Characterization of NaCl tolerance mechanism and its relation with the antioxidant mechanisms in the acidophilic bacterium *Leptospirillum ferriphilum* DSM 14647

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Javier Ignacio Rivera Araya

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APPROVAL REPORT

DOCTORAL THESIS

The Graduate School of the Faculty of Sciences is informed that the Doctoral Thesis presented by the candidate.

Javier Ignacio Rivera Araya

has been approved by the Thesis Evaluation Commission as a requirement to qualify for the Doctor of Science degree with a mention in Microbiology, in the exam of Private Thesis Defense rendered on September 24th, 2019.

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To my family.

BIOGRAPHY

My name is Javier Ignacio Rivera Araya; I am 29 years old. I was born on February 26th, 1990, in the city of Copiapó, Atacama, Chile. For elementary school, I went to Pedro León Gallo school from 1995 to 2003, and for high school, I went to Escuela Técnico Profesional of Copiapó during 2004-2007. I started University in 2008 for Biotechnology Engineer at Universidad de Santiago de Chile, year when I moved on Santiago. In 2013 I obtained the degree of Biotechnology Engineer after developing my thesis in the Laboratory of Basic and Applied Microbiology under the tutelage of Dr. Gloria Levicán. In 2015 I enrolled in the PhD program in Sciences with mention in Microbiology imparted by the Universidad de Chile – Universidad de Santiago de Chile. For four years I developed my Thesis in the same laboratory and I am currently presenting my work to apply for the degree of PhD in Science with mention in Microbiology.

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LIST OF ABBREVIATIONS

AMD	Acid mine drainage
bp	Base pair
CcP	Cytochrome *c* peroxidasae
cDNA	Complementary DNA
cm	Centimetre
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ε	Extinction coefficient
eV	Electronvolt
GC-MS	Gas chromatography mass spectrometry
H_2DCFDA	Probe 2′,7′-dichlorodihydrofluorescein diacetate
HPLC	High performance liquid chromatography
IStd	Internal standard
l	Litter
mg	Miligram
min	Minutes
ml	Mililitter
mM	Milimolar
MSTFA	*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide
nm	Nanometre
ORF	Open reading frame
MIC	Minimal inhibitory concentration
PCR	Polymerase chain reaction
pH_{in}	Intracellular pH
pH_{ex}	Extracellular pH
qPCR	Quantitative PCR
rDNA	Ribosomal DNA
RISCs	Reduced inorganic sulfur compounds
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT-qPCR	Real time qPCR
s	Second
TAE	Tris-acetate-EDTA
Tris	Trisaminomethane (2-Amino-2-hydroxymethyl-propane-1,3-diol)
Trx	Thioredoxin
U	Enzymatic unit
µg	Micrograms
µl	Microlitter
µM	Micromoles
UV/VIS	Visible ultraviolet
vol	volume
z	Charge

RESUMEN

La biolixiviación con cloruro se considera un método alternativo prometedor para recuperar el cobre de la calcopirita y otros minerales sulfurados de cobre, porque favorece la cinética de la lixiviación y evita la pasivación de los minerales. Sin embargo, los iones cloruro son altamente tóxicos para los microorganismos biolixiviantes. Además del desequilibrio osmótico, el cloruro también puede inducir la acidificación del citoplasma en estos microorganismos. Por consiguiente, es posible predecir que la acidificación intracelular produce un aumento de la frecuencia respiratoria y la generación de especies reactivas de oxígeno, por lo que se podría inducir estrés oxidativo. El objetivo general de este estudio fue caracterizar el mecanismo molecular de tolerancia al NaCl y establecer su relación con el mecanismo antioxidante en *Leptospirillum ferriphilum* DSM 14647.

Inicialmente, se estudió la participación de los sistemas canónicos de tolerancia al estrés osmótico y los sistemas antioxidantes en la respuesta temprana. Utilizando herramientas bioinformáticas, se determinó que la mayoría de los microorganismos acidófilos oxidantes de hierro poseen un *set* completo o parcial de genes para los transportadores de K^+ y las vías de síntesis y transportadores de los solutos compatibles (hidroxi)ectoína y trehalosa. También la exposición de *L. ferriphilum* a 100 mM de NaCl reguló de forma positiva los genes *kdpC* y *kdpD* que codifican para transportadores de potasio. La exposición prolongada a NaCl llevó también a la expresión de genes para la biosíntesis de los solutos compatibles (hidroxi)ectoína (*ectC* y *ectD*) y trehalosa (*otsB*). Como consecuencia, los niveles intracelulares de estos solutos aumentaron significativamente, lo que sugiere una respuesta para mantener la homeostasis osmótica. Por otro lado, el pH intracelular disminuyó significativamente de 6.7 a pH 5.5 y el

consumo de oxígeno aumentó cuando las células se expusieron al estrés con NaCl. Además, esta condición de estrés llevó a un aumento significativo del contenido intracelular de especies reactivas de oxígeno (ROS), y a un aumento de las actividades antioxidantes citocromo *c* peroxidasa (CcP) y tiorredoxina (Trx). En concordancia con estos resultados, los genes *ccp* y *trx* fueron sobre-expresados bajo esta condición, lo que sugiere que esta bacteria muestra una respuesta regulada transcripcionalmente contra el estrés oxidativo inducido por el cloruro.

En paralelo, se adaptó *L. ferriphilum* a NaCl 180 mM para identificar la estrategia de respuesta tardía. El análisis del perfil transcriptómico reveló que los principales mecanismos involucrados en la adaptación estaban relacionados con los genes asociados a la integridad de la membrana celular, la respiración y las proteínas antioxidantes, probablemente para mantener el pH y la homeostasis redox. El estudio de las células adaptadas demostró un aumento en la tasa respiratoria, sin embargo, el nivel de ROS intracelular se mantuvo en niveles basales. Por el contrario, los genes asociados con la biosíntesis de hidroxiectoína (*ectB*, *ectC*, *ectD*) fueron reprimidos, lo cual coincide con la falta de detección de este compuesto en extractos celulares. Por consiguiente, estos datos indican que las células no se encontrarían expuestas a un estrés osmótico.

Finalmente, de acuerdo con estos resultados, se puede concluir que el cloruro tiene un efecto multifacético dramático en la fisiología de acidófilos que involucra estrés osmótico, ácido y oxidativo. El mecanismo de respuesta temprana estuvo compuesto por la respuesta osmótica, la homeostasis de pH a través de la respiración celular y la respuesta antioxidante. En su lugar, el mecanismo de respuesta tardía involucró la homeostasis de pH y la respuesta antioxidante.

ABSTRACT

Chloride bioleaching is considered a promising alternative method to recover copper from chalcopyrite and other primary copper sulfides, because it favors the leaching kinetics and avoids passivation of minerals. Nevertheless, chloride ions are highly toxic for iron-oxidizing microorganisms that participate in the bioleaching process. In addition to the osmotic imbalance, chloride can also induce acidification of the cytoplasm in these microorganisms. We predicted that intracellular acidification produces an increase in respiratory rate and reactive oxygen species generation, and therefore oxidative stress can also be induced. The general goal of this study was to characterize the NaCl tolerance molecular mechanism and establish its relation with the antioxidant mechanism in *Leptospirillum ferriphilum* DSM 14647.

First, the participation of canonical systems of tolerance to osmotic stress and the antioxidant systems, as an early response mechanism, were studied. By bioinformatic analysis, it was determined that genes for a complete or partial repertoire of K^+ transporters, the biosynthesis pathways and transporter for compatible solutes (hidroxi)ectoine and trehalose were found in most of the acidophilic iron-oxidizing microorganisms. Additionally, the exposition of *L. ferriphilum* to 100 mM NaCl immediately up-regulated *kdpC* and *kdpD* genes coding for potassium transporters. A prolonged exposure to NaCl also increased the expression of genes encoding for biosynthesis of compatible solutes (hydroxy)ectoine (*ectC* and *ectD*) and trehalose (*otsB*). As a consequence, the intracellular levels of both hydroxyectoine and trehalose increased significantly, suggesting a strong response to keep osmotic homeostasis. On the other

hand, the intracellular pH significantly decreased from 6.7 to pH 5.5 and oxygen consumption increased significantly when the cells were exposed to NaCl stress. Furthermore, this stress condition led to a significant increase of the intracellular content of reactive oxygen species, and to a rise of the antioxidative cytochrome *c* peroxidase (CcP) and thioredoxin (Trx) activities. In agreement with these results, *ccp* and *trx* genes were up-regulated under this condition, suggesting that this bacterium displays a transcriptionally regulated response against oxidative stress induced by chloride.

In parallel, *L. ferriphilum* was adapted to 180 mM NaCl to identify the late response strategy. The analysis by transcriptomic profile revealed that the principal mechanisms involved in the adaptation were related with genes associated to the cell membrane integrity, respiration and antioxidant proteins, probably to conserve the pH and redox homeostasis. Inspection of these parameters in the adapted culture proved an increase in the respiratory rate and the maintain of the intracellular ROS levels. On contrary, genes associated with biosynthesis of hydroxyectoine (*ectB*, *ectC*, *ectD*) were repressed, coincident with the lack of detection of this compound in cell extract. Thus, these data suggest that the cells were not under osmotic stress.

Finally, according with these results, we were able to conclude that chloride has a dramatic multifaceted effect on acidophile physiology that involves osmotic, acidic and oxidative stresses. The early response mechanism was composed by the osmotic response, pH homeostasis by cell respiration and antioxidant response. Instead, the late response mechanism involved pH homeostasis and antioxidant response.

1. INTRODUCTION

1.1 Bioleaching of sulphide ores and acidophilic microorganisms

Bioleaching consists in the conversion of an insoluble valuable metal to a soluble form using microorganisms, and the subsequent recovery of the metal from the medium with conventional methods (Brandl and Faramarzi, 2006; Schippers et al., 2013; Mishra and Rhee, 2014). The bioleaching process is developed by an indirect action of the iron-oxidizing microorganisms, because ferric iron (Fe^{3+}) produced by the oxidation and the protons contained in the medium (H^{+}) are the oxidant agents for the minerals (Rawlings, 2005).

In nature, metals like copper (Cu), zinc (Zn) and nickel (Ni) are founded as sulphide ores, which are insoluble under environmental conditions and low acidity. However, the natural and industrial bioleaching environments are characterized for being acidic (pH<3) and, thus, facilitate the metal dissolution in high concentrations. Therefore, the microorganisms who live in these environments are extreme acidophiles (Baker-Austin and Dopson, 2007), able to tolerate high concentration of metals (>260 mM of copper) and have a chemolitotrophic metabolism, because they oxidize Fe^{2+} and/or reduced inorganic sulphur compounds (RISCs) to obtain energy.

With the development of new molecular biotechnological tools, it has been possible to make progress in the understanding of the diversity and ecology of acidophilic microorganisms in acid mine drainages (AMD) and industrial processes, where microorganisms from all three domains has been described to be present. In mesophilic conditions, the bioleaching process is mainly dominated by bacteria from the

Acidithiobacillus and *Leptospirillum* genus (Mishra and Rhee, 2014). Nevertheless, *Leptospirillum* spp. has been accepted as a predominant specie because can grow even in the late state of the process, when the pH is very low, there is a high redox potential and high concentrations of metals (Watling, 2011).

In our laboratory, a research area based on the hypothesis that *Leptospirillum* spp. dominance is provided by its capacity of tolerate the highly oxidant conditions from these environments has been developed. This idea has been supported with genomic studies, which have shown a higher presence of genes coding for lipid and DNA repair systems in *Leptospirillum,* in contrast with *Acidithiobacillus* (Cárdenas et al, 2012). In addition, it was determined that *L. ferriphilum* has a highly efficient antioxidant system, mediated by thioredoxin (Trx/TR) (Norambuena et al., 2012). Besides, this bacterium possesses the antioxidant enzymes Dyp-type peroxidase (DyP) and cytochrome *c* peroxidase (CcP) to reduce the hydrogen peroxide generated at cytoplasmic and periplasmic level, respectively (Contreras et al., 2015; Zapata et al., 2017). Finally, it has been established that *Leptospirillum* sp. CF-1 use a mechanism based on the cobalamin (vitamin B_{12}) to protect itself against oxidant conditions (Ferrer et al., 2016b).

1.2 Effect of NaCl in the bioleaching process.

Bioleaching has been established as a successful technology for the extraction of metals from low grade, polymetallic and gold refractory ores. However, the treatment of sulphide ores, highly refractories in the process, is a challenge that remain unsolved (Johnson, 2014). For example, the efficient treatment of abundant, but refractory sulfides

such as chalcopyrite ($CuFeS_2$) and enargite (Cu_3AsS_4) belong to a principal focus of development for the biomining (Johnson 2014, Wang et al., 2012).

The low kinetic of bioleaching and little recovering of metal during the treatment of chalcopyrite is mainly due to the production of a "passivation layer", which consists in an accumulation of sulphur compounds on the surface of the mineral. This layer becomes a wall that avoids the mineral attacks, and thus, the bacterial oxidation (Hirato et al., 1987; Wang et al., 2012). Interestingly, it has been reported that the addition of sodium chloride (NaCl) increases the dissolution kinetic of chalcopyrite, because reduces the activation energy (Carneiro and Leão, 2007; Yoo et al., 2010; Wang et al., 2012). Additionally, several studies have shown that chloride ion can increase the superficial area and the porosity of the mineral, improving the solubilization and the metal recovery (Carneiro and Leão, 2007). Nevertheless, the chloride ion is particularly toxic for acidophilic microorganisms who participate in the bioleaching process, thus, it has not been possible yet apply NaCl in industrial bioleaching process of sulphide ores.

On the other hand, all microorganisms involved in the dissolution process work in an aqueous phase, thus the availability of water is a critical point for this industrial process (Gahan et al., 2009). In addition, since the principal copper-rich deposits are situated in areas where the water sources are scarce, e.g., Western Australia and Northern Chile (Davis-Belmar et al., 2014, Gahan et al., 2010), the application of biohydrometallurgy using seawater (Cl^- concentration overcomes 500 mM in the process) has become a long-term goal of the mining industry.

The two aspects described above reveal the necessity of improving the industrial bioleaching process in presence of chloride. But, with exception of halotolerant

microorganisms, the addition of NaCl in the culture medium is damaging for the microorganisms. The acidophiles have been reported as being extremely sensitive to chloride and other anions (excluding sulphate) (Watling et al., 2016). It has been demonstrated that 200 mM chloride inhibits the bioleaching ability of an undefined acidophilic consortium (Shiers et al., 2005), and also the pyrite biooxidation and ferrous-iron oxidation by a *Leptospirillum ferriphilum*-dominated culture (Gahan et al., 2010). These findings significantly limit the applicability of these microorganisms in the bioleaching of $CuFeS_2$ or other refractory sulfides in saline water as well as bioleaching when copper chlorides like atacamite ($Cu_2Cl(OH)_3$) or chloride-containing gangue material comprise a major part of the ore (Cameron et al., 2007). Therefore, it raises the necessity to understand the molecular basis of the low tolerance of acidophilic iron-oxidizing microorganisms against chloride and characterize the mechanism of response under salt-stress conditions in these microorganisms.

1.3 Toxicity of chloride ion in acidophilic microorganisms.

Acidophilic microorganisms use ferrous iron as primary energy source, and thus, they re-generate ferric (Fe^{3+}) iron, which is assumed to have a relevant role as oxidant for dissolution of the minerals in the bioleaching process (Johnson, 2014). In bioleaching industrial environments, cations can be additionally accumulated due to the water source, recycling of processed waters, evaporation, and because of gangue mineral dissolution (Rea et al., 2015). Consequently, these microorganisms have an unusual tolerance to cations, however as mentioned before, they are extremely sensitive to anions (Alexander et al., 1987; Suzuki et al., 1999; Rea et al., 2015). Especially, chloride has showed to be

highly inhibitory for a number of acidophiles such as *Acidithiobacillus ferrooxidans*, *Acidithiobacillus caldus*, *Leptospirillum ferrooxidans*, *Leptospirillum ferriphilum*, *Sulfobacillus thermosulfidooxidans* (Simmons and Norris, 2002; Marhual et al., 2008; Gahan et al., 2009; Gahan et al., 2010; Wang et al., 2012; Guo et al., 2014; Galleguillos et al., 2018; Huynh et al., 2019). In a study of *At. ferrooxidans*, this bacterium was exposed to both chloride and sulphate, establishing that the chloride ion is the toxic agent which damages Fe^{2+} oxidation activity (Kieft and Spence, 1988). Additionally, it was stablished that both the iron and sulphur oxidation can be selectively inhibited by different anions, inhibitors (cyanide and sodium azide) and the osmotic pressure (Harahuc et al., 2000). Studies using *At. ferrooxidans* as acidophile model have allowed to establish that osmotic imbalance induced by chloride and other anions disrupt the internal positive membrane potential, and lead to an influx of protons in the cytoplasm (Alexander et al., 1987; Zammit and Watkin, 2016).

Acidophiles use a series of mechanisms to maintain the intracellular pH close to neutrality, even when the proton concentration is different in 10^5-folds between the extra- and intra-cellular space (Zammit and Watkin, 2016). In *At. ferrooxidans*, for example has been estimated that the intracellular pH is ~6.5 when oxidyzing pyrite (FeS_2), Fe(II) or inorganic sulphur and in an acidogenic medium (pH~1) (Cox et al., 1979). The pH difference between the external medium and the cytoplasm is kept by the following characteristics of acidophilic microorganisms: 1) changes to the cell membrane; 2) the cell keeps the internal potential membrane in a positive charge to avoid the massive entrance of protons, this condition is stimulated by the presence of membrane proteins with high isoelectric-point values (pI) (Alexander et al., 1987; Zammit et al., 2012); 3)

by reactions of proton consumption, such as the metabolism by carboxylases; 4) modification and repair of DNA; and finally, 5) the respiration is one of the principal mechanisms to keep intracellular pH close to neutrality where protons are extruded to the exterior or consumed at cytoplasmic level during NAD(P)+ and O_2 reduction to form NAD(P)H and H_2O (Baker-Austin and Dopson, 2007; Levicán et al. 2012; Quatrini et al. 2009).

Since acidophiles have a positively-charged cell surface, the exposure to chloride lead the influx of these anions to the intracellular space. Consequently, they disrupt the positive internal membrane potential, the protons may enter to the cells and acidification of the cytoplasm (Alexander et al., 1987; Suzuki et al., 1999; Dopson et al., 2017). Because of the decrease in the intracellular pH, a decreasing in the membrane potential can be produced with subsequent detrimental effect on the ATP and NAD(P)H synthesis. In our interpretation, in order to compensate this effect and restore pH homeostasis, cells should increase respiratory rate. However, an increase in oxygen consumption also increases the probability of electrons leakage from electron transfer chain, and thus reactive oxygen species (ROS), toxic for the cell, might be produced (Ziegelhoffer and Donohue, 2009). These theoretical considerations suggest that exposure to anions and, in particular, chloride could induce a condition of oxidative stress that would partly explain the highly toxic effect of this anion.

1.4 NaCl response in acidophilic microorganisms.

Nowadays, the biomining industry is developing methods to solubilize copper of refractory and low grade sulphide ores, like the chalcopyrite. From all over solutions

exposed, the addition of NaCl or, specifically, the use of sea water in the process is a promising option. However, as previously discussed, it has not been possible to implement this technique at industrial level. Since the sensitivity of acidophilic microorganisms to chloride, there is a necessity of new isolate NaCl-tolerant strains or to understand why they are so sensitive, to provide them the optimal conditions. Until now, no extreme halotolerant acidophilic microorganisms have been described, however moderately halotolerant *Leptospirillum ferriphilum* Sp-Cl (12 g L^{-1} NaCl) and *Acidihalobacter prosperus* DSM 5130 (20 g L^{-1} NaCl) were recently isolated (Issotta et al., 2016; Dopson et al., 2017). The mechanisms that these or other acidophilic microorganisms use to withstand the osmotic stress are just recently being investigated. However, evidences derived from various studies have showed they use canonical mechanisms of protection. For example, *Leptospirillum* sp. uses the accumulation of potassium and compatible solutes (hydroxy)ectoine and trehalose, as strategy to tolerate high osmolarity (Parro et al., 2007; Mosier et al., 2013; Galleguillos et al., 2018). *At. ferrooxidans* uses proline and betaine as osmoprotectans (Kieft and Spence, 1988). Synthesis of proline and DNA binding proteins have been also described as a NaCl adaptation mechanism for *At. caldus* (Guo et al., 2014). Furthermore, a proteomic study conducted to evaluate the response of the moderately halotolerant *Ac. prosperus* to chloride-stress suggested a response based on the synthesis and ectoine uptake (Dopson et al., 2017). In addition, this microorganism also seems to develop a more specific adaptive response that involves changes in amino acid composition of the rusticyanin protein. When compared with rusticyanin from non-halotolerant *At. ferrooxidans*, rusticyanin from *Ac. prosperus* showed to have a more negative surface potential which

is predicted contribute to protect the environment of the copper ion that form the redox center of the protein (Dopson et al., 2017).

Interestingly, proteomic studies carried out in *Ac. prosperus* also suggested an increased activity of iron oxidation pathway which is believed to be associated to high proton extrusion. These findings support the idea of the acidophilic microorganisms increase the respiratory rate under saline conditions to regulate pH homeostasis, and consequently, this leads to the production of intracellular ROS. In the same line, Dopson et al. (2017) observed an increase in the activity of the antioxidant proteins rubrerythrin and Dyp-type peroxidase, probably to compensate the ROS produced. In agreement with these fact, a meta-transcriptomic analysis performed in Rio Tinto by Parro et al. (2007), detected an increase in the expression of the genes related with oxidative-stress response (synthesis of carotenoids) in samples obtained from a saline environment. These findings suggest that exposure to anions could induce a condition of oxidative stress that would partly explain its highly toxic effect of acidophilic microorganisms. Although, in recent years the response to oxidative stress has been studied in some acidophiles (Ram et al., 2005; Norambuena et al., 2012; Contreras et al., 2015; Ferrer et al., 2016b; Zapata et al., 2017; Bellenberg et al., 2019), a relation between saline stress and the response to oxidative stress has not been established yet. Therefore, in this work, it is proposed to study the chloride susceptibility of iron-oxidizing acidophilic microorganisms as a widespread multifactorial phenomenon that involve osmotic imbalance, acidification of the cytoplasm, increase in the respiratory rate and oxidative stress induction. Thus, representing parameters of each condition were evaluated in *Leptospirillum ferriphilum* DSM 14647 exposed and adapted to NaCl stress.

2. HYPOTHESIS

The chloride susceptibility of *Leptospirillum ferriphilum* DSM 14647 is due to a widespread multifactorial phenomenon that involves osmotic imbalance, acidification of the cytoplasm and oxidative stress induction.

3. GOALS

3.1 General Goal

Characterize the NaCl tolerance molecular mechanism and establish its relation with the antioxidant mechanism in the acidophilic bacterium *Leptospirillum ferriphilum* DSM 14647.

3.2 Specific Goals

3.2.1 Evaluate the participation of canonical systems of tolerance to osmotic stress in cells exposed to NaCl.

3.2.2 Evaluate the participation of the antioxidant response in the protection of cells exposed to NaCl.

3.2.3 Identify the transcriptomic profile of *Leptospirillum ferriphilum* DSM 14647 adapted to NaCl.

4. MATERIALS AND METHODS

4.1 Evaluate the participation of canonical systems of tolerance to osmotic stress in cells exposed to NaCl.

To investigate the early osmotic response mechanism that acidophilic microorganisms may use to tolerate the presence of the anion, a bioinformatic analysis was done in different acidophiles and then these mechanisms were evaluated in cells exposed to saline shock with NaCl.

4.1.1 Bioinformatic analysis.

To elucidate a general mechanism of osmotic stress response in acidophiles, the complete genomic sequences from twelve aerobic iron-oxidizing microorganisms were examined using the sequences available in the DDBJ/EMBL/GenBank databases. Proteins related to NaCl tolerance were obtained from the KEGG and MetaCyc databases. The predicted genes for these proteins were identified in each genome using the Artemis Bioinformatics Tool (https://www.sanger.ac.uk/science/tools/artemis). To infer homology, BLASTp parameters that included 30% identity, 80% coverage and the presence of conserved domains were used (Pearson, 2013). The analysis was performed on genome sequences from members of the following phyla or classes, respectively: Nitrospirae (represented by *L. ferriphilum* DSM 14647 [NCBI accession number: PGK00000000], *L. ferriphilum* Sp-Cl [NCBI accession number: LGSH00000000] and *Leptospirillum* sp. CF-1 [NCBI accession number: CP012147]), Firmicutes (represented by *Alicyclobacillus ferrooxydans* TC-34 (Jiang et al., 2008) [NCBI accession number

LJCO00000000] and *Sulfobacillus thermosulfidooxidans* DSM 9293 [NCBI accession number: NZ_FWWY00000000.1]), Actinobacteria (represented by *Ferrimicrobium acidiphilum* DSM 19497 [NCBI accession number: JXUW00000000.1]), Beta-proteobacteria (represented by "*Ferrovum myxofaciens"* P3G (Moya-Beltrán et al., 2014), Gamma-proteobacteria (represented by *Acidihalobacter prosperus* V6 [NCBI accession number: CP017448), Acidithiobacillia (represented by *At. ferrooxidans* ATCC 23270 [NCBI accession number: CP001219] and *Acidithiobacillus ferrivorans* SS3 [NCBI accession number: CP002985]), Euryarchaeota (represented by "*Ferroplasma acidarmanus"* fer1 [NCBI accession number: CP004145]) and Crenarchaeota (represented by *Metallosphaera sedula* DSM 5348 [NCBI accession number: CP000682]).

4.1.2 Culture of bacterial strains

L. ferriphilum DSM 14647 was cultured aerobically in flasks at 37ºC in DSMZ 882 medium pH 1.8 supplemented with 20 g l^{-1} ferrous sulphate with constant stirring of 180 rpm as described in Norambuena et al. (2012). *At. ferrooxidans* ATCC 23270 was grown aerobically at 30°C in modified 9K medium [0.1 g/l $(NH_4)_2SO_4$, 0.01 g/l $MgSO_4 \cdot 7\ H_2O$; 0.04 g/l K_2HPO_4, pH 1.6, supplemented with 33.3 g/l ferrous sulphate] with constant stirring at 180 rpm.

4.1.3 Determination of minimal inhibitory concentration (MIC) for NaCl.

To estimate the concentration of NaCl that could induce stress in *L. ferriphilum*, the minimal inhibitory concentration (MIC) for NaCl was determined. This assay was carried out on planktonic cells of *L. ferriphilum* and *At. ferrooxidans* according to Bobadilla-Fazzini et al. (2014). Briefly, each bacterium was cultured in presence of different NaCl concentrations, ranging from 0 to 400 mM. The experiments were carried out in triplicate in 6-well plates, containing 5 ml of the respective culture medium. Strains were inoculated to a concentration of $1x10^6$ cells ml^{-1} and later incubated until the control sample (0 mM of NaCl) reached stationary phase. The MIC value corresponds to the minimal NaCl concentration where no bacterial growth was observed.

4.1.4 Determination of expression of K-transporters and compatible solutes biosynthesis genes in cells exposed to NaCl.

To detect the effect of NaCl on the mRNA levels of K-transporters (KdpABCD, TrkA and YggT), the (hydrox)ectoine transporter (ProP) and compatible solutes biosynthesis genes (EctABCD, TreXYZ, TreS and OtsAB), and to elucidate whether these can be upregulated in a condition of chloride stress, a transcription analysis by RT-qPCR was performed in *L. ferriphilum* DSM 14647.

RNA Isolation and cDNA Synthesis.

L. ferriphilum was grown until late exponential phase. 350 ml of cells were harvested by centrifugation at 8,000 x *g* for 15 min, and washed once with acid water (10 mM H_2SO_4) and twice with 10 mM sodium citrate pH 7.0. Washed cells were suspended in DSMZ 882 medium and incubated with 100 mM NaCl for the times indicated. Cells

were collected by centrifugation at 8,000 x *g* for 10 min, and washed twice with 10 mM sodium citrate pH 7.0. RNA was isolated using the RNeasy Mini Kit (Qiagen). DNA was removed by DNase I treatment (New England, Biolabs) according to the manufacturer's instructions. cDNA synthesis was carried out with the AffinityScript qPCR cDNA Synthesis kit (Agilent Technologies). The reaction mixture (20 µl) contained First Strand master mix, 0.1 µg/µl random primers, Affinity Script RT/RNase Block enzyme mixture and 1 µg of RNA. The synthesis was carried out at 25ºC for 5 min and after that at 42ºC for 15 min. Then the enzyme was inactivated at 95ºC for 5 min. cDNA was stored at -80ºC until further use.

qPCR Reaction.

Primers for qPCR reactions were designed using the available gene sequences of *L. ferriphilum* (Cárdenas et al., 2014). For details of specific primers used in each RT-qPCR experiment, see Table 1. Then, KAPA SYBR FAST qPCR kits (Kapa Biosystems) was used for qPCR amplification according to the manufacturer's instructions. Reactions were performed in 20 µl reaction volumes containing 10 µl of KAPA SYBR Fast RT-qPCR Master Mix 2X (Kapa Biosystems), 0.4 µl of each primer (at a concentration of 10 µM each), 0.4 µl of 50x ROX high, 7.8 µl of nuclease-free water, and 1 µl of cDNA. The qPCR conditions were an initial denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation (95ºC for 30 s), annealing (60ºC for 20 s) and extension (72ºC for 10 s). All these reactions were performed in a StepOne Real-Time PCR system (Applied Biosystems). The relative abundance of each gene versus a constitutively expressed gene (16S rDNA) was determined. Appropriate negative controls were included. For each gene expression analysis, three replicates were performed.

Table 1. Primers used for RT-qPCR.

Gene	Gene product	Primer (5'-3')	Amplicon (bp)
ectC	L-ectoine synthase	(F)ACGACCATCTACGCCAATACC (R)TGTCTCGACCTCTCCTTCTCC	93
ectD	Ectoine hydroxylase	(F)AGCTGTTCCATCCTCCTGA (R)ACGCCATACTCCTGTTTTCG	94
proP	L-Proline Glycine Betaine transporter ProP	(F)TCCTTTTCCTTGCCCGTCT (R)GCCATTCTCCCTCTTCTCTTGT	109
treZ	Malto-oligosyltrehalose trehalohydrolase	(F)TCCGACGACTTCCACCAT (R)GCAAACTGTCCCTGATAGACG	131
treS1	Trehalose synthase	(F)GTATCAGGACCACAAGGACGA (R)CGACATCCAGATTGAACCAGT	101
treS2	Trehalose synthase	(F)GGTGACGATGCTGTTTTCCT (R)CGACGAAGCGACAAGATAGTG	132
otsB	Trehalose-6-phosphate phosphatase	(F)GACATTCCTCCAGGCAAACA (R)ACTTCCTCCGCTTGCTTCTT	142
kdpC	Potassium-transporting ATPase C	(F)CGGGTCGAGTCCTCTTCCTTA (R)GGCAGTCCGTTTTCTTTTCG	126
kdpD	Osmosensitive K^+ channel histidine kinase kdpD	(F)GCTTCTGGTCCTTCATCTTCG (R)ACATCTTCCCTCCATTTTCG	114
trkA1	Potassium channel	(F)CTTTTGGTGACGCCTTCTTC (R)CAGGGCAATAATGGACAGC	93
trkA2	Potassium channel	(F)GTCCCGAAAACCGATGATG (R)ACAGATGCCGCACACGAT	124
yggT	Integral membrane protein YggT	(F)CTCGCCGGATCCCTACAAT (R)GCTTTTCGGGAGGAACCAGT	95
ccP	Cytochrome *c* peroxidase	(F)ATGCCGCCTACTTTCCTCT (R)TAGTTGGGGTTAGCCATTTCC	106
trxB	Thioredoxin reductase	(F)GCTCTTTTCCTGACCCGTTT (R)GAGATTTTCTTGTTTGCCTTGG	110
trx1	Thioredoxin	(F)TCGGAAGAGTATAAAGGCAAGG (R)AAAACATCAGAGTGGGAATGC	106
trx4	Thioredoxin	(F)CTGGAAATCCCTGAGAAACG (R)GGAACCGACTGAATGGAGTG	105
trx6	Thioredoxin	(F)TGGATGAAAACCCCTACACC (R)ATAGGCACCCACCAGTCG	104
rrsB	RNA ribosomal 16S	(F)ACGGGTGAGTAGACATGGG (R)GGTAGGGTGCAAACGGG	105

F: Forward. R: Reverse

4.1.5 Determination of intracellular compatible solutes content in *L. ferriphilum* DSM 14647 exposed to NaCl, by HPLC and GC-MS.

To study the late response mechanism of *L. ferriphilum* against saline shock, the intracellular level of compatible solutes ectoine, hydroxyectoine and trehalose detected by the bioinformatic analysis were measured. Hence, the cells were stressed with 100 mM NaCl for 90 min, harvested and then the metabolites were extracted.

Metabolite extraction.

The metabolite extraction was performed using a modified version of the methods described by Mosier et al. (2013). Briefly, three replicates with approximately 75 mg (wet weight) of biomass were used from each sample and were extracted in 700 µl of methanol-isopropanol-water (3:3:2 [vol/vol/vol]). Samples were then sonicated at maximum amplitude for 10 minutes in steps of 30 s and further centrifuged for 10 min at 18,000 × *g*. The supernatant was transferred to a clean tube, to which 5 mg of ionic exchange resin (BioRex RG 50l-XB) were added, and the mixture was shaken at 600 × *g* for 30 min at room temperature. Then, samples were centrifuged for 5 min at 15,000 × *g*, the supernatant was taken and saccharose was added to 500 µM final concentration as an internal standard (IStd) for GC-MS analysis. Finally, these mixtures were dried using a SpeedVac (reduced pressure typically 4-8 hours). Extraction blank controls also followed this complete procedure precisely, which began by adding the extraction solvent to the empty tubes.

Analysis of intracellular (hydroxy)ectoine content.

The compatible solutes ectoine and hydroxyectoine were quantified by HPLC analysis, using an Ultimate 3000-2015 HPLC (Thermo Scientific) system with a 250 mm × 4.6 mm Hypurity Aquastar C-18 column with particle size of 5 µm (Thermo Scientific). Chromatography was performed with a gradient of two solutions as mobile phase (Table 2), eluent A (0.8 mM KH_2PO_4/6.0 mM Na_2HPO_4, pH 7.6) and eluent B (acetonitrile), at a flow rate of 1.0 ml/min at 25°C. The presence of compatible solutes was monitored at 215 nm by a UV/VIS detector. The retention time of ectoine and hydroxyectoine were determined using commercially available compounds (purity ≥ 95%, Sigma-Aldrich, USA). Intracellular ectoine and hydroxyectoine content were calculated as nanograms per mg of wet biomass, using a calibration curve (Figure S1)

Table 2. Elution gradient of HPLC runs.

Time [min]	%Eluent A	%Eluent B
0.00	100	0
1.50	100	0
3.00	60	40
4.50	100	0
10.00	100	0

Analysis of intracellular trehalose content.

The trehalose content was quantified by gas chromatography associated with mass spectrometry (GC-MS) on samples derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) according to Medeiros and Simoneit (2007) with some modifications. Briefly, dry extracts were suspended in 200 µl of pyridine, 25

µl of MSTFA were added and the mixture were heated at 60ºC for 1 h. After that, they were centrifuged at maximum speed for 5 min at room temperature and the supernatants were analyzed.

Aliquots of 1µl of silylated total extracts, as well as standard solutions of 500 µM trehalose/saccharose, were analyzed within 24 h, using a TRACE 1310 gas chromatograph interfaced with an (ISQ) mass-selective detector (GC–MS). A TRACE ™ TR-5 capillary column (30 m × 0.25 mm I.D. and film thickness of 0.25 µM (Thermo Scientific) was used with helium (Airgas) as the carrier gas at a constant flow rate of 1.3 ml min^{-1}. The injector and MS source temperatures were maintained at 280 and 230ºC, respectively. The column temperature program consisted of injection at 65ºC, held for 2 min, temperature increase from 6ºC min^{-1} to 300ºC, followed by an isothermal hold at 300°C for 8 min. The MS was operated in the electron impact mode with ionization energy of 70 eV. The measurement was set to 361 m/z for 5 min.

Data were acquired and processed with the Xcalibur 2.2 software. Compound identification was performed by comparison with the chromatographic retention characteristics and mass spectra of authentic standards, reported mass spectra and the mass spectral library of the GC–MS data system. Compounds were quantified using the total ion current (TIC) peak area converted to compound mass using calibration curves of internal standards with 500 µM saccharose (Figure S2). Procedural blanks were run in sequence on the samples in order to monitor significant background interferences.

4.2 Evaluation of the participation of the antioxidant response in the protection against NaCl.

To investigate the relation that may exist between the osmotic stress in saline shock and oxidant conditions induction in acidophilic microorganisms, the intracellular pH, respiratory rate, ROS production, antioxidant protein activities and gene expression related with these proteins were evaluated.

4.2.1 Measurement of the intracellular pH in *L. ferriphilum* DSM 14647 exposed to NaCl.

The intracellular pH of *L. ferriphilum* exposed to 100 mM NaCl was determined by the fluorescent probe pH rodo^{TM} Green (Life Technologies, USA) according to the manufacturer's instructions. Briefly, cells derived from the different treatments were centrifuged at 8,000 x *g* for 15 min, washed and resuspended in staining solution (pH rodo Green and Power Load, dissolved in 10 mM HEPES buffer pH 7.4). These suspensions were incubated for 30 min at 37°C in darkness under agitation at 180 rpm. Subsequently, the cells were washed in the same 10 mM HEPES buffer and incubated with 10 mM each valinomycin/nigericin stock solution at 37°C for 10 min. Finally, the fluorescence of 100 µl of each sample was measured using excitation/emission wavelengths of 509 and 533 nm, respectively. The relative fluorescence values obtained were related to pH by means of the pH calibration kit (Life Technologies, USA). The calibration curve is in the Figure S3. The assay was performed in triplicate.

4.2.2 Measurement of the oxygen consumption in *L. ferriphilum* DSM 14647 exposed to NaCl.

The oxygen consumption of *L. ferriphilum* exposed to 1, 12.5, 25, 50 100 and 150 NaCl was measured. The respiratory rate was determined by means of optodes (Fibox 3, PreSens-Precision Sensing GmbH, Regensburg, Germany) (Giebner et al., 2017). In short, fresh iron-grown cultures of *L. ferriphilum* DSM 14647 were harvested by centrifugation at 8,000 x *g* for 15 min and, after removal of supernatant, a volume of 0.1 ml resuspended cells was added to a 3 ml measuring cuvette containing 2.6 ml of DSMZ 882 culture medium (pH 1.8) with increasing NaCl concentrations, ranging from 0 to150 mM NaCl. Afterward, 0.15 ml of ferrous iron solution were added into the cuvette to give 20 g/l final concentration and the suspension was mixed cautiously. The cuvette was then carefully closed with a glass lid. An oxygen sensing optode spot had previously been embedded inside the measuring cuvette. Fibre-optics located outside of the cuvette on the opposite side of the oxygen sensor spot was connected with a 4-channel fiber–optics oxygen meter (Firesting O_2), also equipped with a receptacle for a temperature sensor. The optode signal was evaluated using the software Pyro Oxygen Logger. Noticeably, due to the strong temperature dependence of fluorescence, measurements had to be made in a thermostated cabinet (UVP Hybridizer HB-1000). Optode measurements were done in triplicate.

4.2.3 Determination of total ROS levels in *L. ferriphilum* DSM 14647 exposed to NaCl.

As a consequence of the cytoplasm acidification and the increase in the respiratory rate, production of reactive oxygen species (ROS) may be triggered, and therefore oxidative stress can be induced. Thus, the intracellular level of total ROS in *L. ferriphilum* DSM 14647 exposed to 50, 100 and 150 mM NaCl for 60 min or 100 mM NaCl for 30, 60 and 90 min was measured using the oxidant-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Davidson et al., 1996). For ROS determination, 300 ml of cells were centrifuged and washed with 10 mM sodium citrate pH 7.0 and incubated for 30 min in 100 mM potassium phosphate pH 7.4, containing 10 μM final concentration of H_2DCFDA (from a 1 mM stock solution dissolved in dimethyl sulfoxide). After washing, the cells were suspended in 100 mM potassium phosphate pH 7.4, disrupted by sonication, and centrifuged at 18,000 × *g* for 20 min. Aliquots of cell extracts (100 μl) were obtained and the fluorescence intensity was measured using a fluorescence reader (Synergy HT, BioTek) and excitation at 498 nm. Emission values recorded at 522 nm were normalized to the respective protein concentration. Protein concentration was determined as described by Bradford (1976).

4.2.4 Measurement of antioxidant protein activities CcP and Trx of *L. ferriphilum* DSM 14647 exposed to NaCl.

To complement the assay of the section 4.2.3, the antioxidant protein activities cytochrome *c* peroxidase (CcP) and thioredoxin (Trx) were measured. Since it has been

previously demonstrated that they play a key role in *Leptospirillum* spp. against oxidant conditions (Norambuena y cols., 2012; Ferrer y cols., 2016b; Zapata y cols., 2017).

Whole-cell extracts preparation.

L. ferriphilum was grown until late exponential phase and 350 ml of culture were harvested by centrifugation at 8,000 x g for 15 min, washed once with acid water (pH 1.4) and twice with 10 mM sodium citrate pH 7.0 to eliminate the remaining iron. The washed cells were suspended in 882 medium and incubated with 50, 100 and 150 mM NaCl for 60 min or 100 mM NaCl for 30, 60 and 90 min. After exposure to chloride, cells were harvested and washed twice with 10 mM sodium citrate pH 7.0. Bacterial extracts were prepared by ultrasonic disruption in buffer containing 30 mM Tris-HCl pH 8.0, 30 mM NaCl, 1 mM dithiothreitol (DTT), followed by centrifugation for 30 min at 20,000 × *g* at 4◦C twice and supernatant was recovered. As a negative control, the activities of a protein extract inactivated at 65ºC for 15 min were followed. Protein concentration was determined as described by Bradford (1976).

Thioredoxin (Trx) activity.

Thioredoxin activity was assayed by the reduction of disulfides of free chain insulin B by DTT and measured spectrophotometrically as turbidity formation from the protein precipitation. The assay was carried out as described Norambuena et al. (2012). Using a reaction mixture (500 ml) contained TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.5), 0.16 mM insulin (Sigma) and cell extract (25 mg of total protein extract from *L. ferriphilum*). The reaction was started by adding of 0.33 mM DTT. The absorbance at 650 nm was monitored using an UVmini-1240 spectrophotometer (Shimadzu) at room

temperature. Blank contained all the components except DTT and the mixture without protein extract was the negative control.

Cytochrome *c* peroxidase (CcP) activity.

This activity was assayed as described Yonetani and Ray (1966). 50 mg of horse heart cytochrome *c* (Merck) were dissolved in 2 ml of 10 mM potassium phosphate pH 7.0 and 1 mM EDTA. To reduce ferricytochrome *c*, the reaction mixture was incubated with 10 mM sodium dithionite for 2 min. Salt excess was removed by gel filtration in Micro Bio-Spin columns (BioRad) packed with Bio-GelP6 (molecular exclusion limit of 1-6 kDa) (BioRad). Reduced cytochrome *c* was estimated spectrophotometrically at 550 nm. An aliquot of 10 µl was mixed with 490 µl of phosphate buffer pH 7.0 and absorbance was measured at 550 nm. The absorbance of a ferricyanide-oxidized cytochrome *c* was also determined. The percentage of cytochrome *c* reduction was estimated according to Matthis and Erman (1995) using an extinction coefficient (ε) of 27.7 mM^{-1} cm^{-1}. To measure CcP activity, the reaction mixture (500 µl) contained 10 mM potassium phosphate pH 7.0, 25 mM ferrocytochrome *c* and 50 µg protein extract. The reaction started by adding 200 mM H_2O_2. The enzyme assay was performed by measuring the oxidation rate of ferrocytochrome *c* every 10 s for 3 min. Blank contained all the components except ferrocytochrome *c* and the mixture without H_2O_2 was used as the negative control.

4.2.5 Effect of the external antioxidant (cobalamin) and compatible solute (hydroxyectoine) supplementation in the antioxidant response of *L. ferriphilum* DSM 14647 exposed to NaCl.

In the case of the antioxidant agent, prior to the induction of stress with 100 mM NaCl for 90 min, the cells were pre-treated with 5 nM cobalamin for 1 hour. For the compatible solute, 0.5 mM hydroxyectoine was added at the same time to the stress was applied with 100 mM NaCl for 90 min. After that, the ROS production and antioxidant activities were evaluated according with sections 4.2.3 and 4.2.4, respectively.

4.2.6 Determination of expression of antioxidant protein encode genes in cells exposed to NaCl.

To detect the effect of NaCl on the mRNA levels of genes for thioredoxin system (TrxA and TrxB) and cytochrome *c* peroxidase (CcP), a transcription analysis by RT-qPCR was performed in *L. ferriphilum* DSM 14647 according with the methodology described in the section 4.1.4.

4.3 Identification of the transcriptomic profile of *Leptospirillum ferriphilum* DSM 14647 adapted to NaCl.

To investigate the late response mechanism that acidophilic microorganisms susceptible to chloride may use to tolerate the presence of the anion, the cells were adapted to NaCl. This experiment was done with a culture adapted to 180 mM NaCl.

4.3.1 Adaptation of *L. ferriphilum* DSM 14647 to 180 mM NaCl, supplementing the medium with compatible solutes.

In order to improve the osmotic tolerance of microorganisms, the adaptation was performed with the supplementation of ectoine and trehalose. When a 180 mM NaCl adapted culture was obtained, the compatible solutes were gradually removed from the medium. The adaptation of these cells started with 50 mM NaCl and were exposed to increasing concentrations of NaCl with 3 passages per every salt concentration.

4.3.2 Growth curve of *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

The experiment was carried out in 250 mL Erlenmeyer flasks. Each flask contained 100 mL of 882 medium with 0 - 180 mM NaCl for non-adapted and adapted cells, respectively. Samples were taken periodically for determination of cell growth, which were measured by direct microscopic counting by using a modified Neubauer chamber. The initial cell density was $1x10^6$ cells ml^{-1}.

4.3.3 Determination of minimal inhibitory concentration (MIC) for NaCl of *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

This assay was carried out on planktonic cells of non-adapted and adapted cells of *L. ferriphilum* cultured in 882 medium at pH 1.4, 1.8, 2.4 and 3.0, according with the methodology described in the section 4.1.3.

4.3.4 Evaluation of the transcriptomic profile of *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl by RNAseq.

cDNA library preparation and Illumina sequencing.

Total RNA extraction was performed in cells cultured in 1 L of 882 medium with addition of 180 mM NaCl, using the commercial kit RNeasy® mini (Qiagen) and later quantified through photometric analysis. Quality and Integrity were evaluated using an Agilent Bioanalyzer 2100 and a RNA 600 Nano Kit (Agilent Technologies), where the samples of total RNA with a RIN (RNA Integrity Number) above 7 were approved to be used in the sequencing. Three RNA preparations of high quality were then pooled together and submitted for transcriptome analysis as previously described (Zhu et al. 2016; Hosseinpour et al. 2018). Before library preparation, rRNA was depleted by using the MICROBExpress kit (Thermo Fisher). Then, a TruSeq stranded mRNA library prep kit (Illumina) was used to generate cDNA libraries for whole transcriptome analysis. The resulting libraries were sequenced on an Illumina MiSeq system with v3 chemistry and 2 x 75-nucleotide read length (paired end).

Functional annotation.

The draft reference genome of *L. ferriphilum* DSM 14647 [NCBI accession number: PGK00000000] was re-annotated by RAST to identify genes, and a function for each was provided according to the public Gene Ontology (GO) database by Go feat tool (Araujo et al., 2018).

Differential expression analysis.

To obtain reads of high quality, the total raw reads were filtered to exclude adaptors, and to select reads with quality higher than Q20 using the CLC Genomics

Workbench software. Then, the filtered reads were aligned into the draft reference genome of *L. ferriphilum* DSM 14647 re-annotated by RAST using CLC Genomics Workbench software.

From the alignment, the raw counts table was obtained, and then, it was analyzed by the statistic R software, using the package DESeq2 (Love et al., 2014). This package take as input the raw counts, then normalize them, estimate dispersion, and run the differential expression analysis. The output of this software was a p-value and a fold change for each gene. It was considered differentially expressed a gene with a p-value<0.05.

4.3.5 Measurement of the oxygen consumption in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

To evaluate if the cellular respiration was determinant in the adaptation of *L. ferriphilum*, the oxygen consumption of non-adapted and adapted *L. ferriphilum* exposed to 180 mM NaCl was measured as explained in section 4.2.2.

4.3.6 Determination of total ROS levels in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

To evaluate whether the oxidative response may be involved in the adaptation of *L. ferriphilum*, the intracellular ROS levels was measured in non-adapted and adapted cells cultured in 882 medium supplemented with 180 mM NaCl. The measurement of the total ROS levels was carried as explained in section 4.2.3.

4.3.7 Determination of intracellular (hydroxy)ectoine content in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl, by HPLC.

To evaluate whether the osmotic response mechanism was part of the adaptation of *L. ferriphilum*, the intracellular content of ectoine and hydroxyectoine was measured in non-adapted and adapted cells cultured in 882 medium supplemented with 180 mM NaCl. The measurement of the compatible solutes was performed according with the method described in the section 4.1.5.

5. RESULTS

5.1 Evaluation of the participation of canonical systems of tolerance to osmotic stress in cells exposed to NaCl.

5.1.1 *In silico* analysis of putative saline stress tolerance in acidophilic iron-oxidizing microorganisms.

To get insights into the mechanisms involved in the saline stress response in iron-oxidizing bacteria and archaea, and to compare them among different phyla, a bioinformatic search was performed using complete genome sequences of twelve aerobic acidophilic microorganisms (10 bacteria and 2 archaea) as described in Materials and Methods. In this analysis was included the moderately halotolerant acidophilic bacteria *Ac. prosperus* V6 and *L. ferriphilum* Sp-Cl. The search comprised genes coding for transporters involved in the uptake of potassium, and genes for biosynthesis and uptake of compatible solutes.

Table 3 summarizes the main characteristics of the microorganisms and their respective genomes. Bacteria and archaea under study are chemolithotrophs able to oxidize iron. However, some of them can also oxidize elemental sulfur and reduced sulfur compounds and use organic carbon sources alternatively to CO_2 fixation. The genomes of these microorganisms have a size in a range from 1.94 to 5.04 Mb, the G+C content ranges from 36.5 to 62.2%, and the ORF number ranges from 1963 to 4541.

Table 3. Comparison of the genome features of acidophilic microorganisms under study.

Phyla	Microorganism	Strain	Energy source	Genome size (MB)	%GC	ORF number	Ref
Nitrospirae	*Leptospirillum ferriphilum*	DSM 14647	Iron	2.41	54.2	2270	Cárdenas et al., 2014
	Leptospirillum ferriphilum	Sp-Cl	Iron	2.48	54.4	2882	Issota et al., 2016
	Leptospirillum sp.	CF-1	Iron	2.71	54.6	2736	Ferrer et al., 2016a
Firmicutes	*Sulfobacillus thermosulfidooxidans*	DSM 9293	Iron/sulfur	3.86	49.7	3875	DS
	Alicyclobacillus ferrooxydans	TC-34	Iron	5.04	48.6	4541	Jiang et al., 2008
Actinobacteria	*Ferrimicrobium acidiphilum*	DSM 19497/T23	Iron	3.09	55.3	3015	Eisen et al., 2015
Proteobacteria	*"Ferrovum myxofaciens"**	P3G	Iron	2.70	54.9	2694	Moya-Beltran et al., 2014
	*Acidihalobacter prosperus***	V6	Iron/sulfur	3.36	62.2	3241	Khaleque et al., 2017
	*Acidithiobacillus ferrooxidans****	ATCC 23270	Iron/sulfur	2.98	58.8	3217	Valdés et al., 2008
	*Acidithiobacillus ferrivorans****	SS3	Iron/sulfur	3.21	56.5	3206	Liljeqvist et al., 2011
Euryarchaeota	*"Ferroplasma acidarmanus"*	fer1	Iron	1.94	36.5	1963	Yelton et al., 2013
Crenarchaeota	*Metallosphaera sedula*	DSM 5348	Iron/sulfur	2.19	46.0	2330	Auernik et al., 2008

DS: Direct submission reference sequence NZ_FWWY01000001.

(*): Betaproteobacteria, (**): Gammaproteobacteria, or (***): Acidithiobacillia classes.

5.1.1.1 K^+ uptake systems.

Potassium transporters have a critical role in initial response to osmotic stress and high salinity (Roessler and Müller, 2001). Bacteria accumulate K^+ by a number of

different transporters that vary in kinetics, energy source, and regulation (Csonka, 1989). In most species two or more independent saturable K^+-transport systems are present. In *E. coli*, Kup and Trk are proton motive force-driven K^+-uptake systems that are constitutively expressed (Csonka, 1989). Kup, is a single-protein transporter known as low-level K^+-uptake system, which exhibits a high transport velocity, but a rather a low affinity. K^+-uptake activity of Kup is not influenced by osmolarity of the medium (Kempf and Bremer, 1998). However, this system has been reported to play a major role in adaptation to osmotic stress at low pH (Trchounian and Kobayashi, 1999). Trk from *E. coli* is a K^+ transporter with modest affinity that is composed of the NADH-binding TrkA, the ATP-binding TrkE, and the transmembrane K^+-path subunits TrkH/G (Bossemeyer et al., 1989, Schlösser et al., 1993). This system plays an important role when the cell is exposed to K^+ concentrations higher than 1 mM (Bakker, 1993). In contrast to housekeeping systems from *E. coli*, potassium uptake via the multicomponent Kdp is increased upon osmotic shock, thus indicating that its expression is regulated by environmental conditions (Csonka, 1989, Ito et al., 2009). The transporter KdpFABC is an ATP-driven K^+ pump with high affinity and specificity, which is only found in bacteria and archaea (Greie, 2011). KdpFABC synthesis is governed by the membrane-bound sensor kinase KdpD and the response regulator KdpE, and it takes place when potassium concentration of the medium falls below 100 µM. Finally, a fourth uptake system represented by the YggT protein drives K^+ uptake and osmotic regulation when *E. coli* cells are exposed to osmotic stress with high concentrations of NaCl or mannitol (Ito et al., 2009).

In-silico comparative analysis revealed that the constitutive low-affinity Kup and Trk systems are not well represented in genomes of acidophiles (Table 4 and Table S1). In fact, the presence of a Kup system seems to be an exception among acidophilic iron oxidizers, since *kup* genes could be detected only in the genomes of the Proteobacteria "*F. myxofaciens", Ac. prosperus, At. ferrooxidans* and *At. ferrivorans. Kup* genes appear to be missing in genomes of acidophiles belonging to the phyla Nitrospirae, Firmicutes, Actinobacteria, Euryarcheota and Crenarcheaota. The lack of the Kup system is apparently in contradiction to the reported functionality of this system in the osmoadaption at low pH (Trchounian and Kobayashi, 1999). However, this fact suggests that low-affinity systems may not be enough to satisfy potassium requirements in natural environments where low K^+ concentrations are usually found (Ashley et al., 2006, Dutrizac, 2008). In the same line, although genes homologous to *trkA* are present on most of the chromosomes, genes predicted to encode the specific potassium transmembrane transporter TrkH/G and the ATP-binding protein TrkE were not detected on most chromosomes. Exceptions are the firmicute *Alb. ferrooxydans* TC-34, which harbors *trk*A and *trk*G genes, and the proteobacterium *Ac. prosperus* V6 in which *trkA* and *trkH* are adjacent in the chromosome and likely form an operon.

The situation is very different regarding the high-affinity Kdp system. In agreement with the wide distribution previously reported for this system (Epstein, 2003), most of the genomes analyzed in this study contain a whole or partial repertoire of the *kdpFABC* genes for the Kdp transporter, and of the *kpdDE* genes encoding a two-component regulator, controlling *kdp* expression (Greie, 2011). The *kdp* genes appear to form putative operons (Figure 1) and to be conserved among acidophiles, since their

corresponding products show significant similarity with other known Kdp P-type ATPases. Thus, the products of the *kdpABC* genes share 39-56%, 47-71%, and 34-52% sequence identity with the *E. coli* KdpA, KdpB, and KdpC subunits, respectively. Furthermore, the KdpDE proteins display 33-52% and 38-63% identity with the *E. coli* KdpD and KdpE subunits, respectively. In addition, it is remarkable that *Alb. ferrooxydans* TC-34, *Fm. acidiphilum* and *At. ferrooxidans* have two or more copies of *kdp*ABC and *kdp*ED genes indicating that they may be highly relevant for osmotic adaptation of these microorganisms. On the other hand, we could identify only in four genomes ("*F. myxofaciens*", *Ac. prosperus*, *At. ferrooxidans* and *At. ferrivorans*) a gene homologous to *kdpF*, encoding the fourth component of the Kdp system in *E. coli*. Similarly, this gene has also been reported as missing in the genome of *Sinorhizobium meliloti* (Domínguez-Ferreras et al., 2009). It is interesting to note that the *kdp* genes were found on the genomes of the halotolerant bacteria *Ac. prosperus* V6 and *L. ferriphilum* Sp-Cl.

Finally, comparative analysis showed that genes for an YggT-type K^+ transporter are widespread and highly conserved (56-70% similarity with YggT of *E. coli*) and presumably enable acidophiles among Nitrospirae, Firmicutes, Actinobacteria, and Proteobacteria to take up K^+ from the medium. In contrast, a YggT encoding gene was not detected in the archaea *M. sedula* and "*F. acidarmanus"*. Since the YggT system has been reported as having a role in osmoadaptation at high NaCl concentration (Ito et al., 2009), it may confer increased tolerance to chloride and therefore an adaptive advantage to those microorganisms that carry it. Co-localized to YggT also the YggS gene was found which has been reported as part of the same operon in *E. coli* (Ito et al., 2009). The role

of YggS in osmotic regulation has not been determined experimentally, but its broad conservation suggests that it might be playing some complementary role to YggT functionality.

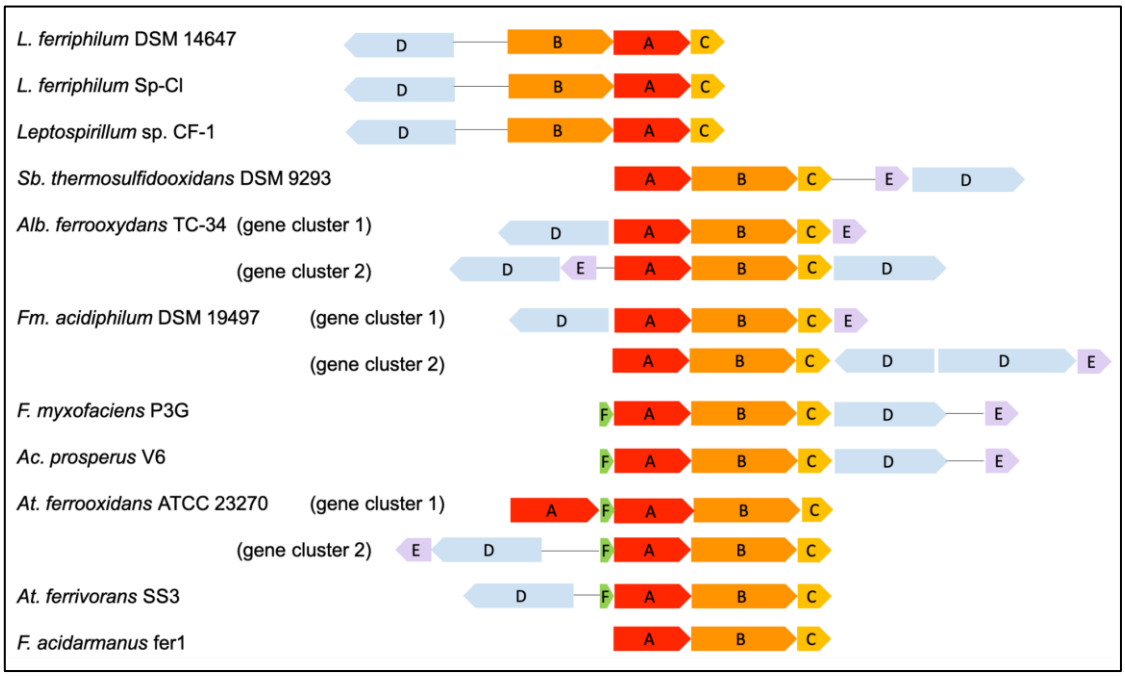

Figure 1. Organization of *kdp* gene cluster detected in iron-oxidizing acidophiles. The KdpFABC is an ATP-dependent multicomponent K^+ transporter, KdpD a sensor kinase, and KdpE a response regulator (Greie, 2011).

Table 4. Osmotic stress-related genes in iron-oxidizing bioleaching acidophiles.

Microorganism	Potassium uptake														Trehalose synthesis								Trehalose uptake								Ectoine synthesis				Ectoine uptake					
	kup	*kdp*						*trk*		*ygg*		*tre*			*treS*	*ots*		*sug*			*mal*				*ect*				*proP*	*proU*	*ehu*			
		A	*B*	*C*	*D*	*E*	*F*	*A*	*G/H*	*T*	*S*	*X*	*Y*	*Z*		*A*	*B*	*A*	*B*	*C*	*E*	*F*	*G*	*K*	*A*	*B*	*C*	*D*			*A*	*B*	*C*	*D*
L. f. DSM 14647	-	1	1	1	1	-	-	2	-	1	1	2	1	1	3	1	1	-	-	-	-	-	-	-	1	1	1	1	1	-	-	-	-	-
L. f. Sp-Cl	-	1	1	1	1	-	-	2	-	1	1	2	1	1	1	1	1	-	-	-	-	-	-	-	1	1	1	1	-	-	-	-	-	-
L. sp. CF-1	-	1	1	1	1	-	-	2	-	1	1	3	1	1	3	1	1	-	-	-	-	-	-	-	1	1	1	1	2	-	-	-	-	-
Sb. t. DSM 9293	-	1	1	1	1	1	-	2	-	1	1	-	-	-	1	1	1	-	-	-	1	1	1	1	-	-	-	-	-	-	-	-	-	-
Alb. f. TC-34	-	2	2	2	3	2	-	4	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	-	-	1	-	-	-	-
Fm. a. DSM 19497	-	2	2	2	3	2	-	1	-	1	-	-	-	-	1	-	-	1	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F. m. P3G	1	1	1	1	1	1	1	-	-	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ac. p. V6	1	1	1	1	1	1	1	1	2	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	1	-	1	1	1	1	1
At. f. ATCC 23270	1	3	2	2	1	1	2	2	-	1	1	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
At. f. SS3	1	1	1	1	1	1	1	2	-	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F. a. fer1	-	1	1	1	-	-	-	-	-	-	-	1	-	-	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. s. DSM 5348	-	-	-	-	-	-	-	1	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

L. f: *Leptospirillum ferriphilum*; *L.* sp*.: Leptospirillum* sp.*; Sb. t*: *Sulfobacillus thermosulfidooxidans*; *Alb. f*: *Alicyclobacillus ferrooxydans*; *Fm. a*: *Ferrimicrobium acidiphilum*; *F. m*: "*Ferrovum myxofaciens*" *Ac. p*: *Acidihalobacter prosperus*; *At. f*: *Acidithiobacillus ferrooxidans*; *F. a*: "*Ferroplasma acidarmanus"*; *M. s*: *Metallosphaera sedula*

5.1.1.2 Biosynthesis and uptake of compatible solutes

Bacteria accumulate various organic molecules such as trehalose, ectoine, glycine betaine, and others, to provide the necessary osmotic balance in different external salt concentrations. The repertoire of enzymes used for production and/or uptake of compatible solutes in an organism is indicative for its strategy of adaptation to changes in the salinity of the medium.

Trehalose synthesis.

A typical compatible solute prevailing in mesophilic as well as in thermophilic microorganisms is the non-reducing disaccharide trehalose. Its uptake, accumulation, and synthesis have been studied in detail in several bacterial representatives (Ruhal et al., 2013). Three major pathways for biosynthesis of trehalose have been described, and they include the multistep routes OtsAB (also called TPS/TPP) (Kaasen et al., 1992) and TreXYZ (Maruta et al., 2000), and the TS pathway in which trehalose synthase (TreS) catalyzes the one-step conversion of maltose to trehalose (Nishimoto et al., 1996).

As is shown in Table 4 and Table S1 based on the bioinformatic analysis, with exception of *Ac. prosperus* V6, all genomes analyzed harbor a total or partial gene repertoire for trehalose biosynthesis, Nitrospirae being the only phylum with predicted genes for all three bacterial trehalose pathways, which agrees with observations recently reported for *L. ferriphilum* Sp-Cl (Issota et al., 2016, Galleguillos et al., 2018). In addition, the TreS-encoding gene appeared to be contained on the genome of the firmicute *Sb. thermosulfidooxidans*, which agrees with what has previously been described (Galleguillos et al., 2018). The *treS* gene was also detected in the actinobacterium *Fm. acidiphilum*, the betaproteobacterium "*F. myxofaciens",* the acidithiobacillium *At.*

ferrivorans, the euryarchaeon "*F. acidarmanus",* and the crenarchaeon *M. sedula*. The TreS proteins deduced from the respective gene sequences showed high similarities (43 - 60%) to their homolog TreS from *E. coli*. On the other hand, besides in Nitrospirae, the genes for components of the TreXYZ system were only found in the Betaproteobacteria ("*F. myxofaciens")* and in the Acidithiobacillia (*At. ferrivorans*). Although the *tre*X gene was detected on the genomes of the firmicute *Alb. ferrooxydans* and of the archaea *M. sedula* and "*F. acidarmanus"*, a functional TreXYZ system seems unlikely, since no *tre*Z and *tre*Y genes were detected in these microorganisms. Altogether these results suggest that, with exception of Firmicutes (*Alb. ferrooxydans)* and Gammaproteobacteria (*Ac. prosperus)*, microorganisms from all studied lineages have the potential capability to synthesize trehalose to respond to osmotic stress. This is in line with the widespread presence and general role of trehalose and of other sugars to protect cells by preventing denaturation of lipid membranes and membrane proteins (Pereira and Hünenberger, 2008). This also agrees with the recent identification of trehalose as a compatible solute in different species of acidophilic bacteria including members of the genus *Leptospirillum* and of the species *At. ferrooxidans*, *Sb. thermosulfidooxidans* and *Acidiphilium cryptum* (Galleguillos et al., 2018).

Trehalose uptake.

In bacteria intracellular accumulation of trehalose can proceed through the incorporation from the environment by the activities of the ABC-type transporter SugABC and the widely-described maltose transporter MalEFGK. Functionality of the SugABC system in trehalose uptake has been shown in *Mycobacterium tuberculosis*, where it has proved to be involved in the formation of the mycolic acid-containing cell envelope and

in virulence (Kalscheuer et al., 2010). A function of MalEFGK in trehalose uptake has been proven in several microorganisms including thermophiles (Haardt et al., 1995, Nanavati et al., 2005, Silva et al., 2005).

Unlike genes encoding proteins of trehalose biosynthesis, prevalence of genes encoding trehalose uptake systems is lower among the analyzed acidophile genomes. As is shown in Table 4 and Table S1, the trehalose transporter SugABC was found in the representative of the Actinobacteria (*Fm. acidiphilum*), but predicted *sug*ABC genes were not detected in any representative from the other phyla. In addition, the widely-described maltose transporter MalEFGK was only found in the firmicute species *Sb. thermosulfidooxidans*. In *M. sedula*, genes for an ABC transporter closely related to the *malEFGK* genes were identified. This transporter has been reported to have a role in sn-glycerol-3-phosphate (Ugp) import. Complementation studies have revealed that the *ugp*C gene restores the phenotype of a *mal*K(-) mutant of *E. coli* (Hekstra and Tommassen, 1993). In addition, a hybrid MalFG-UgpC proved to be functional with specific activity comparable to that of the native maltose transporter (Wuttge et al., 2012). Whether the predicted complex Ugp encoded in *M. sedula* is capable of importing trehalose into the cytoplasm is still an open question.

These findings are consistent with the fact that bioleaching environments are in general depleted of organic matter (Wang et al., 2014). So it is expected that trehalose and other sugars are poorly available for microorganisms inhabiting these environments. According to these data, uptake of these sugars might not be a strategy widely used for osmodaptation in acidophiles. In contrast to our results, enzymes of trehalose metabolism have been detected in extracellular polymeric substances of a *Leptospirillum* group II-

dominated biofilm from an acid mine drainage (Jiao et al., 2011), therefore indicating that this sugar may be a component of the EPS generated by this community or a product generated from such a component. Thus, the role of trehalose as a protective or structural sugar in acidophilic microorganisms needs to be particularly studied.

Ectoine synthesis.

Ectoine is a common compatible solute in mesophilic organisms, but it has appeared absent in (hyper)-thermophilic microorganisms (Empadinhas and Da Costa, 2006). Ectoine biosynthesis is strongly enhanced under high-osmolality growth conditions (Calderón et al., 2004, Bursy et al., 2008). The enzymes for ectoine biosynthesis are encoded by a highly conserved gene cluster, *ectABC* (Bursy et al., 2008). In addition, some microorganisms may synthesize 5-hydroxyectoine through a hydroxylation reaction catalyzed by ectoine hydroxylase EctD (Bursy et al., 2008). This derivative has also been shown to serve as a compatible solute essential for salt stress tolerance (Bursy et al., 2008, Tao et al., 2016).

A complete set of genes *ectABC* for ectoine biosynthesis was found in the genomes of the Nitrospirae *L. ferriphilum* DSM 14647, *L. ferriphilum* Sp-Cl and *Leptospirillum* sp. CF-1, of the firmicute *Alb. ferrooxydans* TC-34, and of the proteobacterium *Ac. prosperus* V6. In addition, in *Leptospirillum* strains, the *ectABC* genes are followed in the same transcriptional direction by the *ectD* gene which encodes an ectoine hydroxylase EctD (51% identical amino acids with EctD of *E. coli*). The presence of the *ectABCD* gene cluster in the genome suggested that the Nitrospirae representatives have the capability to synthesize both compatible solutes ectoine and 5-hydroxyectoine. In other microorganisms, it has been established that ectoine represents the major compatible

solute under conditions of high salinity, while 5-hydroxyectoine is a minor solute. However, the ratio of these solutes (ectoine:5-hydroxyectoine) can increase with NaCl concentration, and it is dependent on the growth phase (Tao et al., 2016). After the cell has been exposed to an osmotic shock, 5-hydroxyectoine seems to be preferentially synthesized in the late growth phase and later than ectoine. Thus, a functional differentiation of the compounds seems to take place during stress response. Furthermore, a wealth of evidence has indicated that compatible solutes as ectoine and 5-hydroxyectoine are involved in other responses than just osmotic adjustment, and they have been shown to have a role in the protection of cells under cold and heat shock, and under oxidative conditions (Kuhlmann et al., 2011, Bownik and Stępniewska, 2016). Therefore, it may be important to determine the role of both ectoine and 5-hydroxyectoine in osmotic-shock response and protection against other types of environmental stress in acidophilic microorganisms.

Ectoine uptake.

Several ectoine-uptake systems that belong to different transporter families have been identified in various microorganisms and they include, for example, ProP, ProU (ProVWX), EctT, Ehu, and OpuA/C transporters (Haardt et al., 1995, Smits et al., 2008, Kuhlmann et al., 2011). Most of these transporters function in the scavenging of ectoine as osmoprotectant or as recycling systems for excreted ectoine. The *proP*-encoded transporter is a single polypeptide embedded in the cytoplasmic membrane and the *proU* operon (*proVWX*) encodes a multicomponent, binding-protein-dependent transport system (Haardt et al., 1995). Both ProP and ProU were originally identified as transporters for the osmoprotective solute proline, but subsequent studies showed that both can also

take up and export glycine betaine, taurine, and ectoine (Haardt et al., 1995). In addition, a high-affinity ectoine ABC transport system (K_D= 0.5±0.2 μM), named Ehu for ectoine/hydroxyectoine uptake, encoded by the *ehuABCD* genes, has been reported to be responsible for ectoine uptake in *Sinorhizobium meliloti* (Jebbar et al., 2005).

The genomic inspection revealed that transport systems for ectoine (and proline, and glycine betaine) are not widely represented among acidophiles, since they were detected only in members of the phylum Nitrospirae where a predicted ProP (65% similarity to ProP from *E. coli*) encoding gene was detected, and in the firmicute *Alb. ferrooxydans* where the ProU system was detected (72%, 69% and 63% identical positions with ProV, ProW and ProX from *E. coli*, respectively). In addition, BLAST analyses showed that the gammaproteobacterium *Ac. prosperus* V6 contains a gene cluster which includes three putative ProU-system genes highly similar to *proVWX* genes of *E. coli* (65%, 70% and 59% identical amino acids, respectively) and the opuA/C gene (59% similarity to OpuA/C from *E. coli*), suggesting these systems may importantly contribute to osmotic tolerance. This microorganism also has genes that encode proteins sharing considerable sequence similarity with proteins of the ABC transport system Ehu for (hydroxy)ectoine uptake. All four predicted Ehu proteins exhibit high similarities to their counterpart in *S. meliloti* (76%, 49%, 69%, and 66% similitude to EhuA, EhuB, EhuC and EhuD, respectively). These results agree with recently reported ectoine transporters in *Ac. prosperus* (Khaleque et al., 2019).

Finally, together these results show that canonical genes for osmotic-stress response are contained within the genomes of acidophilic iron-oxidizing microorganisms, and in case of Nitrospirae and Firmicutes the deduced proteins are highly similar to

proteins found in neutrophilic microorganisms. In addition, according to our predictions, the representatives of Nitrospirae, Firmicutes and Actinobacteria have more systems to synthesize or uptake compatible solutes than the representatives of the other phyla suggesting they are better adapted to tolerate osmolarity upshifts.

5.1.2 Determination of minimal inhibitory concentration (MIC) for NaCl.

To evaluate the tolerance of microorganisms from different phyla present in our laboratory, the minimum inhibitory concentration (MIC) of NaCl for *L. ferriphilum* DSM 14647 (Nitrospirae) and *At. ferrooxidans* ATCC 23270 (Proteobacteria) was determined. As described in Materials and Methods, NaCl MIC determinations were made on planktonic cultures that were kept in mineral media in presence of NaCl concentrations ranging from 0 to 400 mM. Optimal culture media used for microorganisms under study contained ferrous iron concentrations ranging from 20 to 33.3 g/l, a pH range between 1.6 – 1.8, and a growth temperature range of 30ºC – 37ºC. The time for cultivation was between 72 and 86 h.

Non-halophilic microorganisms grow optimally at NaCl concentrations below 200 mM (Buetti-Dinh et al., 2016). As shown in Table 5, MIC values of the bacteria investigated were 150 and 225 mM, considering to *L. ferriphilum* DSM 14647 and *At. ferrooxidans* ATCC 23270 as sensitive to NaCl. However, *L. ferriphilum* turned out to be significantly ($p < 0.001$) more tolerant to NaCl than *At. ferrooxidans*, a fact which is consistent with the low number of genes for osmotic defense detected in this last bacterium and with published observations (Dopson et al., 2017). The MIC values obtained in this study for *L. ferriphilum* DSM 14647 and *At. ferrooxidans* ATCC 23270 are below the

optimal NaCl concentrations reported for the moderate halotolerant *Ac. prosperus* (342 mM) (Dopson et al., 2017).

Table 5. Minimum inhibitory concentration of NaCl in iron-oxidizing bacteria.

Microorganism	NaCl MIC [mM]
L. ferriphilum DSM 14647	225
At. ferrooxidans ATCC 23270	150

In consequence, since the genetic characteristics and the better tolerance to NaCl, *L. ferriphilum* will continue being studied in more detail using 100 mM NaCl (approximately 50% of the MIC value).

5.1.3 Determination of the mRNA level of genes coding for potassium transporters and compatible solutes biosynthesis in *L. ferriphilum* DSM14647 exposed to NaCl.

In order to evaluate the adaptive response predicted by bioinformatic analysis in *L. ferriphilum* DSM 14647, the mRNA levels of key genes associated with K^+-uptake, and synthesis and uptake of compatible solutes were determined upon osmotic stress conditions. Total RNA was isolated from cells exposed to 100 mM NaCl for indicated times, and the mRNA levels of the housekeeping gene *rrsB* were quantified as internal expression control.

K^+-transporters (*kdpABCD*, *trkA* and *yggTS*), (hydroxy)ectoine (*ectABCD*, *proP*) and trehalose (*treXYZ*, *treS* and *otsAB*) biosynthesis genes have been determined as possible osmotic response mechanisms in *Leptospirillum* spp. Thus, to assess the first response, the mRNA level of genes encoding for KdpC, KdpD, TrkA1, TrkA2 and YggT

were quantified in cells treated with NaCl for 0, 5, 20 and 50 min. The real-time PCR data showed that *trkA* and *yggT* genes were, in fact, transcribed in this bacterium exposed to NaCl, but they did not show significant changes in their transcript level between treated and untreated cells (Figure 2.A). However, *kdpC* and *kdpD* genes encoding for the Kdp-transport system were significantly ($p<0.0001$) up-regulated in response to NaCl-induced stress at 5 min of exposure. The increase in the *kdpC* and *kdpD* genes were 25 and 50-fold change at 5 min, respectively. On the contrary, at 20 and 50 min of stress the mRNA levels of *kdpCD* genes were decreased to normal condition. These results suggest that in the strain DSM 14647, potassium is important to balance the osmotic pressure under saline shock with NaCl as a first response, and KdpCD transporter might to play a key role in its uptake from the medium as suggested the *in-silico* analysis for mostly acidophiles.

Accumulation of compatible solutes are part of the late-response mechanism to saline stress in microorganisms (Wood, 2015). To evaluate whether this response is also present in *L. ferriphilum*, the mRNA levels of genes associated to uptake (*proP*) and synthesis (*ectC*, *ectD*, *treZ*, *treS1*, *tres2*, and *otsB*) of these compounds from cells exposed to NaCl for 0 and 80 min were measured. As is shown in Figure 2.B, although the mRNA levels of ProP (hydroxy)ectoine transporter encoding gene tended to decrease, the data analysis showed no significant differences between stressed and non-stressed cells. On the other hand, when mRNA levels of *ectC* and *ectD* genes encoding for ectoine synthase (EctC) and ectoine hydroxylase (EctD), respectively, were measured (Figure 2.B), a significant 4-fold increase was observed, suggesting that ectoine and/or hydroxyectoine may be used by *L. ferriphilum* to keep the osmotic pressure under prolonged saline stress.

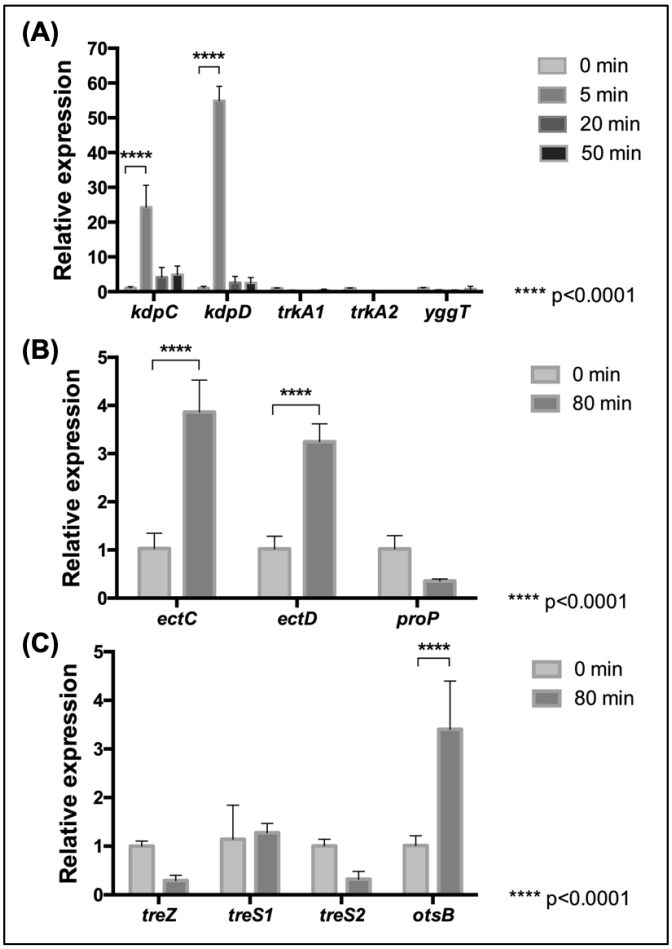

Figure 2. Relative mRNA levels of osmotic stress related genes in *L. ferriphilum* DSM 14647. Relative expression of K^+ transporter genes was evaluated **(A)** in cells treated with 100 mM NaCl for 5, 20 and 50 min. Relative expression of genes for biosynthesis of (hydroxy)ectoine **(B)** and trehalose **(C)** was evaluated in cells treated with 100 mM NaCl for 0 and 80 min. Data were normalized by the 16S rRNA gene (*rrsB*). Data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA and T Test.

In addition, in *L. ferriphilum* the genes for three described trehalose synthesis routes were predicted, thus the mRNA levels of genes code for key enzymes from each pathway were evaluated in cells exposed to 100 mM NaCl for 0 and 80 min. The studied

genes encode for malto-olygosil trehalose trehalohydrolase (*treZ*), trehalose synthase (*treS1*, *treS2*) and trehalose-6-phosphate phosphatase (*otsB*). The results showed that *treZ* and *treS* genes are expressed in this bacterium under tested experimental conditions (Figure 2.C), tough significant differences were not detected between cells from treated and untreated conditions. Interestingly, as is shown in Figure 2.C the *otsB* gene resulted to be significantly upregulated at 80 min of stress exposure, as compared to the control, suggesting that this bacterium synthesizes trehalose by the Ots pathway under stress conditions studied here. Altogether, these results suggest that *L. ferriphilum* develops a cell-safety mechanism when is exposed to long-term osmotic stress, leading the biosynthesis of (hydroxy)ectoine and trehalose.

5.1.4 Determination of the intracellular levels of ectoine, hydroxyectoine and trehalose in *L. ferriphilum* DSM14647 exposed to NaCl.

Since (hydroxy)ectoine and trehalose synthesis genes were up-regulated in *L. ferriphilum* exposed to NaCl, the intracellular concentration of the compatible solutes ectoine, hydroxyectoine and trehalose were monitored. To quantify the compatible solutes, the cells were cultivated in medium 882 in the presence of 100 mM NaCl for 90 min. After that, the cells were harvested, the compatible solutes were extracted and then analyzed by HPLC (ectoine, hydroxyectoine) or GC-MS (trehalose) as described in Materials and Methods. As is shown in the Figure 3.A, the ectoine was not detected, however the molecule hydroxyectoine was detected in both treated and untreated cells. Under control conditions, the intracellular content of hydroxyectoine was 20 nmol/mg wet biomass. Interestingly, when the cells were exposed to NaCl, the intracellular content of

this compatible solute significantly increased to 100 nmol/mg wet biomass. On the other side, the intracellular trehalose content also increased under salinity conditions (100 mM NaCl) reaching a concentration of 0.47 nmol/mg wet biomass, 2.3-fold the content of this molecule in control condition (Figure 3.B). These results showed that the concentrations of hydroxyectoine and trehalose are clearly salt dependent, and the hydroxyectoine is the major compatible solute for *L. ferriphilum*, while trehalose would be the minor component.

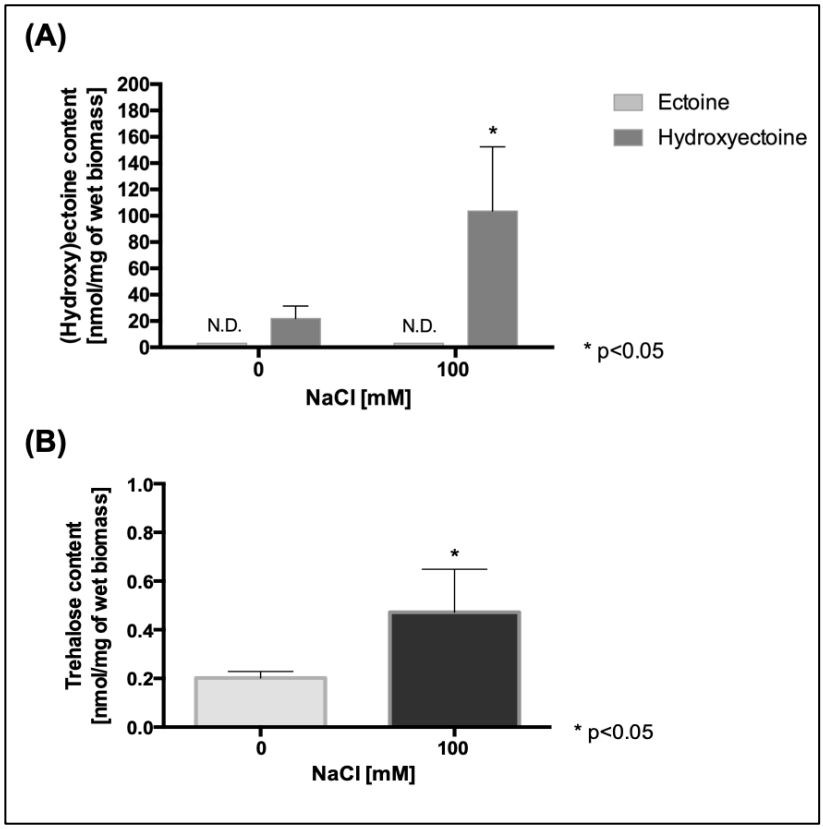

Figure 3. Effect of NaCl exposure on intracellular content of compatible solutes in *L. ferriphilum* DSM 14647. Content of ectoine and hydroxyectoine **(A)**, and trehalose **(B)** in cells exposed to 100 mM NaCl for 90 min. The data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA and T Test. N.D.: not detected.

5.2 Evaluation of the participation of the antioxidant response in the protection against NaCl.

While acidophilic bacteria can grow in acidogenic environment, they are able to keep the intracellular pH close to neutrality. The pH-homeostasis in these microorganisms is mainly kept by the respiratory rate, because the electron transport chain exports and consumes protons during its activity. However, one of the most severe effects of chloride exposition described in acidophiles is the cytoplasm acidification. Thus, to bring back the intracellular pH close to neutrality, acidophiles could increase the activity of the respiratory chain and subsequently oxygen consumption. Consequently, the increase in the respiratory rate might favor the production of reactive oxygen species (ROS). In order to evaluate whether chloride ion favors ROS production by the increase in the respiratory rate as a consequence of the cytoplasm acidification in *L. ferriphilum* DSM 14647, the intracellular pH, oxygen consumption, the intracellular total ROS production and the activity of antioxidant proteins were measured upon saline stress.

5.2.1 Measurement of the intracellular pH in *L. ferriphilum* DSM 14647 exposed to NaCl.

It has been described that the presence of anions in the culture medium of the acidophiles *At. ferrooxidans* and *At. thiooxidans* reduces their iron oxidation activity and their intracellular pH (pH_{in}) (Alexander et al., 1987; Suzuki et al., 1999). Thus, it raised the question whether there is a similar effect when *L. ferriphilum* is exposed to high chloride conditions. According to the Figure 4, these cells preserve an intracellular pH of 6.7 under standard culture conditions (pH_{medium}=1.8). However, when the cells were

exposed to 100 and 150 mM NaCl, intracellular pH significantly decreased close to 6.0 ($p<0.01$) and 5.5 ($p<0.001$), respectively (Figure 4.A). In addition, as it is shown in Figure 4.B, the exposure of the cells to 100 mM NaCl for different times showed that at 60 and 90 min, the pH_{in} significantly decreased until approximately 6.0 ($p<0.05$) and 5.7 ($p<0.01$), respectively. These results suggest the chloride ion induces similar effect in *L. ferriphilum* than other acidophiles, acidifying the intracellular pH and thus likely impairing cellular functioning.

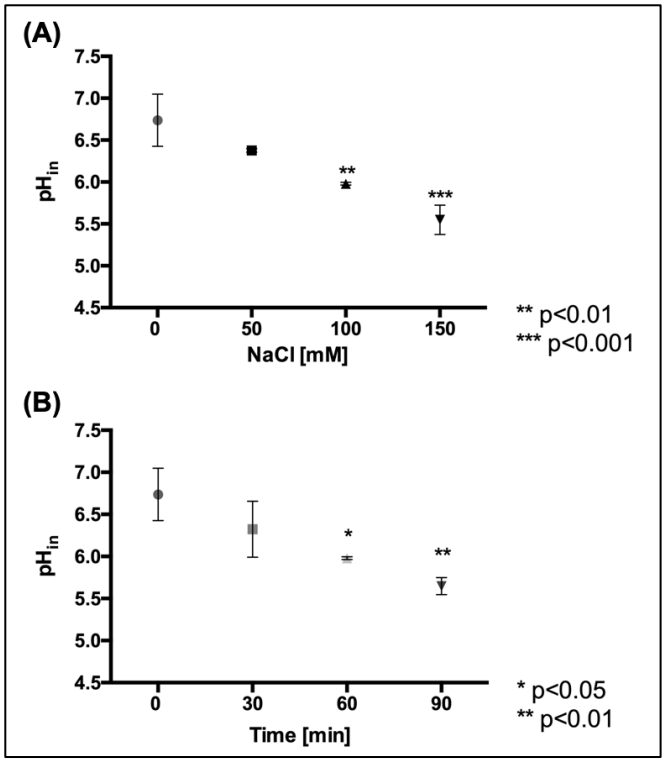

Figure 4. Effect of NaCl exposure on intracellular pH (pH_{in}) in *L. ferriphilum* DSM 14647. Effect of NaCl concentration on pH_{in} of the cells exposed for for 60 min to the stress condition **(A).** Effect of the time exposure in cells exposed to 100 mM NaCl **(B)**. The data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA Test.

5.2.2 Measurement of the oxygen consumption in *L. ferriphilum* DSM 14647 exposed to NaCl.

To evaluate the effect of NaCl on the respiratory rate, the oxygen consumption rate was estimated in cells exposed to a range of 0-150 mM NaCl for 30 min. As it is shown in Figure 5, the values of oxygen consumption proved to be significantly higher (from $1.2x10^{-9}$ to $2x10^{-9}$ ppm O_2 $s^{-1}cell^{-1}$, $p<0.001$) in cells exposed to range of 1 – 50 mM NaCl, as compared to the control condition. Interestingly, at 150 mM NaCl only extremely low respiration rate was observed, indicating that this might be a concentration which *L. ferriphilum* cannot cope with.

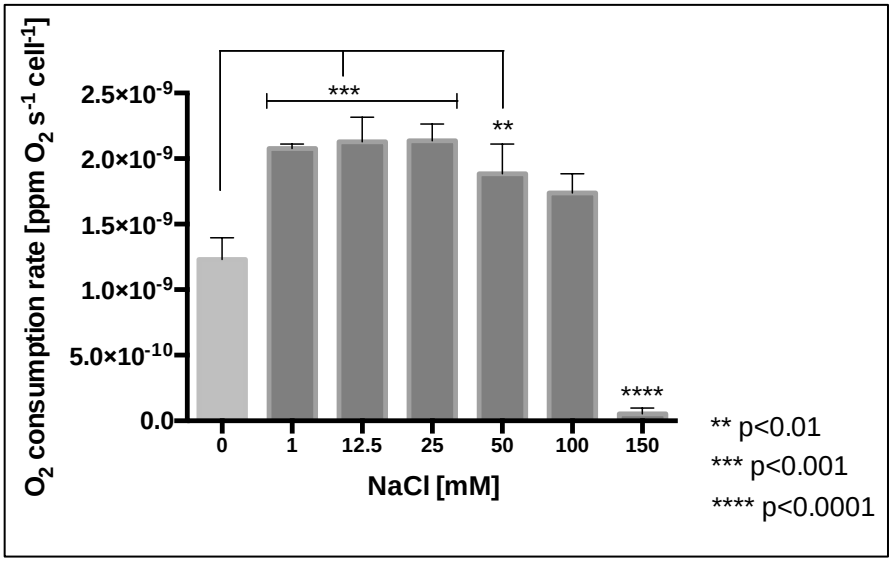

Figure 5. Effect of NaCl concentration on oxygen consumption rate in *L. ferriphilum*. The data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA Test.

5.2.3 Determination of total ROS levels in *L. ferriphilum* DSM 14647 exposed to NaCl.

To determine whether the increase in the respiratory rate, resulting from chloride stress, can induce an increase of reactive oxygen species (ROS); the intracellular content of ROS was measured in cells treated with NaCl and compared to the content in non-stressed control cells. As shown in Figure 6.A, with mere presence of 50 mM NaCl for 60 min in *L. ferriphilum*, the intracellular content of ROS started to increase compared with its control.

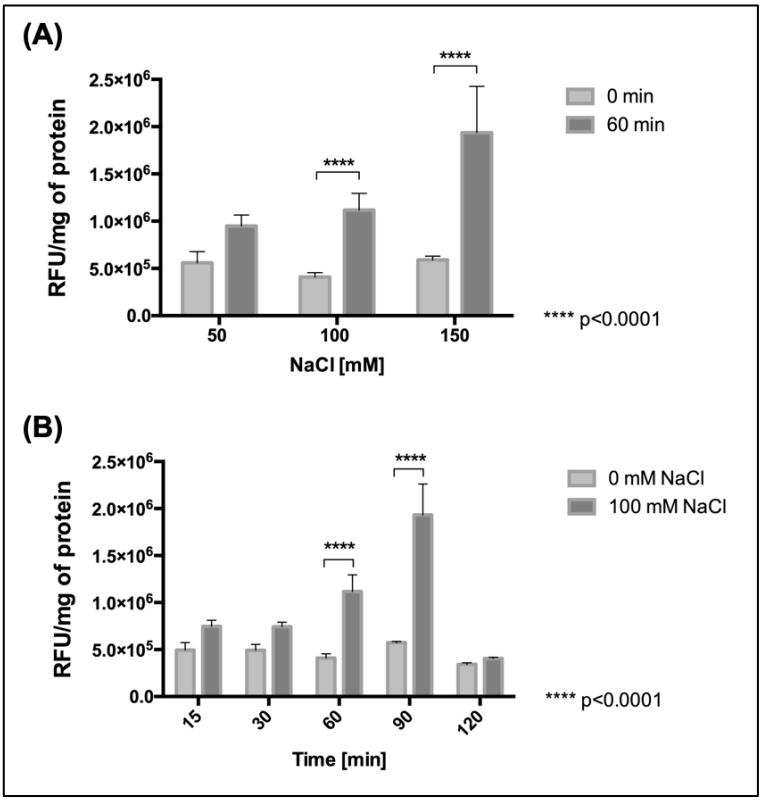

Figure 6. ROS generation in *L. ferriphilum*. Effect of NaCl concentration **(A)** and time exposure **(B)** on intracellular ROS generation in the bacteria. Concentration effect was evaluated in cells exposed to NaCl for 60 min. The effect of time exposure was evaluated in cells exposed to 100 mM NaCl. Cytoplasmic ROS content was expressed as relative fluorescence units (RFU) of the activated fluorescent probe H_2DCFDA per mg of protein.

Cells exposed to 100 and 150 mM NaCl for 60 min showed significantly increased total ROS content (to 274% and 328%, respectively) as compared to control cells (100%). Additionally, Figure 6.B shows that cells exposed to 100 mM NaCl for 90 min also had a significantly increased total ROS content of 336% compared with control cells. Altogether these results suggest that the presence of NaCl in the culture medium produces a severe oxidative condition in cells from *L. ferriphilum* DSM 14647.

5.2.4 Measurement of antioxidant protein activities CcP and Trx of *L. ferriphilum* DSM 14647 exposed to NaCl.

As previously described, *Leptospirillum* sp. activates peroxidase and thioredoxin-based thiol/disulfide system to face oxidative stress (Norambuena et al., 2012; Contreras et al., 2015; Zapata et al., 2017). In order to determine whether NaCl-induced ROS was accompanied by an increase in activity of antioxidant proteins, the activities of cytochrome *c* peroxidase (CcP) and of thioredoxin (Trx) were measured in whole cell extracts derived from cells exposed to 100 mM NaCl for 0, 30, 60 and 90 min. As it is shown in Figure 7.A, at 30 min, 60 and 90 min of exposure to NaCl, the cytochrome *c* peroxidase activity resulted to be significantly higher (4-fold) compared to the control ($p<0.01$). The exposure of the cells to chloride stress also lead to an increase (3.5-fold, $p<0.001$) in thioredoxin activity at 30 and 90 min. However, this activity appeared declined at 60 min (1.5-fold), respect to the control condition (Figure 7.B). It is likely part of the natural redox cycle of thioredoxins that occurs during the antioxidant response and which, in turn, is dependent on the activity of thioredoxin reductase (Norambuena et al., 2012). These results clearly indicate that peroxidase and thioredoxin systems are activated

as part of the antioxidant response of *L. ferriphilum* DSM 14647 to fight against severe oxidative conditions induced by chloride exposure.

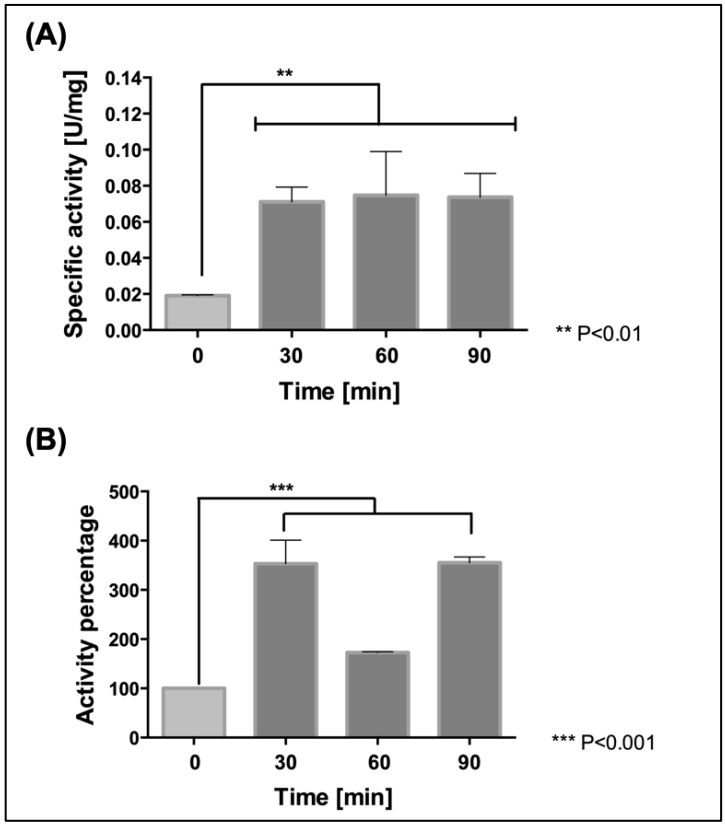

Figure 7. Antioxidant protein activity in *L. ferriphilum*. The cytochrome *c* peroxidase **(A)** and thioredoxin activity **(B)** in cells exposed to 100 mM NaCl. The data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA Test.

5.2.5 Effect of the external antioxidant (cobalamin) and compatible solute (hydroxyectoine) supplementation in the antioxidant response of *L. ferriphilum* DSM 14647 exposed to NaCl.

To determine the relation between chloride stress and oxidative stress in acidophilic microorganisms, the effect of the addition of the antioxidant cobalamin and

the compatible solute hydroxyectoine were evaluated on the ROS levels and antioxidant protein activities of *L. ferriphilum* exposed to NaCl 100 mM.

Evaluation of the effect of cobalamin and hydroxyectoine on the intracellular ROS levels.

Since the exogenous addition of cobalamin (vitamin B12) reduces the levels of intracellular ROS and the damage to biomolecules in *Leptospirillum* strain CF-1 (Ferrer et al., 2016b) and since the compatible solute hydroxyectoine protects biomolecules from high osmolarity conditions and maintains their native functions (Widderich et al., 2014), to get additional insights into response to NaCl, here the effect of the external supplementation of cobalamin and hydroxyectoine in the culture medium of *L. ferriphilum* on total ROS generation was studied. To evaluate the effect of cobalamin, the culture was pre-treated with 5 nM of this vitamin for 60 min and, after that, treated with 100 mM NaCl for 90 min. As expected, NaCl exposure led to a significant increase in total ROS generation (254%), compared with cells grown under control condition (100%). Interestingly, pre-treatment with cobalamin significantly reduced the ROS level from 254% (in NaCl-stressed cells) to 137% (in NaCl-stressed and cobalamine pre-treated cells), a level that is comparable to those observed under control condition without stress (100%) (Figure 8.A). On the other hand, to evaluate the effect of hydroxyectoine, the culture exposed to 100 mM NaCl for 90 min was treated with 0.5 mM of this compound. The result showed that NaCl-stressed cell significantly increased total ROS level (240%), however, the stressed cells treated with hydroxyectoine had a decreased in the ROS production (155%, $p<0.05$) respect to those stressed cells, but not treated with this compatible solute (Figure 8.B). These results suggest that a relation between chloride

stress and the generation of oxidative stress by NaCl may exist in acidophilic microorganisms, since the stress may partially be alleviated with an antioxidant or a compatible solute.

Evaluation of the effect of cobalamin and hydroxyectoine on the antioxidant protein activities.

Additionally, the effect of cobalamin and hydroxyectoine on the activity of antioxidant proteins was also evaluated. Interestingly, the pre-treatment with 5 nM cobalamin led to a significant additional increase of both CcP (613%) and Trx (527%) activity in cells exposed to NaCl compared to activity measured in salt exposed cells, but not cobalamin-pretreated (CcP: 317% and Trx: 362%) (Figure 8.C). On the contrary, the treatment with 0.5 mM hydroxiectoine led to a decreased the activity of CcP (160%) and Trx (218%) in cells exposed to NaCl stress compared to stressed cells that were not treated with this compatible solute (CcP: 317% and Trx: 362%) (Figure 8.D). Thus, although cobalamin and hydroxyectoine can significantly reduce the intracellular ROS level under chloride stress, according to these results they would exert their antioxidant effect by mechanisms operating at different level of the ROS control machinery.

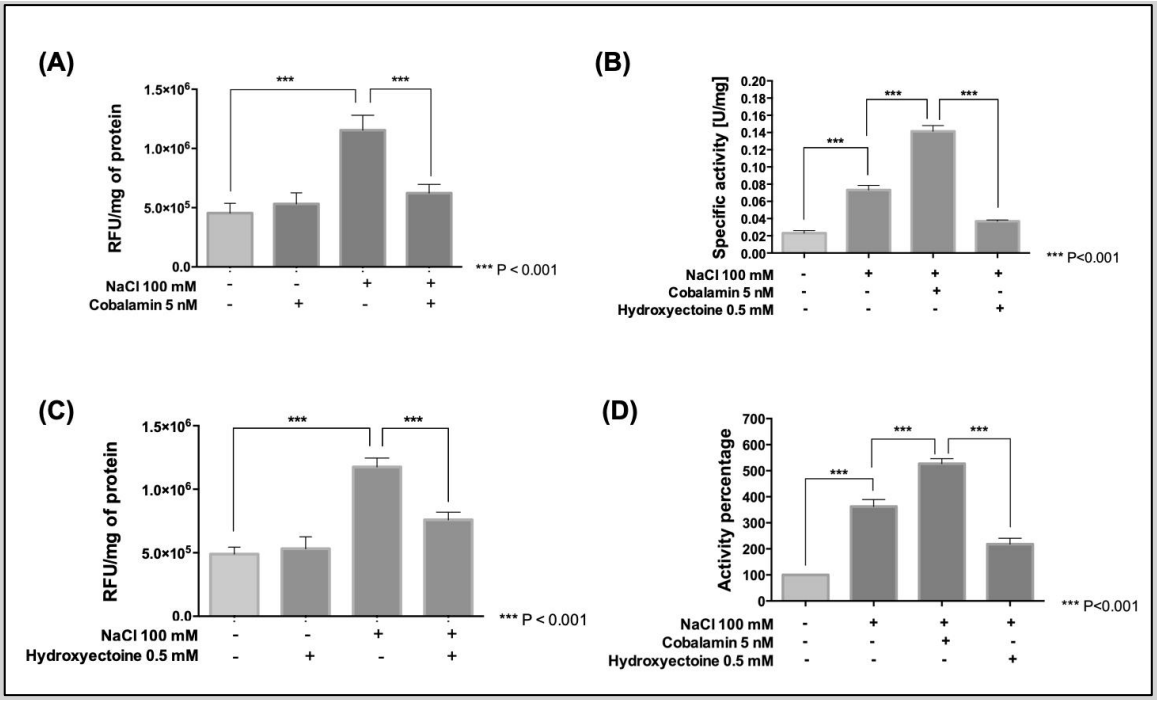

Figure 8. Effect of cobalamin and hydroxyectoine on ROS generation and antioxidant protein activities. Effect of antioxidant cobalamin **(A)** and osmoprotectant hydroxyectoine **(B)** on ROS generation when *L. ferriphilum* was exposed to 100 mM NaCl for 90 min. Effect of cobalamin and hydroxyectoine on cytochrome *c* peroxidase (CcP) activity **(C)** and thioredoxin (Trx) activity **(D)** in the bacteria exposed to 100 mM NaCl for 90 min. The data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA Test.

5.2.6 Determination of expression of antioxidant protein encode genes in cells exposed to NaCl.

Since chloride-mediated stress results in increased activity of cytochrome *c* peroxidase and thioredoxin proteins of *L. ferriphilum* DSM 14647, the transcriptional response of this bacterium under NaCl exposure was assessed. The mRNA level profiles of *ccp*, *trx1*, *trx4* and *trx6* encoding for CcP (Zapata et al., 2017), and Trxs (Norambuena

et al., 2012), respectively, were analyzed. Expression level of each gene was quantified at 0, 20, 50 or 80 min after exposure to the chloride stress condition using RT-qPCR assays. As is shown in Figure 9, the mRNA level of *ccp* gene was up-regulated at all the assayed times, but it resulted be significantly higher at 20 and 80 min ($p<0.05$ and $p<0.0001$, respectively). The expression profile of *ccp* gene is in agreement with detected high activity of CcP enzyme described above (Figure 7.A).

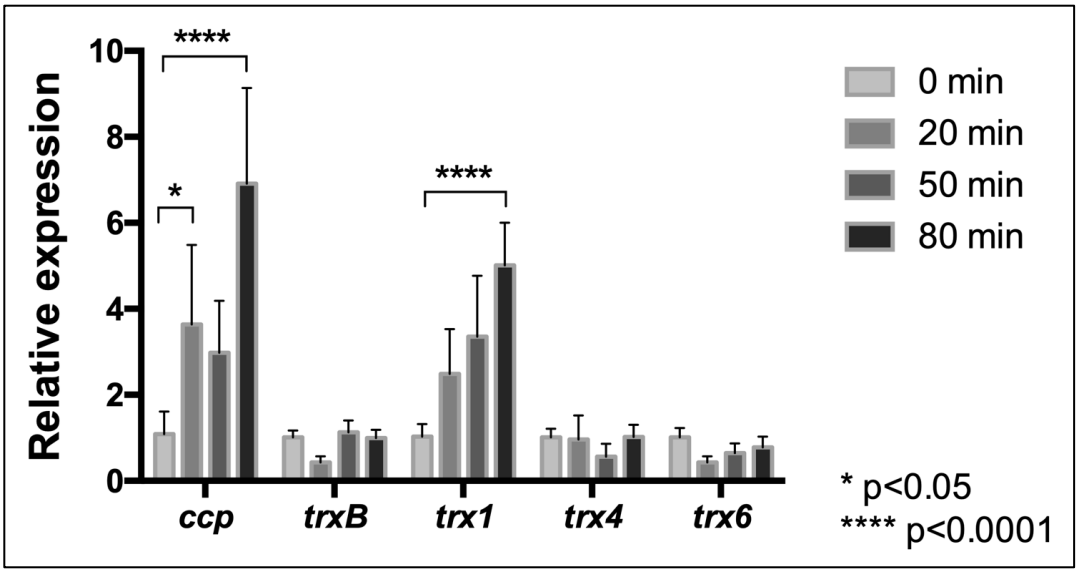

Figure 9. Relative mRNA levels of oxidative stress related genes in *L. ferriphilum* DSM 14647. Relative expression of cytochrome *c* peroxidase (*ccp*) and thioredoxin (*trx*) encoding genes was evaluated in cells treated with 100 mM NaCl. Data were normalized by the 16S rRNA gene. Data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA Test.

Concerning to thioredoxin encoding genes, the obtained results (Figure 9) showed that although the mRNA levels from *trx1*, *trx4* and *trx6* genes were detected in cells exposed to 100 mM NaCl at all exposure times, but only the mRNA level of *trx1* significantly increased (5-fold) in response of chloride stress respect to the control

conditions ($p<0.0001$). It is interesting to note that detected increase of *trx1* gene was in agreement with detected increase of Trx activity in cells exposed to NaCl (Figure 7.B). Altogether, the expression profile of the *ccp* and *trx1* gene suggests that oxidative stress induced by NaCl triggers a transcriptional response that contribute to increase activity of antioxidant proteins as a strategy to endure the oxidative condition.

5.3 Identification of the transcriptomic profile of *Leptospirillum ferriphilum* DSM 14647 adapted to NaCl.

As a way to characterize the NaCl tolerance mechanism of *L. ferriphilum* DSM 14647 in the late phase, this strain was adapted to 180 mM (80% of the MIC value determined in section 5.1.2) in order to favor the activation of the mechanisms involved. Subsequently, the identification of the mechanism through transcriptomic profile, respiratory rate, total ROS level determination and (hydroxy)ectoine measurement was performed.

5.3.1 Adaptation of *L. ferriphilum* DSM 14647 to 180 mM NaCl, supplementing the medium with compatible solutes.

To study the late response mechanism of *L. ferriphilum* against saline conditions, the cells were adapted to NaCl. The adaptation of the strain was carried out sequentially in the presence of compatible solutes ectoine and trehalose for 24 months and reached up to 180 mM NaCl (80% of the MIC value).

As a preliminary result of the adaptation, the effect of compatible solutes on the growth of *L. ferriphilum* cultured in presence of 100 mM NaCl was determined using a

modified Neubauer chamber. As shown in Figure 10, the microorganisms in presence of NaCl showed significantly decreased cell growth (*L. ferriphilum*: 22.9%) at 48 h as compared to control cells (100%). Interestingly, a significant increase of cell density was observed in cultures of *L. ferriphilum* supplemented with 0.5 mM ectoine. As mentioned above this bacterium is predicted to be a (5-hydroxy)ectoine and trehalose producing bacterium that is also capable to import ectoine, but not trehalose, from the environment. Consistent with these predictions, ectoine partially restored the growth of *L. ferriphilum* exposed to 100 mM of NaCl (69.6% at 48 h), while trehalose did not exert any growth-recovery effect. Consequently, the supplementation with 0.5 mM ectoine favored the growth of this bacterium until 180 mM NaCl. When the growth of *L. ferriphilum* was stable under this NaCl concentration, the ectoine started to be sequentially removed from the medium till the supplementation was not necessary.

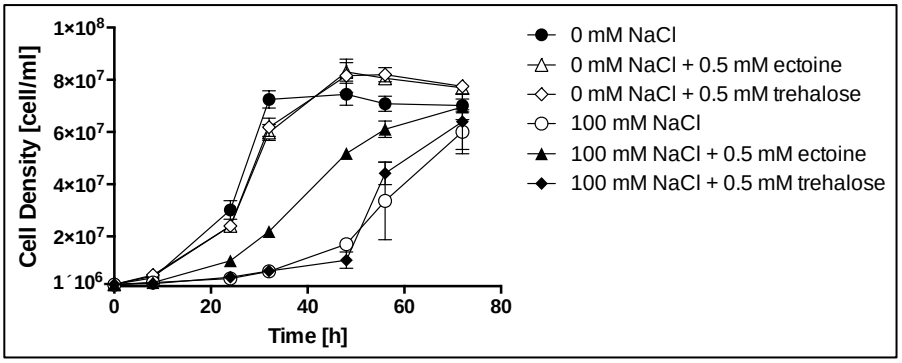

Figure 10. Effect of NaCl on growth of *L. ferriphilum* DSM 14647 adapted to 100 mM NaCl in presence of ectoine or trehalose. Data represent the average of 3 independent experiments (bar indicates the value range). Initial cell density $1x10^6$ cells/ml.

5.3.2 Growth curve of *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

After the cells were able to grow in a medium with 180 mM NaCl, the growth curve was plotted following the procedure described in the section 4.3.2 of Material and Methods. It should be noted that at this concentration the cell culture grew steadily and without ectoine supplementated. The adaptation to 180 mM allowed the cells to obtain the same cell density as the non-adapted cells (aprox. $8x10^7$ cell/ml), but in 96 hrs (Figure 11). This condition tripled the time of cellular duplication (T_d) from 6 to 17 h.

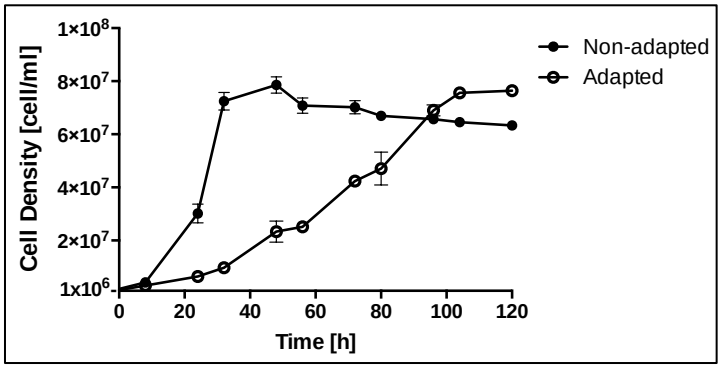

Figure 11. Growth curve of *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl. Data represent the average of 3 independent experiments (bar indicates the value range). Initial cell density $1x10^6$ cells/ml.

5.3.3 Determination of minimal inhibitory concentration (MIC) for NaCl of *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

As it has been described among this manuscript, the pH is an important parameter for acidophiles when they are exposed to saline conditions, thus, it raises the question whether the adapted cells could be better prepared to deal with the chloride. For this reason, the minimal inhibitory parameters of *L. ferriphilum* adapted to 180 mM NaCl and cultured at different external pH (pH_{ex}) values was compared with its counterpart non-adapted cells. The table 6 shows that the adaptation of *L. ferriphilum* have allowed to

increase the MIC values at all pH_{ex} conditions. Additionally, the toxic effect of chloride was directly dependent on the pH_{ex}, because when the external pH is more acidic the NaCl is more toxic for the cells, as it was expected for the largest pH gradient trough the cellular membrane (Alexander et al., 1987; Suzuki et al., 1999; Zammit and Watkin, 2016).

Table 6. Minimum inhibitory concentration of NaCl in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl at different external pH (pH_{ex}).

pH_{ex}	NaCl MIC [mM]	
	***L. f.* non-adapted**	***L. f.* adapted to 180 mM NaCl**
1.4	175	350
1.8	225	375
2.4	350	425
3.0	400	500

5.3.4 Evaluation of the transcriptomic profile of *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl by RNAseq.

The adaptation of *L. ferriphilum* to 180 mM NaCl was planed to study the late response mechanism to saline stress in low-tolerant acidophiles, therefore to characterize its mechanism the transcriptomic profile was evaluated.

In this study, after filtering the reads to eliminate adaptors and sequences of low quality (Q20 score, with an error probability of 0.01), a total of 1,392,138 and 878,046 clean reads of high quality were generated from cDNA libraries for non-adapted and adapted cells, respectively. These clean reads were then aligned against the reference

genome data (physically re-annotated by RAST, functionally annotated using the database Gene Ontology (GO) and conformed by 2,736 genes), using the CLC Genomics Workbench software. The differential expression analysis was performed comparing the 180 mM NaCl adapted *L. ferriphilum* respect to the control condition (non-adapted strain), using the statistic R software and the package DESeq2 as it was mentioned in Materials and methods. From this analysis, 99 genes were found with differential expression, of which 69 and 30 were significantly ($p<0.05$) up-regulated and down-regulated, respectively.

The Table 7 shows the up-regulated genes (excluding 43 ORFs predicted as hypothetical proteins, check in Table S2) in a range of 4.1 to 91.7-fold change, comparing the adapted respect to non-adapted cells. Due the function was given for every gene, the genes were clustered in 5 groups to favor the study of the late response mechanism to NaCl stress: "Metabolism and energy conservation" (3 genes), "Osmoregulation and membrane-associated proteins" (5 genes), "Stress response" (8 genes), "Signal transduction" (4 genes) and "Others" (6 genes). From the results in Table 7 there are two genes up-regulated into "Metabolism and energy conservation" and belonging to the electron transport chain, cytochrome c oxidase (4.9 ± 2.2-fold) and sulfide-quinone reductase (4.6 ± 2.1-fold). This likely reflected the need to exclude protons from the cytoplasm to regulate pH and provides reducing power for the stress response. Another known response to osmotic stress is the production of proteins involved in the maintenance of the cell membrane integrity, in consequence, the gene encode for the cell membrane integrity Tol-Pal system (TolC protein, 7.4 ± 2.3-fold), the regulator gene of membrane proteins integrity with protease activity (HflC protein, 6.6 ± 2.3-fold) and the

gene supervisor of the membrane fluidity (fatty acid desaturase, 4.9 ± 2.2-fold) were up-regulated in the adapted to 180 mM NaCl strain. Interestingly, considering the production of oxidative stress under high salt condition, the "Stress response" resulted in the up-regulation of genes encoding for antioxidant proteins flavohemoprotein (14.3 ± 2.5-fold), cytochrome *c* peroxidase (10.9 ± 2.4-fold), FAD-dependent pyridine nucleotide-disulphide oxidoreductase (5.6 ± 2.2-fold), radical s-adenosyl-methionine (SAM, 5.2 ± 2.1-fold), and mercuric reductase *merA* gene (4.3 ± 2.1-fold) from *mer* operon. In addition, related with "Signal transduction" 3 genes coding for diguanylate cyclase phosphodiesterase (5.2 ± 2.1 to 11.2 ± 2.4-fold) and 1 gene for DSF synthase (10.7 ± 2.4-fold) were over-expressed, suggesting the necessity of a cell communication to coordinate a physiological response.

On the other hand, the Table 8 shows the down-regulated genes (excluding 8 ORFs predicted as hypothetical proteins) in a range of -4.31 to -9.3 – fold change, comparing the adapted respect to non-adapted cells. These genes are represented in 3 groups associated to the late response mechanism to NaCl stress: "Metabolism and energy conservation" (5 genes), "Osmoregulation and membrane-associated proteins" (15 genes) and "Stress response" (2 genes). Metabolic and energy conservation genes involved in the gluconeogenesis with lower expression in adapted cells included, glycosyl transferase (-5.3 ± 2.3-fold), UDP-glucose dehydrogenase (-6.4 ± 2.2-fold) and UTP-glucose-1-phosphate uridylyltransferase (-9 ± 2.3-fold), indicating that the energy production was likely utilized for cellular growth rather than as a response to osmotic stress. An interesting gene repressed in high salt condition was glutamine-fructose-6-phosphate aminotransferase (-6 ± 2.3-fold), involved in the biosynthesis of glutamate, suggesting the

unnecessary production of contra ions for K^+ related with osmoadaptation. In the same line, the results in Table 8 shows that genes related with "Osmoregulation, cell wall and membrane-associated proteins" were over-represented, indicating that should exist a higher control at level of membrane under saline conditions. Mostly genes are related with biosynthesis of membrane, including the repression of genes encode for undecaprenyl-phosphate galactose phosphotransferase (-4.8 ± 2.2-fold), capsular polysaccharide biosynthesis protein I (-5 ± 2.2-fold), putative membrane-fusion protein (-5.4 ± 2.1-fold), phospholipid-lipopolysaccharide ABC transporter (-5.9 ± 2.3), glycosyltransferase involved in cell wall biosynthesis (-6 ± 2.3-fold) and eight transmembrane protein EpsH (-9 ± 2.4-fold). It is very important notice, that 3 genes involved in the biosynthesis pathway of (hydroxy)ectoine were repressed, Diaminobutyrate-2-oxoglutarate transaminase (*ectB*, -6.4 ± 2.2-fold change), L-ectoine synthase (*ectC*, -5.6 ± 2.2-fold change) and ectoine hydroxylase (*ectD*, -8 ± 2.3-fold change). This result was unexpected since the hydroxyectoine has an important role protecting the cells against saline stress, therefore, the analysis of hydroxyectoine content of adapted cells could be an interesting aim that should be addressed.

Table 7. Up-regulated genes in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

Code[a]	Gene product	Fold change[b]	SE[c]
Metabolism and energy conservation			
fig_6666666.437888.peg.2314	Carbonic anhydrase	8.1	2.3
fig_6666666.437888.peg.1060	Cytochrome c oxidase subunit CcoN	4.9	2.2
fig_6666666.437888.peg.1718	Sulfide-quinone reductase	4.6	2.1
Osmoregulation and membrane-associated proteins			
fig_6666666.437888.peg.1048	TolC family protein	7.4	2.3
fig_6666666.437888.peg.745	Regulator of protease activity HflC	6.6	2.3
fig_6666666.437888.peg.1610	Methyl-accepting chemotaxis protein	5.4	2.2
fig_6666666.437888.peg.480	Methyl-accepting chemotaxis protein	5.1	2.2
fig_6666666.437888.peg.1785	Fatty acid desaturase	4.9	2.2
Stress response			
fig_6666666.437888.peg.700	Flavohemoprotein	14.3	2.5
fig_6666666.437888.peg.1049	Cytochrome *c* peroxidase	10.9	2.4
fig_6666666.437888.peg.2031	Heat-shock protein Hsp20	8.1	2.2
fig_6666666.437888.peg.693	Transcriptional Regulator IscR	7.2	2.2
fig_6666666.437888.peg.2315	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	5.6	2.2
fig_6666666.437888.peg.500	Radical SAM domain protein	5.2	2.1
fig_6666666.437888.peg.1084	Periplasmic serine protease DO (HtrA-1)	4.9	2.2
fig_6666666.437888.peg.1862	Mercuric reductase	4.3	2.1
Signal transduction			
fig_6666666.437888.peg.753	Diguanylate cyclase/phosphodiesterase	11.2	2.4
fig_6666666.437888.peg.1805	DSF synthase (RpfF)	10.7	2.4
fig_6666666.437888.peg.1893	Diguanylate cyclase/phosphodiesterase	6.6	2.2
fig_6666666.437888.peg.1778	Diguanylate cyclase/phosphodiesterase	5.2	2.1
Others			
fig_6666666.437888.peg.1453	Transposase	7.7	2.3
fig_6666666.437888.peg.1965	Phage related integrase	5.4	2.2
fig_6666666.437888.peg.2126	Flagellin protein FlaB	4.4	2.0
fig_6666666.437888.peg.2237	DNA-binding protein HU	4.3	2.0
fig_6666666.437888.peg.2659	Prokaryotic ubiquitin-like protein Pup	4.2	2.1
fig_6666666.437888.peg.1177	Shufflon-specific DNA recombinase	4.2	2.1

[a] Code refers to the identified gene within the re-annotated genome of *L. ferriphilum* DSM 14647 by RAST.
[b] Average fold up-regulation of the comparison between non-adapted and adapted *L. ferriphilum* DSM 14647 transcriptomic profile.
[c] Standard error of the mean of the average fold up-regulation for the comparisons between treatments.

Table 8. Down-regulated genes in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

Code[a]	Gene product	Fold change[b]	SE[c]
Metabolism and energy conservation			
fig_6666666.437888.peg.1846	glycosyl transferase, group 1 family protein	-5.3	2.3
fig_6666666.437888.peg.1886	Glutamine--fructose-6-phosphate aminotransferase	-5.7	2.2
fig_6666666.437888.peg.1830	glycosyltransferase family 2 protein	-6.0	2.3
fig_6666666.437888.peg.1887	UDP-glucose dehydrogenase	-6.4	2.2
fig_6666666.437888.peg.1834	UTP--glucose-1-phosphate uridylyltransferase	-9.0	2.3
Osmoregulation, cell wall and membrane-associated proteins			
fig_6666666.437888.peg.1852	Undecaprenyl-phosphate galactose phosphotransferase	-4.8	2.2
fig_6666666.437888.peg.1851	Polysaccharide export protein	-4.9	2.2
fig_6666666.437888.peg.1874	Capsular polysaccharide biosynthesis protein I	-5.0	2.2
fig_6666666.437888.peg.2214	Outer membrane efflux protein	-5.1	2.1
fig_6666666.437888.peg.2215	Putative membrane-fusion protein	-5.4	2.1
fig_6666666.437888.peg.2670	L-ectoine synthase (EctC)	-5.6	2.2
fig_6666666.437888.peg.1822	Phospholipid-lipopolysaccharide ABC transporter	-5.9	2.3
fig_6666666.437888.peg.1829	Glycosyltransferase involved in cell wall bisynthesis	-6.0	2.3
fig_6666666.437888.peg.2219	RND family efflux transporter MFP subunit	-6.2	2.2
fig_6666666.437888.peg.2671	Diaminobutyrate-2-oxoglutarate transaminase (EctB)	-6.4	2.2
fig_6666666.437888.peg.2218	Cobalt-zinc-cadmium resistance protein CzcA	-6.5	2.2
fig_6666666.437888.peg.1853	Tyrosine-protein kinase EpsD	-6.8	2.2
fig_6666666.437888.peg.2669	Ectoine hydroxylase (EctD)	-8.0	2.3
fig_6666666.437888.peg.1848	Polysaccharide deacetylase	-8.4	2.6
fig_6666666.437888.peg.1833	Eight transmembrane protein EpsH	-9.3	2.4
Stress response			
fig_6666666.437888.peg.2216	Two component sigma54 specific transcriptional regulator Fis family	-4.3	2.1
fig_6666666.437888.peg.1832	Sigma-54 dependent transcriptional regulator	-10.2	2.4

[a] Code refers to the identified gene within the re-annotated genome of *L. ferriphilum* DSM 14647 by RAST.
[b] Average fold down-regulation of the comparison between non-adapted and adapted *L. ferriphilum* DSM 14647 transcriptomic profile.
[c] Standard error of the mean of the average fold down-regulation for the comparisons between treatments.

5.3.5 Measurement of the oxygen consumption in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

To evaluate whether the cellular respiration was determinant in the adaptation of *L. ferriphilum*, as it was suggested in the results of the transcriptomic profile from section 5.3.4. The oxygen consumption of non-adapted and adapted cells of *L. ferriphilum* exposed to 180 mM NaCl was measured. The Figure 12 shows that *L. ferriphilum* non-adapted and exposed to 180 mM was not available to respire under this salty conditions. Interestingly, the adapted culture and treated with 180 mM NaCl significantly ($p<0.01$) increased the O_2 consumption rate from $1.2x10^{-9}$ to $2.2x10^{-9}$ ppm O_2 $s^{-1}cell^{-1}$, compared with the non-adapted and untreated cells. This agrees with the up-regulation of the iron oxidation genes encoding for cytochrome c oxidase and sulfide-quinone reductase (Table 7), observed in the 180 mM NaCl adapted *L. ferriphilum* respect to non-adapted cells.

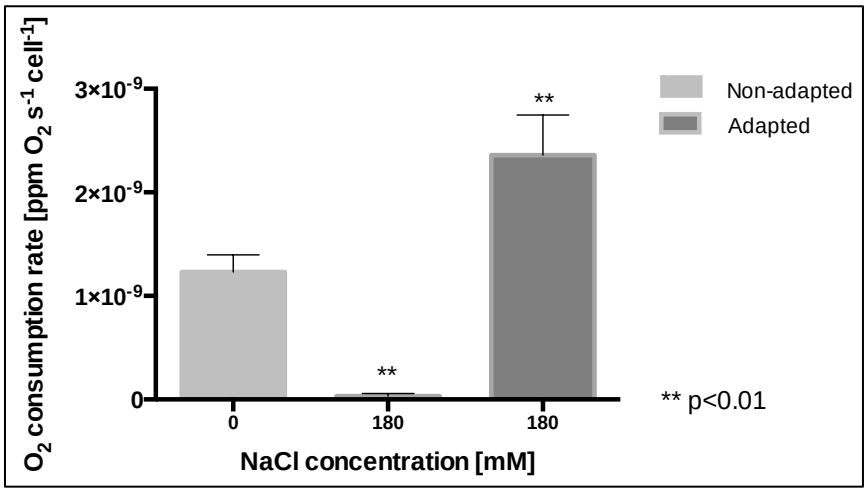

Figure 12. Oxygen consumption rate in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl. The data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA Test.

5.3.6 Determination of total ROS levels in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

As it is proposed in this work, it should be expected that the oxidative response should be involved in the adaptation of *L. ferriphilum*, since the transcriptomic profile revealed an up-regulation of the cytochrome *c* peroxidase (CcP) gene (10.9-fold change, Table 7). Thus, the intracellular ROS levels was measured in non-adapted and adapted cells cultured in 882 medium supplemented with 180 mM NaCl. The Figure 13 shows that non-adapted cells and exposed to 180 mM NaCl significantly increased the intracellular ROS levels ($p<0.01$), compared with the control condition (non-adapted and untreated). Interestingly, *L. ferriphilum* adapted and treated with 180 mM NaCl showed similar intracellular ROS levels than the control condition, which agrees with the transcriptomic profile.

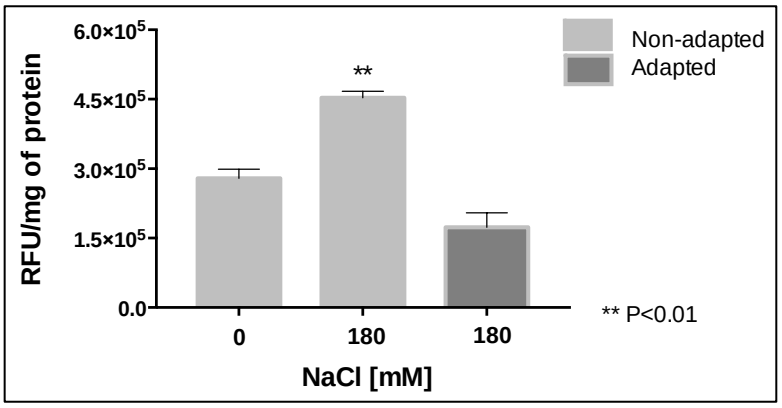

Figure 13. Effect of NaCl on ROS generation in *L. ferriphilum* adapted to 180 mM NaCl. Cytoplasmic ROS content was expressed as relative fluorescence units (RFU) of the activated fluorescent probe H_2DCFDA per mg of protein.

5.3.7 Determination of intracellular (hydroxy)ectoine content in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl, by HPLC.

Since the transcriptomic profile showed that the genes diaminobutyrate-2-oxoglutarate transaminase (*ectB*), L-ectoine synthase (*ectC*) and ectoine hydroxylase (*ectD*) were significantly ($p<0.05$) down regulated, the intracellular content of ectoine and hydroxyectoine was measured in non-adapted and adapted cells. As it is shown in the Figure 14, the ectoine was not detected in any of both adapted and non-adapted cells. On the other hand, the concentration of hydroxyectoine in non-adapted cells cultured without NaCl was 20 nmol/mg of wet biomass (Figure 14, $p<0.01$). However, in agreement with the results of the transcriptomic analysis, the hydroxyectoine was not detected in the extract from *L. ferriphilum* adapted to 180 mM NaCl.

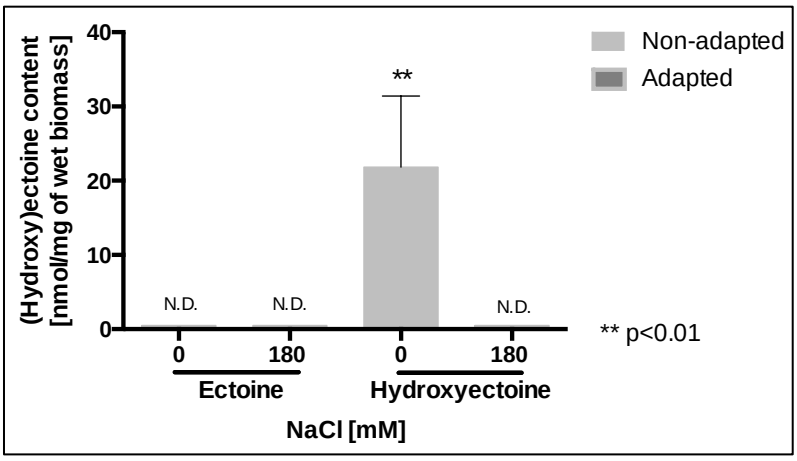

Figure 14. Intracellular content of compatible solutes in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl. The data represent the average of 3 independent experiments (bar indicates the value range). Statistical analysis was carried out by ANOVA and T Test. N.D.: not detected.

6. DISCUSSION

In this section the main results obtained during this thesis will be discussed, starting with the first response mechanism of acidophilic iron-oxidizing bacteria and archaea upon saline stress, including a better characterization at genetic and metabolite level of osmotic tolerance in *L. ferriphilum* exposed to NaCl. Additionally, the characterization of the oxidative stress production as a consequence of the NaCl exposure in *L. ferriphilum* will be analyzed in detail, where the cytoplasmic acidification, increase in the respiratory rate, ROS production and activation of the antioxidant proteins will be discussed and compared with the current literature. Finally, the late response mechanism against saline stress, based on a transcriptomic profile from an adapted to 180 mM NaCl *L. ferriphilum*, will be discussed.

6.1 Evaluation of the participation of canonical systems of tolerance to osmotic stress in cells exposed to NaCl.

The genomic analysis performed on the twelve genomes show that canonical genes for osmotic-stress response are contained within the genomes of acidophilic iron-oxidizing microorganisms (Figure 15). The well-represented set of the high-affinity K^+ transport systems Kdp and Ygg suggests that K^+ uptake may represent a general strategy to face high levels of external soluble anions in environments where extremely low pH and low concentrations of K^+ are usually present (Dutrizac 2008, Ito et al., 2009). However, since in acidophiles an internal positive membrane potential formed by K^+ ions has been suggested as a strategy to maintain pH homeostasis (Cox et al., 1979, Buetti-Din

et al., 2016), the wide distribution of potassium transport systems could also be indicative of a generalized role of this ion in the regulation of intracellular pH. On the other hand, the fact that the Kdp and YggT transport systems seem to be absent in acidophilic archaeal models suggests that there are various possibilities regarding the evolution of acidophile genomes to handle osmotic balance. Finally, it is interesting to remark that findings in this work suggest that acidophilic bacteria seem to be by far better equipped with K^+ uptake systems than archaea. Whether different or non-canonical systems are present in acidophilic archaea should be addressed; since it has been described that archaea synthesize unusual solutes such as ß-aminoacids, N-acetyl-ß-lysine, mannosylglycerate and di-*myo*-inositol phosphate (Müller et al., 2005).

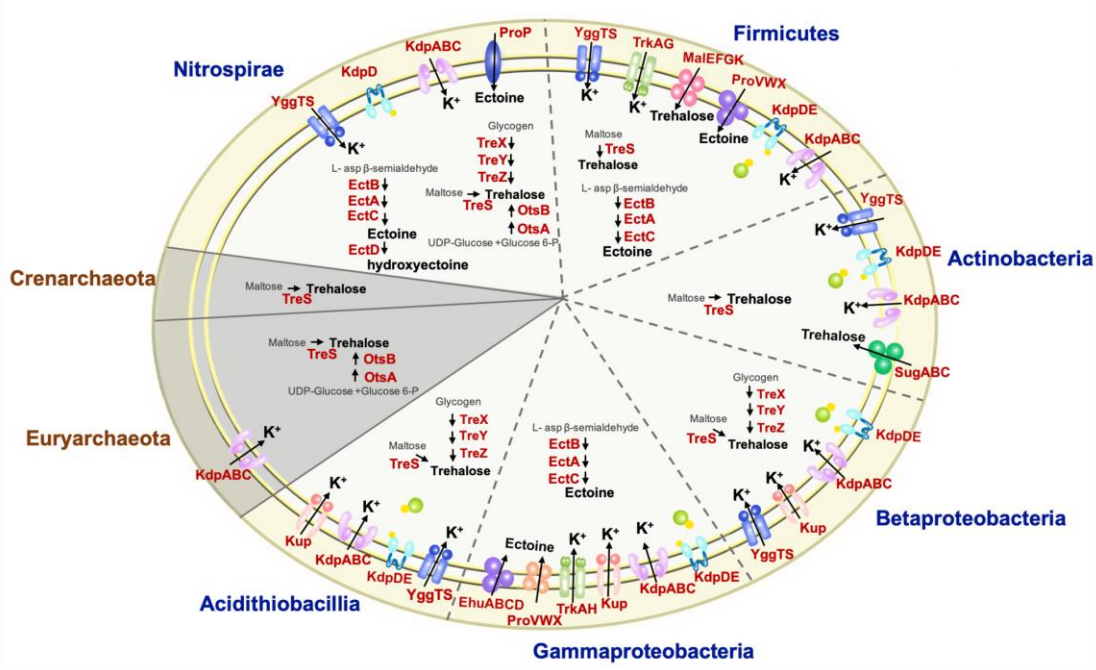

Figure 15. Schematic representation of predicted genes for NaCl tolerance. The prediction of genetic products was derived from 12 genomic sequences of aerobic acidophilic iron-oxidizing microorganisms.

The results also support a general role of the compatible solute trehalose, highlighting the members of the Nitrospirae phylum due they are the only ones that have genes for all three described routes for trehalose biosynthesis, which agrees with observations recently reported for *L. ferriphilum* Sp-Cl (Issota et al., 2016, Galleguillos et al., 2018). In contrast, (5-hydroxy)ectoine seems to be restricted to acidophiles from the phyla Nitrospirae and Firmicutes, and the proteobacterium *At. prosperus*. Although the use of uptake systems for trehalose and ectoine appeared to be present in some acidophilic representatives, the uptake of compatible solutes from the medium seems not to be an extended strategy in these microorganisms due to the reduced presence among different phyla. The moderately halotolerant strains *L. ferriphilum* Sp-Cl and *Ac. prosperus* V6 possess most of the known genes for osmotic-stress response with a similar genetic dosage to that detected in sensitive strains of acidophiles, thus suggesting that they have built up a response also based on additional systems. Interestingly, although genes for the ectoine uptake were bioinformatically detected in members of the genus *Leptospirillum* (*proP*) and in *Alb. ferrooxydans* (*proVWX*), *Ac. prosperus* was the only one that showed the possession of *ehuABCD* genes, suggesting these could to be highly relevant genes for the tolerance described in this bacterium.

Experimental determinations of tolerance to NaCl in *L. ferriphilum* DSM 14647 (MIC of 225 mM) and *At. ferrooxidans* ATCC 23270 (MIC of 150 mM), and the optimal NaCl concentrations reported for the moderate halotolerant *L. ferriphilum* Sp-Cl (205 mM) and *Ac. prosperus* (342 mM) (Issota et al., 2016; Khaleque et al., 2018), suggest that acidophiles differ in their sensitivity. Tolerances seem to fit well to the number of mechanisms predicted to exist by the bioinformatics approach. In this way, the

microorganisms predicted as being better equipped with osmotic tolerance mechanisms *L. ferriphilum* and *Ac. prosperus* seem to be, in fact, more tolerant to NaCl than *At. ferrooxidans*. However, it is remarkable that although *Leptospirillum* and *Acidihalobacter* strains have a plethora of canonical genes for proteins related to the maintenance of osmotic balance, their tolerance levels determined here remain below those reported for non-halophilic neutrophilic bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Cebrián et al., 2015). As mentioned before, it is a relatively known fact that external pH contributes importantly to the toxicity of anions for acidophilic bacteria (Zammit and Watkin, 2016), and certainly it may contribute to the observed sensitivity of *Leptospirillum* sp. and of other acidophiles to NaCl.

When studying the osmoprotection mechanism, the results showed that the exposition of *L. ferriphilum* to NaCl produced osmotic stress, as judged by transcription of genes related with canonical osmoregulation. Such that, according to the characteristics of a first response against saline stress described for neutrophilic and halophilic microorganisms (Kempf and Bremer, 1998; Csonka, 1989; Krämer, 2010; Wood, 2015; Kindzierski et al., 2017), the mRNA level of two genes associated with the regulated K^+ uptake system Kdp exhibited a significant increase immediately after exposure to NaCl. Additionally, in a longer-term response, the expression of *kdp* genes was again reduced and instead the transcription level of genes associated with de novo biosynthesis of (hydroxy)ectoine and trehalose showed a significant increase, suggesting the up-regulation of the EctABCD and OtsAB pathways. In agreement with these facts, after exposure to NaCl hydroxyectoine and trehalose exhibited significantly enhanced intracellular content. Interestingly, hydroxyectoine is preferentially produced over ectoine

and trehalose in *L. ferriphilum* DSM 14647. In agreement with these results, an early meta-transcriptomic analysis performed in acidic water from Rio Tinto (Spain), dominated by *Leptospirillum ferrooxidans*, showed that the gene clusters *ectABCD*, *otsAB* and *kdpABCD* were up-regulated in a place with high presence of NaCl (Parro et al., 2007). Additionally, previous proteomic studies showed that in AMD biofilms with *Leptospirillum* group II bacteria, there was a high production of proteins necessary for ectoine and hydroxyectoine biosynthesis (Goltsman et al., 2009; Belnap et al., 2010; Mosier et al., 2013). Moreover, Mosier et al. (2013) detected the presence of only hydroxyectoine and not ectoine in the AMD biofilm by stable isotope labeling coupled with HPLC. For other organisms, early studies have suggested that the hydroxylation of ectoine has always been linked to extreme conditions, as this may be a survival strategy (García-Estepa et al., 2006; Van-Thuoc et al., 2013; Tao et al., 2016), which is consistent with the findings in this study. Despite their closely related chemical structures, 5-hydroxyectoine often possesses a superior protective effect than its precursor ectoine (Van-Thuoc et al., 2013). Therefore, hydroxyectoine may be the main osmolyte against osmotic stress under the extreme conditions encountered in acidic saline environments.

6.2 Evaluation of the participation of the antioxidant response in the protection against NaCl.

Acidophilic sulfur- and iron-oxidizing bacteria have been described as very sensitive to the presence of anions, in particular chloride. Until now, the reasons of this have been ascribed mainly to two aspects: the osmotic imbalance and the acidification of the cytoplasm (Zammit and Watkin, 2016). Here, it proposes that as a consequence of a

decrease in the intracellular pH and in order to restore the pH homeostasis, the cells respond by increasing the respiratory rate which could favor generation of ROS.

Measurements of intracellular pH (pH_{in}) of *L. ferriphilum* DSM 14647 performed in this work, showed a significant decrease of pH_{in} upon exposure to NaCl and the effect was even a function of the time of exposure and of salt concentration. These results agree with a previous report for *At. ferrooxidans,* where exposure of this bacterium to 100 mM NaCl for 1 hour reduced the cytoplasmic pH to close to 5.1 (Alexander et al., 1987). The acidification of the cytoplasm in presence of NaCl is predicted to be a consequence of a massive chloride entry which would be favored by the positive membrane potential. A drop in the membrane potential due to the movement of anions will favor the entry of protons and concomitant acidification (Alexander et al., 1987; Zammit and Watkin, 2016). In acidophiles, the effect of chloride is exacerbated by the high external proton concentration in the medium and the large pH gradient over the cellular membrane. Thus, it is possible to predict that the toxic effect of chloride is inversely dependent on the external pH. This relationship was, in fact, described for *Acidithiobacillus thiooxidans*, where permeability to chloride and its toxicity were shown to increase as the external pH decreases (Suzuki et al., 1999). A similar effect was also detected in *L. ferriphilum* DSM 14647, since the change of pH of the media from 3.0 to 1.8 had a dramatic effect on minimal inhibitory concentration (2.3 folds lower), and therefore, on the tolerance of this bacterium (Table 6). Finally, the effect of lowering the intracellular pH suggests that during chloride stress the cell must also face a situation of acidic stress and therefore, the mechanisms that facilitate pH homeostasis in acidophiles could play an important role in salt tolerance. Thus, although some studies have approached this topic (Mangold et al.,

2013), more efforts should be made to understand the mechanisms that allow tight control and maintenance of intracellular pH (around 6.5) in microorganisms that inhabit extremely acidic environments (pH < 3).

As predicted, exposure of *L. ferriphilum* to chloride stress significantly increases the respiratory rate, as deduced from oxygen consumption values. In addition, cells showed a significant increase in the intracellular ROS level, suggesting that a generalized oxidative condition has been elicited by chloride exposure. The detected increase in ROS content is due to the increase in the rate of oxygen consumption and the predicted increase in the activity of the electron transport chain between Fe (II) and O_2. However, the cell is also under a strong condition of osmotic and pH stresses that could damage macromolecules, favoring the release of metal ions from metalloproteins abundant in these microorganisms (Yarzábal et al., 2002, Potrykus et al., 2011, Levicán et al., 2012). The relationship between osmotic and pH stresses in the induction of redox stress has previously been reported for neutrophilic microorganisms (Wilks et al., 2009, Mols et al., 2010).

In agreement with the accumulation of intracellular ROS, real time RT-qPCR and protein activity assays showed that the antioxidant protein activities CcP and Trx were elevated upon saline exposure. The increase in mRNA levels of the corresponding *ccp* and *trx* genes suggests that, at least in part, this activation is supported at the transcriptional level. In general terms, these results are consistent with increased oxygen consumption reported for *Acidithiobacillus thiooxidans* upon exposure to the chloride salts KCl, NaCl and LiCl exposure (Suzuki et al., 1999). In the same line, a proteomic analysis performed in *Acidimicrobium ferrooxidans* under saline stress with 100 mM NaCl revealed the up-

regulation of a peroxidase encoding gene (Zammit et al., 2012). Furthermore, a proteomic analysis carried out in *Acidithiobacillus caldus* exposed to 500 mM NaCl showed up-regulation of a gene for a thiol-peroxidase (Guo et al., 2014). Additionally, a proteomic study showed that *Ac. prosperus* over-expressed rubrerythrin and Dyp-type peroxidases upon saline stress induction (Dopson et al., 2017). Thus, these findings suggest that in acidophilic microorganisms, saline stress triggers a cellular antioxidant response that mainly involves the activation of proteins for the scavenging of inorganic peroxide, thereby avoiding Fenton chemistry and the generation of highly deleterious hydroxyl radical (Ferrer et al., 2016c). According to this results, *L. ferriphilum* DSM 14647 displays a similar response that involves activation of cytochrome *c* peroxidase activity. However, the system in charge of the thiol/disulfide balance also seems to have a role in the maintenance of redox homeostasis in this bacterium under NaCl stress.

The external supplementation of cobalamin in *Leptospirillum* group II strain CF-1 has shown a reduction in the levels of intracellular ROS, suggesting the uptaking of this antioxidant by the bacterium (Ferrer et al., 2016b). In addition, the presence of the ProP transporter in *L. ferriphilum* DSM14647, determined by transcriptomic analysis, provides evidence to the incorporation of the compatible solute hydroxyectoine in this strain. Consequently, an interesting finding of this work, was that the external supplementation of cobalamin or hydroxyectoine attenuates chloride-induced ROS accumulation. These compounds do, however, have a totally opposite effect on the activity of antioxidant proteins of strain DSM 14647. The external addition of cobalamin to the cell culture clearly increased the activity of the antioxidant proteins CcP and Trx, while the addition of hydroxyectoine did not exert any significant effect on the activity of these proteins. As

previously described (Ferrer et al., 2016b), the effect of cobalamin involves the activation of antioxidant proteins that will subsequently exert an effect by reducing the level of reactive oxygen species. For its part, hydroxyectoine exerts a protective effect on biomolecules in response to osmotic stress. It can be predicted that during chloride-induced stress, the uptake and accumulation of this compatible solute attenuated the release of metals from metalloproteins and so also the generation of ROS in the cytoplasm. In addition, at potential effect of hydroxyectoine to reduce ROS through other mechanisms, like a direct antioxidant activity, cannot be excluded (Sajjad et al., 2018). Altogether, these data give us a wider view about how operating mechanisms supporting different adaptive responses can contribute to regulate cytoplasmic ROS concentration. At the same time, these results pointed out the fact that chloride exposure can induce different stresses to the cell, and the way in which the cell responds to all of them as a whole will be a determining factor of NaCl tolerance in acidophilic microorganisms.

Finally, these results allow to better understand the multifactorial effect of chloride-stress in acidophilic bacteria, and therefore decipher the molecular basis of the extreme sensitivity of these microorganisms to saline shock with chloride and other anions. Based on the obtained results it proposes a model for chloride-shock effects on *Leptospirillum* DSM 14647 (Figure 16**)**.

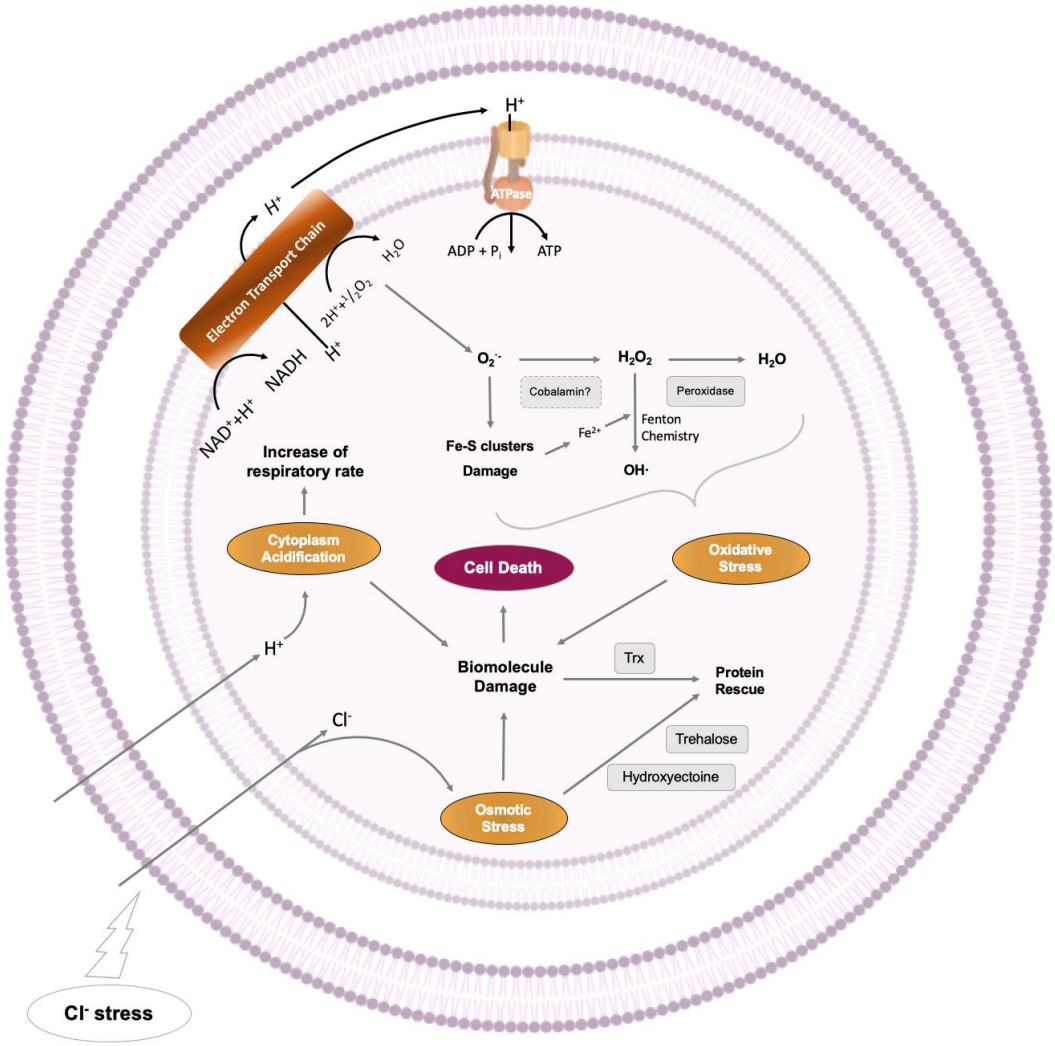

Figure 16. Multifaceted effect of NaCl in *L. ferriphilum* DSM 14647.

6.3 Identification of the transcriptomic profile of *Leptospirillum ferriphilum* DSM 14647 adapted to NaCl.

Non-acidophilic halotolerant microorganism can grow under saline conditions, but they are not restricted to environments of high salt concentration, in consequence, these microorganisms may encounter transient changes in NaCl concentration outside their

optimum growth conditions (Zammit and Watkins, 2016). This property is sustained primarily through the amassing of physiologically compliant organic ions (K^+) to counteract the negative effects of high external osmolality on cellular hydration. However, as a sustained stress response these bacteria prefer the accumulation of compatible solutes, allowing the reduction of the ionic strength of the cytoplasm through export of K^+ (Bremer and Krämer, 2019). For acidophilic microorganisms, despite these two response mechanisms have been identified and their function under saline conditions has been approached, the way that they behave according with the permanence under stress conditions has not been characterized yet. Additionally, as it has been evaluated and discussed in this work, acidophiles should deal with more than osmotic stress under chloride exposure. For this reason, the adaptation of the bacterium *L. ferriphilum* DSM 14647 was crucial to decipher the response mechanism of NaCl-susceptible acidophiles against steady presence of salt.

The adaptation of *L. ferriphilum* to NaCl was favored by the supplementation of ectoine, which agreed with the presence of the ProP transporter detected by the bioinformatic analysis and its expression studied here by RT-qPCR. The external concentration of 180 mM NaCl obtained on adapted *L. ferriphilum* DSM 14647 was close to the optimal concentration of 205 mM NaCl reported for the moderate halotolerant *L. ferriphilum* Sp-Cl (Issota et al., 2016), suggesting that to increase the tolerance to anions is not required new genetic information. After the adaptation process was finished, the growth was stable but longer than the non-adapted cells (3-times higher), probably for the requirements that implies growing under these conditions. A similar effect upon iron oxidation and growth rate has been observed in different studies of susceptible acidophilic

microorganisms, including also *L. ferriphilum* and other examples like *At. ferrooxidans* and *S. thermosulfidooxidans* (Dopson et al., 2017, Dieu et al., 2019). As it is mentioned above, the external pH contributes importantly to the toxicity of anions in acidophilic bacteria (Zammit and Watkin, 2016), hence the evaluation of the NaCl tolerance by measurement of the MIC confirmed that when the pH decreases the chloride ion is more harmful for both non-adapted and adapted cells. Moreover, the adaptation conferred more tolerance to *L. ferriphilum* against NaCl, since their MIC value increased in all pH conditions respect to non-adapted cells. This result suggests that the response mechanism addressed toward the osmotic stress is closely related with acidic stress in these bacteria.

The highest number of differentially expressed genes in the transcriptomic profile were related with metabolism and energy conservation, osmoregulation, cell wall and membrane-associated proteins, stress response and signal transduction. This likely reflected the need to adjust the increased energy required to maintain pH and osmotic balance, cell envelope to maintain cellular integrity, and finally, the status redox (reviewed in Slonczeski et al., 2009; Ferrer et al., 2016b; Zammit and Watkin, 2016). Among the results obtained with up-regulation under high salt condition, the genes encode for proteins belonged to the electron transport chain were over-expressed, suggesting an increase in the respiratory rate of this bacterium to provide energy and reducing power to deal with the stress, and mainly for the necessity of extrude protons. Fortunately, in this work was possible evaluate the respiratory rate in the adapted culture and support that under salty conditions there is an increase in the respiration. The same effect was observed also in the proteomic study of *Ac. prosperus* where a high concentration of proteins cytochrome c_1, rusticyanin and ATP synthase subunit b were over-expressed in the

presence of 500 mM NaCl (Dopson et al., 2017), indicating that the proton extrusion by respiration may be a general anions response mechanism in acidophiles. Consistent with the upper oxygen consumption, some of the genes over-expressed and related with stress response were linked to oxidative stress, including cytochrome *c* peroxidase, flavohemoprotein (a NO detox) and a protein with domain SAM (precursor for the biosynthesis of the antioxidant cobalamin). The development of this mechanism was supported by the measurement of intracellular ROS level in *L. ferriphilum* adapted, which was keep it around normal concentrations. Proteomic profiles have detected high levels of antioxidant proteins as a part of the osmotic stress response in several studies with acidophilic microorganisms (Zammit et al., 2012; Guo et al., 2014; Dopson et al., 2017). These findings agree with the proposal that an osmotic stress triggers antioxidant response in acidophilic microorganisms for scavenging of ROS. In the same line, in this work was observed an increase in the expression of the transcriptional regulator IscR, potentially involved in the biosynthesis of proteins with [Fe-S] cluster (Py and Barras, 2010). The [Fe-S] cluster are susceptible of being oxidized by anion superoxide, releasing Fe^{3+}, thereby it can trigger Fenton chemistry and the generation of highly harmful hydroxyl radical (Imlay, 2006; Ferrer et al., 2016b). Therefore, this results sustain that upon high salt conditions proteins with [Fe-S] cluster have been oxidized and, in consequence, the cell is compensating the loss trough the regulation of the biosynthesis pathway and modulating the antioxidant response. Interestingly, the transcription of the heavy metal resistance gene, *merA*, that encodes a mercuric reductase was over-expressed under an adaptation to NaCl. The mercury-resistance (*mer*) genes are activated and repressed by the MerR metalloregulatory protein, which have shown high degree of selectivity to

mercury ($HgCl_2$), but can additionally be partially stimulated by a variety of transition metals such as Cd^{2+}, Zn^{2+}, Ag^{+}, Au^{+}, and Au^{3+} (Ralston and O'Halloran, 1990). In the metal-tolerant bacteria *Cupriavidus metalliduran*s the expression of the *merA*, *mer*T and *mer*P genes were upregulated when this bacterium was exposed to a condition of stress by cadmium (Rojas, 2011; Alviz-Gazitua et al., 2019). A similar phenomenon has been described in *Nitrosomonas europaea*, since the *mer* operon was also induced by cadmium (Park and Ely, 2008). This background suggests that the chloride stress in *L. ferriphilum* can cause oxidation of metalloproteins, releasing oxidized metals and probably activating the mercuric reductase (MerA) to reduce them in order to avoid intracellular oxidative damage. Such is the case of ferric iron (Fe^{3+}) that could be released from [Fe-S] cluster- or heme-containing proteins. Whether MerA is being used by the *L. ferriphilum* as a metal tolerance mechanism remains to be tested.

Another known response to osmotic and acidic stress is the modification of the cell membrane, that was importantly controlled since genes coding for proteins of the Tol-Pal system and the regulator of protease activity (HflC) were over-expressed. The high expression of these two genes suggests a quality control for the cell membrane integrity. Notwithstanding the above, a gene coding for the fatty acid desaturase was also increased in high salt condition and its protein product catalyze the reaction that produce double bond C-Cs allowing the membrane to become more fluid (Aguilar and Mendoza, 2006). This result contrasts with the acidic tolerance mechanisms described in acidophilic bacteria and archaea, which involves the modification to a rigid and impermeable cytoplasmic membrane that limit the influx of protons into the cytoplasm (Jones et al., 2012; Mangold et al., 2013; Zammit and Watkin, 2016). Similarly, 6 genes involved in

the biosynthesis and glycosilation of the cell wall had a higher concentration in low salt conditions, potentially contradictory to the increasing number of sugar moieties, ciclopentane rings, presence of glycolipids and hopanoid lipids in acidophilic archaea and bacteria (Chong et al. 2010; Jones et al., 2012). Suggesting that probably there is no osmotic stress condition in adapted culture. In the same line, the canonical osmoregulation mechanism detected by the transcriptomic profile showed a gene involved in the biosynthesis of glutamate and it was repressed in the NaCl adapted cells, suggesting that the K^+ uptake was not necessary to control the osmotic pressure in the late phase response. On the other hand, three of four genes involved in the (5-hydroxy)ectoine biosynthesis pathway were also down-expressed in the adapted strain. Consistent with this result the evaluation of the ectoine and hydroxyectoine intracellular content demonstrated there was no presence of these compatible solutes under high salt conditions. Concordantly, in the proteomic analysis of the moderated halotolerant *Ac. prosperus* grown under 500 mM NaCl was not possible detect proteins involved in the biosynthesis of ectoine, even when this bacterium has been reported as a producer of this osmoprotectant (Dopson et al., 2017; Khaleque et al., 2019). Other studies performed in bacterial consortia with *At. ferrooxidans, At. caldus, Am. ferrooxidans* and *Sb. thermosulfidooxidans* have shown similar results, where the osmoregulation was not wholly observed in proteomics studies potentially due to the primary effect of chloride on pH homeostasis rather than osmotic stress (Zammit et al., 2012; Guo et al., 2014).

Other mechanism well represented in the transcriptomic analysis was the signal transduction. The gene coding for the DSF synthase (RpfF) is being over-expressed in adapted culture. Diffusible Signal Factor (DSF) is synthesized by RpfF, which has 3-

hydroxyacyl-acyl dehydratase activity in unsaturated fatty acids. When DSF reaches a threshold concentration outside the cell, the bacteria activate their cognate receptor RpfC, a hybrid membrane sensor kinase that phosphorylates the intracellular response regulator RpfG. This regulator then converts the intercellular signal into an intracellular signal through its c-di-GMP phosphodiesterase (PDE) activity, which in turn alters the expression of target genes (He et al., 2006a; He et al., 2006b; Ionescu et al., 2013). In agreement with this finding, the over-expression of the diguanylate cyclase phosphodiesterase proteins suggests a very controlled synthesis and degradation of bis-(3´-5´)-cyclic dimeric guanosine monophosphate (c-di-GMP). This unusual cyclic nucleotide has been widely described as an inductor of cell-to-cell communication, photosynthesis, cell morphology, exopolysaccharide production biofilm formation and stimulation of motility. In addition, this molecule is emerging as an important bacterial second messenger that appear to alter the expression of genes involved in protein composition of membranes, virulence, resistance to bacteriophages and heavy metal resistance (Römling et al., 2005). Therefore, given the conditions worked here, it is possible to infer that in this case DSFs could be forming part of the cell-cell communication to modulate the expression of genes, including tolerance to metal ions, by c-di-GMP.

Finally, taking together these results it is possible approach the late response mechanism of *L. ferriphilum*, and potentially mostly acidophiles, implicated in the adaptation to high concentration of chloride, considering: the cell membrane adaptations to maintain the pH homeostasis and osmotic pressure, the respiration to produce energy and export protons, and the antioxidant response to avoid the oxidative stress developed.

7. CONCLUSIONS

The exposition of *L. ferriphilum* DSM 14647 to salt shock induces canonical systems of tolerance to osmotic stress, responding immediately with K^+ uptake by the Kdp transporter and, later, by the biosynthesis pathways of hydroxyectoine (EctABCD) and trehalose (OtsAB).

Additionally, the exposition of *L. ferriphilum* to salt shock triggers the cytoplasm acidification, increase of the cell respiration, ROS production and, in consequence, the antioxidant response by antioxidant proteins CcP and Trx.

The adaptation of *L. ferriphilum* to 180 mM NaCl develops a late response mechanism that involve cell wall and membrane integrity, increase in the cell respiration to extrude protons, and the antioxidant response to keep the homeostasis redox.

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SUPLEMENTARY MATERIAL

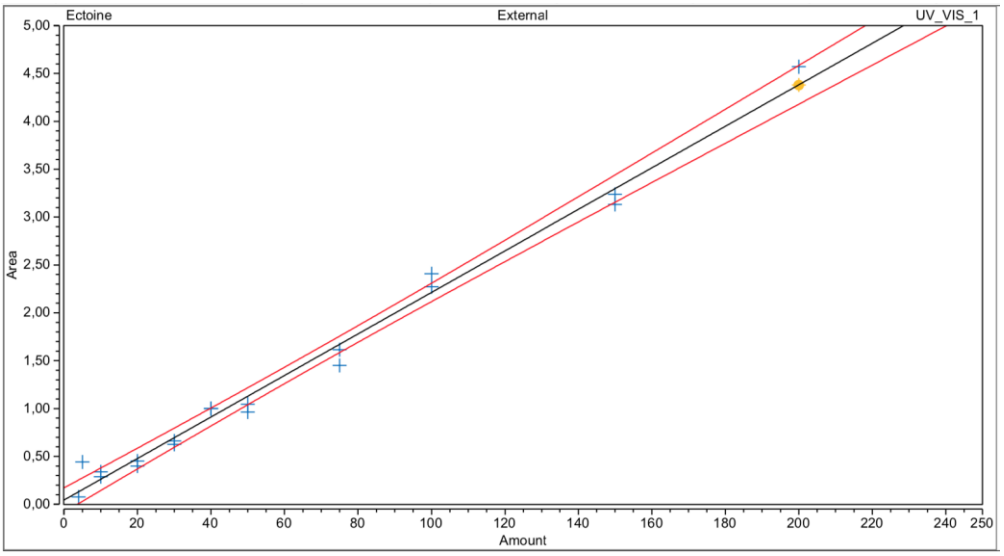

Figure S1. Ectoine and Hydroxyectoine Calibration curve for (Hydroxi)ectoine content assay. Area of ectoine and hydroxyectoine values were interpolated in the linear regression equation.

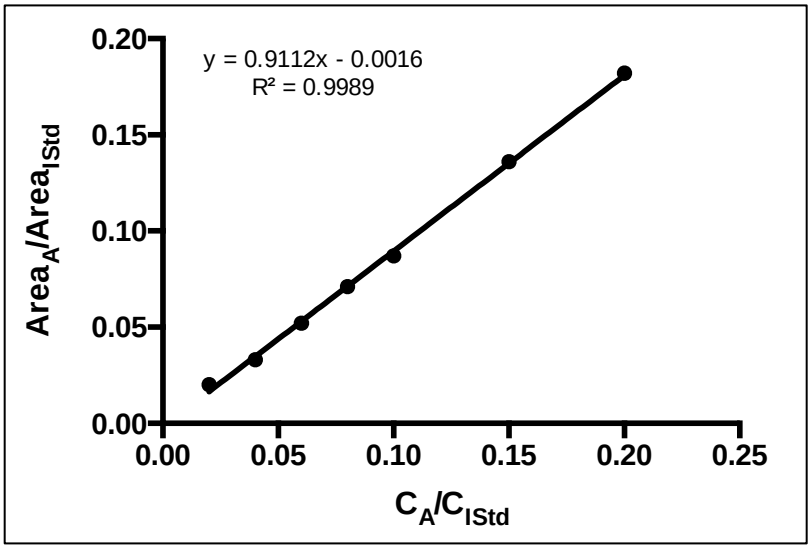

Figure S2. Trehalose (A)/Saccharose (IStd) calibration curve for trehalose content assay. Area of trehalose/Area of saccharose values were interpolated in the linear regression equation to obtain concentration of trehalose (C_A)/concentration of saccharose (C_{IStd}).

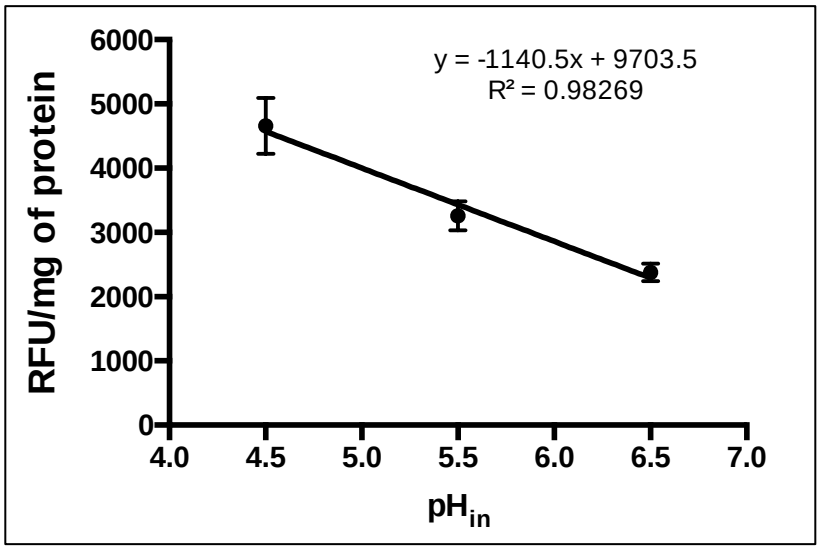

Figure S3. Intracellular pH Calibration curve. Relative fluorescence of each sample was interpolated in the linear regression equation to obtain the intracellular pH (pH_{in}) value. The standard deviation is a consequence of three independent samples.

Table S1. Accession numbers of predicted proteins involved in osmoprotection in iron-oxidizing acidophiles.

(Attached excel file)

Table S2. Complete set of up-regulated genes in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

Code[a]	Gene product	Fold change[b]	SE[c]
fig_6666666.437888.peg.756	Uncharacterized protein	91.7	7.9
fig_6666666.437888.peg.340	Uncharacterized protein	48.5	4.6
fig_6666666.437888.peg.535	Uncharacterized protein	45.8	3.1
fig_6666666.437888.peg.2427	Uncharacterized protein	36.2	2.8
fig_6666666.437888.peg.1735	Uncharacterized protein	32.0	3.2
fig_6666666.437888.peg.540	Uncharacterized protein	25.3	3.0
fig_6666666.437888.peg.342	Uncharacterized protein	22.9	2.7
fig_6666666.437888.peg.537	Uncharacterized protein	22.3	2.6
fig_6666666.437888.peg.541	Uncharacterized protein	22.2	2.7
fig_6666666.437888.peg.538	Uncharacterized protein	20.5	2.5
fig_6666666.437888.peg.1450	Uncharacterized protein	20.4	3.8
fig_6666666.437888.peg.2442	Uncharacterized protein	20.3	2.6
fig_6666666.437888.peg.706	Uncharacterized protein	20.1	2.5
fig_6666666.437888.peg.705	Uncharacterized protein	18.7	2.5
fig_6666666.437888.peg.539	Uncharacterized protein	14.8	2.5
fig_6666666.437888.peg.748	Uncharacterized protein	14.6	2.4
fig_6666666.437888.peg.700	Flavohemoprotein	14.3	2.5
fig_6666666.437888.peg.754	Uncharacterized protein	12.9	2.4
fig_6666666.437888.peg.1992	Uncharacterized protein	11.3	2.6
fig_6666666.437888.peg.2367	Uncharacterized protein	11.3	2.4
fig_6666666.437888.peg.753	Diguanylate cyclase/phosphodiesterase	11.2	2.4
fig_6666666.437888.peg.2429	Uncharacterized protein	11.2	2.7
fig_6666666.437888.peg.460	Uncharacterized protein	11.0	3.0
fig_6666666.437888.peg.1049	Cytochrome *c* peroxidase	10.9	2.4
fig_6666666.437888.peg.1805	DSF synthase	10.7	2.4
fig_6666666.437888.peg.1558	Uncharacterized protein	10.5	2.7
fig_6666666.437888.peg.757	Uncharacterized protein	10.4	2.6
fig_6666666.437888.peg.222	PKD domain protein	9.8	2.3
fig_6666666.437888.peg.695	Uncharacterized protein	9.1	2.4
fig_6666666.437888.peg.1806	Uncharacterized protein	9.0	2.4
fig_6666666.437888.peg.682	Uncharacterized protein	9.0	2.3
fig_6666666.437888.peg.478	Uncharacterized protein	8.1	2.3
fig_6666666.437888.peg.2031	Heat-shock protein Hsp20	8.1	2.2
fig_6666666.437888.peg.2314	Carbonic anhydrase	8.1	2.3
fig_6666666.437888.peg.1453	Transposase	7.7	2.3
fig_6666666.437888.peg.1087	Uncharacterized protein	7.5	2.3
fig_6666666.437888.peg.1048	Outer membrane protein	7.4	2.3
fig_6666666.437888.peg.693	Nitrite-sensitive transcriptional repressor NsrR	7.2	2.2
fig_6666666.437888.peg.1893	Diguanylate cyclase/phosphodiesterase	6.6	2.2
fig_6666666.437888.peg.745	Putative stomatin/prohibitin-family membrane protease	6.6	2.3
fig_6666666.437888.peg.1717	Uncharacterized protein	6.5	2.5

[a] Code refers to the identified gene within the re-annotated genome of *L. ferriphilum* DSM 14647 by RAST.
[b] Average fold up-regulation of the comparison between non-adapted and adapted *L. ferriphilum* DSM 14647 transcriptomic profile.
[c] Standard error of the mean of the average fold up-regulation for the comparisons between treatments.

(Continuation)

Code[a]	Gene product	Fold change[b]	SE[c]
fig_6666666.437888.peg.694	Uncharacterized protein	6.3	2.3
fig_6666666.437888.peg.1621	Uncharacterized protein	6.0	2.4
fig_6666666.437888.peg.1082	Sel1 repeat-containing protein	6.0	2.2
fig_6666666.437888.peg.310	Uncharacterized protein	5.9	2.2
fig_6666666.437888.peg.1086	Uncharacterized protein	5.9	2.2
fig_6666666.437888.peg.2315	Sulfide-quinone reductase	5.6	2.2
fig_6666666.437888.peg.1610	Methyl-accepting chemotaxis protein	5.4	2.2
fig_6666666.437888.peg.1965	Phage related integrase	5.4	2.2
fig_6666666.437888.peg.2717	Uncharacterized protein	5.4	2.3
fig_6666666.437888.peg.750	Uncharacterized protein	5.3	2.1
fig_6666666.437888.peg.1964	Uncharacterized protein	5.2	2.3
fig_6666666.437888.peg.1778	Diguanylate cyclase	5.2	2.1
fig_6666666.437888.peg.500	Radical SAM domain protein	5.2	2.1
fig_6666666.437888.peg.1085	Uncharacterized protein	5.1	2.2
fig_6666666.437888.peg.1081	Uncharacterized protein	5.1	2.2
fig_6666666.437888.peg.480	Methyl-accepting chemotaxis protein	5.1	2.2
fig_6666666.437888.peg.1060	Cytochrome c oxidase subunit CcoN	4.9	2.2
fig_6666666.437888.peg.1785	Fatty acid desaturase	4.9	2.2
fig_6666666.437888.peg.1084	Periplasmic serine protease DO (HtrA-1)	4.9	2.2
fig_6666666.437888.peg.2258	Uncharacterized protein	4.8	2.1
fig_6666666.437888.peg.501	Uncharacterized protein	4.7	2.1
fig_6666666.437888.peg.1718	Sulfide-quinone reductase	4.6	2.1
fig_6666666.437888.peg.2126	Flagellin	4.4	2.0
fig_6666666.437888.peg.2237	DNA-binding protein HU	4.3	2.0
fig_6666666.437888.peg.1862	Mercuric reductase	4.3	2.1
fig_6666666.437888.peg.2659	Ubiquitin	4.2	2.1
fig_6666666.437888.peg.1177	Shufflon-specific DNA recombinase	4.2	2.1
fig_6666666.437888.peg.1777	Uncharacterized protein	4.1	2.1

[a] Code refers to the identified gene within the re-annotated genome of *L. ferriphilum* DSM 14647 by RAST.
[b] Average fold up-regulation of the comparison between non-adapted and adapted *L. ferriphilum* DSM 14647 transcriptomic profile.
[c] Standard error of the mean of the average fold up-regulation for the comparisons between treatments.

Table S3. Complete set of down-regulated genes in *L. ferriphilum* DSM 14647 adapted to 180 mM NaCl.

Code[a]	Gene product	Fold change[b]	SE[c]
fig_6666666.437888.peg.2216	Two component sigma54 specific transcriptional regulator	-4.3	2.1
fig_6666666.437888.peg.76	Uncharacterized protein	-4.6	2.2
fig_6666666.437888.peg.1852	Sugar transferase	-4.8	2.2
fig_6666666.437888.peg.1851	Polysaccharide export protein	-4.9	2.2
fig_6666666.437888.peg.1874	Capsular polysaccharide biosynthesis protein I	-5.0	2.2
fig_6666666.437888.peg.2214	Outer membrane efflux protein	-5.1	2.1
fig_6666666.437888.peg.1846	Glycosyl transferase group 1 family protein	-5.3	2.3
fig_6666666.437888.peg.1830	Putative glycosyltransferase	-5.3	2.2
fig_6666666.437888.peg.2215	Putative membrane-fusion protein	-5.4	2.1
fig_6666666.437888.peg.1817	Uncharacterized protein	-5.6	2.2
fig_6666666.437888.peg.2670	L-ectoine synthase (EctC)	-5.6	2.2
fig_6666666.437888.peg.1886	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	-5.7	2.2
fig_6666666.437888.peg.1720	Uncharacterized protein	-5.9	2.3
fig_6666666.437888.peg.1822	Phospholipid-lipopolysaccharide ABC transporter	-5.9	2.3
fig_6666666.437888.peg.1829	Glycosyltransferase involved in cell wall bisynthesis	-6.0	2.3
fig_6666666.437888.peg.2219	RND family efflux transporter MFP subunit	-6.2	2.2
fig_6666666.437888.peg.1887	UDP-glucose dehydrogenase	-6.4	2.2
fig_6666666.437888.peg.2671	Diaminobutyrate--2-oxoglutarate transaminase (EctB)	-6.5	2.2
fig_6666666.437888.peg.2218	Cobalt-zinc-cadmium resistance protein CzcA	-6.8	2.2
fig_6666666.437888.peg.1853	Tyrosine-protein kinase EpsD	-6.9	2.2
fig_6666666.437888.peg.1831	Uncharacterized protein	-7.1	2.3
fig_6666666.437888.peg.1721	Uncharacterized protein	-7.3	2.4
fig_6666666.437888.peg.1500	Uncharacterized protein	-7.5	2.3
fig_6666666.437888.peg.1888	Uncharacterized protein	-7.9	2.4
fig_6666666.437888.peg.2669	Ectoine hydroxylase (EctD)	-8.0	2.3
fig_6666666.437888.peg.1848	Polysaccharide deacetylase	-8.4	2.6
fig_6666666.437888.peg.1813	Uncharacterized protein	-8.5	2.3
fig_6666666.437888.peg.1834	UTP--glucose-1-phosphate uridylyltransferase	-9.0	2.3
fig_6666666.437888.peg.1833	Eight transmembrane protein EpsH	-9.3	2.4
fig_6666666.437888.peg.1832	Sigma-54 dependent transcriptional regulator	-10.2	2.4
fig_6666666.437888.peg.2216	Two component sigma54 specific transcriptional regulator	-4.3	2.1
fig_6666666.437888.peg.76	Uncharacterized protein	-4.6	2.2
fig_6666666.437888.peg.1852	Sugar transferase	-4.8	2.2
fig_6666666.437888.peg.1851	Polysaccharide export protein	-4.9	2.2

[a] Code refers to the identified gene within the re-annotated genome of *L. ferriphilum* DSM 14647 by RAST.
[b] Average fold up-regulation of the comparison between non-adapted and adapted *L. ferriphilum* DSM 14647 transcriptomic profile.
[c] Standard error of the mean of the average fold up-regulation for the comparisons between treatments.