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ESCUELA DE INGENIERÍA

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JAVIERA M. ANGUITA LEYTON

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science in Engineering

Advisor:

IGNACIO T. VARGAS

Santiago de Chile, (May, 2016)

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Dedicated to my family for their love and support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF ILLUSTRATIONS

Page

ABSTRACT

Biological arsenic oxidation is a key biogeochemical process that controls its mobilization and fate in aqueous environments. According to our knowledge, only four chemolithoautotrophic arsenite oxidizing (CAO) bacteria have been characterized by describing the essential genes for chemolithoautotrophic arsenic oxidation while coupling bacterial growth with arsenic oxidation. Moreover, considering our literature review, CAO bacteria have not been reported as electrochemically active microorganisms, which could be a key aspect for potential applications in enhanced arsenic attenuation techniques.

In this study, a new chemolithoautotrophic arsenite-oxidizing bacterium was isolated from a hydrothermal source where natural dissolved arsenic attenuation was observed, and its electrochemical activity was studied. The bacterial isolate, denominated TS-1, owns the essential genes for chemolithoautotrophic arsenite oxidation. In addition, the dependence on bacterial growth and arsenite consumption was experimentally demonstrated, and a mathematical model is suggested for obtaining kinetic parameters by simultaneously fitting bacterial growth and arsenite depletion curves. In addition, the electrochemical capacity of TS-1 was supported by electrochemical tests, evidencing an increase of the cathodic current in a chronoamperometry test, and therefore, the catalysis of the electron transference from the electrode to the culture media.

Keywords: chemolithoautotrophic arsenite-oxidizing (CAO) bacteria, electrochemically active bacteria (EAB), modeling of arsenic oxidation.

RESUMEN

La oxidación biológica de arsénico es un proceso biogeoquímico clave que controla la movilización y destino del arsénico en ambientes acuáticos naturales. Acorde a nuestro conocimiento, sólo cuatro bacterias quimiolitoautotróficas oxidantes de arsénico (CAOs, por su abreviatura en inglés) han sido identificadas incluyendo evidencia experimental de una directa relación entre crecimiento microbiano y oxidación de arsénico, y describiendo los genes necesarios para la oxidación de arsénico de forma quimiolitoautotrófica. Además, considerando nuestra revisión bibliográfica, bacterias con metabolismo CAO no han sido reportadas como microorganismos electroquímicamente activos, lo que podría ser un aspecto clave para potenciales aplicaciones en el mejoramiento de técnicas de atenuación de arsénico.

En este estudio, una nueva bacteria quimiolitoautotrófica oxidante de arsénico fue aislada desde una fuente hidrotermal donde atenuación natural de arsénico disuelto fue observada, y su capacidad electroquímica fue estudiada. La bacteria aislada, denominada TS-1, posee los genes esenciales para la oxidación de arsénico quimiolitoautotrófica. También, la dependencia entre el crecimiento del microorganismo y el consumo de arsénico fue experimentalmente demostrada, y un modelo dinámico es sugerido para la obtención de parámetros cinéticos a través de un ajuste simultáneo entre el crecimiento y el consumo de arsénico. Además, la capacidad electroquímica de TS-1 fue soportada por ensayos electroquímicos que evidencian un aumento de la corriente catódica y, por tanto, una catálisis en la transferencia de electrones desde el electrodo hacia el medio de cultivo.

Palabras claves: bacterias quimiolitoautotróficas oxidantes de arsénico, bacterias electroquímicamente activas, ajuste acoplado para biomasa y sustrato.

1 BACKGROUND

Arsenic (As) is a toxic metalloid widely distributed in the world. In the Earth's crust, As is principally found as inorganic compounds associated with igneous and sedimentary rocks (Tamaki and Frankenberger Jr, 1992). In the biosphere, As is released due to natural processes (e.g. volcanic eruptions, geothermal sources, aquifers) and human activities (i.e. mining and smelting activities to recover base metals) (Drewniak and Sklodowska, 2013). As is mainly transported in the environment by water (WHO, 2000). More than 150 million people worldwide are exposed to As concentrations over 10 μg L^{-1}, the maximum level for drinking water, established by the World Health Organization (WHO, 2011) (Ravenscroft et al., 2009).

In natural environments, As mobilization depends on physical, chemical and biological processes (Wang and Mulligan, 2006). The oxidized state of As [As(V), arsenate] is considered less mobile and less toxic than its reduced state [As(III), arsenite] (Cullen and Reimer, 1989). In oxic environments, some microorganisms can oxidize As, increasing its sorption onto Fe(III)oxy(hydro)oxides (Wang and Mulligan, 2006). Natural attenuation of dissolved As mediated by arsenite oxidizing bacteria has been reported in different places (Connon et al., 2008; Gihring et al., 2001; Leiva et al., 2014; Salmassi et al., 2006).

Two bacterial groups have the capacity of arsenite oxidation: heterotrophic arsenite oxidizers (HAOs) and chemolithoautotrophic arsenite oxidizers (CAOs). HAOs oxidize As(III) as a detoxification strategy and use organic matter as a source of energy and carbon (Oremland and Stolz, 2003), while CAOs use arsenite as an energy source and carbon dioxide as a carbon source (Santini et al., 2000). Around 60 bacterial strains have demonstrated the capacity of arsenite oxidation (Table A-1). According to our knowledge, just 12 bacterial strains have been reported as CAOs, and in only four of them, a direct relationship between bacterial growth and As(III) oxidation has been demonstrated. Therefore, the identification and characterization of new CAOs will

contribute to a better understanding of their role in the biogeochemical processes controlling As speciation and mobilization in water sources, and also to the development or improvement of sustainable solutions to treat this contaminant.

Recent studies have reported that chemolithoautotrophic bacteria are good candidates to be electrochemically active microorganisms (EAM) in biocathodes. The capacity of chemolithoautotrophs to use different sources of electrons and carbon, make it possible to speculate that these microorganisms can grow using an electrode as an electron source concurrently with carbon dioxide fixation (Sydow et al., 2014). This electrochemical activity can be used as an enrichment and culture tool (Summers et al., 2013), and for treating oxidized contaminants, such as chromium (VI) (Tandukar et al., 2009), and perchlorate (Shea et al., 2008). Webster *et al.* (2014) studied a modified strain of *Shewanella oneidensis*, which produces an electrical current in response to As. However, CAOs have not been reported as EAM according to our review.

Sites with high dissolved As concentration are excellent niches for the prospection of CAOs. The firsts known CAOs were isolated from a gold mine, using water (Ilialetdinov and Abdrashitova, 1980) and arsenopyrite (FeAsS) containing minerals (Santini et al., 2000). In northern Chile, previous research reported microbial As(III) oxidation in a hydrothermal source with high As concentration, resulting in natural attenuation by the immobilization of As(V) in a solid matrix of Fe(III)oxy(hydro)oxides (Leiva et al., 2014). These previous results positioned this site as an ideal location to find arsenite oxidizing microorganisms.

This thesis integrates the use of molecular, bioinformatics, mathematical, and electrochemical tools, to isolate and characterize a new CAO isolated from a hydrothermal source in northern Chile.

1.1 Objectives

The main purpose of this thesis was the isolation of a new chemolithoautotrophic arsenite oxidizing bacterium, its characterization and the study of its electrochemical capacity.

The specific objectives of this research are:

1) To isolate and identify a potential new arsenic oxidizing microorganism.
2) To determinate the genetic capacity for chemolithotrophic arsenic bio-oxidation of the isolate.
3) To estimate kinetic parameters for arsenite oxidation and biomass growth of this bacterial isolate through mathematical modeling.
4) To study the electrochemical capacity of this isolate.

2 A NEW CHEMOLITHOAUTOTROPHIC ARSENIC OXIDIZING MICROORGANISM ISOLATED FROM A HYDROTHERMAL SOURCE EVIDENCING NATURAL ARSENIC ATTENUATION

2.1 Abstract

Biological arsenic oxidation has been suggested as a key biogeochemical process that controls the mobilization and fate of this metalloid in aqueous environments. To the best of our knowledge, only four chemolithoautotrophic arsenite-oxidizing (CAO) bacteria have been shown to be able to grow via direct arsenic oxidation, and to have the essential genes for chemolithoautotrophic arsenite oxidation. In this study, a new chemolithoautotrophic arsenite-oxidizing bacterium was isolated from a hydrothermal source evidencing natural dissolved arsenic attenuation. The bacterial isolate, denominated TS-1, is closely related to the *Ancylobacter* genus, in the *Alphaproteobacteria* class. Results showed that TS-1 owns both the gene for arsenite oxidation and carbon fixation. The dependence of bacterial growth from arsenite oxidation was demonstrated. A mathematical model was suggested to obtain kinetic parameters by simultaneously fitting the growth and arsenite depletion curves. This research increases the knowledge of chemolithoautotrophic arsenic oxidizing microorganisms and contributes proposing a methodology to determinate the kinetic parameters of CAOs.

Keywords: chemolithoautotrophic arsenite-oxidizing (CAO) bacteria, electrochemically active bacteria (EAB), modeling of arsenic oxidation.

2.2 Introduction

Arsenic (As) is a toxic metalloid widely distributed around the world (Smedley and Kinniburgh, 2002). Chronic exposure to this contaminant in drinking water has been associated with adverse effects on health, such as skin lesions and different types of cancer (e.g. skin, lung, and liver) (Brown and Ross, 2002; Hughes, 2002;

Jomova et al., 2011). Drinking water has been an important exposure route of humans to As (Kapaj et al., 2006). The World Health Organization defines the maximum limit of 10 µg L^{-1} of As in drinking water (WHO, 2011). However, As concentrations as high as 100 mg L^{-1} have been measured in groundwater(Welch et al., 2000).

The mobility of As in aqueous environments depends on physicochemical and biological processes. In general, arsenate [As (V)] is more easily immobilized onto solid phases than arsenite [As(III)] (Bowell, 1994). As oxidation can be mediated by abiotic agents, including hypochlorite, permanganate, or ozone (Mondal et al., 2013); and biotic agents such as bacteria. Previous studies have shown that bacteria play a significant role in As(III) oxidation and its immobilization in surface waters (Hamamura et al., 2009; Leiva et al., 2014).

The bacterial As(III) oxidation can be used as a detoxification strategy to survive in polluted environments, or to obtain energy directly from reduced As (Yamamura and Amachi, 2014). While detoxification is performed mainly by heterotrophic arsenite oxidizers (HAOs) using organic matter as an electron donor and carbon source (Oremland and Stolz, 2003); chemolithoautotrophic arsenite oxidizers (CAOs) obtain energy by using As(III) as an electron donor and carbon dioxide as a carbon source (Santini et al., 2000). CAOs grow by As(III) oxidation through the arsenite oxidase enzyme, and fix carbon by using the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme.

In two recent reviews, Andres and Bertin (2016) and Yamamura and Amachi (2014) published more than 130 homologs genes of arsenite oxidase distributed in strains among *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Aquificae, Deinococcus-Thermus, Chlorobi, Chloroflexi, Nitrospira,* and *Crenarchaeota* (Andres and Bertin, 2016; Yamamura and Amachi, 2014) (Table A-1). By using qualitative or quantitative tests, around 60 bacterial strains have

shown As oxidation, all of them belonging to *Proteobacteria, Deinocci, and Crenarchaeota phyla.* Among this group, only 12 strains in these phyla have been reported as CAOs, and a direct relationship between bacterial growth and As oxidation has only been demonstrated for 4 of them.

Properly identified CAOs have been isolated from As-rich environments associated with mining activity. *Rhizobium* sp. NT-26 (Santini et al., 2000), *Thiomonas arsenivorans* DSM16361 (Battaglia-Brunet et al., 2006) and *Sinorhizobium* sp. M14 (Drewniak et al., 2008) were isolated from a gold mine (Santini et al., 2000). *Ancylobacter dichloromethanicus* As 3-1b was isolated from the rhizosphere of an agricultural soil with high As content derived from mining wastes (Andreoni et al., 2012). Despite that biological As oxidation is considered a key biogeochemical process controlling the fate and the mobilization of this element in natural environments, CAO research is still in its infancy. There is a lack of information on CAO diversity, metabolism and kinetic parameters for growth and As oxidation. The benchmarking of kinetic parameters is difficult because: (i) few experiments have been reported, (ii) different experimental conditions were devised, and (iii) not clear fitting methodologies have been used.

The purposes of this research were (i) to isolate a new chemolithoautotrophic arsenic oxidizing microorganism from a hydrothermal source in northern Chile, where dissolved As attenuation driven by bacteria has been previously reported (Leiva et al., 2014), and (ii) to describe robustly the capacity for As(III) oxidation. For this last objective, molecular tools and dynamic modeling were used to support the chemolithoautotrophic arsenic oxidizing capacity of the isolated microorganism.

2.3 Materials and methods

2.3.1 Study site and sample collection

Sediment samples were obtained from a hydrothermal source located in the upper section of the Lluta River (Arica and Parinacota Region) in northern Chile (17°43´12´´ S and 69°49´18´´ W). Natural attenuation of dissolved As has been previously detected at the site, which explains high As contents observed in sediment samples (6.4 ± 1.7 mg kg^{-1}) (Leiva et al., 2014). For this study, sediments were aseptically collected in 50 mL polypropylene tubes (BD Biosciences, Mountain View, CA) in December 2013 and saved for ~ 3 months at 4°C until they were used.

2.3.2 Enrichment and isolation

The inoculum was a mix (3:1 v/v) of 1 g of sediment and water extracted from the same site. The inoculum was cultivated in 10 mL of a liquid chemically defined medium (CDM, composition is given below) for final As(III) concentrations of 0.01; 0.1; and 1 mM (added as $NaAsO_2$). The enrichments were incubated in a rotatory shaker (at 120 rpm and 30°C) for ~ 3 months until microorganisms were observed by optic microscopy using an Olympus CX31 microscope (Olympus, Japan). After incubation, aliquots (100 µL of liquid culture) were spread on the CDM solidified with bacto agar (1.5%) and isolated by successive culture in plates. Isolates were incubated in liquid CDM for two weeks and saved in a glycerol stock at -80 °C. A preliminary screening analysis of As(III) oxidation was performed to isolates in liquid culture using the $KMnO_4$ method, described by Salmassi et al. (2002).

The CDM consisted (per liter) of: 30 mg Na_2SO_4; 100 mg KCl, 80 mg $MgCl_2$; 100 mg $CaCl_2 \cdot 2H_2O$; 100 mg $(NH_4)_2SO_4$; 6.8 mg KH_2PO_4; and trace elements from stock 1000X added according to Bahar et al (2012), including 0.018 mg

$AlCl_3 \cdot 6H_2O$; 0.2 mg Na_2EDTA. 0.025 Na_2MoO_4 $2H_2O$, 0.034 mg Na_2SeO_3 $5H_2O$, 0.03 mg $Na_2WO_4 \cdot 2H_2O$ and 1.9 mg $FeCl_2$. This base medium was autoclaved at 121°C for 20 min and supplemented with filtered stock solutions (PTFE filters, pore size 0.22, Clarinert) under sterile conditions, in order to obtain (per liter): 840 mg $NaHCO_3$, 129.9 $NaAsO_2$, and vitamins according to Santini (Santini et al., 2000). HCl was added for adjusting pH at 6.3.

2.3.3 Identification and general characterization

2.3.3.1 16S rDNA amplification and sequencing

DNA extraction from isolates was done using a Genomic DNA Kits PureLink®, following the manufacturer´s instructions. DNA from isolates was subjected to 16S rDNA PCR amplification using the primer set 8F / 1392R. PCR products were purified and sequenced by Macrogen Inc. (Korea). The isolate sequence was compared to the GenBank database using Blastn for taxonomic assignments (Altschul et al., 1990). One isolated was selected for the next analysis.

2.3.3.2 Identification of arsenite oxidase gene

Bacterial capacity for As oxidation was evaluated through the amplification of partial subunit A of the arsenite oxidase (AioA) gene (Lett et al., 2012). Primer set: aoxBM1-2F / aoxBM3-2R was used according to Quemeneur (2008). PCR product was identified with agarose gel 1.5%. Amplicons were purified and sequenced by Macrogen Inc. (Seoul, Korea). The protein sequence was deduced using Expasy Translate Tool (Gasteiger et al., 2003). The Partial AioA gene was compared to the GenBank database using Blastp for taxonomic assignments (Altschul et al., 1990).

2.3.3.3 Identification of RuBisCO gene

To confirm the autotrophic capacity of the isolate, PCR amplification of RuBisCO gene were executed using the primer set RBCO-1Cf / RBCO-1Cr with the program described by Alfreid et al. (Alfreider et al., 2003). The purified amplicon was cloned into a plasmid with backbone pU0002 in SwaI site (Hansen et al., 2011), and inserted into competent cells of *E. coli* TOP10F`, using for both previously described protocols (Warr et al., 1990). Plasmids were sequenced with the primers used for the PCR amplification (Macrogen, Korea). The protein sequence was deduced from nucleotide sequence using Expasy Translate Tool (Gasteiger et al., 2003). Sequences were compared to the GenBank database using Blastp for taxonomic assignments (Altschul et al., 1990).

2.3.3.4 Phylogenetic analyses based on 16S rRNA gene and arsenite oxidase

Phylogenetic analyses were done separately for the partial 16S rRNA genes, and the arsenite oxidase proteins using CAOs and HAOs belong to *Alphaproteobacteria* and *Betaproteobacteria* classes.

For the 16S rRNA genes, multiple sequence alignments (MSAs) were executed with the T-COFFEE algorithm and General Time Reversible with invariable sites and gamma distribution (GTR+I+G) model of evolution. The algorithm was selected from T-COFFEE (Notredame et al., 2000), MUSCLE (Edgar, 2004), MAFFT (Katoh et al., 2002) algorithms using the T-COFFEE program; through the sum of pairs score (sp-score) with NUC44 scoring matrix for nucleotide using the bioinformatics toolbox of Matlab (MathWorks, 2016). The best-fit of the model evolution was defined through Bayesian Information Criterion (BIC) and likelihood ratio tests (hLRT) using jModeltest v 2.1.7 (Posada and Crandall, 1998).

For the arsenite oxidases, MSAs were performed with T-COFFEE and Le and Gascuel with invariable sites and gamma distribution (LG+I+G). The selection of

algorithm was performed similar to the 16S rRNA but using the Blosum62 scoring matrix to calculate the sp-score (MathWorks, 2016). The best-fit model of evolution was selected applying the BIC criteria using Prottest v 3.4 (Darriba et al., 2011). The MSAs were automatically cured with Gblocks v 0.91b (Castresana, 2000), using a minimum length block of 10 and with half gap positions allowed (Gao et al., 2009). The best MSAs were defined as the highest sp-score with Blosum62 scoring matrix (MathWorks, 2016).

Phylogenetic trees were constructed by Maximum Likelihood (ML) using RAxML v8.0 (Stamatakis, 2014), and Bayesian inference (BI) implemented in MrBayes v3.2.3 (Ronquist and Huelsenbeck, 2003). Support analyses were presented with posterior probability for BI and bootstrap values for ML. Sequences with less than 50 % of bootstrap in guide trees were taken out, and the MSA and guide trees were remade. Trees were visualized with FigTree v1.4.2 (http://www.tree.bio.ed.ac.uk).

2.3.3.5 Morphology characterization

The bacterial isolate from the liquid culture was visualized by scanning electron microscopy. A JEOL JSM-IT300LV was used for microscopic analyses. Aliquots extracted from liquid cultures (during the exponential stage) were centrifuged and resuspended in 1 mL of 2.5 % glutaraldehyde, and cacodylate 10 mM. The resuspended pellets were filtered through 0.05 µm pore size polycarbonate filters (ISOPORE, Millipore, Ireland). The filters were dehydrated with ethanol, water was removed by using critical point drying, and coated with gold before microscopic observation (Fischer et al., 2013).

2.3.3.6 Determination of minimal inhibitory concentration (MIC)

Bacterial resistance to As was determined using the MIC, defined as the lowest metalloid concentration that causes no visible growth (Valenzuela et al., 2009). 5 µL of culture suspended adjusted to an optical density at 600 nm of 0.3 was

(Achour et al., 2007) spread on LB agar plates supplemented with different concentrations of As(III) (1-10 mM) and As(V) (1-100 mM). The plates were incubated for seven days at 30 °C.

2.3.4 Chemolithotrophic and mixotrophic As(III) oxidation

2.3.4.1 Cultures

The isolate was cultured in batch reactors using three different media: oligotrophic [modified CDM (mCDM)], chemolithoautotrophic [mCDM with As(III)], and mixotrophic [mCDM with As(III) plus yeast extract (YE)]. The mCDM was the previously described but adjusted to pH 7.2 with phosphate buffer (final concentration per liter of 750 mg KH_2PO_4; 3,950 mg $Na_2HPO_4 \cdot 7H_2O$; and HCl). Initial As(III) concentration in chemolithoautotrophic culture (i) was ~2.5 mM, and in mixotrophic culture (ii) was ~3.2 mM. All biological experiments were inoculated with $2 \cdot 10^7$ cells and performed in duplicates with 100 mL of culture medium in flasks of 250 mL, at 34°C and 200 rpm. Abiotic controls were performed for all culture media.

Samples from liquid cultures were taken for measurements of biomass, As, and pH. Biomass was measured through direct cell count. For this, the cells were stained with a 0.002 % acridine orange solution (Lunau et al., 2005) and observed in a Neubauer chamber with a fluorescence microscope (Olympus CX31 microscope; Olympus, Japan) at 40X. Total As was measured by using an inductively coupled plasma mass spectrometer (ICP-MS) (Elan 9000, Perkin-Elmer, Canada). As(III) was measured using speciation cartridges (MetalSoft Center, USA), according to manufacturer's specifications (Meng and Wang, 1998) and ICP-MS. pH was monitored using a 420Aplus pHmeter with an Orion 8175BNWP electrode (Thermo Scientific, USA).

2.3.4.2 Mathematical modeling for kinetic parameters estimation

To estimate kinetic parameters and to demonstrate the relationship between bacterial growth and As(III) decrease, experimental curves of chemolithoautotrophic culture were simultaneously fitted. Biomass growth (equation 1) and substrate consumption (equation 2), both using Monod equations for specific growth rate (equation 3), were fitted by the weighted least square method (equation 4), estimating the maximum specific growth rate (μ_{max}) and the yield of biomass from As(III) utilization ($Y_{As(III),X}$). The saturation constant (k_s) used was of 0.1483 mM, which was reported for a similar CAO (Garcia-Dominguez et al., 2008). Initial parameters were obtained by linearization of equations 1 and 2.

$$\frac{dX}{dt} = \mu X \qquad \text{(Equation 1)}$$

$$\frac{dAs(III)}{dt} = -\mu \frac{X}{Y_{As(III),X}} \qquad \text{(Equation 2)}$$

$$\mu = \mu_{max} \frac{As(III)}{k_S + As(III)} \qquad \text{(Equation 3)}$$

The weighted least square method (equation 4) was performed although the minimization of the sum of the weighted root mean square error for biomass ($WRMSE_X$) and As(III) ($WRMSE_{As(III)}$). To normalize the biomass residuals, the weights for biomass ($w_{x,i}$) were defined as the square of the estimated biomass in time 'i' ($x_{model,\ i}$) divided by the mean of the square of the model biomass in all measured time. Considering the As(III) residuals, weights for As(III) ($w_{As(III),i}$) were '1.' To equalize the errors of biomass and As(III), these also were weighted for the sum of modeling biomass and modeling As(III), respectively.

$$\text{Min}\left(\frac{1}{\sum_{j=1}^{n} X_{mod,j}} \sum_{i=1}^{n} \sqrt{\frac{(X_{mod,i} - X_{exp,i})^2}{W_{X,i}}} + \frac{1}{\sum_{j=1}^{n} As(III)_{mod,j}} \sum_{i=1}^{n} \sqrt{\frac{(As(III)_{mod,i} - As(III)_{exp,i})^2}{W_{As(III),i}}}\right)$$

(Equation 4)

Matlab R2015b was used for parameters estimation, confidence intervals, and prediction bounds. Differential equations were solved by using the *'ode45'* function. Minimization was performed with the *'fminsearch'* function. Confidence intervals of parameters were found with the *'nlparci'* function. Prediction bounds of biomass and As(III) were done with 95% confidence level using the equation 5. Jacobian was evaluated using the *'lsqcurvefit'* function, using one iteration. The estimation of the variance and covariance matrix were made by equations 6 and 7, respectively (Seber and Wild, 2003).

$$\left[z_{model} \pm t_{DF,0.975} \sqrt{w_z {S_z}^2 + J(\mu_{max}, Y_{SX})_z \, Cov(\mu_{max}, Y_{SX})_z \, J(\mu_{max}, Y_{SX})_z{}^T} \right] \quad \text{(Equation 5)}$$

$$S_z{}^2 = \frac{\sum_{i=1}^{n} \frac{(z_{mod,i} - z_{exp,i})^2}{W_{z,i}}}{n-p} \quad \text{(Equation 6)}$$

$$Cov(\mu_{max}, Y_{SX})_z = S_z{}^2 \left(J(\mu_{max}, Y_{SX})_z{}^T \, W_z{}^{-1} \, J(\mu_{max}, Y_{SX})_z \right)^{-1} \quad \text{(Equation 7)}$$

Where:

z: X or As(III)

z_{model}: Vector of model X or As(III)

z_{exp}: Vector of experimental X or As(III)

S_z: Variance of the sample "z".

w_z: Vector with the weights for "z"

W_z: Diagonal matrix with in the position "i, i" the weight $w_{z,i}$

$J(\mu_{max}, Y_{SX})_z$: Jacobian evaluated at a specified predictor value.

$Cov(\mu_{máx}, Y_{SX})_z$: Covariance matrix

$t_{DF,0.975}$: Student's t-distribution with "DF" degree freedom and, 0.975 confidence levels

n: Number of samples

p: Number of parameters

$Cov(\mu_{máx}, Y_{SX})_z$: Covariance matrix

To obtain μ_{max} of the cultures in oligotrophic (mCDM), and heterotrophic (mCDM with As (III) and YE), the exponential phases of growth were fitted using a

linearization of equation 1 and 2 (considering μ equal to μ_{max}, in the exponential phase of growth).

2.4 Results

2.4.1 Isolation and characterization

2.4.1.1 Isolation and general characterization

Four strains were isolated from enrichments with different As concentrations using an inoculum from a hydrothermal source in northern Chile with high As concentration. The strains were denominated TS-1 (from 1 mM As(III) enrichment), TS-2 and TS-3 (from 0.1 mM As(III) enrichment), and TS-4 (from 0.01 mM As(III) enrichment). All strains were Gram-negative, positive for the preliminary As(III) oxidation test, and the 16S rRNA gene sequences show an identity of more than 99.9% (1177 bp). The 16S rRNA gene sequences were submitted to NCBI with access number: KX139404 (TS-1), KX139405 (TS-2), KX256157 (TS-3), KX139406 (TS-4). The strain isolated from the enrichment with the highest As concentration (i.e. TS-1) was selected for further analysis and characterization.

TS-1 cells were visualized as small rods (~ 0.5 by 1.0 μm) in cultures with As(III) as the electron donor (Figure 2-1a) and cultures with As(III) and YE (Figure 2-1b). TS-1 has a 5 mM MIC for As(III), and over a 100 mM MIC for As(V).

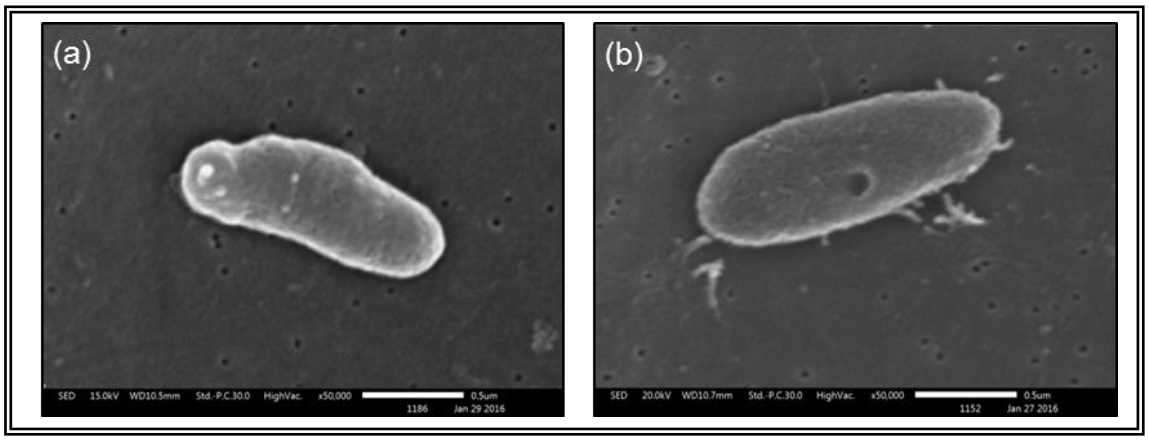

Figure 2- 1: The scanning electron micrograph of TS-1 in (a) mCDM with 2.5 mM of As (III) and (b) mCDM with 3.2 mM of As (III) plus 0.004 % YE, both at exponential phase.

2.4.1.2 Genetic characterization and phylogenetic analysis of TS-1

The 16s rRNA, arsenite oxidase, and RuBisCO genes of TS-1 were amplificated, sequenced, and submitted to NCBI database with the access number: KX139404, KX250215, KX250216; respectively.

The taxonomic analysis of the partial 16S rRNA gene showed affiliation of TS-1 to the genus *Ancylobacter* of the *Alphaproteobacteria* class. Considering the 15 highest identity of sequences TS-1 did not present a close phylogenetic relation with previously reported species and strains (Figure A-1). The highest identity of TS-1 was with the *Ancylobacter aquaticus strain* DH5 (99.0% of identity, and 97% of coverage), which has not been observed to have the As(III) oxidizing capacity. Additionally, TS-1 showed a high sequence identity with previously reported CAO *Ancylobacter dichloromethanicus* As3-1b (98.9% of identity, and 97% of coverage). Unfortunately, the reported 16S rRNA sequence of *A. dichloromethanicum* As3-1b is very short (357 bp, FN392676.1), limiting this comparison. In consequence, TS-1 cannot be classified as a previously described species. Then, the new isolate obtained in this study was named *Ancylobacter* sp. TS-1. Further efforts should be aim to the classification of the existing

Ancylobacter species, such as DNA-DNA hybridization of microorganisms with high 16s rRNA identity, or complete sequencing of a model microorganism.

The deduced aminoacid sequence of arsenite oxidase had the best alignment with the large subunits of arsenite oxidase-1 of *Ancylobacter dichloromethanicus* As3-1b (95.8% of identity, 90% of coverage) and *Ancylobacter* sp. strain OL1 (93.0% of identity, 94% of coverage). The deduced aminoacid sequence of RuBisCO reported the highest identity to the deduced RuBisCO sequence of *Ancylobacter dichloromethanicus* As3-1 (97% of identity, 93% of coverage).

The phylogenetic analysis of the 16S rRNA genes and arsenite oxidase protein from CAOs and HAOs performed with ML algorithm presented a division of *Alphaproteobacteria* and *Betaproteobacteria* (Figure 2-2 and A-2). The similar topologies for trees constructed with 16S rRNA and arsenite oxidase indicate that the principal way of gene transference of the arsenite oxidase gene is vertical. The exception of this similitude is *Hydrogenophaga* sp. CL3, a *Betaproteobacteria* but linked to *Alphaproteobacteria* for arsenite oxidase gene, indicating a possible horizontal transference of genes. In addition, for the 16S rRNA genes and the arsenite oxidase protein trees, *Ancylobacter* sp. TS-1 is close to a group of CAOs (bold font) indicating a possible common ancestor and suggesting the role of the environmental geochemistry (i.e. high As concentration) as pressure driver for evolutive selection of microorganisms in almost non-disturbed unique extreme environments on Earth.

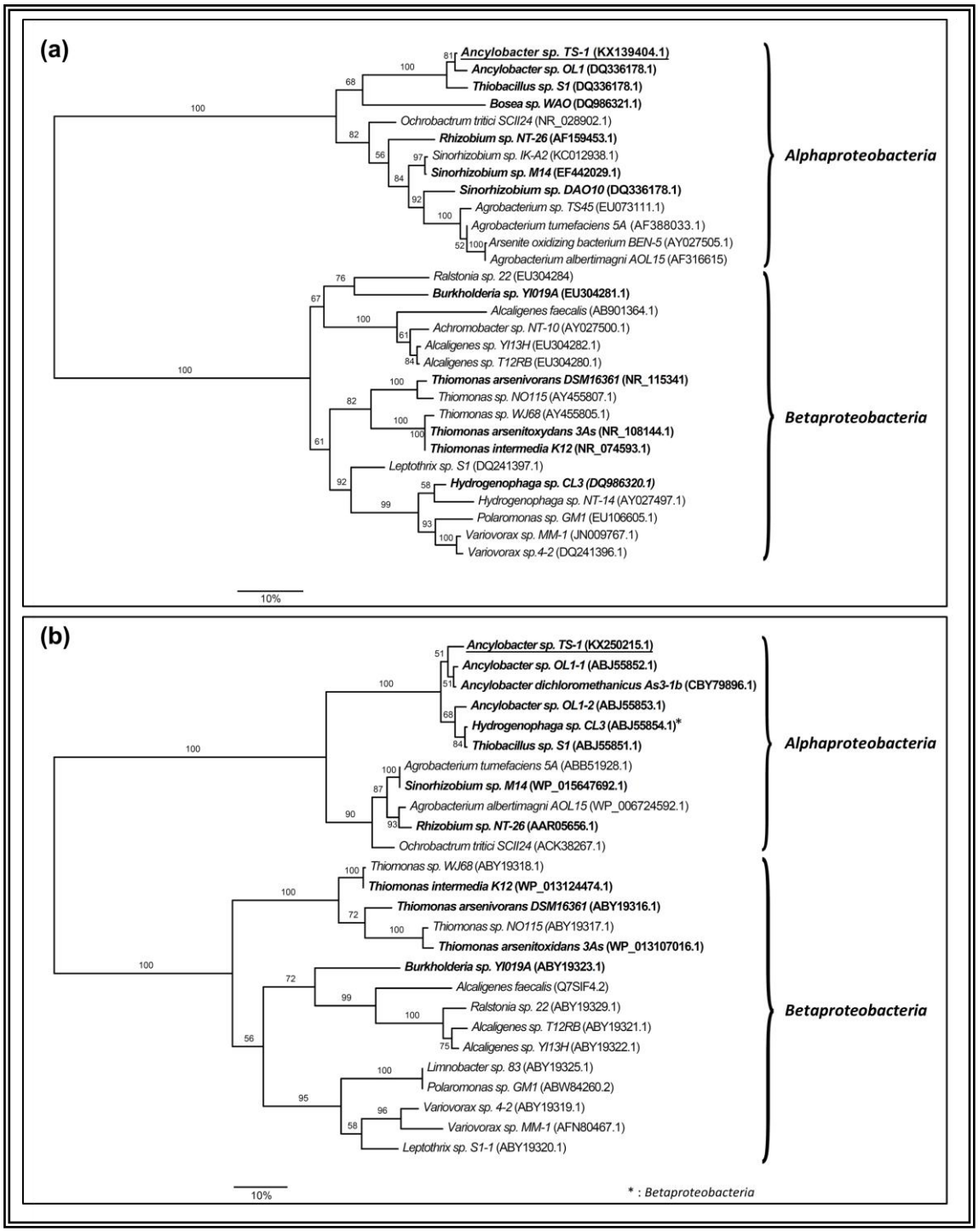

Figure 2- 2: Guide trees of multiple sequence alignments of (a) 16S rRNA genes (~1200 bp) and (b) arsenite oxidases (~330 residues), using CAOs and HAOs. The bars show the percentage of substitutions; the numbers at the branch show the bootstrap percentage of 1000 resampling. CAOs are shown in bold type, and the isolate proposed as *Ancylobacter* sp. TS-1 is in bold underlined font.

2.4.2 *Ancylobacter* sp. TS-1 growth in different culture media and kinetic parameter estimations

Chemolithoautotrophic, mixotrophic and oligotrophic cultures were conducted. Curve fitting was performed to obtain the kinetic parameters related. The normalized root mean squared error of measures were 10% for the As concentration and 9% for biomass.

2.4.2.1 Chemolithoautotrophic culture in mCDM with As(III)

Ancylobacter sp. TS-1 showed growth in mCDM with As(III) as an electron donor and limiting substrate, and CO_2-HCO_3^- as carbon source (Figure 2-3). The total As concentration in liquid phase did not show significant changes in *Ancylobacter* sp. TS-1 culture, discarding sorption process (Figure A-3a). In abiotic cultures, total As and As(III) did not present significant changes; refuting abiotic oxidation (Figure A-3b). Results from the nonlinear estimation of Monod growth kinetic parameters suggested values for μ_{max} of 0.119 ± 0.003 h^{-1} and for $Y_{As(III),X}$ of $1.69 \cdot 10^8$ ± $0.04 \cdot 10^8$ cells ml^{-1} mM^{-1} ($1.69 \cdot 10^{11}$ ± $0.04 \cdot 10^{11}$ cells $mmol^{-1}$) ($WRMSE_X$: 0.10, $WRMSE_{As(III)}$: 0.25).

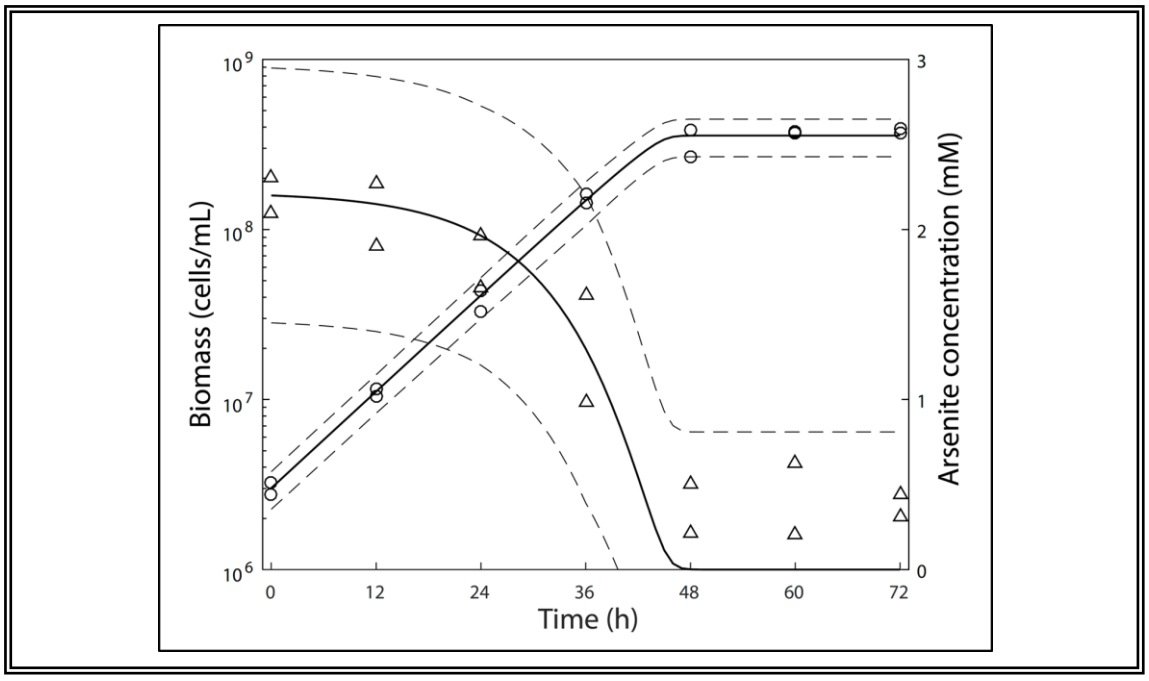

Figure 2- 3: *Ancylobacter* sp. TS-1 growth and arsenite depletion curve in culture with mCDM and arsenite. The fitting was performed for time range when arsenite was depleted with nonlinear estimation of Monod growth kinetic parameters from a single substrate depletion curve. Experimental duplicates are plotted for each sample times. The observed concentration of arsenite (triangle), the observed cell number per milliliter (circle), the fitting curves (continuous lines), and the 95 % prediction bounds (dashed lines).

2.4.2.2 Mixotrophic culture in As(III) plus YE

Ancylobacter sp. TS-1 showed depleted As(III) while grew in mCDM with As(III) and YE. In abiotic cultures, As(III) oxidation was not observed. The total As concentration did not change significantly in *Ancylobacter* sp. TS-1 culture (Figure A-3c). In abiotic cultures, As(III) and total As did not present significantly alteration over time (Figure A-3d). Bacterial growth was fitted for exponential phases, separating the growth curve in three stages: before As(III) oxidation (stage I), during As(III) oxidation (stage II), and after As(III) oxidation (stage III) (Figure A-4a). The μ_{max} for each stage were estimated as 0.075 ± 0.006 h^{-1} (stage I); 0.170 ± 0.009 h^{-1} (stage II); and 0.047 ± 0.008 h^{-1} (stage III); with R^2 of 0.98; 0.98; and 0.84, respectively. For the stage II, $Y_{As(III),X}$ was estimated as $1.821 \cdot 10^8$ ±

$0.141 \cdot 10^8$ cells ml^{-1} mM^{-1} ($1.821 \cdot 10^8 \pm 0.141 \cdot 10^{11}$ cells $mmol^{-1}$) considering the initial and final values. This experiments showed that the growth rate decrease when the As(III) was consumed in mCDM plus As(III) and YE. Moreover, this indicates its capacity to use different electron sources for growth (i.e. As(III) and YE), at the same time.

2.4.2.3 Oligotrophic cultures in mCDM

Ancylobacter sp. TS-1 showed oligotrophic growth in mCDM without As(III) or YE. In the exponential phase of growth, the fitting showed a $\mu_{max} = 0.048 \pm 0.002$ h^{-1} (R^2 of 0.99) with a cellular concentration in the stationary phase of $1.3 \cdot 10^7$ cells mL^{-1} (Figure A-4b). The μ_{max} in this phase was similar to the reported for the stage III (where As(III) was not consumed), supporting the oligotrophic growth.

2.4.3 Kinetic parameters estimation of previously reported CAOs

The coupled fit performed for reported CAOs estimated the kinetic parameters considering that As(III) were to be the limiting substrate (Figure A-5). The reported parameters and the estimated parameters using the fit described in this study for CAOs are showed in Figure 2-4a and 2-4b (for more details view Table A-2). With the coupled fit for biomass and As(III) was possible estimate the $Y_{As(III),X}$ for microorganisms with this not reported (*Ancylobacter dichloromethanicus* As3-1b and *Sinorhizobium* sp. M14). For some kinetic parameters, the reported and estimated values were similar and for others were statistically different. This result highlights the importance to describe the fit methodology including the experimental data, for future comparison. Although the fit methodology was not described in the previous CAOs papers, it is likely that uncoupled fits were done for biomass and As(III). We propose that parameters should be estimated through a coupled fit with the weighted least square method, using the $WRMSE_x$ and the $WRMSE_{As(III)}$ as indicators of the fit goodness and reliability of the parameters (Table A-2). Consequently, these indicators could be

used as a standard estimation of the coupling between bacterial growth and As(III) consumption.

Considering the reported duplication and the estimated duplication times (t_d) in this study (Figure 2-4a and 2-4b), *Ancylobacter* sp. TS-1, *Ancylobacter dichloromethanicus* As3-1b, and *Rhizobium* sp. NT-26 had similar t_d; the lowest t_d was described for *Thiomonas arsenivorans* DSM16361; and the highest t_d was described for *Sinorhizobium sp.* M14 (t_d could not be estimated in this study because experimental values were not reported). The similitude of *Ancylobacter* sp. TS-1, *Ancylobacter dichloromethanicus* As3-1b, and *Rhizobium* sp. NT-26 could be because the arsenite oxidase enzymes are related for these three microorganisms belong to *Alphaproteobacteria* (Figure 2-2b). The small observed difference could be due to the pHs of the culture medium, *Ancylobacter* sp. TS-1 was growing in culture medium with lower pH and its t_d was the lower of the three. This is support for the lower t_d of the five, *Thiomonas arsenivorans* DSM16361, because the experiment was conducted at pH 5.5, close to the optimum pH for the arsenite oxidase enzyme. On the contrary, the highest duplication time was described for *Sinorhizobium* sp. M14, cultivated without vitamins, which probably increases the duplication time mainly due to the shortage of cofactors and trace elements.

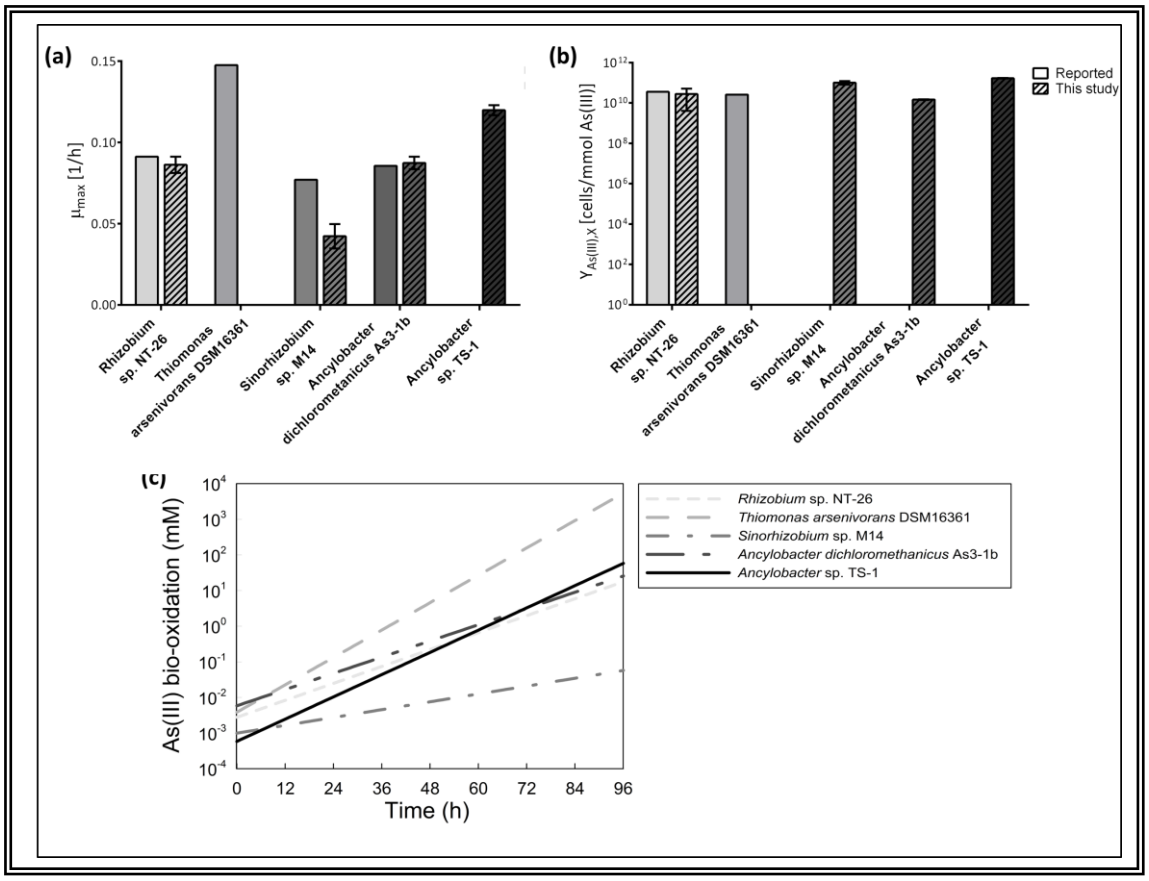

Figure 2- 4: The kinetic parameters and As(III) oxidation of CAOs.(a) μ_{max} and (b)$Y_{As(III),X}$ reported and estimated in this study. (c) As(III) oxidation by CAOs calculated with initial biomass concentration of 10^5 cells mL^{-1}, and the kinetic parameters estimated in this study, with the exception of *Rhizobium* sp. NT-26 and *Thiomonas arsenivorans* DSM1636, because not significate $Y_{As(III),X}$ and not reported values for the estimation, respectively.

The difference between yields of CAOs (Figure 2-4b) could be due to metabolic differences, cell size variances, and the experimental factors previously mentioned in this article. A useful way to compare CAOs is by estimating the As(III) bio-oxidation over time (equation 8). To determinate the equation 8, biomass (X) in equation 1 was integrated in time (considering constant μ, and equal to μ_{max}) and, replaced in equation 2. Then, As(III) bio-oxidation was obtained by the subsequent integration in time of equation 2. This was calculated by normalizing the initial biomass concentration (X_0) during the exponential phase (Figure 2-4c). The *Ancylobacter* sp. TS-1 bio-oxidation was in the range of the discussed CAOs (with

the exception of *Thiomonas arsenivorans* DSM16361). Among this group, TS-1 shows the second highest As(III) bio-oxidation rate at 72 h due to its $\mu_{max.}$, this result not only positions *Ancylobacter* sp. TS-1 as a good candidate to be selected as a model CAO, but also support the importance of its role in biogeochemical processes in natural and engineered systems. An example of the possible role of *Ancylobacter* sp. TS-1 in natural As(III) oxidation is that *Ancylobacter* sp. TS-1 was isolated from a site where natural As attenuation have been reported for microorganisms closed to *Thiobacillus* sp. S1 (Leiva et al., 2014). For the similitude between *Thiobacillus* sp. S1 and *Ancylobacter* genus is probable that TS-1 plays a significant role in As(III) oxidation of this site.

$$As(III)_{bio-oxidation} = \frac{X_0\, e^{\mu max\, t}}{Y_{As(III),X}} \quad \text{(Equation 8)}$$

2.5 Conclusions

A new microorganism, *Ancylobacter* sp. TS-1, was isolated from a hydrothermal source of northern Chile. Molecular, kinetic, and phylogenetic analysis showed that *Ancylobacter* sp. TS-1 is a chemolithoautotrophic arsenite oxidizing microorganism closely related to the *Ancylobacter* genus. Furthermore, this study presents a coupled fitting using the weighted least square method as a methodology to obtain the kinetic parameters (μ_{max} and $Y_{As(III),X}$). Considering the μ_{max} and $Y_{As(III),X}$ obtained with a coupled fit for CAOs, the specific As(III) bio-oxidation of *Ancylobacter* sp. TS-1 is in the range of previously reported CAOs. Therefore, *Ancylobacter* sp. TS-1 may be a potential catalyst for As(III) oxidation in natural and engineered systems, being this study the first and fundamental stage for its application.

2.6 Acknowledgements

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We thanks to Dr. Álvaro Videla from The Department of Mining Engineering at The Pontificia Universidad Católica de Chile, for ICP-MS measurements. We also express gratitude to Paulina M. Torres and Rodrigo A. Santibañez, both from The Department of Chemical and Bioprocess Engineering at The Pontificia Universidad Católica de Chile, for assistance in dynamic modeling and vector cloning, respectively.

3 A NEW ELECTROCHEMICALLY ACTIVE CHEMOLITHOAUTOTROPHIC ARSENIC OXIDIZING BACTERIUM *ANCYLOBACTER* SP. TS-1

3.1 Abstract

The study of electrochemically active microorganisms (EAMs) has revealed the metabolic versatility of chemolithoautotrophic microorganisms in bioelectrochemical systems (BESs). However, direct electron uptake from electrodes to support bacterial growth has been suggested only for the iron oxidizing bacteria *Mariprofundus ferrooxydans* and *Acidithiobacillus ferrooxidans*, and the sulfur oxidizing bacterium *Thiobacillus denitrificans.* To our knowledge, chemolithoautotrophic arsenite oxidizers (CAOs) have not been reported as EAM. This paper studies the electrochemical activity of *Ancylobacter* sp. TS-1, a new CAO. The electrochemical capacity of TS-1 was evidenced by linear sweep voltammetry tests, revealing a peak of cathodic current dependent on biomass concentration at -500 mV (vs. Ag/AgCl, at pH 7.2); and by chronoamperometry, showing an increase in the cathodic current over time. Cathodic currents obtained for TS-1 (9.5 ± 2.4 μA cm^{-2}) are comparable with values previously reported for *A. ferrooxidans* (~27 μA cm^{-2}) and *M. ferrooxydans* (~8 μA cm^{-2}). Scanning electron micrographs show TS-1 cells on electrodes during the chronoamperometry, suggesting the development of a TS-1 biofilm using the cathode as only electron donor. Hence, TS-1 not only enlarges the list of known EAMs, expanding this phenotype to the *Alphaproteobacteria* class, but also paves the way for novel BES applications on arsenic electro-bioaugmentation and bioremediation.

Keywords: electrochemically active microorganism (EAM), bioelectrochemical systems, biocathode, chemolithoautotrophic arsenite oxidizer (CAO).

3.2 Introduction

Electrochemically active microorganisms (EAMs) have the capacity to exchange electrons with a conductive surface (electrodes) in bioelectrochemical systems (BESs). Chemolithoautotrophic microorganisms are good candidates to be classified as EAM; because they could replace their natural electron donors (such as FeS_2) by electrodes while they fix inorganic carbon (Carbajosa et al., 2010). These EAMs can be found in metal-rich contaminated environments such as soils and sediments. If they are used in BESs, EAMs can modify biogeochemical processes that control the mobilization and fate of contaminants (Tandukar et al., 2009).

Recent studies on EAMs have revealed the metabolic versatility of chemolithoautotrophic microorganisms in biocathodes, being principally oxidizers of iron, sulfur, and hydrogen. BES with iron oxidizing microorganisms, such as *Mariprofundus ferrooxydans* PV-1 (Summers et al., 2013), and *Acidithiobacillus ferrooxidans* (Carbajosa et al., 2010) have been studied as a strategy to enrich and culture, where Fe^{2+} is replaced by an electrode in a BES. The sulfur oxidizing microorganisms *Thiobacillus denitrificans* and *Pseudomonas denitrificans* can remove nitrate in a BES (Parvanova-Mancheva and Beschkov, 2009; Pous et al., 2014; Yu et al., 2015). Some hydrogen oxidizers have been used in biocathodes for their capacity of treating contaminants, including chromium (VI) (Tandukar et al., 2009), perchlorate (Shea et al., 2008), sulfate (Su et al., 2012), and nitrate (Mousavi et al., 2012), among others. In 2014, Webster *et al.* published a study of a genetically modified strain of *Shewanella oneidensis*, which produces increased current in response to arsenic (Webster et al., 2014). However, to our knowledge, wild type strains with the capacity of arsenic oxidation have not been reported as EAM.

Ancylobacter sp. TS-1 is a recently isolated microorganism with the capacity of chemolithoautotrophic arsenic oxidation (chapter 2). TS-1 was isolated from a site with high iron, sulfur, and arsenic content in a hydrothermal source in Northern Chile (Leiva et al., 2014). The purpose of this study is to evaluate the electrochemical activity of TS-1 in a BES.

3.3 Materials and methods

The batch culture and abiotic controls were conducted in a chemical defined medium (CDM) with 2.5 mM of arsenite. CDM was prepared using (per liter): 30 mg Na_2SO_4; 100 mg KCl, 80 mg $MgCl_2$; 100 mg $CaCl_2 \cdot 2H_2O$; 100 mg $(NH_4)_2SO_4$; 840 mg $NaHCO_3$, trace elements (2012), and vitamins (Santini et al., 2000). The pH was adjusted to 7.2 with phosphate buffer (final concentration per liter of 750 mg KH_2PO_4; 3,950 mg $Na_2HPO_4 \cdot 7H_2O$; and HCl). Cultures were performed in duplicate with 100 mL of liquid medium in flasks of 250 mL, at 34 °C and 200 rpm. Microbial growth was quantified through direct cell count. Bacterial cells were stained with 0.002 % acridine orange solution (Lunau et al., 2005) and observed in a Neubauer chamber with a fluorescence microscope (Olympus CX31 microscope; Olympus, Japan) at 40X. Total arsenic was measured by using an ICP-MS (Elan 9000, Perkin-Elmer). Arsenite was measured using cartridges of speciation (Metalsoft Center) (Meng and Wang, 1998) and ICP-MS.

A guide tree of multiple sequence alignment (MSA) was done with biocathodic EAM previously reported in different studies and the bacterial isolate presented in this article (de Campos Rodrigues and Rosenbaum, 2014; Huang et al., 2011; Rosenbaum et al., 2011; Summers et al., 2013; Xafenias et al., 2013). The MSA was performed with the MAFFT algorithm (Katoh et al., 2002) using the T-COFFEE program (Notredame et al., 2000). The alignment was automatically cured with Gblocks v0.91b, with recommended parameters for rDNA (Castresana,

2000). The tree was constructed with the Neighbors Joining algorithm using MEGA 6 (2013).

The electrochemical activity of TS-1 was studied using a 40 mL (effective volume) three-electrode cells with graphite rods (projected area of 3.4 cm^2) as working electrode, a counter electrodes of platinum (CHI115, CH Instruments Inc.), and silver/silver chloride (Ag/AgCl; 3M KCl; 0.21 V vs. SHE) reference electrodes (CHI111, CH Instruments Inc.). The electrochemical measurements were performed under dark conditions, and all experiments were performed in duplicate.

A TS-1 culture was prepared for linear sweep voltammetry (LSV) tests using Luria-Broth (LB) [15.5 g L^{-1}] with 1 mM of arsenite at 30 °C and 120 rpm, for five days. LB was used to obtain optical density over 0.4 at 600 nm. The bacteria were washed three times with PBS and centrifuged at 5,000 g for 10 min. Aliquots were used to inoculate the electrochemical cells to reach the desired optical density. LSVs were performed from 0.2 to -1.0 V (vs. Ag/AgCl) at 1 mV s^{-1} using a potentiostat Reference 600 (Gamry Instrument Inc.). Experiments were conducted in PBS (10 mM) using two different bacterial concentrations 1X (corresponding to an optical density at 600 nm of 0.4) and 10X. Abiotic controls were performed using (i) only PBS, (ii) PBS with arsenite (1 mM), (iii) PBS with arsenate (1 mM), and (iv) PBS with LB (15.5 g L^{-1}).

A TS-1 culture was prepared using a CDM with arsenite 2.5 mM, at 30 °C and 120 rpm for 10 days. The bacterial cells were washed 3 times with CDM and centrifuged at 5,000 g for 10 min. Chronoamperometry analyses were performed with initial TS-1 concentration of $5 \cdot 10^8$ cells mL^{-1} at a poised potential of -500 mV (vs. Ag/AgCl) using a multichannel potentiostat Interface 1000 (Gamry Instrument Inc.), in CDM without arsenite (pH 7.2; ~24 °C), with horizontal agitation (100 rpm), for three days of operation. During the chronoamperometry test, CDM was used as a culture medium to accomplish nutritional requirements for supporting

bacterial growth without arsenite and using the cathode electrode as only electron donor. Two control types were performed: (i) abiotic control without TS-1; and (ii) negative control, not connected to the potentiostat.

The batch cultures and the graphite rod electrodes used in the chronoamperometry test were visualized by using scanning electron microscopy. A JEOL JSM-IT300LV was used for microscopic analyses. The samples were fixed with 2.5% glutaraldehyde in cacodylate 10 mM, dehydrated with ethanol, water was removed by using critical point drying, and coated with graphite before microscopic observation (Fischer et al., 2013).

3.4 Results and discussion

TS-1 was characterized as a Gram-negative small rod (~0.5 by 1.0 µm) bacterium. After 48 hours of batch culture, TS-1 shows 84(±8)% of arsenite oxidation and the cell concentration increased two orders of magnitude from 3.02(±0.37)·106 to 3.28(±0.84)·108 cells mL^{-1}. In the same culture period, but in a batch culture medium without arsenite, the growth was from 1.58(±0.02)·106 to 1.16(±0.03)·107 cells mL^{-1}, indicating its capacity for oligotrophic growth (mainly due to trace elements present in the culture medium), and the positive effect of arsenite on the growth rate.

The guide tree of the MSA (Figure 3-1) shows the phylogenetic diversity of EAMs identified in biocathodes, being dominated for the Proteobacteria phylum. Considering the metabolism of biocathodic EAMs, only a part of this selected group has reported the chemolithoautotrophic capacity (bold font), and only a few studies have experimentally demonstrated biofilm development together with an increases in the cathodic current in an electrochemical cell using the cathode electrode as only electron donor (de Campos Rodrigues and Rosenbaum, 2014; Summers et al., 2013). Indeed, according to our knowledge and following Figure

3-1, this capacity has been reported only for the iron oxidizing bacteria *M. ferrooxydans* (*Zetaproteobacteria*) and *A. ferrooxidans* (*Gammaproteobacteria*), and for the sulfur oxidizing bacteria *T. denitrificans* (*Betaproteobacteria*).

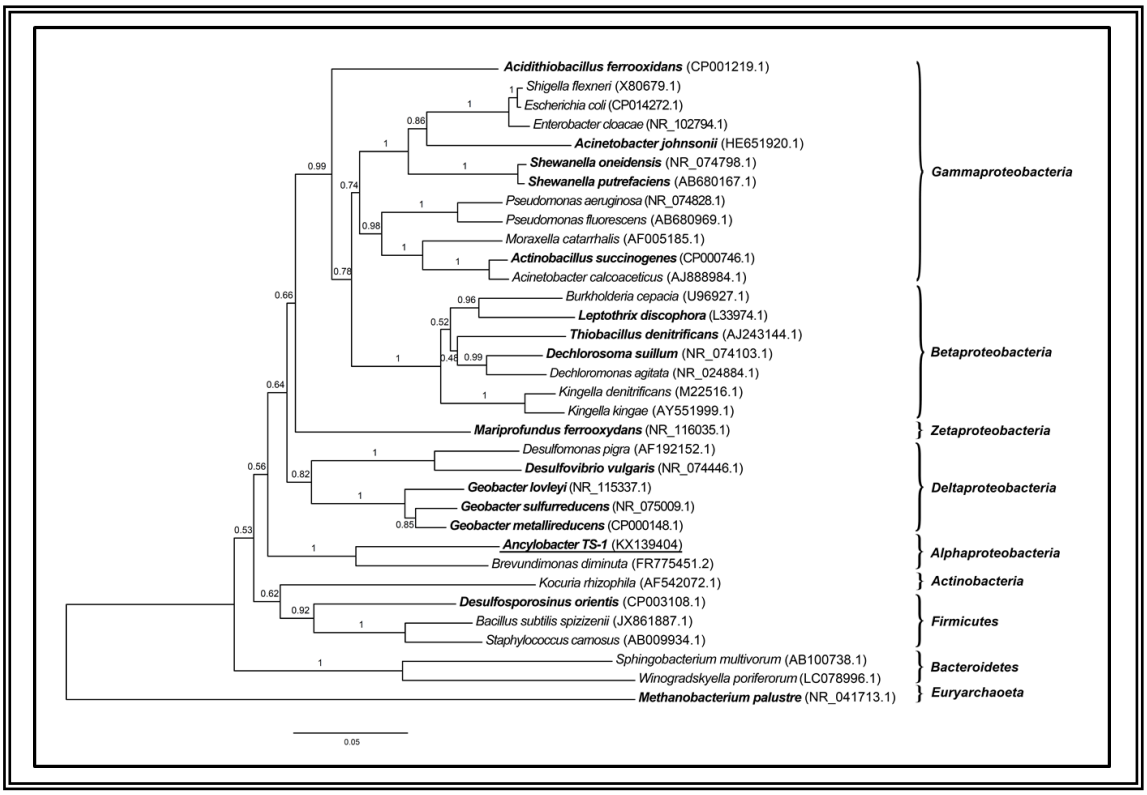

Figure 3- 1: The guide tree of MSA with biocathodic EAMs. The MSA was performed with MAFFT, and the guide tree was conducted with The Neighbors Joining Algorithm (1213 bp). EAMs with chemolithoautotrophic capacity are in bold font. The percentage of samples that recovered specific clades on this topology represents that clade's posterior probability. The bars represent the percentage of substitutions; the numbers at the branch show the posterior probability of branches.

The LSV and chronoamperometry analyses support the electrochemical capacity of TS-1 to catalyze electron transfer from electrodes to the culture medium. The LSV test provides preliminary information regarding the electrochemical activity of TS-1 without the necessity to culture microorganisms on the electrode. Figure 3-2a shows a clear peak at -500 mV (vs. Ag/AgCl) suggesting a cathodic catalysis of electron transfer. The LSV tests suggest a direct relation between biomass and

current, through the intensification in the observed current density peak with an increase in the concentration of biomass from 1X to 10X. In abiotic controls, no significant peaks were observed (Figure 3-2a and B-1) and the current densities were similar to the previously reported for abiotic controls (Summers et al., 2013). Interestingly, the current peak observed in the LSV test at -500 mV (~ 130 mV vs. SHE at pH 7.2) is close to arsenite oxidase enzymes, 204 mV (vs. SHE) for *Rhizobium* sp. NT-26 (Kalimuthu et al., 2014), and 215 mV (vs. SHE) for *Alcaligenes faecalis* (Hoke et al., 2004), both at pH 7.2. Considering dissolved oxygen as the final electron acceptor of the used BES, the overpotential of the system for oxygen reduction was about +680 mV (vs. SHE at pH 7.2). This value is less than the reported potential for oxygen reduction using a graphite rod electrode (without any chemical catalyzer), estimated in over +900 mV (vs. SHE at pH 7.2) (Freguia et al., 2010). These results suggest that TS-1 is acting as biocatalyst by reducing the overpotential for oxygen reduction. In the abiotic LSV controls, not significative peaks were observed, indicating that compounds of the culture media and PBS are not responsible for the observed peak.

The chronoamperometry confirms that TS-1 is catalyzing the cathodic reaction, probably due to bacterial growth and settlement on the cathode surface. Over three days of operation, the cathodic current density increased in 9.5(± 2.4) μA cm^{-2} (Figure 3-2b). This value has a similar magnitude to the increase reported for *M. ferrooxydans* PV-1 (~8 μA cm^{-2}) (Summers et al., 2013) and *A. ferrooxidans* (~27 μA cm^{-2}) (de Campos Rodrigues and Rosenbaum, 2014), after three days of BES incubation using graphite rod electrodes as cathodes; and the increase reported for *T. denitrificans* (~3 μA cm^{-2}) (Yu et al., 2015), after 15 days in BES with carbon cloth as cathode.

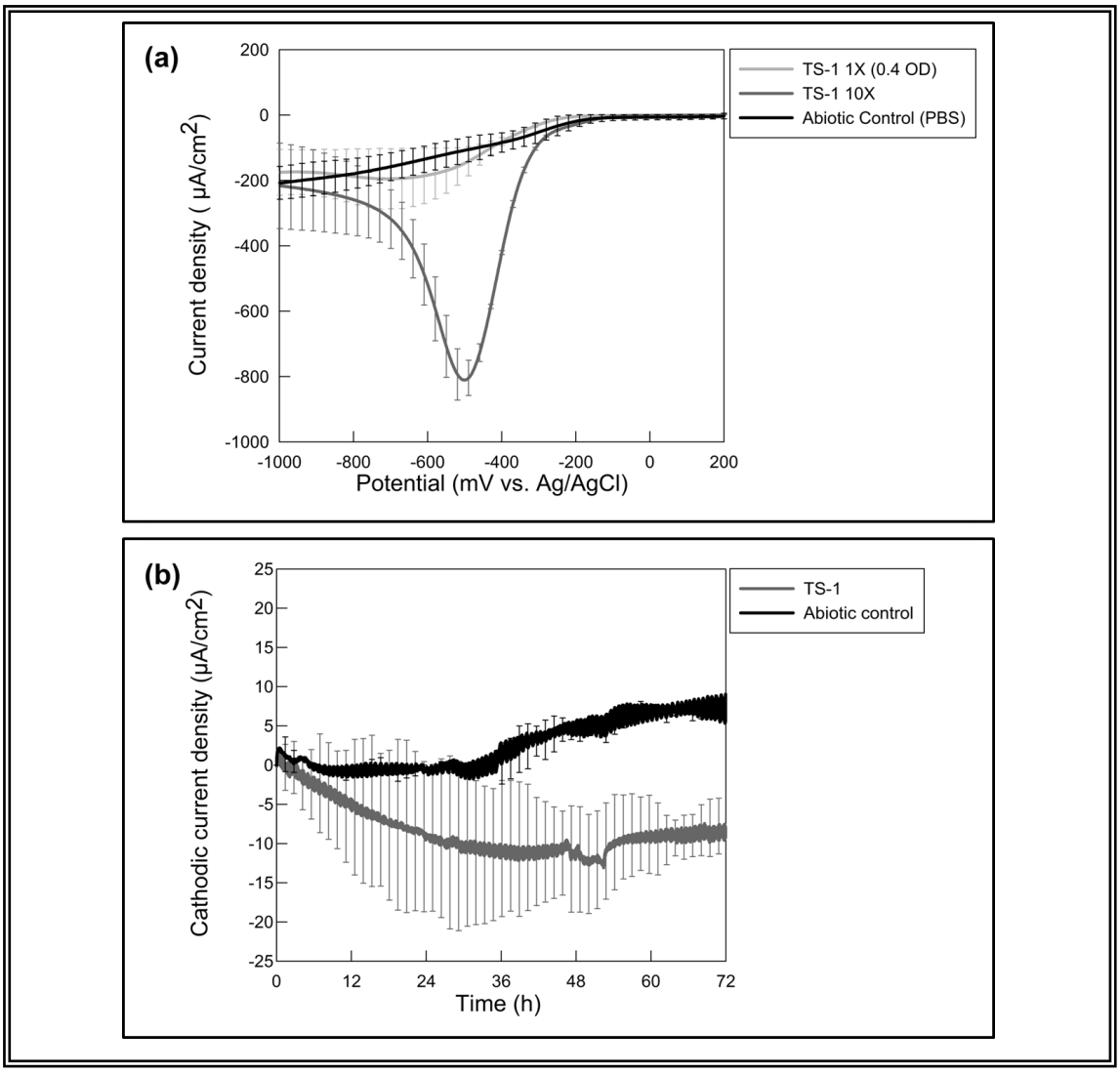

Figure 3- 2: Electrochemical evaluation of *Ancylobacter* sp. TS-1. (a) The LSV tests suggest the electrochemical activity of *Ancylobacter* sp. TS-1. Comparison between two different cellular concentrations (1X and 10X). The error bars represent the standard deviation (n=2). (b) The chronoamperometry tests of *Ancylobacter* sp. TS-1 at -500 mV. The cathodic current density was calculated by the subtraction between the measured current over time and the initial measured current and, then it was normalized by the projected area of the electrode. The experiments (biotic and abiotic controls) were performed in CDM, at ~24 °C and 100 rpm. The experiments conducted with an initial cell concentration of $5 \cdot 10^8$ cells mL^{-1}. The error bars represent the standard deviation (n=2).

Assuming that the specific electrode bio-oxidation rate is similar to the specific arsenite bio-oxidation rate of TS-1, estimated in chemolithoautotrophic cultures as

$7 \cdot 10^{-13}$ mmol arsenite $cell^{-1}$ h^{-1} ($1.4 \cdot 10^{-12}$ mmol e- $cell^{-1}$ h^{-1}), ~$2 \cdot 10^{5}$ cells cm^{-2} are needed to produce the observed increase in current density. This number is reasonable, considering the initial cell concentration in solution ($5 \cdot 10^{8}$ cells mL^{-1}). In contrast, the abiotic controls show decreases in cathodic current over time of +8.5(±2.0) µA cm^{-2} (Figure 3-2b). The decline in the cathodic current density is probably due to the consumption and not regeneration of trace chemical catalysts present in the medium. Summers *et al.* reported a similar behavior using the same electrode material (Summers et al., 2013). The observed difference between the initial current density measured in the chronoamperometry could be due to differences in the effective area of the electrodes, produced by a variation on electrode porosity.

The scanning electron micrographs show more bacterial cells on the graphite rod electrodes connected to the potentiostat after the chronoamperometry test with TS-1 (Figure 3-3a) than the electrode not connected to a potentiostat but cultured in the same electrochemical cell and during the same period of time (Figure 3-3b). The abiotic control (Figure 3-3c) does not show significant features over the electrode surface. Figure 3-3d shows a magnification of Figure 3-3a, revealing the early biofilm formation observed for the chronoamperometry test with TS-1. Cell agglomeration and cell division were visualized suggesting that the biofilm is growing on the electrode surface. Similar morphology and size were observed for bacteria grown in the cathodic biofilm (Figure 3-3d) and in batch liquid cultures using arsenic as electron donor (Figure 3-3e). Hence, this result enforces the idea of a biofilm developed over connected cathodes and electron uptake to support growth.

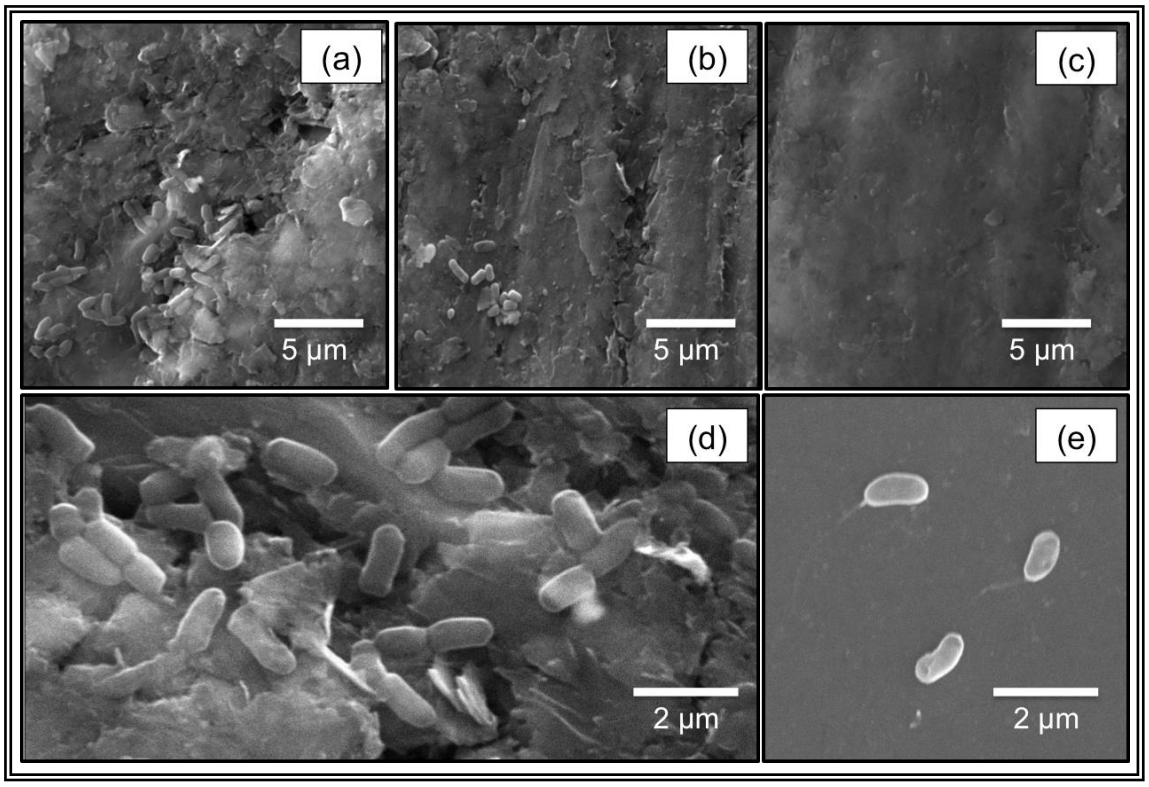

Figure 3- 3: The scanning electron micrographs of *Ancylobacter* sp. TS-1 biofilms developed on graphite electrodes (GE) and batch cultures. (a) GE tested in chronoamperometry with TS-1. (b) The GE tested in electrochemical cells not connected to the potentiostat with TS-1. (c) The GE tested in chronoamperometry without TS-1. (d) Magnification of biofilm formed on (a) showing bacterial growth and cell division. (e) TS-1 cells cultured in a liquid medium using arsenite as electron donor.

3.5 Conclusions

The experimental analyses indicate that the chemolithoautotrophic arsenite oxidizing microorganism *Ancylobacter* sp. TS-1 can catalyze cathodic reactions in a BES. The results of this research suggest that TS-1 not only enlarges the list of known EAM, expanding this phenotype to the *Alphaproteobacteria* class, but also could be the first reported arsenic oxidizer EAM. This finding could expand BES applications for arsenic electro-bioaugmentation and bioremediation in natural and bioengineered environments. Specific analyses are needed to understand the mechanism of this catalysis and the capacity of TS-1 to grow using the electrode as

energy source. Further research would be aimed to study the inhibition of the electron transport chain of TS-1 or detailed electrochemical analyses at cell scale.

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4 CONCLUSIONS AND PERSPECTIVES

A new bacterium, *Ancylobacter* sp. TS-1, was isolated from a hydrothermal source in northern Chile, where previously biological arsenite oxidation was described. The chemolithoautotrophic arsenite oxidation capacity of TS-1 was supported and demonstrated by the combination of molecular techniques, direct evidence, mathematical modeling, and bioinformatics tools. In addition, the electrochemical tests supported the hypothesis that *Ancylobacter* sp. TS-1 could be the first known As(III) oxidizer EAM.

The kinetic parameters of TS-1 were useful to understand and support the reported arsenite oxidation observed in the hydrothermal source used as inoculum (Leiva et al., 2014). TS-1 showed a specific bio-oxidation rate (μ_{max} $Y_{As(III),X}^{-1}$) in the chemolithoautotrophic and mixotrophic cultures of 7.0 and 9.4 10^{-13} mmol As(III) $cell^{-1}$ h^{-1}, respectively. Considering the reported As removal (~ 8 µM) and the residence time (1 min) in the hydrothermal source (Leiva et al., 2014), the number of TS-1 cells necessary to account the reported biological attenuation is $5 \cdot 10^8$ and $7 \cdot 10^8$ cells mL^{-1}, considering the chemolithoautotrophic and the mixotrophic specific bio-oxidation rates, respectively. These cell concentrations are in the range of the total microorganisms present in similar environmental soils, estimated between 10^8 to 10^9 cells mL^{-1} (Edwards et al., 1999). In consequence, the kinetic parameters obtained by the mathematical model implemented in this thesis support the reported biogeochemical process of dissolved As attenuation.

In addition, the electrochemical analyses suggest that the electrochemically active capacity of TS-1 for using an electrode as electron source could be utilized as a bioaugmentation tool to strengthen the arsenite oxidation in natural environments. Further research is needed to evaluate the applicability of this innovative bioremediation strategy in contaminated water sources.

Considering the chemolithoautotrophic arsenite oxidation capacity of TS-1, this bacterium has the potential to be used in the treatment of As in drinking water. While the use of bacteria have an acceptance barrier, because the goal of drinking water treatment is clean and remove bacteria; the biologically active filters have shown the arsenite oxidation capacity in drinking water treatment (Lytle et al., 2007; Zhu et al., 2010). To obtain arsenite oxidation rates equivalent to conventional chemical agents (e.g. chlorine, hydrogen peroxide, and permanganate) cell concentrations between 10^5 to 10^{10} cells mL^{-1} are needed (Table 4-1). In this thesis, the cells concentrations achieved in oligotrophic, chemolithoautotrophic, and mixotrophic cultures of TS-1 are about 10^7, 10^8, and 10^9 cells mL^{-1}, respectively. According to the results, theoretically, TS-1 could provide an oxidation rate equivalent to the reported for chlorine, ozone, hydrogen peroxide, and permanganate. The cell concentration obtained in chemolithoautotrophic culture could oxidize 50µg L^{-1} in less than one minute. However, more experimental analyses of TS-1 are required, such as the study of growth and As oxidation in drinking water with typical As concentration.

Table 4- 1: Arsenite oxidation using conventional agents and the equivalent cell concentration estimated to obtain similar oxidation rates. Cell concentrations were calculated using the specific arsenic bio-oxidation in chemolithoautotrophic culture, 8.2 $\cdot 10^{-13}$ mmol As(III) cell^{-1} h^{-1}.

Oxidation method	Type of water	Concentration of oxidizing agent added	Concentration of oxidized arsenic	time	Cell concentration (cells mL^{-1}) to obtain similar oxidation rate
Free available chlorine (Dodd et al., 2006)	Real water pH ~ 7	0.1 mg L^{-1}	From 50 µg L^{-1} to < 1 µg L^{-1}	10 s	$3.0 \cdot 10^{8}$
O_3, pure air and oxygen (Kim and Nriagu, 2000)	Groundwater pH (7.6-8.5)	saturated with the O_3	From 50 µg L^{-1} to 1 µg L^{-1}	15 min	$3.3 \cdot 10^{6}$
Hydrogen peroxide (Pettine et al., 1999)	Sea and river water pH 8.3	910 µ mol L^{1}). with 0.01 mol L^{1} NaCl pH 8.3	From 50 µg L^{-1}to 13 µg L^{-1}	260 min	$1.9 \cdot 10^{5}$
Permanganate (Ghurye and Clifford, 2004)	Synthetic Water pH 8.3	0.2 mg L^{-1}	50 µg L^{-1} to < 3 µg L^{-1}	20s	$1.5 \cdot 10^{8}$
FeO_4^{2-} (Lee et al., 2003)	River water pH 7.8	2.0 mg L^{-1}	From 517 µg L^{-1} to below 50 µg L^{-1}	< 1s	$3.0 \cdot 10^{10}$

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A P P E N D I C E S

APPENDIX A: SUPPLEMENTAL MATERIAL OF "A NEW CHEMOLITHOAUTOTROPHIC ARSENIC OXIDIZING MICROORGANISMS ISOLATED FROM A HYDROTHERMAL SOURCE WITH NATURAL ARSENIC ATTENUATION"

Table A- 1: Bacteria with the subunit A of arsenite oxidase gene, reported in (Yamamura and Amachi, 2014) and (Andres and Bertin, 2016).

Metabolism	Phylogenetic affiliation	Strain	Aio gene Acc. No. (Genbank)	Methodology for the determination of arsenite oxidation	Reference
HAO	*Alphaproteobacteria*	*Aminobacter* sp. 86	ABY19334	quantitative	(Quemeneur et al., 2008)
		Sinorhizobium sp. IK-A2	AGC82137	quantitative	(Hamamura et al., 2013)
		Arsenite oxidizing bacterium BEN-5	ABD72612	quantitative	(Santini et al., 2002)
		Agrobacterium tumefaciens 5A	ABB51928	quantitative	(Macur et al., 2004)
		Agrobacterium albertimagni AOL15	WP_006724592	quantitative	(Salmassi et al., 2002)
		Agrobacterium sp. TS45	ACB05955	qualitative	(Cai et al., 2009)
		Ochrobactrum tritici SCII24	ACK38267	qualitative	(Branco et al., 2009)
	Betaproteobacteria	*Achromobacter* sp. NT-10	ABD72610	quantitative	(Santini et al., 2002)
		Alcaligenes sp. YI13H	ABY19322	quantitative	(Quemeneur et al., 2008)
		Alcaligenes sp. T12RB	ABY19321	quantitative	(Quemeneur et al., 2008)
		Achromobacter sp. WA20	ABD72615	not reported	(Inskeep et al., 2007)
		Ralstonia sp. 22	ABY19329	quantitative	(Lieutaud et al., 2010)
		Alcaligenes faecalis	Q7SIF4	quantitative	(Philips and Taylor, 1976)
		Herminiimonas arsenicoxydans ULPAs1	AAN05581	quantitative	(Weeger et al., 1999)
		Thiomonas sp. WJ68	ABY19318	quantitative	(Bryan et al., 2009)
		Thiomonas sp. NO115	ABY19317	quantitative	(Coupland et al., 2004)
		Hydrogenophaga sp. str. NT-14	ABD72609	quantitative	(Santini et al., 2002)
		Hydrogenophaga WA13	ABD72613	not reported	(Inskeep et al., 2007)

Table A-1 continued.

Metabolism	Phylogenetic affiliation	Strain	Aio gene Acc. No. (Genbank)	Methodology for the determination of arsenite oxidation	Reference
HAO	*Betaproteobacteria*	*Limnobacter* sp.83	ABY19325	quantitative	(Quemeneur et al., 2008)
		Polaromonas sp.GM1	ABW84260	quantitative	(Osborne et al., 2010)
		Variovorax sp. 4-2	ABY19319	quantitative	(Battaglia-Brunet et al., 2006)
		Variovorax sp. MM-1	AFN80467	quantitative	(Bahar et al., 2013)
		Leptothrix sp. S1-1	ABY19320	quantitative	(Battaglia-Brunet et al., 2006)
		Ralstonia sp. R229	CCA82914	quantitative	(Quemeneur et al., 2008)
	Gammaproteobacteria	*Marinobacter santoriniensis* NKSG1	ACF09051	quantitative	(Handley et al., 2009)
		Pseudomonas sp. 72	ABY19330	quantitative	(Quemeneur et al., 2008)
		Pseudomonas sp. 89	ABY19328	quantitative	(Quemeneur et al., 2008)
		Pseudomonas sp. 1	ABY19326	quantitative	(Quemeneur et al., 2008)
		Pseudomonas sp. 73	ABY19327	quantitative	(Quemeneur et al., 2008)
		Acidovorax sp. 75	ABY19324	quantitative	(Quemeneur et al., 2008)
		Acinetobacter sp. WA19	ABD72614	Not reported	(Inskeep et al., 2007)
		Acinetobacter sp. 33	ABY19331	quantitative	(Quemeneur et al., 2008)
		Pseudomonas sp. D2OHCJ	ABY19332	quantitative	(Quemeneur et al., 2008)
		Pseudomonas sp. 46	ABY19333	quantitative	(Quemeneur et al., 2008)
		Stenotrophomonas sp. MM-7	AFN80468	quantitative	(Bahar et al., 2012)
		Halomonas sp. HAL1	EHA13972	not reported	(Lin et al., 2012)
	Deinococcus-Thermus	*Thermus thermophilus* HB8	BAD71923	quantitative	(Gihring et al., 2001)
		Thermus sp. HR13	ABB17184	quantitative	(Gihring and Banfield, 2001)
		Thermus aquaticus Y51MC23	EED09253	quantitative	(Gihring et al., 2001)
CAO	*Alphaproteobacteria*	*Ancylobacter* sp. OL1	ABJ55853 ABJ55852	quantitative	(Garcia-Dominguez et al., 2008)
		Ancylobacter dichloromethanicus As3-1b	CBY79896	quantitative	(Andreoni et al., 2012)

Table A-1 continued.

Metabolism	Phylogenetic affiliation	Strain	Aio gene Acc. No. (Genbank)	Methodology for the determination of arsenite oxidation	Reference
CAO	*Alphaproteobacteria*	*Rhizobium sp*. NT-26	AAR05656	quantitative	(Santini et al., 2000)
		Bosea sp. WAO	ABJ55855	quantitative	(Rhine et al., 2008)
		Sinorhizobium sp. DAO10	ABJ55850	quantitative	(Rhine et al., 2006)
		Sinorhizobium sp. M14	WP_015647692	quantitative	(Drewniak et al., 2008)
CAO	*Betaproteobacteria*	*Thiomonas arsenivorans* DSM16361	ABY19316 ADE33058 ADE33057	quantitative	(Battaglia-Brunet et al., 2011)
		Burkholderia sp. YI019A	ABY19323	quantitative	(Quemeneur et al., 2008)
		Thiobacillus sp. S1	ABJ55851	quantitative	(Garcia-Dominguez et al., 2008)
		Hydrogenophaga sp. CL3	ABJ55854	quantitative	(Garcia-Dominguez et al., 2008)
		Thiomonas arsenitoxidans 3As	WP_013107016	quantitative	(Duquesne et al., 2008)
		Thiomonas intermedia K12	WP_013124474	quantitative	(Freel et al., 2015)
Arsenite oxidizer without reported metabolism	*Alphaproteobacteria*	*Methylobacterium* sp. S47	ADF47200	qualitative	(Sultana et al., 2012)
	Betaproteobacteria	*Alcaligenes* sp. S46	ADF47192	qualitative	(Sultana et al., 2012)
		Achromobacter arsenitoxydans SY8	ABP63660	qualitative	(Cai et al., 2009)
		Burkholderia sp. S31R	ADF47190	qualitative	(Sultana et al., 2012)
		Burkholderia sp. S32	ADF47191	qualitative	(Sultana et al., 2012)
	Gammaproteobacteria	*Halomonas* sp. A3H3	CDG56105	not reported	(Koechler et al., 2013)
Not Arsenite oxidizer	*Alphaproteobacteria*	*Bosea* sp. S41RM2	ADF47199	qualitative	(Sultana et al., 2012)
		Bosea sp. S41RM1	ADF47198	qualitative	(Sultana et al., 2012)
	Betaproteobacteria	*Burkholderia* sp. S232	ADF47189	qualitative	(Sultana et al., 2012)
		Burkholderia sp. S222	ADF47188	qualitative	(Sultana et al., 2012)
	Gammaproteobacteria	*Pseudomonas stutzeri TS44*	ACB05943	qualitative	(Shakya and Pradhan, 2009)
	Actinobacteria	*Micromonospora* sp. X14	CCD32987	quantitative	(Delavat et al., 2012)
Not determined	*Alphaproteobacteria*	*Caulobacter segnis* ATCC 21756	WP_013079615	N.A.	Unpublished

Table A-1 continued.

Metabolism	Phylogenetic affiliation	Strain	Aio gene Acc. No. (Genbank)	Methodology for the determination of arsenite oxidation	Reference
Not determined	*Alphaproteobacteria*	*Acidiphilium multivorum* AIU301	GAN73887	N.A.	Unpublished
		Rhodobacter sp. SW2	WP_008027033	N.A.	Unpublished
		Pseudovibrio sp. FO-BEG1	AEV39545	N.A.	(Bondarev et al., 2013)
		Roseovarius sp. 217	WP_009817403	N.A.	Unpublished
		Starkeya novella DSM 506	WP_013166106	N.A.	Unpublished
		Xanthobacter autotrophicus Py2	WP_012115922	N.A.	Unpublished
		Polymorphum gilvum SL003B-26A1T	YP_004304060	N.A.	(Li et al., 2011)
		Bosea sp. 43AGV	AEL22179	N.A.	(Heinrich-Salmeron et al., 2011)
		Bosea sp. 7AGV	AEL22177	N.A.	(Heinrich-Salmeron et al., 2011)
		Bosea sp. 8AGV	AEL22178	N.A.	(Heinrich-Salmeron et al., 2011)
		Nitrobacter hamburgensis	YP_571843	N.A.	(Starkenburg et al., 2008)
		Methylocystis sp. SC2	CCJ06851	N.A.	(Dam et al., 2012)
		Mesorhizobium sp. DM1	ABD35887	N.A.	(Inskeep et al., 2007)
		Ensifer adhaerens	CBY79895	N.A.	(Andreoni et al., 2012)
		Roseobacter litoralis Och 149	YP_004691143	N.A.	(Cai et al., 2013)
		Sulfospirillum barnesii SES-3	WP_014770544	N.A.	Unpublished
	Betaproteobacteria	*Rhodoferax ferrireducens* T118	WP_011465357	N.A.	Unpublished
		Acidovorax sp. NO-1	WP_008904937	N.A.	(Slyemi et al., 2013)
		Burkholderia oklahomensis C6786	WP_010121987	N.A.	Unpublished
		Burkholderia multivorans ATCC 17616	WP_012218063 WP_012218087	N.A.	Unpublished
		Achromobacter sp. 40AGIII	AEL22195	N.A.	(Heinrich-Salmeron et al., 2011)
		Variovorax sp. RM1	ABD35886	N.A.	(Inskeep et al., 2007)
	Gammaproteobacteria	*Ralstonia solanacearum* PSI07	YP_003749888	N.A.	(Remenant et al., 2010)
		Ralstonia syzygii R24	CCA86643	N.A.	(Remenant et al., 2010)

Table A-1 continued.

Metabolism	Phylogenetic affiliation	Strain	Aio gene Acc. No. (Genbank)	Methodology for the determination of arsenite oxidation	Reference
Not determined	*Gammaproteobacteria*	*Achromobacter* sp. 38AGIII	AEL22188	N.A.	(Heinrich-Salmeron et al., 2011)
		Vibrio splendidus LGP32	CAV26302	N.A.	Unpublished
		Halomonas boliviensis sp. LC1	WP_008959505	N.A.	Unpublished
		Acinetobacter sp.18AAV	AEL22197	N.A.	(Heinrich-Salmeron et al., 2011)
		Acinetobacter sp.16AAV	AEL22196	N.A.	(Heinrich-Salmeron et al., 2011)
		Pseudomonas sp.5AAV	AEL22194	N.A.	(Heinrich-Salmeron et al., 2011)
		Pseudomonas sp. 15AGV	AEL22189	N.A.	(Heinrich-Salmeron et al., 2011)
		Pseudomonas sp. 20AAIII	AEL22181	N.A.	(Heinrich-Salmeron et al., 2011)
		Pseudomonas sp. 25AAIII	AEL22184 AEL22187	N.A.	(Heinrich-Salmeron et al., 2011)
		Pseudomonas sp. 24AGIII	AEL22186	N.A.	(Heinrich-Salmeron et al., 2011)
		Vibrio tasmaniensis LGP32	WP_012600566	N.A.	Unpublished
	Epsilonproteobacteria	*Sulfurospirillum multivorans* DSM 12446	AHJ14346	N.A.	(Goris et al., 2014)
	Bacteroidetes	*Flavobacterium* sp. 19AAV	AEL22198	N.A.	(Heinrich-Salmeron et al., 2011)
		Flavobacterium sp. 16AGV	AEL22190	N.A.	(Heinrich-Salmeron et al., 2011)
		Flavobacterium sp. 18AGV	AEL22191	N.A.	(Heinrich-Salmeron et al., 2011)
	Actinobacteria	*Rhodococcus* sp. 46AAIII	AEL22193	N.A.	(Heinrich-Salmeron et al., 2011)
		Agromyces sp. 44AGV	AEL22180	N.A.	(Heinrich-Salmeron et al., 2011)
	Firmicutes	*Bacillus* sp. 21AAIII	AEL22182	N.A.	(Heinrich-Salmeron et al., 2011)

Table A-1 continued.

Metabolism	Phylogenetic affiliation	Strain	Aio gene Acc. No. (Genbank)	Methodology for the determination of arsenite oxidation	Reference
Not determined	*Nitrospira*	*Nitrospira defluvii*	YP_003799308	N.A.	(Lücker et al., 2010)
	Deinococcus-Thermus	*Thermus scotoductu* SA-01	ADW22085	N.A.	(Gounder et al., 2011)
	Aquificae	*Chloroflexus aggregans* DSM 9485	YP_002461759	N.A.	Unpublished
		Sulfurihydrogenibium sp. Y04ANG1	ACN59445	N.A.	(Hamamura et al., 2010)
		Sulfurihydrogenibium yellowstonense SS-5	ACN59446	N.A.	(Inskeep et al., 2009)
		Hydrogenobaculum sp. 3684	ACJ04807	N.A.	(Romano et al., 2013)
		Chloroflexus aurantiacus J-10-fl	YP_002569069	N.A.	Unpublished
		Chloroflexus sp. Y-400-fl	YP_002569069	N.A.	Unpublished
		Hydrogenobaculum sp. HO	AGG14649	N.A.	(Romano et al., 2013)
		Hydrogenobaculum sp. SN	AGH92948	N.A.	(Romano et al., 2013)
	Chlorobi	*Chlorobium phaeobacteroides* BS1	YP_001960747	N.A.	Unpublished
		Chlorobium limicola DSM 245	YP_001942454	N.A.	Unpublished
	Crenarchaeota	*Sulfolobus tokodaii str. 7*	BAB67500	N.A.	(Kawarabayasi et al., 2001)
		Pyrobaculum oguniense TE7	AFA38559	N.A.	(Bernick et al., 2012)
		Aeropyrum pernix K1	BAA81573	N.A.	(Muller et al., 2003)
		Aeropyrum camini SY1	BAN91066	N.A.	(Daifuku et al., 2013)
		Cladisphaera lagunensis DSM 15908	AFZ71060* *Small subunit	N.A.	Unpublished
		Pyrobaculum sp. 1860	AET33249	N.A.	(Mardanov et al., 2012)
		Pyrobaculum calidifontis JCM 11548	ABO08790	N.A	(van Lis et al., 2013)
	Euryarchaeota	*Halorubrum* sp. AJ67	WP_048077806	N.A.	(Burguener et al., 2014)

N.A.: Not applicable

Table A- 2: Comparison of duplication time (t_d) and yield of biomass from arsenite ($Y_{As(III),X}$) of CAOs with experimentally demonstrated arsenite oxidation. Reported and estimated parameters are showed. Estimated parameters were calculated considering individual curves of biomass and arsenite plotted in each study.

Microorganism	Culture condition	Reported		Estimated in this study using weighted least square method		
		t_d (h)	$Y_{As(III),X}$ (cells mmol $As(III)^{-1}$)	t_d* (h) (95% confidence)	$Y_{As(III),X}$ (10^{10} cells mmol $As(III)^{-1}$) (95% confidence)	WRMSE
Rhizobium sp. NT-26 (Santini et al., 2000)	Initial pH ~ 8, with vitamins	7.6	$3.6 \cdot 10^{10}$	[7.2-9.1]	[-1,98-7.56]**	X: 0.23 As(III): 0.05
Ancylobacter dichloromethanicus As3-1b (Andreoni et al., 2012)	Initial pH ~ 8, with vitamins	8.1	N.R.P.	[7.3-8.7]	[1.38-1.56]	X: 0.03 As(III): 0.00
Sinorhizobium sp. M14 (Drewniak et al., 2008)	Initial pH ~ 8, without vitamins	9.0	N.R.P.	[12.1-25.5]	[5.90-14.45]	X: 0.36 As(III):0.05
Thiomonas arsenivorans DSM16361 (Battaglia-Brunet et al., 2006)	Initial pH 5.5, with vitamins	4.7	$2.6 \cdot 10^{10}$	N.R.V	N.R.V	N.R.V
Ancylobacter sp. TS-1	Initial pH 7.2 with vitamins	-	-	[5.3-6.9] (exp. 1) [5.6-6.0] (exp. 2) [5.5-6.1] (considering exp. 1 and 2)	[14.84-18.69] (exp. 1) [17.32-17.95] (exp. 2) [16.08-17.73] (considering exp. 1 and 2)	X: 0.08 As(III): 0.24 (exp. 1) X: 0.04 As(III):0.19 (exp. 2) X: 0.10 As(III): 0.25 (considering exp. 1 and 2)

WRMSE: Weighted root mean squared error. Calculated as: $\frac{1}{\sum_{j=1}^{n} z_{mod,j}} \sum_{i=1}^{n} \left(\frac{\sqrt{(z_{mod,i} - z_{exp,i})^2}}{W_{z,i}} \right)$, with z: biomass (X) and arsenite (As(III)), and $w_{z,i}$: weighted, defined in "Materials and method" (Section 2.3.2).

N.R.P: Non-reported parameter

N.R.V: Non-reported values (of biomass and substrate).

(*) t_d calculated as: $\frac{\ln(2)}{\mu_{max}}$

(**) Non-statistically significant parameter (α=0.05)

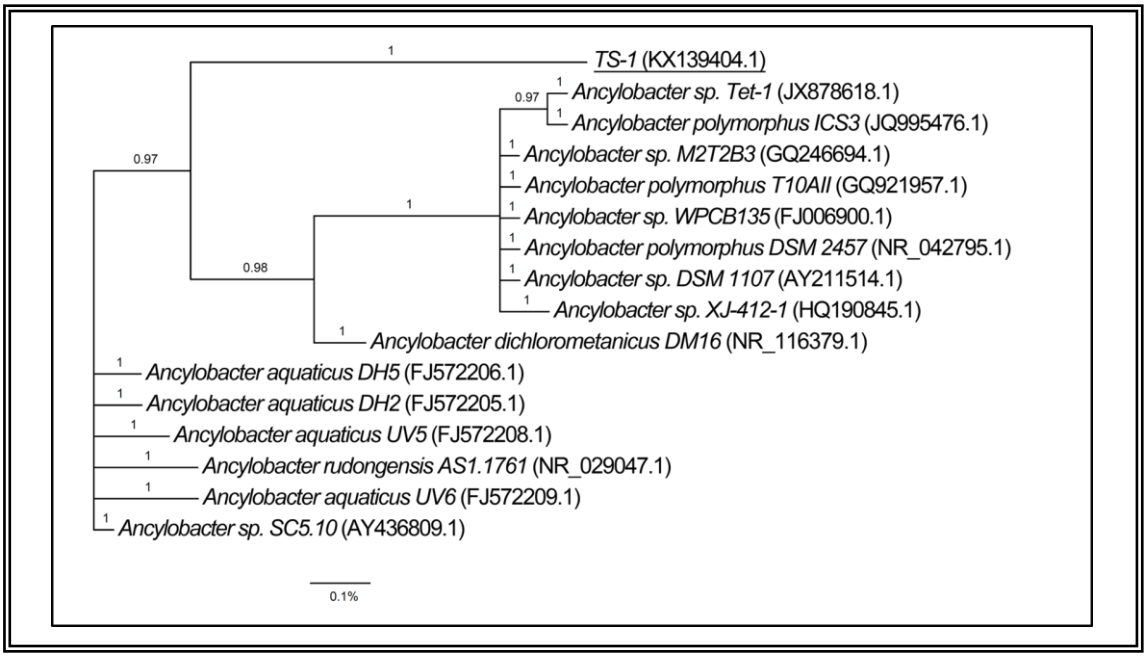

Figure A- 1: Phylogenetic analysis of the 16S rRNA genes of TS-1 and the 15 highest identities of microorganisms. T-COFFEE, MAFFT, and MUSCLE were used for MSA obtaining the same alignment. The best fit of model evolution was obtained with jModeltest v 2.1.7.Trees were constructed with MrBayes v3.2.3, using the evolution model F81+I . MCMC simulations were run with four chains using 1000000 generations sampling at intervals of 250 generations. The first 25% of sampled trees were considered 'burn-in' trees and were discarded prior to the tree reconstruction. The percentage of samples that recovered specific clades on this topology represents that clade's posterior probability. Bars represent the percentage of substitutions; the numbers at the branch show the posterior probability of branches.

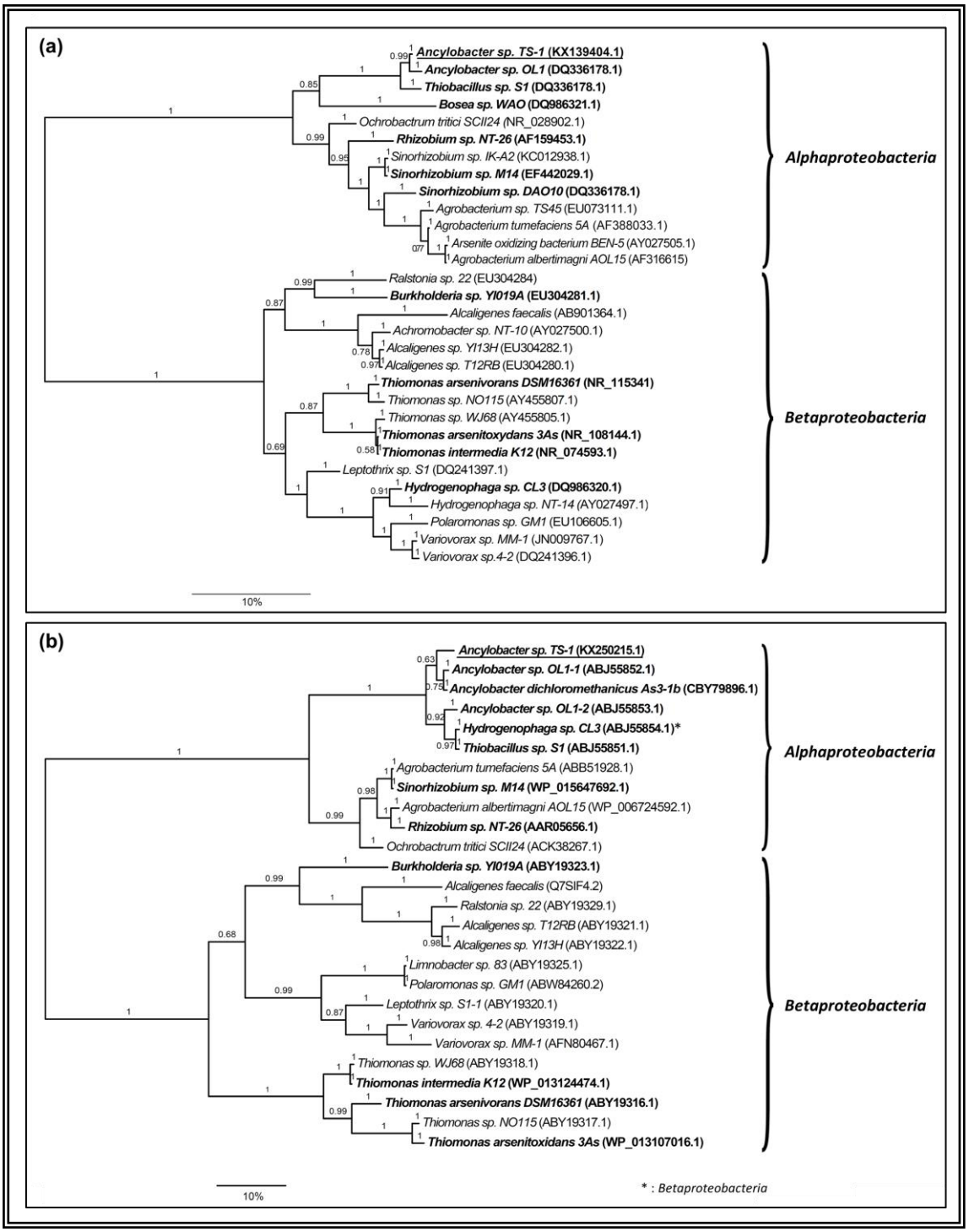

Figure A- 2: Phylogenetic trees of 16s rDNA (a) and arsenite oxidase (b) from CAOs and HAOs belonging to *Alphaproteobacteria* and *Betaproteobacteria*. MUSCLE was used for MSA of 16s rDNA. T-COFFEE was used for MSA of arsenite oxidase. Trees were constructed with MrBayes v3.2.3, using GTR+I+G for 16s rDNA and LG+I+G for arsenite oxidase. MCMC simulations were run with four chains using 1000000

generations sampling at intervals of 250 generations. The first 25% of sampled trees were considered 'burn-in' trees and were discarded prior to the tree reconstruction. The percentage of samples that recovered specific clades on this topology represents that clade’s posterior probability. Bars represent the percentage of substitutions; the numbers at the branch show the posterior probability of branches. CAOs are in bold type, and isolate discussed in this study is in bold underlined type.

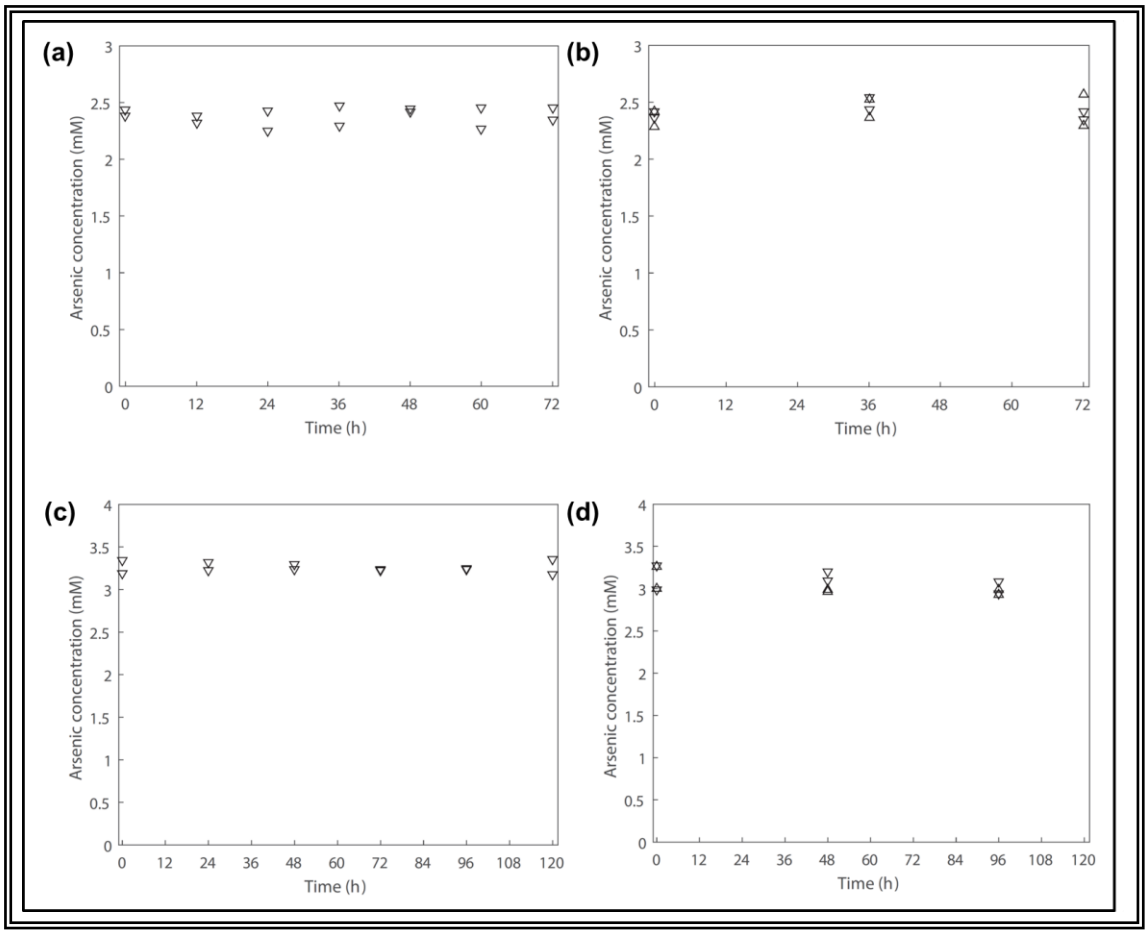

Figure A- 3:Arsenite [As(III)] and total arsenic [As_{total}] in chemolithoautotrophic and mixotrophic cultures. For the chemolithoautotrophic mediums, (a) As_{total} in TS-1 cultures, and (b) As(III) and As_{total} in abiotic cultures. For the mixotrophic mediums, (c) As_{total} in TS-1 cultures, and (d) As(III) and As_{total} in abiotic cultures. As(III) and As_{total} are represented with triangle and inverse triangle, respectively.

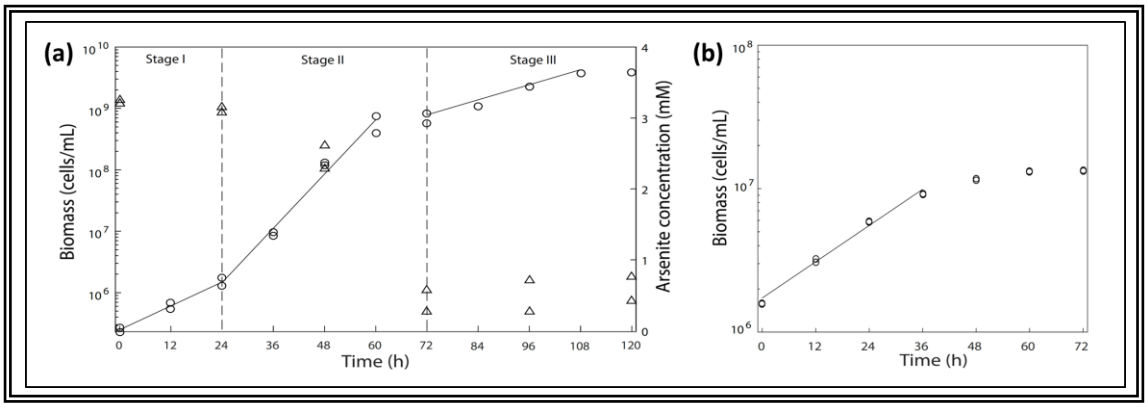

Figure A- 4: *Ancylobacter sp.* TS-1 growth in (a) chemolithoautotrophic and (b) mixotrophic culture. (a) The growth was fit to the exponential function (the integral of equation 1) in three stages: before arsenite oxidation (stage I), on arsenite oxidation (stage II), and after arsenite oxidation (stage III). (b) Fitting was performed with exponential function. For both, experimental duplicates are plotted for each sample times. The observed concentration of arsenite (triangle), the observed cell number per milliliter (circle), and the fitting curves (continuous lines).

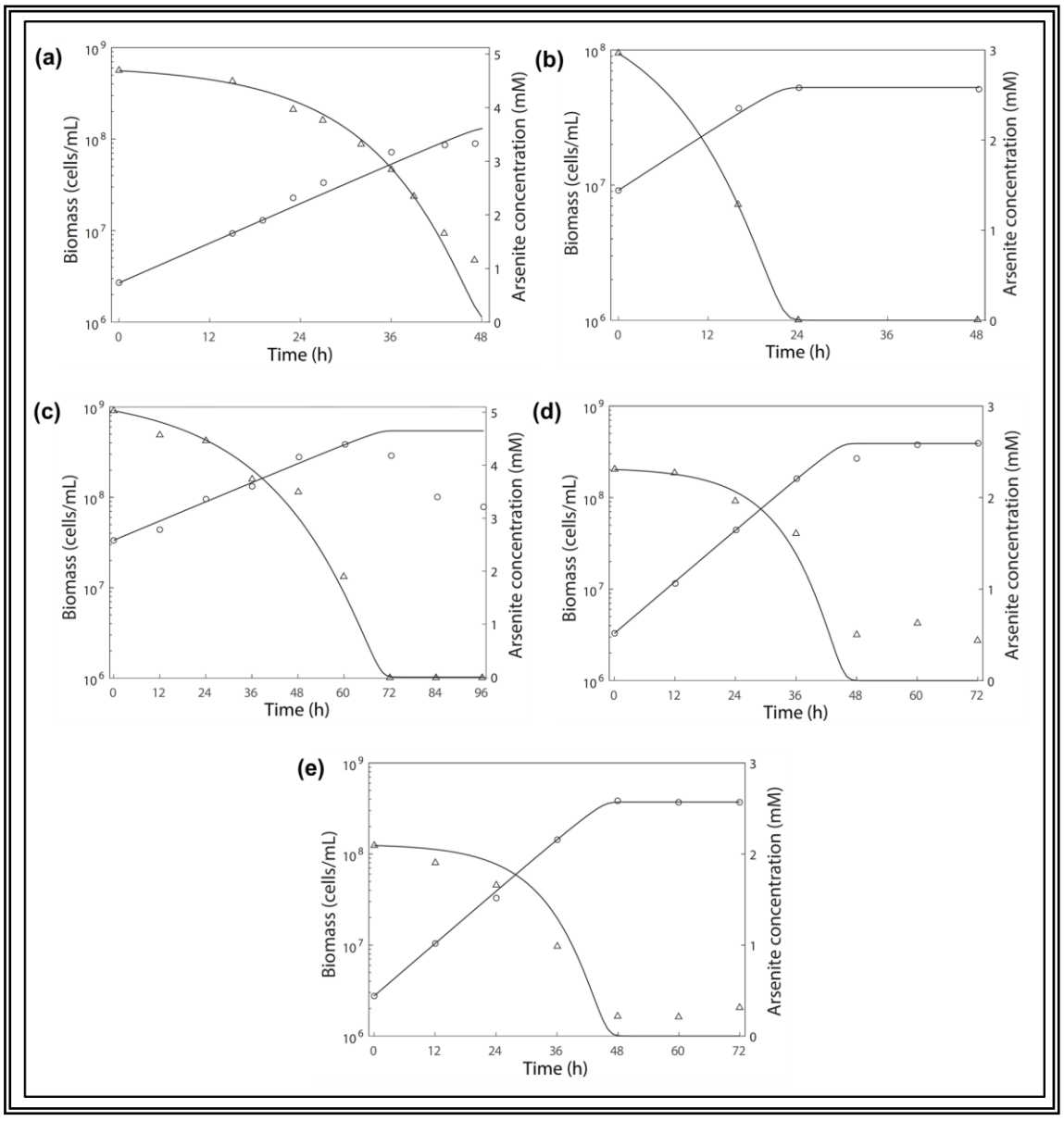

Figure A- 5: CAOs growth and arsenite consume. (a) *Rhizobium* sp. NT-26 (Santini et al., 2000), (b) *Ancylobacter dichloromethanicus* As3-1b (Andreoni et al., 2012), (c) *Sinorhizobium* sp. M14 (Drewniak et al., 2008), (d) TS-1 (experiment 1), (e) TS-1 (experiment 2). Fitted were done with nonlinear estimation of Monod growth kinetic parameters from a single substrate depletion curve.

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APPENDIX B: "A NEW ELECTROCHEMICALLY ACTIVE CHEMOLITHOAUTOTROPHIC ARSENIC OXIDIZING BACTERIUM *ANCYLOBACTER* SP. TS-1"

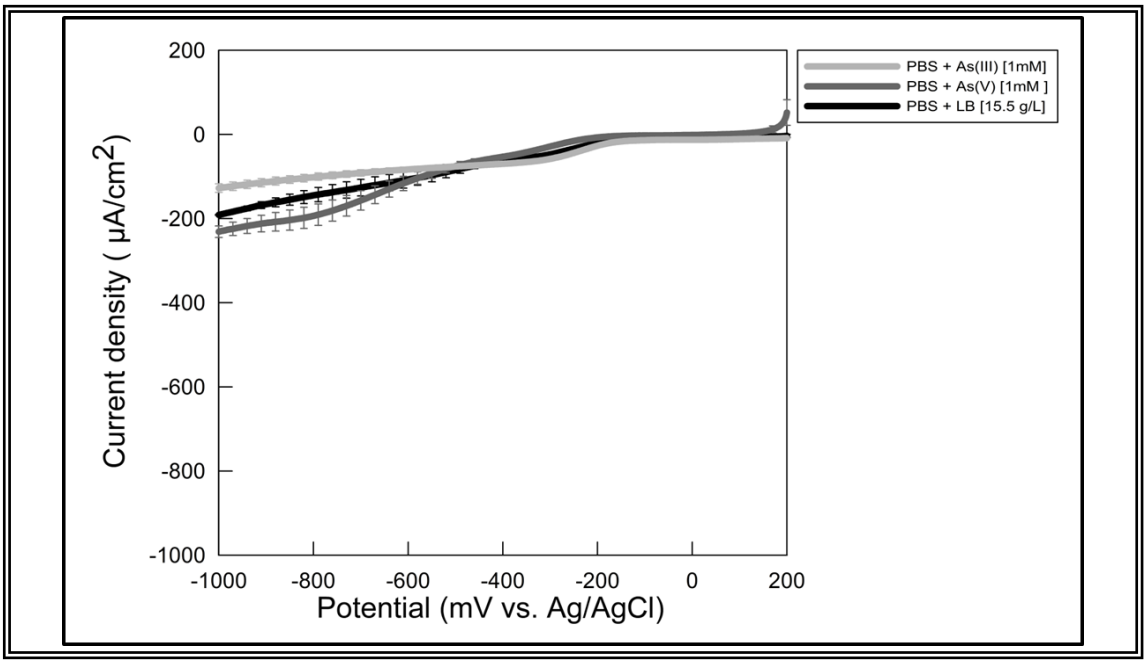

Figure B- 1: The LSV tests of different abiotic controls did not show significant peaks. The error bars represent the standard deviation (n=2).