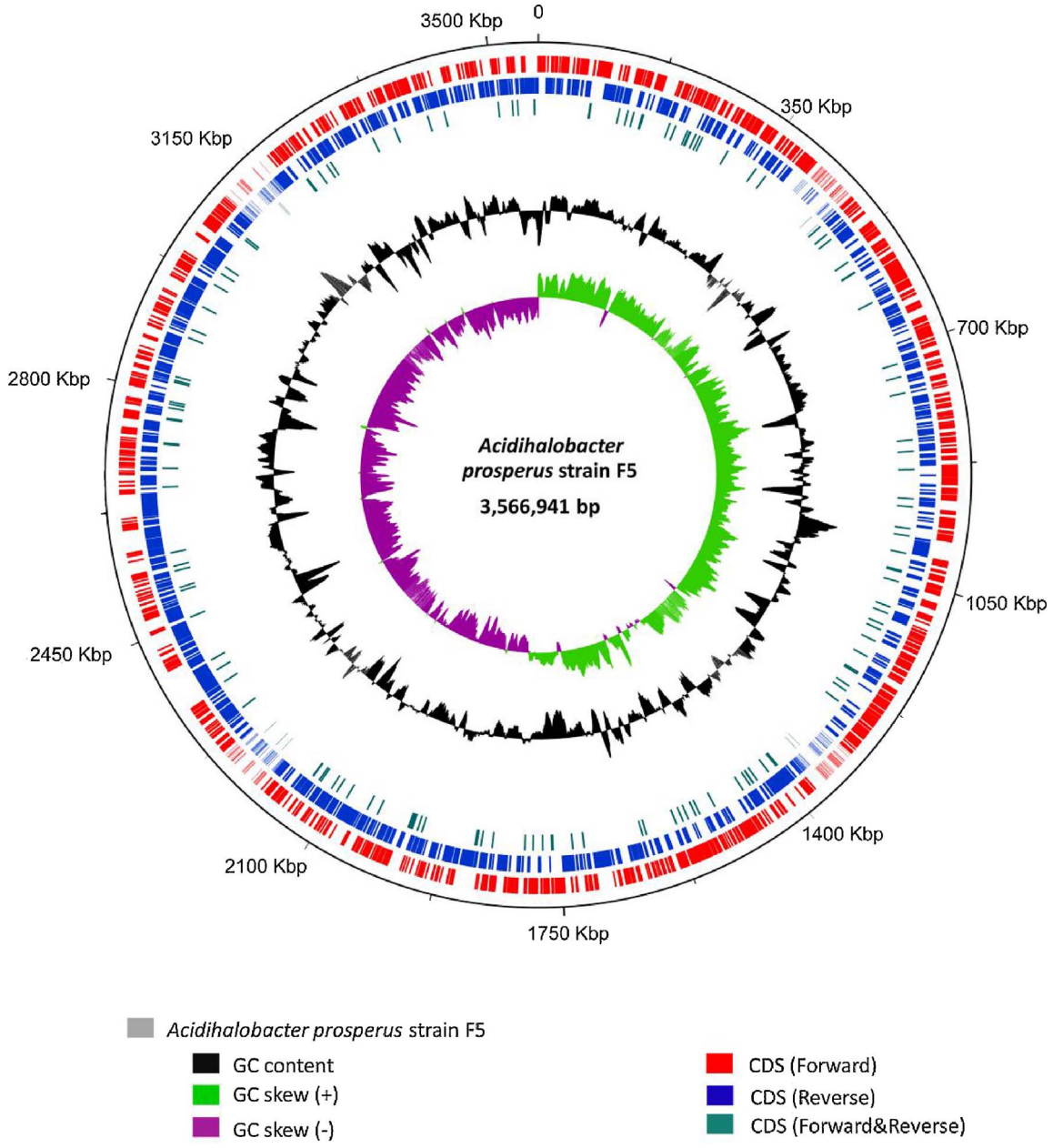

Fig. 2. Circular genome plot of *Acidihalobacter prosperus* strain F5. From innermost to outermost rings: GC skew, where outwards-directed lines represent positive GC skew; GC content, where outwards-directed lines represent higher GC content; Predicted coding sequences (CDS) on both DNA strands (teal), on the reverse DNA strand (blue) and on the forward DNA strand (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

predicted iron transport proteins (accession numbers: WP_070077550.1, WP_070077551.1, WP_070077552.1). Also present were genes encoding various sulfur oxidation, metabolism and transport pathway proteins, including those of the SoxAX, B, YZ complex (Friedrich et al., 2005) (accession numbers: WP_070078781.1, WP_083251331.1, WP_070077629.1, WP_070079247.1). Genes for proteins involved in carbon dioxide fixation (via the Calvin Benson Benham cycle) (Shively et al., 1998) and chemotaxis were found, similar to those described in the type strain *Ac. prosperus* 5130 (Ossandon et al., 2014) and the environmental isolate *Ac. prosperus* DSM 14174 (Khaleque et al., 2017) (accession numbers: JQSG00000000.2, CP017448.1).

Ac. prosperus strain F5 also carried genes encoding for the synthesis and transport of osmoprotectants including N-acetyldiaminobutyrate dehydratase, diaminobutyrate acetyltransferase, ectoine synthase and sucrose synthase (accession numbers: WP_070079295.1, WP_070079297.1, WP_070079295.1, WP_070079442.1). Genes encoding ABC transporters for ectoine and glycine betaine uptake were also present (accession numbers: WP_070078974.1, WP_083251372.1, WP_070078973.1, WP_083251085.1). These proteins have been linked to the survival of acidophiles under high osmotic pressure (Dopson et al., 2017).

Interestingly, the genome of *Ac. prosperus* strain F5 contains multiple genes encoding copper resistance proteins and a copper translocating P-type ATPase, which may be responsible for its copper tolerance and ability to leach chalcopyrite (accession numbers: WP_070078257.1, WP_070078256.1, WP_070079189.1, WP_070077568.1). Genes encoding multiple heavy metal ABC transporters for zinc, nickel, mercury and other heavy metals were also identified (accession numbers: WP_070077548.1,

BI364_RS17635, WP_070078780.1, WP_070079191.1), potentially explaining the ability of F5 to leach pentlandite effectively in the presence of 45 g L^{-1} chloride ion, as shown in Fig. 1.

The information from the bioleaching tests and complete genome analysis of *Ac. prosperus* strain F5 has provided clues to understand mechanisms of salt and copper tolerance in halophilic acidophiles. The isolation of this halophilic, iron- and sulfur-oxidizing acidophile with high heavy-metal tolerance is highly significant due to its potential for use in recovery of metals from low grade ores, particularly chalcopyrite, in regions of high salinity in ores or source water.

Nucleotide sequence accession numbers The bioproject designation for this project is PRJNA344521. The whole-genome of *Acidihalobacter prosperus* strain F5 has been deposited at DDBJ/EMBL/GenBank under the accession no. CP017415. The version described in this paper is CP017415.1.

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