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Diversity of arsenite transporter genes present in microbial populations of the abandoned Uranium mine in Urgeiriça

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Abstract

Arsenic is an ubiquitous toxic metal present in the environment. Microorganisms have co-existed with arsenic for millions of years. This motivated the evolution of arsenic resistance determinants that are now widespread among microbial populations. These determinants can be chromosomally or plasmid encoded. To study the diversity and occurrence of arsenic resistance determinants in the microbial populations of 8 different sites in the abandoned Urgeiriça uranium mines a PCR approach was used. Agar supplemented with 2mM to 20mM sodium arsenite was used to test for arsenic resistance phenotype in isolated strains recovered from mine tailings and surroundings sites. Resistance to antimonite was also ascertained as the mechanism for antimonite extrusion from the cytoplasm are the same as in arsenite. Primers were used to investigate the occurrence of *arsB, ACR3* (arsenite pumps), *arsC* (arsenate reductase), *arrA* (dissimilatory arsenate reduction) *and aioB* (arsenite oxidase) determinants. Several amplifications were obtained regarding determinants *ACR3* and *arsB*. The diversity of organisms present in the sampled sites was high as was the diversity of identified resistant bacteria. Among the 36 arsenic resistant strains, we found representatives for 13 different genera. There were several sites with no uranium or arsenic detected yet arsenic resistance determinants were found in those uncontaminated sites. This indicates that arsenic resistance determinants are ubiquitous and the absence of arsenicals does not necessarily indicate that there are no arsenic resistant bacteria present. Further studies including the cloning of arsenic resistance gene amplicons would enable the distinction between several determinants of the same strain enabling us to identify if there are multiple structures belonging to different ancestors in the same strain or if there is a dominant determinant in a specific population. After phylogenetic analysis we observed that HGT of arsenic

resistance determinants may have occurred in site A1 and A4. There was no detected contamination with uranium in site A1 whereas site A4 had the highest concentration of uranium of all the sites. There were other contaminants present in site A1 that can justify the stress that led to the possible occurrence of the horizontal gene transfer, also, this can also have occurred without the intervention of stress caused by contaminants.

Keywords: arsenic, uranium, horizontal gene transfer, radionuclides, diversity

Resumo

O arsénio é um metal amplamente disseminado na crusta terrestre. Os microrganismos coexistem com este metal há milhões de anos. Este facto motivou o aparecimento de determinantes de resistência a arsénio que estão agora disseminados nas populações microbianas. Estes determinantes podem estar inseridos no DNA cromossómico ou em plasmídeos. Para estudar a existência e diversidade de determinantes genéticos de resistência ao arsénio na população microbiana de 8 locais diferentes nas minas abandonadas da Urgeiriça, foi usado um protocolo de reação em cadeia da polimerase. Agar suplementado com concentrações de 2mM a 20mM foi usado para testar o fenótipo de resistência a arsénio em estirpes recuperadas de efluentes e outros locais na proximidade das minas. O fenótipo de resistência a antimónio foi também testado uma vez que os mecanismos de extrusão de antimonito do citoplasma são os mesmos que os de extrusão de arsénio. Foram utilizados primers para investigar a ocorrência dos determinantes *arsB, ACR3* (bombas de extrusão de arsenito), *arsC* (arsenato reductase), *arrA* (redutase de arsenato) e *aioB* (arsenito oxidase). Houve amplificação em diversas estirpes quando testadas para a presença dos determinantes *arsB* e *ACR3*. A diversidade dos organismos isolados dos locais de amostragem foi elevada assim como a diversidade de organismos resistentes a arsénio. Entre as 36 estirpes resistentes encontrámos representantes de 13 géneros bacterianos diferentes. Havia vários locais de amostragem que não continham contaminação com urânio nem arsénio no entanto, determinantes de resistência a arsénio foram encontrados em estirpes desses locais. Esta observação indica que os determinantes de resistência a arsénio estão bastante disseminados e que a ausência de arsenicais não é sinónimo da não existência de bactérias resistentes a arsénio. Estudos suplementares, incluindo a clonagem dos determinantes de resistência a arsénio de uma

mesma estirpe, permitiriam a distinção entre diferentes determinantes na estirpe permitindo identificar a ocorrência de mais do que um determinante num só organismo ou mesmo saber se existe um determinante que é prevalente numa população num determinado local. Após análise filogenética observámos que é possível que tenha existido transferência horizontal de determinantes de resistência a arsénio no local A1 e A4. Não havia contaminação com arsénio nem urânio no local A1. No local A4 foi detetada a maior concentração de urânio entre os locais analisados no estudo. Estavam presentes outros contaminantes em A1 que podem explicar o stresse que levou à partilha dos determinantes, a qual pode também ter ocorrido sem a intervenção do stresse causado pelos contaminantes.

Palavras-chave: arsénio, urânio, transferência horizontal de genes, compostos radioativos, diversidade

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Chapter 1 Introduction

Introduction

1.1 Metals and microorganisms

Heavy metal contamination has been considered an important and serious universal issue. Events of environmental metals contamination such as metallurgic industry activity, radionuclide contamination derived from nuclear testing in the 50s and 60s of the previous century, the Chernobyl meltdown [1] and more recently the Fukushima disaster, motivated deep discussions to this particular issue in order to further consolidate our understanding of the consequences from heavy metal contamination, in aquifers and soils, on the microflora genomic landscape. Microorganisms, being the most abundant and diverse group of living organisms on the planet, may provide the needed insight on the changes that occur due to natural and anthropogenic alterations on the concentrations of heavy metals in contaminated sites [2]. Microorganisms' capability to cope with changes in the environment coupled with their relative low size (offering the greatest surface to volume ratio of any living organism), provide an ample interface with contaminants, thus they play an important role in the proper understanding of the consequences of the aforementioned contaminations [3]. The knowledge of these events may prove invaluable in evaluating the extent of contaminations and provide useful data for bioremediation strategies.

Despite the lack of a consensual definition to what really is a "heavy metal", it is generally accepted a heavy metal characteristically exhibits density of above $5 g/cm^3$ [4, 5]. Some metals as calcium, cobalt, copper, iron, potassium, magnesium, manganese, sodium, nickel and zinc can be found in several enzymes and play important biological roles in catalytic sites of enzymes. Although these metals are biologically relevant in trace

amounts, in high concentrations they may become toxic and impair enzyme activity, disrupt membrane integrity and damage DNA, severely disrupting biological activity. Other metals such as silver, gold, led, uranium and mercury are potentially toxic and have no known biological role either in trace or high concentrations [6]. In general, metals may permeate the microbe cell and accumulate in the intracellular environment through the action of unspecific constitutively expressed transport systems, whereby exerting their toxicity [7]. As a consequence, microorganisms have been obligated to develop systems to cope with this toxicity[4]. Metal resistance systems may have evolved shortly after the beginning of prokaryotic life and are ubiquitous throughout bacteria. They appeared because microorganisms existed in an environment that always contained metals [6]. In modern times, human activities created environments with high evolutionary pressure for selection of metal resistance mechanisms due to the high level concentrations of metallic ions [7]. There are several mechanisms that convey cellular resistance to metals: a) efflux of the toxic metal through membrane pumps , b) enzymatic conversion, c) intra or extracellular sequestration, d) exclusion by permeability barrier and e) reduction in sensitivity of cellular targets [7]. Bacterial resistance mechanisms generally involve an efflux pump for toxic ion removal from cells [3].

1.2 Arsenic: origin and distribution

Arsenic (As) is a metalloid with atomic number 33, atomic mass 74.92 and four oxidation states: As(-III), As(0), As(III) and As(V) [8]. Despite its low abundance at the surface (0,0001%) it is ubiquitously distributed in the Earth's crust (Table 1). Its main natural source is the erosion of igneous rock and it is commonly found in iron (Fe) complexes or

associated with ores of copper (Cu) gold (Au) and lead (Pb). The main species of arsenic in anaerobic and aerobic environments are arsenite [As(III)] and arsenate [As(V)], appearing as [H_3AsO_3 ; $H_2AsO_3^-$] and [AsO_4^{2-}; $HAsO_4^-$] respectively [8-11].

Table 1: Arsenic concentrations ($mgKg^{-1}$) in the lithosphere and extra-terrestrial objects. [12]

Medium	Concentration	Source
Universe	0.008 (ppm)	Winter (1998)
Sun	0.004 (ppm)	Winter (1998)
Stony meteorites	1.8	Onishi (1969), Winter (1998)
Iron meteorites	11	Onishi (1969), Winter (1998)
Earth crust, total[a]	1.0; 1.7; 1.8	Taylor and McLennan (1995), Wedepohl (1995), Lide (1996)
Upper crust	1.5; 2.0	Taylor and McLennan (1995); Wedepohl (1995)
Ultramafic rocks	0.7	Koljonen (1992)
Ocean ridge basalts (MORB)	1.0	Koljonen (1992)
Gabbros; basalts	0.7	Koljonen (1992)
Granites; granodiorites	3.0	Koljonen (1992)
Sandstones	0.5; 1.0	Onishi (1969);Koljonen (1992)
Shales; schists	13	Onishi (1969);Koljonen (1992)
Carbonates	1.0; 1.5	Onishi (1969);Koljonen (1992) Chester (1993)
Phosphates	12	Onishi (1969)
Metamorphites	0.5−11	Onishi (1969)
Coal	0.34−130; 5−45; 1−10.000;	Piver (1983), Pacyna (1987) Onishi (1969)
Crude oil	0.0024−1.63; 0.134; 0.005−0.14	Pacyna (1987); Piver (1983); Veal (1966) in Onishi (1969)

[a]Mass of the earth crust: 2.13×10^{19} t; Mass of the upper crust: 1.13×10^{19} t

Besides the natural occurrence of arsenic compounds, with varying concentrations as seen on table 1, anthropogenic sources also contribute to the overall distribution of the metalloid, namely: high energy combustion of coal in power plants, incineration, sewage from animal farms, pesticides, disinfectants, medicine for veterinary use, glassware production, semiconductor production, ore production and smelting, metal treatment, wood preservatives, pigment production for paints, pyrotechnics and the release of arsenic from arsenic rich ores originated from mining activity [8, 12].

Arsenic presence in the environment occurs through the cycling between valence states and substitution in organic compounds which is dependent on biotic and abiotic factors. These factors are intrinsically connected most of the time. Thus, understanding the process of arsenic speciation requires the understanding of both chemical and

microbiological inputs in the cycle. This fact motivated several studies in which an arsenic global cycle was described by several authors [12-14]. The cycle encompasses the contribution of the metabolism of arsenic compounds by microbes and the bioaccumulation by higher plants and animals [9].

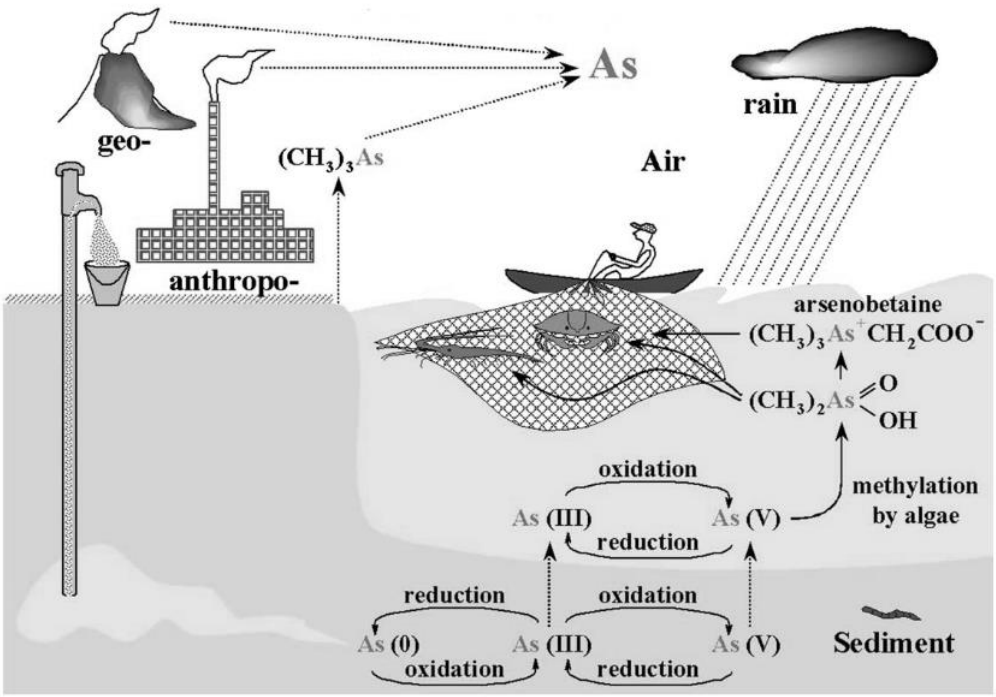

Fig. 1: Arsenic global cycle as proposed by Mukhopadhyay et al. 2002 [14].

Arsenate (the prevalent form in marine environments) is taken up by marine organisms, in their quest for phosphate, and either reduced or converted in organic compounds (these organisms are able to produce organoarsenicals like methylarsonic acid and dimethylarsinic acid, from inorganic arsenic) which are then secreted from the organism. It is assumed that the conversion to methylated arsenic is a form of protection [8]. Some arsenate is also converted into complex organic molecules like arsenolipids. The association with complex lipid soluble molecules can be an adaptation mechanism to the lack of nitrate, since it has been established that arsenic can substitute nitrogen in choline

for structural lipid formation [14]. Phytoplankton and macroalgae are the primary producers of organoarsenicals in marine environments whereas fish and invertebrates retain 99% of accumulated arsenic in organic form. In spite of the high accumulation by both fish and invertebrates the concentration of arsenic in the previous is lower. The main organoarsenical compound found in higher marine organisms is arsenobetaine. The mechanism through which these organisms convert arsenic in arsenobetaine is still unknown. Arsenobetaine is finally converted in methylated arsenic or inorganic arsenic by microorganisms, thus completing the arsenic cycle. [9, 13-15]

Another factor to take into account when studying the cycling of arsenic in nature is the inorganic speciation of arsenic. Complexation with metals and adsorption phenomena play an important role in arsenic bioavailability. Fe, Ca, Mn, Mg, and Al-arsenate salts are usually too soluble to regulate activity of arsenate in soils and natural waters; this activity is more commonly regulated by surface complexation (sorption) with Fe hydroxides and also with Al and Mn hydroxides although to a smaller extent [12, 16]. Arsenite, in contrast, is very selective and exhibits a strong preference for Fe hydroxides. Generally, arsenate has greater sorption capacity than arsenite with the aforementioned metals though arsenite has an equivalent or greater sorption capacity with ferrihydrite ($Fe_2O_3.H_2O$) and goethite (FeO(OH)) [16]. One must note that arsenite sorption is very pH dependant, and the dissolved phase is partially unaffected by the sorption phenomena, contrasting with this property, arsenate adsorption to $Al(OH)_3$ has a low pH dependence and arsenate is completely removed from solution in the pH range of 4-10 [16-18]. Briefly, arsenic sorption phenomena play a crucial role in arsenic mobility and therefore in its availability in soils and waters, in acidic mine tailings, the low pH contributes to the mobilization of arsenic to the solid phase, whereas in regions where the pH rises above 8 arsenic is removed from complexes increasing its bioavailability.

1.3 Toxicity of arsenic compounds

Since antiquity, arsenic has been known to be an effective poison, arsenite trioxide (As_2O_3) was called the "heritage powder", due to its use as a potent poison, that is, until Marsh *et al* developed the first test to detect arsenic in tissues [8]. Nowadays arsenic trioxide is used as a chemotherapeutic agent in some cases of leukaemia [19]. Besides this compound there are more forms of arsenic which are toxic [9].

Methylated forms of arsenic, such as monomethylarsonous acid ($MMAA^{III}$), methylarsonic ($MMAA^{V}$) acid and dimethylarsenic acid ($DMAA^{V}$) are produced by algae and animals and are excretory products of the latter [8, 9]. They occur in low concentrations in the environment and have a variable degree of toxicity which depends on the valence state of the incorporated arsenic [20].

Arsines occur as highly toxic gases, such as H_3As and $(CH_3)_3As$, however very little is known about the natural cycle of these substances because of their scarcity in the environment [20].

Organoarsenicals are naturally occurring compounds commonly found in marine animals. They are not toxic to animals, and there are reports of their intervention in metabolism like choline nitrate substitution for arsenic in structural lipid metabolism [10, 13, 20, 21].

Arsenate [As(V)] can exist ionized in four different forms depending on the hydrogen potential of the solution. It has three pKa values (2.2; 7.0; 11.50) which are very close to those of phosphate (2.1; 7.2; 12.7) resulting in approximately similar quantities of $HAsO_4^{2-}$ and $H_2AsO_4^-$ at physiological pH [13, 22]. This property is determinant in arsenate toxicity, which acts as an analogue to inorganic phosphate in phosphorylation activities, resulting in disruption or reduction of the overall efficiency of the reactions. Its ability to penetrate the intracellular space is also related to its similarities to phosphate. It

enters the cell through specific phosphate transporters, namely the Pit and Pst systems found in the prokaryote *E. coli* and several phosphate transporters in eukaryote cells, as reported for *Saccharomyces cerevisiae* (Fig. 2) [10, 13, 23-25].

Arsenite [As(III)] is another oxanion of arsenic. It appears mostly in its unionized [$As(OH)_3$] form at neutral pH [13, 14] . In this form it resembles an inorganic analogue to glycerol and it is believed to enter the intracellular space through aqua-glyceroporins in bacteria, yeast and mammals [10, 14, 26]. The glycerol facilitator protein GlpF of *E. coli* was reported to allow translocation of antimonite $Sb(OH)_3$, an ion which is very similar to $As(OH)_3$. Furthermore, deletion of the gene Fps1 of *S. cerevisiae*, an analog of GlpF, confers some resistance to arsenite which leads to believe that this channel may be a facilitator to arsenite entry [10, 14, 24]. Upon entry in the cytoplasm, arsenite readily reacts with thiol residues of cysteine which may inhibit function of proteins like pyruvate dehydrogenase and ultimately lead to cell death [8, 9, 20, 27, 28]. There is a difference in the degree of toxicity between arsenite and arsenate. Because arsenite's mechanism of action interferes in a non selective way with the action of enzymes, by binding to thiol groups, whereas arsenate is similar to phosphate and its toxicity is more dependant of the pathways in which phosphate intervenes, arsenite has a higher degree of toxicity than arsenate. It is also believed that some of the effects of arsenate toxicity derive from its conversion into arsenite within the cell[29].

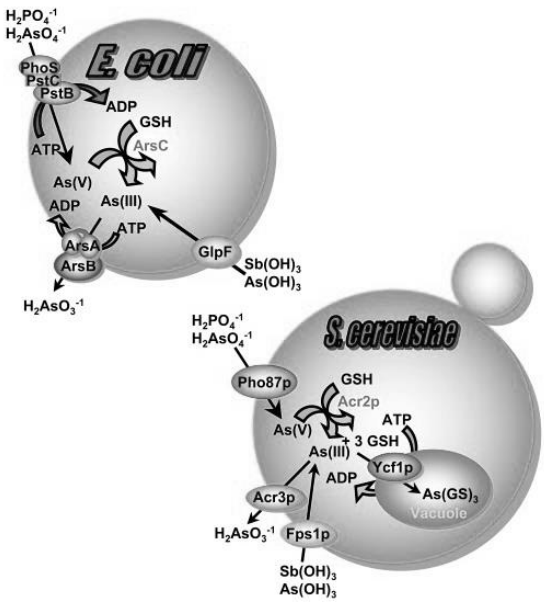

Fig. 2: Schematical representation of proposed arsenite and arsenate uptake and detoxification mechanisms in *E. coli* and *S. cerevisiae*.[10]

1.4 Arsenic resistance genes and enzymes

Due to arsenic toxicity, several organisms have evolved various resistance strategies to cope with the presence of this metalloid such as, reduction, extrusion or methylation of arsenic. Genes for bacterial resistance to arsenic present in plasmids were first reported in a group of β-lactamase plasmids from *Staphylococcus aureus* that determine resistance to heavy metals and antibiotics [10, 14, 30]. After these initial studies, a huge number of arsenic resistance related genes have been found in a diversity of organisms. There are several configurations for operons of arsenic resistance genes [23], however the most thoroughly studied is *E. coli* plasmid R773. It features five genes: *arsA*, *arsB*, *arsC*, *arsD* and *arsR* [8, 31]. The gene *arsA* codes for an ATPase, which binds to an arsenite/antimonite efflux pump, *ArsB*. This transmembrane efflux pump removes arsenite and antimonite from the cytoplasm and is coded by the gene *arsB* [24, 32]. When

ArsA protein is not present, the energy for arsenite extrusion is provided by the membrane potential [33]. In general, there is great similarity among *ArsB* proteins. *ArsB* from *E. coli* pR773 and *S. aureus* pI258 share 58% identity in amino acid sequence [34] and they are part of the arsenite transporter *ArsB* protein family, which is prevalent in *Firmicutes* and *γ-proteobacteria*. Other family of arsenite transporters, the *ACR3* carrier protein family, are found mainly in *Actinobacteria*, *α-proteobacteria* and also in *S. cerevisiae*.[8, 14, 23, 35, 36]. This protein is a member of the BART superfamily (bile/arsenite/riboflavin transporters) and is more widely distributed than the members of *ArsB* family [36, 37]. The first identified member of this family was found in the *ars* operon of the skin element K of *Bacillus subtilis* [38] and has since been found in several organisms such as plants (*Pteris vitata*) and animals (*Danio rerio*).

The gene *arsC* codes for the cytoplasmatic arsenate reductase protein responsible for arsenate reduction to arsenite. There are three distinct unrelated families of *ArsC* reductases: the *E. coli* plasmid R773 family, the *S. aureus* plasmid pI258 family and the ACR2p reductase family from *S. cerevisiae* [14]. They share mechanisms based on cysteine thiol redox cycling. These mechanisms are either coupled to Glutaredoxin (Grx) or Thioredoxin (Trx), though their sequences allow their subdivision in the aforementioned families [14, 23, 35]. The R773 reductase family needs Grx and glutathione (GSH) in the arsenate reduction. The pI258 family couples to Trx in the reduction of arsenate and also needs GSH. The *S. cerevisiae* family of reductases has a similar catalysis mechanism to the R773 reductases [14, 39-41]. The existence of these three distinct families with similar function but unrelated in sequence suggests that it is a case of convergent evolution.

A small chaperone, previously reported as being an upper level transcription regulatory protein [27], is coded by the gene *arsD*. Recent studies have shown that the product of

arsD acts as a metallochaperone protein that facilitates the binding of arsenite to the ArsAB complex increasing the affinity of ArsA for As(III) thus increasing the rate of extrusion [42, 43].

ArsR is a dimeric regulatory repressor coded by the gene *arsR*. The variety of *ars* operons usually start with an *arsR* gene and are regulated by the As(III)/Sb(III) responsive regulator ArsR [44]. ArsR proteins are part of a dimeric helix-turn-helix repressor family and are closely related to each other and to repressors of other metal resistance clusters including those of Pb(II) and Zn(II) [45].

The five *ars* genes (functionally organized as *arsRDABC*) have been found in several plasmids of gram-negative bacteria like *E. coli* R773 and R46 [46], *Acidophulus multivurum* AIU301 and pKW301 among others and are greatly distributed among several phylogenetic divisions [47]. Besides the well studied *ars* operon of plasmid R773, there is the widely found arsenic resistance *arsRBC* operon. This operon can be found in plasmid pI258 of *S. aureus* [34, 46] and in the genome of several other bacteria [23, 32, 46]. This operon contains only the genes that code for regulatory, transmembrane antiporter and reductase proteins. In the numerous of *ars* operons existent in bacteria there are also those containing the gene *arsH*, like those of *Acidithiobacillus ferrooxidans* (*arsHB, arsRC*). ArsH function is still unknown, though recent studies place it in the family of the NADPH-dependent FMN (flavin mononucleotide) reductases [48-50].

When considering the different *ars* operons it seems clear that the efflux pumps and reductases have evolved more than once. Different genes evolved convergently to provide similar function and protection against environmental arsenic [14]. First resistance probably appeared in the primitive anaerobic environment of primordial earth where the main species of arsenic would be arsenite. With the gradual change of the atmosphere to an oxidative nature, arsenate became the prevalent species of arsenic ion which explains

why the cell machinery first reduces arsenate in order to use available arsenite extrusion pumps [27]. Initially *ArsB* would be sufficient to confer resistance to trivalent metalloids, and the operons would probably be composed of only the regulator protein ArsR and the extrusion pump, a pair which is prevalent in almost every operon [14, 27]. With the shift in redox nature of the atmosphere an extra gene is required, the *arsC*, in order to provide resistance to arsenate. Acquisition of either of the *arsC* genes would provide evolutionary advantage. The fact that there are different families of arsenate reductases indicates convergent evolution of these families of genes [27].

The *arsA* and *arsD* genes are probably a recent addition to the *ars* operons. The three gene chromosomal *ars* operon of *E. coli* is more closely related to the R773 *ars* operon than to the three gene operon of *S. aureus*, suggesting that the acquisition of the *arsAD* genes was posterior.

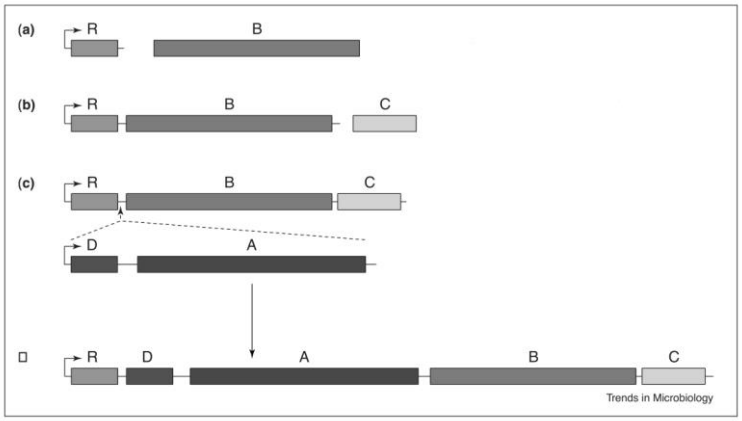

Fig 3: (a) Original resistance genes, extrusion pump and regulator (b) Acquisition of *arsC* also provided response to pentavalent metals (c) acquisition of *arsAD* transformed *arsB* in a high efficiency active transport channel for arsenite. (figure adapted from [27]).

One question arises, though, about the appearance of *arsAD* genes: Was the addition sequential or synchronous? One clue to the answer may lie in the pNRC100 megaplasmid of archaeon *Halobacterium* sp. strain NRC-1. There are four genes corresponding to *ars* genes in a cluster, *arsAD* in one orientation and *arsRC* transcribed divergently. The

presence of both *arsA* and *arsD* in a two gene cluster suggests that they were acquired simultaneously and not sequentially [27, 51].

1.5 Arsenate, beyond the *ars* operon

Several bacteria isolated from mine tailings, river sediments, geothermal springs and soils have shown arsenate oxidizing activity [23, 52-56]. In most of the reported cases the oxidation did not appear to yield energy, nevertheless there are several organisms that are able to utilize arsenate as an electron acceptor in anaerobic autotrophic metabolism [57-60]. A hypothetical electron transport chain was proposed by Santini and collaborators 2004 [57] for the energy yielding aerobic microbial arsenite oxidation. This oxidation is thermodynamically favoured and the conversion of arsenite to arsenate in aerobic conditions has a high equilibrium constant when pe+pH*[1] > 9 [61].

Up to date, the arsenite oxidases already studied are made of a pair number of two subunits, subunit A and subunit B coded by the genes *aioA* and *aioB* respectively (Fig. 5) [14, 62]. *Aio*A is the biggest subunit with approximately 90 kDa, and exhibits a heterodimeric structure with a molybdenium-pterin. It is part of a new subgroup of DMSO reductases, an evolutionary superfamily of proteins that vary in substrate, midpoint electric potential and reaction course since some function as oxidases and others function as reductases [14, 52]. The *aioB* genes code for the small subunit with a Rieske 2Fe-2S centre which probably serves as an electron shuttle accepting the electrons of the large subunit subsequent to arsenite oxidation and then transferring the electrons to an acceptor, probably cytochrome *c* or azurin [14, 55, 57]. In addition to reduction of arsenate

[1] pe is the negative logarithm of electron activity and is obtained by dividing the reduction potential Eh by 59.2. pe+pH represent redox conditions of a system.

catalyzed by the proteins coded by the well known *ars* operon, some microorganisms can also respire arsenate. It is believed that arsenate respiring are spread through several phylogenetic groups [20, 23] and the acronym DARPs, dissimilatory arsenate respiring prokaryotes, refers to prokaryotes with the ability to utilize arsenate as an electron acceptor in respiratory metabolism [63, 64]. Microorganisms with this ability can utilize either solved or sorbed arsenate, which is very important in arsenic speciation in the environment because they are able to mobilize solid state arsenate and convert it to the soluble ion arsenite.

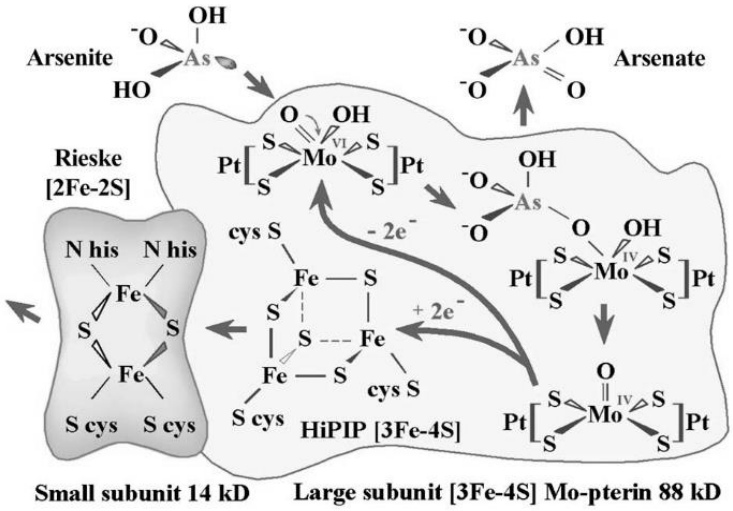

Fig. 4: Structure and hypothetical reaction cycle of arsenite oxidase. Steps: (1) Binding of arsenite to the enzyme, (2) two electron transfer to Mo, oxidizing As(III) to As(V), (3) release of arsenate, (4) two electron transfer from Mo(IV) to 3Fe-4S centre, regenerating Mo(IV) reaction centre to Mo(VI), (5) two electron transfer from 3Fe-4S centre in AioA subunit to 2Fe-2S Rieske centre of *AioB* subunit, electron transfer from the Rieske centre of the oxidase to the respiratory chain [14].

The ability to mobilize arsenate from solid state possibly arose from the localization in the periplasmic membrane of the respiratory arsenate reductase [64, 65]. The respiratory reductase is coded by a two gene cluster, the *arrA* and *arrB* genes. The Arr protein is a heterodimeric periplasmic protein composed of two subunits, the ArrB and ArrA subunits

(Fig. 7) [56, 63]. ArrB is a 29 kDa protein that possesses a Fe-S centre that is not homologous to the Rieske subunit of *AioB*. The ArrA subunit, with approximately 87 KDa, is part of the same DMSO reductase superfamily as AioA though it belongs to a different branch [56, 63].

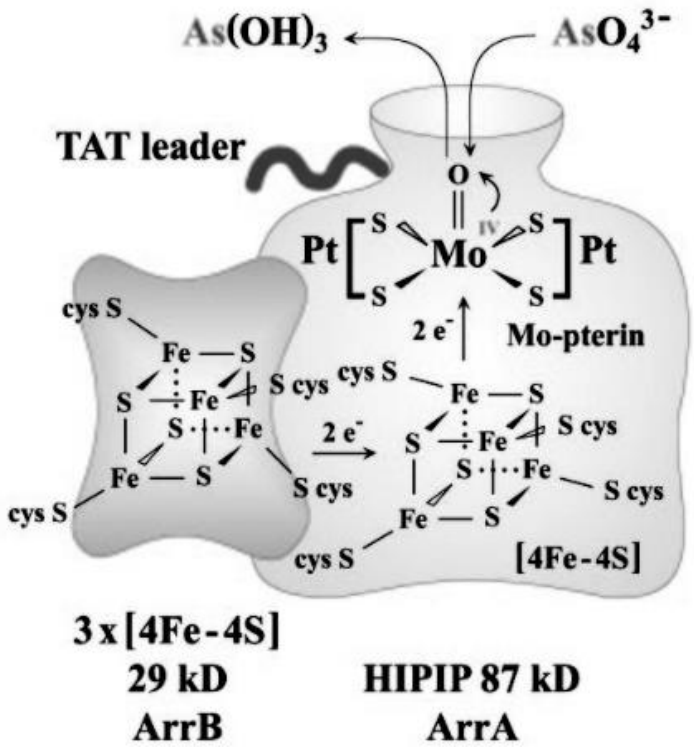

Fig. 5: Schematic representation of the respiratory arsenate reductase as found in [56]

Catalysis performed by these enzymes appears to be of great importance in arsenic speciation in anaerobic conditions.

1.6 HGT events in heavy metal contaminated environments

Horizontal gene transfer of determinants encoded in mobile genetic elements rapidly alters the genetic landscape of contaminated sites, contributing to genome diversification. This diversification may alter the physiology of microorganisms changing their metabolism, pathogenicity and ecological role. Thus, mobile genetic elements give

microbial communities a great adaptation capacity and enable them to survive in rapidly changing environments and explore new ecological niches [66]. Metals and radionuclides often pose a challenge for the survival of microbial communities as they cannot be transformed or broken down as a strategy for detoxification as happens with organic xenobiotic contaminants as such microbes need a strategy to cope with the presence of these contaminants. Metal resistance genes were first detected in plasmids occurring in diverse bacteria, plasmid *ars* operons of *E. coli* and *S. aureus* were described in 1980. (Silver *et al*) [67]. These elements often co-occur with antibiotic resistance determinants. This suggests that some determinants may be transmitted horizontally in tandem, which poses an interesting perspective for the work at hand as the sampled environment has multiple contaminants, mostly of anthropogenic origin as is the case of uranium derived from mining [66].

Chapter 2 Objective

2 Objective

The overall objective of this study was to competently describe microbial diversity of arsenic resistance genes in an area partially contaminated with radioisotopes derived from anthropogenic sources, namely mining activities to extract uranium in the Urgeiriça mines, as this environment exhibits a widely distributed degree of uranium concentrations, which is an very interesting environment to test the hypothesis that contamination with metals, in this particular case radionuclides, promotes horizontal gene transfers in microbial populations. In depth, we set out to evaluate the dispersion of arsenite resistance genes in the microflora, relate it with the strains phylogeny, and try to relate its distribution with the presence of radioisotopes. This should enable us to infer if the stress caused by the presence of radioisotopes is sufficient to promote horizontal gene transfer among the microbial population, namely, arsenic resistance genes.

Chapter 3 Materials and Methods

3 Materials and Methods

3.1 Sampling

The samples were collected in the vicinity of the abandoned Urgeiriça uranium mines and its water treatment plant. The sampling spots were divided in eight distinct areas: A1, A2, A3, A4, A5, A6, 7A e 7B (Fig. 8). This work was performed by Marques, João [68].

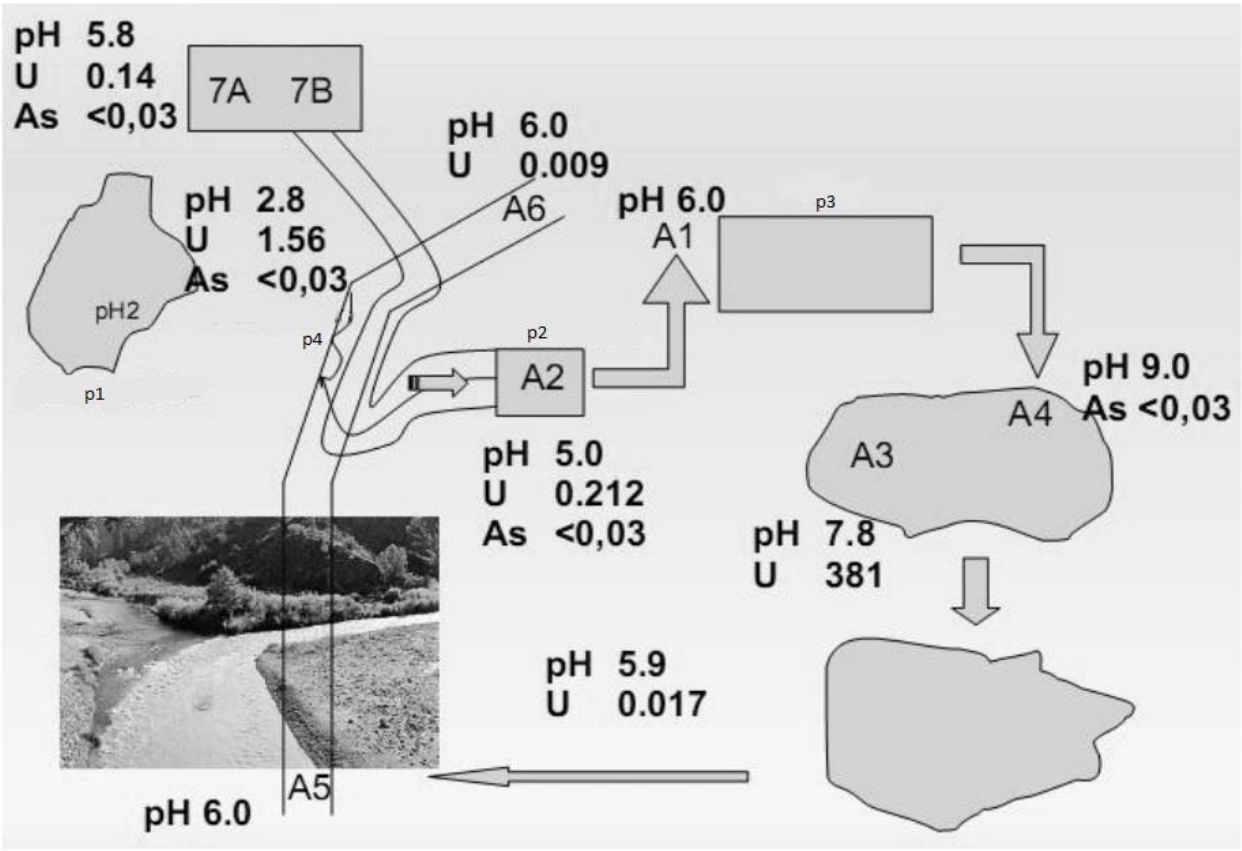

Fig. 6: Sampling places in "Minas de Urânio da Urgeiriça". p1) ""Barragem velha"; p2) "Poço das Cobras"; p3) Water treatment plant; p4) creek :

A1) Sampling area A1: untreated mine tailing without biofilms; pH 6.0. The water was collected from the efflux of a tube, in this point the water is treated with barium and calcium. The collection was made pre-treatment.

A2) Sampling area A2: well with three different water influx sources; pH 5.0. Water was collected from the well which is constantly filled by mine tailings and exsurgences.

A3) Sampling area A3: Sedimentation pool sludge, pH 7.0-8.0. Sludge was gathered from the sedimentation pool.

A4) Sampling area A4: Water from the sedimentation pool; pH 9.0. Water was collected from the sedimentation pool in the influx area from the first step of the water treatment.

A5) Sampling area A5: Water and sludge collected from the stream; pH 6.0. The water was collected from the creek near the exit from the mine.

A6) Sampling Area A6: Water and sludge collected from the stream; pH 6.0. The water was collected from the creek before the mine.

A7) Divided in two sampling sites 7A and 7B:

7A: Water collected from the well in 7A; pH 6.0. Water was collected from the surface of the well; water with some suspended particles.

7B: Water collected from the well in 7B; pH 6.0. Water and sediments collected from greater depth than 7A.

Of all the sampled places A2, A4 and A7 were the only that exhibited arsenic though it was in concentrations below 0.03 g/L. All the remaining sites did not exhibited arsenic contamination. Uranium was the more pressing contaminant detected with concentrations of up to 381ppm which is a significant enough amount to cause variations in microbial populations[69].

3.2 Strain collection

The prokaryote strains used in this work were obtained through isolation and purification from the samples collected in the different mine sites and water treatment plant at Urgeiriça. The isolated strains were then stored at -80ºC in medium "Luria Broth" supplemented with 15% glycerol and sterile glass beads. This work was performed by Marques, João [68].

3.3 Culture of isolated strains

All strains were defrosen and cultured on R2A agar (Difco R2A Agar®: yeast extract 0.05%, protease peptone nº3, casaminoacids 0.05%, dextrose 0.05%, soluble starch 0.05%, dipotassium phosphate 0.03%, magnesium sulfate 0.05% and agar 1.5%) and incubated at 30 º C for 48 h. This work was performed by Marques, João [68].

3.4 16 rRNA amplification of isolated strains.

DNA was extracted from cultured strains and rRNA 16S was amplified and sequenced. This work was performed by Marques, João [68].

3.5 Sterilization conditions

The sterilization of culture media was accomplished through autoclaving at 121ºC for 15 minutes. The stock metallic solutions used were prepared as: 1 M of sodium arsenite ($NaHAsO_2$, Merck), 100 mM of potassium antimony tartarate ($C_4H_4KO_7Sb$, Sigma) and were sterilized by filtration with cellulose acetate membranes through 0.45µm pores (VWR) and then added to the media.

3.6 Metalloid resistance and phenotype tests:

3.6.1 Arsenite resistance

The arsenic resistance of the isolated strains was tested through their cultivation in R2A agar supplied with increasing concentrations of arsenite of 2, 5, 10, 15 and 20 mM. The strains were then incubated at 30 ºC and the growth was assessed at 72 h and 120 h of incubation time.

3.6.2 Antimonite resistance

The antimonite resistance assays were performed by cultivation of the isolates in R2A medium with 1, 3, and 5 mM of antimonite. Then, the strains were incubated at 30 ºC and the growth was assessed at 120 h and 168 h incubation time.

3.6.3 Arsenite oxidation test

A phenotypic test to verify the strain's ability to oxidize arsenite was performed cultivating selected strains in a chemically defined medium (CDM). The medium was prepared containing agar (20 g/l), yeast extract (0,05%), $NaHCO_3$ (10 ml/l) and arsenite (1 mM). The results were obtained by submerging the culture in an $AgNO_3$ (0,1M) that forms a silver sulphate in the presence of arsenate yielding a dark brown colour. In the case of arsenite it yields a yellow color due to the formation of silver sulphite. The strain *Ochrobactrum tritrici* was used as positive control [55].

3.7 Genetic screening for arsenite detoxification determinants

3.7.1 DNA extraction from isolated cultures

DNA extraction and purification was performed by the method described by Pitcher and colleagues [70]. Briefly, cells were gathered from isolated cultures with a sterile inoculation loop and placed in a 1.5 ml Eppendorf type tube with 100 µl of TE with

lysozime for an overnight incubation at 37 ºC. After adding 500 µl of GES the resulting solution was incubated 10 min on ice followed a brief agitation of the solution and 250 µl of ammonium acetate, NH_4Ac (7,5M) were added to improve separation between DNA and cellular components. The solution was then agitated and incubated on ice for 10 min and 500 µl of a 24:1 (v/v) chloroform/isoamyl alcohol was added followed by homogenization of the solution by tube inversion. The solution was then centrifuged and the supernatant was collected with a 1 ml micropipette. The chloroform/isoamyl alcohol extraction was repeated. The DNA was then precipitated by adding 0.54 volumes of isopropyl alcohol (Merck) and then carefully agitated. The solution was then centrifuged at 13200 rpm for 10 min, the isopropyl alcohol was discarded and the DNA was rinsed in 150 µl ethanol (Merck) 70% (v/v) and re-centrifuged for 5 min. Ethanol was discarded and the DNA was dried in an incubator at 70 ºC for 20 min. DNA was ressuspended in 30 µl TE buffer supplemented with RNase to a concentration of 20 µg/ml (tables 1 to 6).

Table 1: TE solution (100x)

Tris/HCL	**121 g**
EDTA solution (0.5 M; pH 8.0)	**20 0ml**
ultrapure H_2O	**up to 1000 ml**

Table 2: EDTA solution

EDTA(Sigma)	**186 g**
ultrapure H_2O	**1000 ml**
pH	**8.0**

Table 3: Lysozime solution

Lysozime	**500 mg**
TE buffer	**500 ml**

Table 4: GES reagent

Guanidine isothiocianate (Sigma)	**60 g**
EDTA solution (0.5M ; pH8)	**20 ml**
N-laurilsarcosine (Sigma)	**1 g**
ultrapure H_2O	**100 ml**

Table 5: NH4Ac solution

NH_4CH_3COO	**121 g**
ultrapure H_2O	**100 ml**

Table 6: RNase solution

RNase	**50 mg**
ultrapure H2O	**4 ml**

3.7.2 *arsB* and *ACR3* gene amplification through a Polymerase Chain Reaction (PCR) approach

The *arsB* and *ACR3* gene amplifications were performed in 200 µl eppendorf type tubes with a final volume of 50 µl of the reaction mixture described in Table 7. The primers used in PCR amplifications are listed in Table 8.

Table 7: PCR reaction mixture for the amplification of *arsB* and *ACR3* genes

ultrapure H_2O	**26.75 µl**
Buffer 10X Mg^{2+} free (DyNAzyme)	**5 µl**
$MgCl_2$ 50mM	**1.5 µl**
dNTP's (1mM)	**10 µl**
Primer "forward"*	**2 µl**
Primer "reverse"*	**2 µl**
DNA sample	**2 µl**
DNA polimerase (DyNAzyme II)	**0.75 µl**

Table 8: Primers for the amplification of *arsB* and *ACR3* genes

Primer	Sequence
***arsB*f**	**5' – GTSATYTGGCARCCSAARGG – 3'**
***arsB*r**	**5' – GTSGGCATRTTRTTCATRAT – 3'**
***ACR3*1f**	**5' – GTSGGBTGYGGMTAYCABGYCTA – 3'**
***ACR3*1r**	**5' – TTGTASGCBGGMCGRTTRTGRAT – 3'**
***ACR3*2f**	**5' – GTSATYTGGCARCCSAARGG – 3'**
***ACR3*2r**	**5' – GTSATYTGGCARCCSAARGG – 3'**

*Primers for the amplification were constructed based on the method described in [71]

The *arsB* PCR reactions were performed in a Biorad's "MyCycler Thermal Cycler" with the following program: 94 ºC for 5 min (initial denaturation) followed by 30 cycles with 90 ºC for 1 min (denaturation), 54 ºC for 1 min (primer annealing) and 72 ºC for 1 min (extension). The final step consisted of 72 ºC for 5 min to assure that the extension of PCR products was complete. A negative control was performed simultaneously to detect an eventual DNA contamination of the master mixture. A positive control consisting of DNA from bacterium *Ochrobactrum tritici* was used to ascertain if the reaction was successful and to establish a reference size for amplified fragments. The *ACR3* PCR reactions were performed in a Biorad's "MyCycler Thermal Cycler" with the following program: 94 ºC for 5 min (initial denaturation) followed by 35 cycles with 90 ºC for 45s (denaturation), 57-52 ºC for 30s with the first ten cycles with -0,5ºC touchdown (primer annealing) and 72 ºC for 30s (extension). The final step consisted of 72 ºC for 5 min to assure that the extension of PCR products was complete. A negative control was performed simultaneously to detect an eventual DNA contamination of the master mixture. A positive control consisting of DNA from bacterium *Ochrobactrum tritici* was

used to ascertain if the reaction was successful and to establish a reference size for amplified fragments.

3.7.3 *arsC* gene amplification through a PCR approach

The *arsC* gene amplifications were performed in 200 µl eppendorf type tubes with a final volume of 50 µl with a mixture equal to the one used in 3.7.2. The program was performed with the same values as the *arsB* program found in 3.7.2. The used primers are listed in Table 9.

Table 9: Primers for the amplification of *arsC* gene

Primer	Sequence
***arsC*f**	**5' – ATYACYATYTAYCACAACCC – 3'**
***arsC*r**	**5' – TCACCRTCYTCYTTSGTGAA– 3'**

*Primers for the amplification were constructed based on the method described in [71]

3.7.4 *aioB* gene amplification through a PCR approach

aioB gene amplification was performed in 200 µl eppendorf type tubes with a final volume of 50 µl and the reaction mixture is described in Table 10. The primers used in PCR amplifications are listed in Table 11.

Table 10: PCR reaction mixture for the amplification of *aioB* gene

ultrapure H_2O	**23.8 µl**
Buffer 10X Mg^{2+} free (DyNAzyme)	**5 µl**
$MgCl_2$ 50mM	**1 µl**
dNTP's (1mM)	**5 µl**
Primer "forward"	**4 µl**
Primer "reverse"	**4 µl**
DNA sample	**7 µl**
DNA polymerase (Invitrogene Taq polymerase Platinum)	**0,2 µl**

Table 11: Primers for the amplification of *aioB* gene

Primer	Sequence
aioBf1*	**5' – GTSGGBTGYGGMTAYCABGYCTA– 3'**
aioBr1*	**5' – TTGTASGCBGGNCGRTTRTGRAT – 3'**
aioBf2*	**5' – GTCGGYTGYGGMTAYCAYGYYTA – 3'**
aioBr2*	**5' – YTCDGARTTGTAGGCYGGBCG – 3'**

The partial arsenite oxidase gene was amplified in Biorad's "MyCycler Thermal Cycler" using the following program: 94ºC for 4min (initial denaturation) followed by 9 cycles with 90ºC for 45s (denaturation), 50º-0.5ºC "touchdown" (per cycle) for 45s ("annealing") and 72ºC for 50s (extension). The first 9 cycles were followed by 25 cycles with 95ºC for 45s (denaturation), 46ºC for 45s ("annealing") and 72ºC for 50s (extension). The final step consisted of 72ºC for 5min step to assure that the extension of PCR products was complete. A negative control was performed simultaneously to detect a possible DNA contamination. A positive control was performed simultaneously to verify the consistency of the reaction.

3.7.5 *arr*A gene amplification through a PCR approach

*arr*A gene amplifications were performed in 200µl eppendorf type tubes with a final volume of 50µl and a reaction mixture equal to the one used in 3.7.2 with the primers listed in Table 12.

Table 12: Primers for the amplification of *arrA* gene

Primer	Sequence
arrAf1*	**5' – CARCARGTGTAYGATCC – 3'**
arrAr1*	**5' – CWTCYCAKGCYACATCACC - 3'**

*Amplification was performed using a protocol adapted from the method described in [64]

Briefly, DNA was amplified in Biorad's "MyCycler Thermal Cycler" with the following program: 94 ºC for 5 min followed by 35 cycles with 94 ºC for 30s (denaturation), 50 ºC for 30s ("annealing") and 72 ºC for 1 min (extension). The final step consisted of 72 ºC for 5 min to assure that the extension of PCR products was complete. A negative control was performed simultaneously to detect an eventual DNA contamination of the master mixture. A positive control consisting of DNA from bacterium *Ochrobactrum tritici* was used to ascertain if the reaction was successful.

3.7.6 Visualization of the amplified products

Agarose was hydrated by addition of the TAE solution. In order to increase hydration efficiency the solution was heated for 4 minutes in a microwave oven with a power of 700 W. After the heating step the volume was adjusted to 100 ml with H_2O. A cooling step followed, always accompanied by slight agitation, then 5µl of ethidium bromide solution were added and the mixture was stirred until the ethidium bromide was completely dispersed (Table 13). The gel was poured in gel rack and the combs were inserted. After polymerization the combs were removed.

Table 13: 1% agarose gel

Agarose (Sigma)	**1 g**
TAE 1x solution	**100 ml**
Ethidium Bromide	**5 µl**

TAE solution (1x) was prepared by dilution of the stock (50x) solution in ultra pure water (Table 14).

Table 14: Stock TAE solution (50x)

Tris (Merck)	**121 g**
Acetic acid (Merck)	**28.55 ml**
Aqueous EDTA solution(0,5 M) (Sigma)	**50 ml**
ultrapure H_2O	**up to 500 ml**

Tris was dissolved in the aqueous EDTA solution and the acetic acid was added. The pH value was adjusted to 8.0 with a NaOH (5 M) solution. The volume was then adjusted to 500 ml with ultra pure water. The stock EDTA solution was stored at room temperature.Ethidium bromide was dissolved in water and the resulting solution was stored in at 4 ºC in an opaque bottle (Table 15).

Table 15: Ethidium bromide solution (5 mg/ml)

Ethidium bromide	**0.5 g**
Ultra pure H_2O	**100 ml**

3.7.7 Electrophoresis

After each amplification reaction, to verify the amplified products, an agarose gel electrophoresis was undertaken. The 1% agarose gel was prepared and submerged on a TAE working buffer solution. The DNA samples (50 µl), with added 5 µl of "*loading buffer*", were applied in the gel and were separated through differential migration speeds through the gel for 45 min at 100 V. The products were visualized using UV light and the

fragments were determined through comparative analysis with the DNA Marker 1 Kb Plus DNA Ladder Invitrogen- (0.1 to 12 Kb).

3.8 Sequencing of the obtained PCR products

3.8.1 Band purification

After UV visualization of the gel, the bands corresponding to molecular weights of the genes being studied were collected. The DNA from the bands was obtained through purification with "JETquick Spin Column Technique – PCR Purification Spin Kit" (Genomed) according to manufacturer protocol. Briefly: The band was dissolved in heated agarose solubilisation solution and the resulting solution was separated through chromatography in a silica gel matrix. After several washing steps, the DNA was collected by addition of 30 µl of TE to the column and posterior centrifugation at 12000 rpm for 1.5 min. The resulting DNA was conserved in TE, the Tris-HCl buffer maintains the pH of the solution near 8 which is optimum for inhibition of nuclease action, and EDTA chelates ions essential for nuclease action, thus increasing the effective conservation time of the samples.

3.8.2 Sequence analysis

The sequences were obtained through the enzymatic method described in [72]. The sequencing reaction was prepared in a 200 uL micro tube with 10 uL of the components listed in Table 16.

Table 16: Sequencing reaction mixture

ultrapure H_2O	**23.8 µl**
Primer solution	**5 µl**
DNA sample	**1 µl**
Sequencing solution (Applied Biosystems)	**5 µl**

The reaction mixture was placed in Biorad's "MyCycler Thermal Cycler" with the following program: 96ºC for 1min (initial denaturation) followed by 26 cycles with 90ºC for 10s (denaturation), 50ºC for 5s ("annealing") and 60ºC for 5min (extension). At the end of the program temperature was maintained at 4ºC.

The purified PCR products sequencing, through capillary electrophoresis, was outsourced and the corresponding .ab1 files, with the resulting chromatograms, were manually analyzed and validated using Applied Biosystems sequence scanner. Obtained sequences were compared to the GeneBank nucleotide data library using the BLAST software (in order to determine their closest phylogenetic relatives) [73]. Sequences were initially aligned with the CLUSTAL X algorithm [74], visually examined, and relocated to allow maximal alignment. The method of Jukes and Cantor (Jukes and Cantor 1969) was used to calculate evolutionary distances. This method measures the evolutionary distances between two or more subjects, based on the sequence homology of the compared sequences. Phylogenetic trees were constructed by the neighbor-joining method and unweighted pair group method with arithmetic mean (UPGMA) using the MEGA5 package. Phylogenetic trees allow the reconstruction of the evolutionary history of a group of species. With this method, it is possible estimate and analyse the ancestry relationship between the individuals from a group. The statistically significance and the topological stability of the obtained groups are confirmed by the bootstrap analysis (1000 replicates) in the MEGA 5. With this analysis, there is a parameter that reflects the robustness of the produced phylogenetic analysis. The bootstrap is generated with the creation of several sequences groups where are randomly chosen columns of the multiple alignment, and are generated new analysis for each new subgroups [75].

3.9 Genetic screening for oxidase genes in whole samples from soil

3.9.1 Soil total DNA extraction:

Total soil/sludge DNA was obtained through extraction with the E.Z.N.A.® Soil DNA kit D5626-01 (Omega Bio-Tek) according to the manufacturer's instructions. Briefly, the water/sludge samples were centrifuged with glass beads and then treated in a specially formulated buffer containing detergent. Proteins, polysaccharides, and other contaminants are subsequently precipitated after a heat-frozen step. Contaminants are further removed by extraction steps. Binding conditions were then adjusted and the samples were applied to a HiBand^{TM} DNA spin-column. Two rapid wash steps removed trace contaminants and pure DNA was eluted in water or low ionic strength buffer.

3.9.2 *aioB* gene amplification through a PCR approach

The *aioB* gene amplification was performed in 200µl eppendorf type tubes with a final volume of 50µl with the same mixture and primers as in section 3.7.4.

3.9.3 Electrophoresis and band purification

Electrophoresis and band purification were achieved through the same methods as the ones described in sections 3.7.7 and 3.8.1 respectively.

3.9.4 Cloning of the amplified PCR products

The purified DNA fragments amplified in the PCR step were cloned in *E. coli* DH5α. Cloning was accomplished through the pCR4-TOPO (Invitrogen) kit according to the

protocol supplied by the manufacturer. Briefly, the PCR fragments were cloned in the plasmid supplied with the kit; cells were then transformed through thermal shock. Transformed cells were then spread and incubated overnight at 37 ºC in LB agar supplemented with 100µg/ml ampicillin, 100µl IPTG (0.1M) and 20µl X-gal (40mg/ml). The positive colonies were selected through blue/white screening and the white colonies were transferred to liquid LB supplemented with 100µg/ml ampicillin and allowed to grow for 12 hours for posterior collection of the plasmids.

3.9.5 Plasmid DNA extraction from positive selected colonies

Plasmid DNA extraction from selected colonies was achieved through the use of the commercial kit JETSTAR Mini (GENOMED). Briefly: *E. coli* cells were harvested, resuspended in cell resuspending buffer with RNase A, and then lysed with lysis buffer. Precipitation buffer was added to the lysate and this was clarified by centrifugation. The cleared lysate was passed through a pre-packed anion exchange column. The negatively charged phosphates on the backbone of the DNA interact with the positive charges on the surface of the resin. The temperature, salt concentration, and pH of the solutions influence binding. Under moderate salt conditions, plasmid DNA remains bound to the resin while RNA, proteins, carbohydrates and other impurities are washed away with wash buffer. The DNA plasmid was eluted under high salt conditions with the elution buffer.. The eluted DNA was desalted and concentrated with an alcohol precipitation step. All these solutions were provided by the purchased plasmid extraction system.

3.10 Sequence analysis

Sequencing and analysis of the cloned sequences was obtained through the same methods as described in 3.8.2.

Chapter 4 Results

4 Results

4.1 Phylogeny of partial 16S sequences of As resistant strains

In order to identify the isolated strains the obtained rRNA 16S sequences were compared to the EZTaxon nucleotide data library using the EZTaxon software (in order to determine their closest phylogenetic relatives). Sequences were initially aligned with the CLUSTAL X algorithm [74], visually examined, and relocated to allow maximal alignment. Phylogenetic trees were constructed by the neighbor-joining method method using the MEGA5 package (Figure 7).

4.2 Identification and characterization of arsenic resistant isolates

The abundance and diversity of arsenic resistant bacteria was ascertained in all soil samples by selection of the bacteria which exhibited growth when cultured in arsenite supplemented R2A agar at 30 ºC. Resistance was defined as the ability to grow in a R2A agar supplemented with a minimum of 2 mM of arsenite (Table 17). Bacterial isolates from the eight sampled areas, were tested for their ability to resist arsenic, as shown on figure 8. Strains were considered resistant if significant growth was observed when cultured on agar supplemented with 2mM sodium arsenite. There were a total of 36 arsenic resistant strains in a universe of 78 cultured strains, which amounts to 46% resistant bacteria. Among the resistant bacteria, 14 were Gram-positive whereas 25 were Gram-negative. 20 mM.

Table 17. Arsenite Resistant Bacterial isolate Characteristics

Isolates	Closest organism when compared to EzTaxon database	Degree of similarity	5 Days resistance to [AsIII]					Arsenic resistance genes					
			2mM	5mM	10mM	15mM	20mM	*arsB*	*ACR3*	*arsC*1	*arsC*2	aio1	arr
A1 652	***Chryseobacterium indoltheticum LMG 4025***	98,09%	+++	+++	-	-	-	-	-	-	-	-	-
A1 653	***Pseudomonas cedrina P515/12***	100%	+++	+++	+++	+++	+++	+	-	-	-	-	-
A1 654	***Chryseobacterium indoltheticum LMG 4025***	98,09%	+++	+++	-	-	-	+	+	-	-	-	-
A1 655	***P. cedrina P515/12***	100%	+++	+++	+++	+++	-	multi	uns	-	-	-	-
A1 662	***P. cedrina P515/12***	100%	+++	+++	+++	+++	+	+	+	-	-	-	-
A1 844	***P. cedrina P515/12***	100%	+++	+++	+++	+++	-	+	+	-	-	-	-
A1 845	***P. cedrina P515/12***	100%	+++	+++	++	+	-	+	+	-	-	-	-
A1 846	***P. cedrina P515/12***	100%	+++	+++	+++	+++	-	-	+	-	-	-	-
A1 847	***P. cedrina P515/12***	100%	+++	+++	+++	+++	+	+	+	-	-	-	-
A1 890	***P. cedrina P515/12***	100%	+++	+++	+++	+++	-	+	+	-	-	-	-
A1 891	***P. cedrina P515/12***	100%	+++	+++	+++	+++	-	+	-	-	-	-	-
A1 892	***P. cedrina P515/12***	100%	+++	+++	++	++	+	+	+	-	-	-	-
A1 893	***P. cedrina P515/12***	100%	+++	+++	+++	++	+	+	+	-	-	-	-
A1 894	***P. cedrina P515/12***	100%	+++	+++	+++	+++	+	+	+	-	-	-	-
A1 977	***Afipia broomeae ATCC49717***	100%	+++	+++	-	-	-	+	-	N.A.	N.A.	N.A.	N.A.
A1 978	***A. broomeae ATCC49717***	100%	+	+	-	-	-	-	-	-	-	-	-
A3 737	***Roseomonas lacus TH-G33***	99,75%	+++	-	-	-	-	-	-	N.A.	N.A.	-	-
A3 738a	***Microbacterium ginsengisoli Gsoil 259***	97,83%	+	-	-	-	-	-	-	N.A.	N.A.	-	-
A3 742a	***Cohnella plantaginis YN-83***	97,76%	+++	-	-	-	-	+	-	N.A.	N.A.	-	-
A3 881	***Bacillus isronensis B3W22***	99,39%	+++	+++	+++	+++	+++	+	-	N.A.	N.A.	-	-
A3 882	***B. isronensis B3W22***	99,39%	+++	+++	+++	-	-	+	-	N.A.	N.A.	N.A.	N.A.
A3 918	***Staphylococcus cohnii ATCC49330***	100%	+++	+++	+++	+++	-	+	-	N.A.	N.A.	-	-
A4 873	***Pseudomonas lurida DSM 15838***	99,58%	+++	+++	+++	-	-	+	-	N.A.	N.A.	-	-

A4 874	***P. lurida DSM 15838***	99,58%	+++	+++	+++	-	-	+	-	N.A.	N.A.	-	-
A4 875	***P. arsenicoxydans VC-1***	99,58%	+++	+++	++	-	-	+	-	N.A.	N.A.	-	-
A5 810	***Burkholderia sabiae Br 3407***	97,64%	+++	-	-	-	-	-	-	N.A.	N.A.	-	-
A5 852	***Rhodococcus globerulus DSM 4954***	100%	+++	+++	+++	+++	+++	-	-	N.A.	N.A.	N.A.	N.A.
A5 925	***Simplicispira limi EMB325***	96,16%	+++	+++	+++	-	-	-	+	N.A.	N.A.	N.A.	N.A.
A5 926	***S. limi EMB325***	96,16%	+	-	-	-	-	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
A5 930	***Paenibacillus taichungensis BCRC 17757***	99,60%	++	+	-	-	-	-	-	N.A.	N.A.	-	-
A5 935	***P. agaridevorans DSM 1355***	97,17%	+++	+++	-	-	-	+	+	N.A.	N.A.	N.A.	N.A.
A6 856	***Rhodococcus globerulus DSM 4954***	100%	+++	+++	+++	++	+++	-	-	N.A.	N.A.	-	-
A6 857	***R. globerulus DSM 4954***	100%	+++	+++	+++	+	+++	-	-	N.A.	N.A.	-	-
A6 863	***Acinetobacter indicus A648***	98,29%	+++	+++	+++	+++	+	+	-	N.A.	N.A.	-	-
7B 581	***Paenibacillus agaridevorans DSM 1355***	100%	+++	+++	+++	-	-	+	+	N.A.	N.A.	N.A.	N.A.
7B 832	***Bacillus drentensis LMG21831***	97,04%	+	++	++	+	-	-	-	N.A.	N.A.	-	-

+,++,+++ represent the degree of observed growth, moderate, average and regular respectively when samples were retrieved after being cultured in arsenite supplemented media.

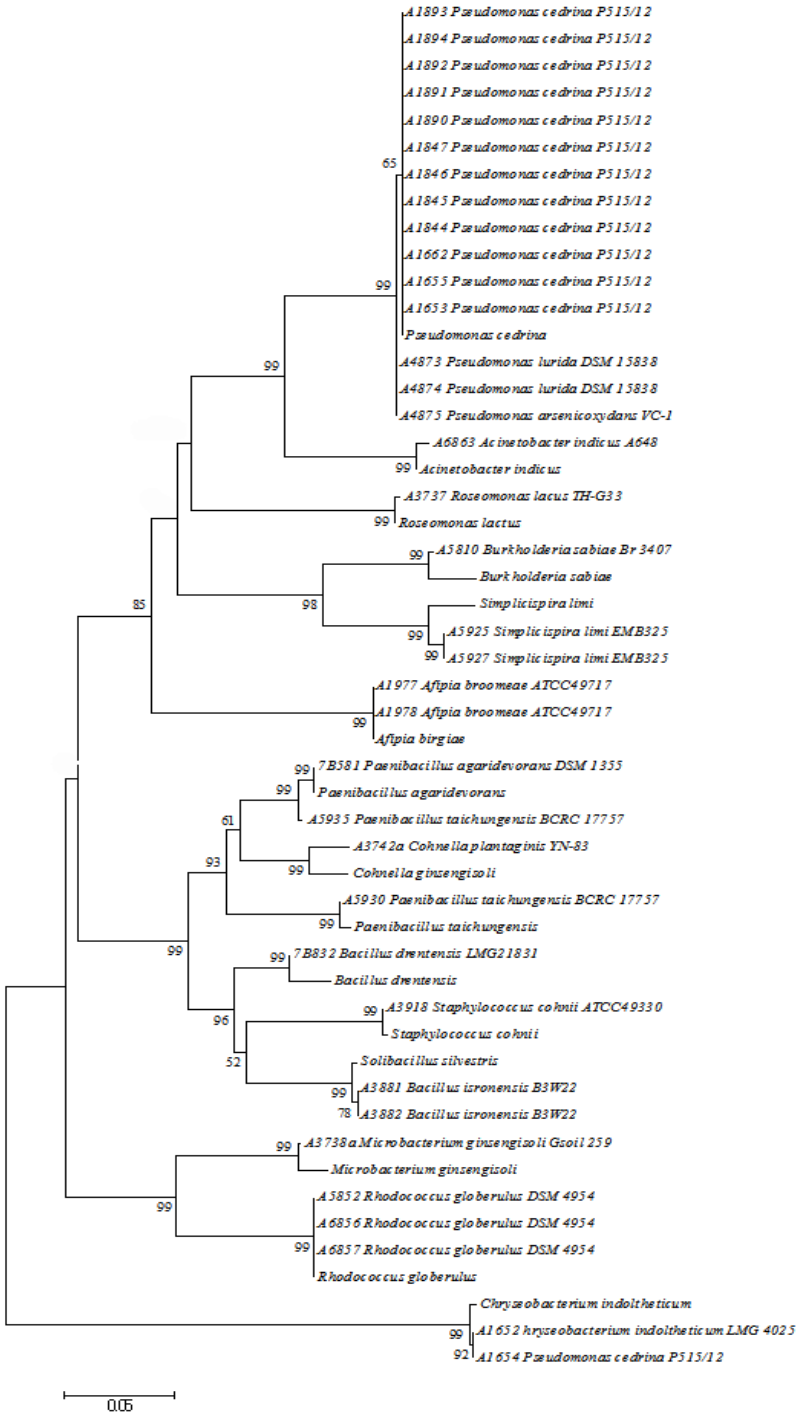

Fig. 7 Phylogeny of partial 16S rRNA (450 bp) of resistante isolates found in this study. Bootstrap values under 50% are not shown. The scale bars indicate substitutions per site. Identifications were retrieved by comparison to the EzTaxon database. Reference closest organisms were added for reference and appear without site number in the identification.

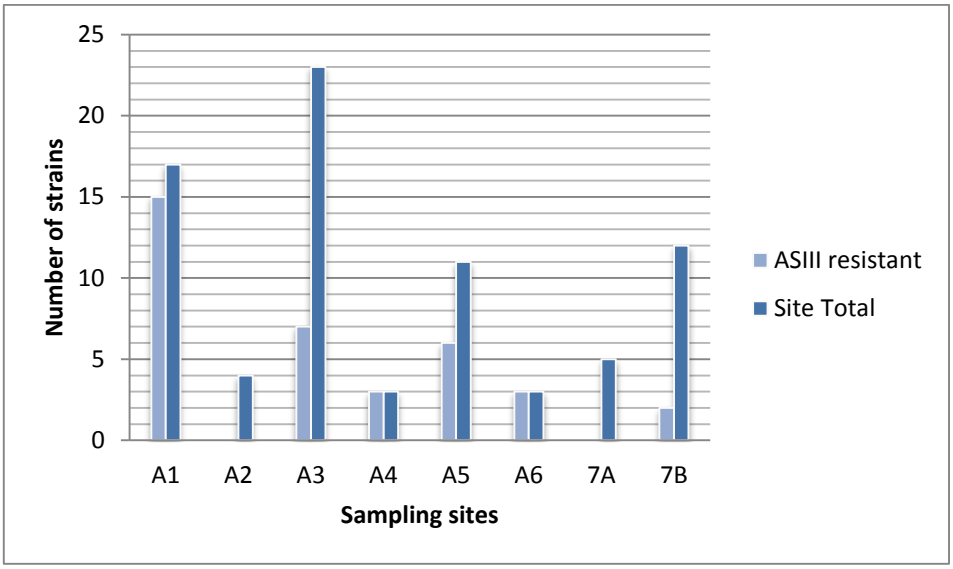

Fig. 8 The number arsenic resistant strains in each sampling site compared to the total number of strains isolated from the site. Strains were considered resistant if significant growth was observed when cultured on agar supplemented with 2mM sodium arsenite.

Of the 78 strains tested for arsenite resistance there was a highly variable minimum inhibitory concentration (MIC), ranging from 2 mM to 20 mM, with approximately 28% of the bacteria exhibiting resistance to the maximum used value of The group of bacteria Gram-staining-positive that exhibited resistance to arsenite was diverse and belonged to genera *Bacillus, Rhodococcus, Staphylococcus, Paenibacillus* and *Microbacterium*. The degree of resistance was variable among the cited genera, with *Rhodococcus* globerulus, highly resistant, exhibiting resistance up to 20mM arsenite and Microbacterium *gisengisoli* only exhibited moderate growth at 2mM arsenite exhibiting no growth at higher concentrations. *Paenibacillus* and *Saphylococcus* strains exhibited resistance up to 15mM arsenite. The Gram-staining-negative bacteria belonged to the genera *Flavobacterium, Chryseobacterium, Pseudomonas, Afipia, Burkholderia, Variovorax* and *Comamonas* and also exhibited resistance to arsenite. These Strains showed variable degrees of resistance, even in individuals of the same generus, as was the case of the *Pseudomonas* isolates retrieved from area A1. The sampling site with the greatest number

of resistant strains was A1, in this site there wasn't a detectable concentration of arsenic ions. In this area, 88% of the strains exhibited resistance to arsenite. Though no arsenic was detected the area was treated with barium and calcium. Despite of the presence of arsenic, in the area A2, no resistant strains were found in the soil samples tested. In area A3, only 30% of the tested strains were resistant to arsenite. In area A4, though the number of cultivated strains was limited, all of the strains belonged to the genus *Pseudomonas* and exhibited resistance to concentrations of 15 mM of arsenite . In area A5, more than 50% of the strains exhibited resistance to arsenic, with one of the representatives of the genus *Rhodococcus* exhibiting resistance up to 20 mM of arsenite when cultivated in supplemented R2A agar. All of the cultivated strains from area A6 exhibited resistance to arsenite. Finally, none of the strains isolated from area 7A exhibited arsenite resistance while, 25% of the bacteria from area 7B exhibited arsenic resistance as seen on figure 9.

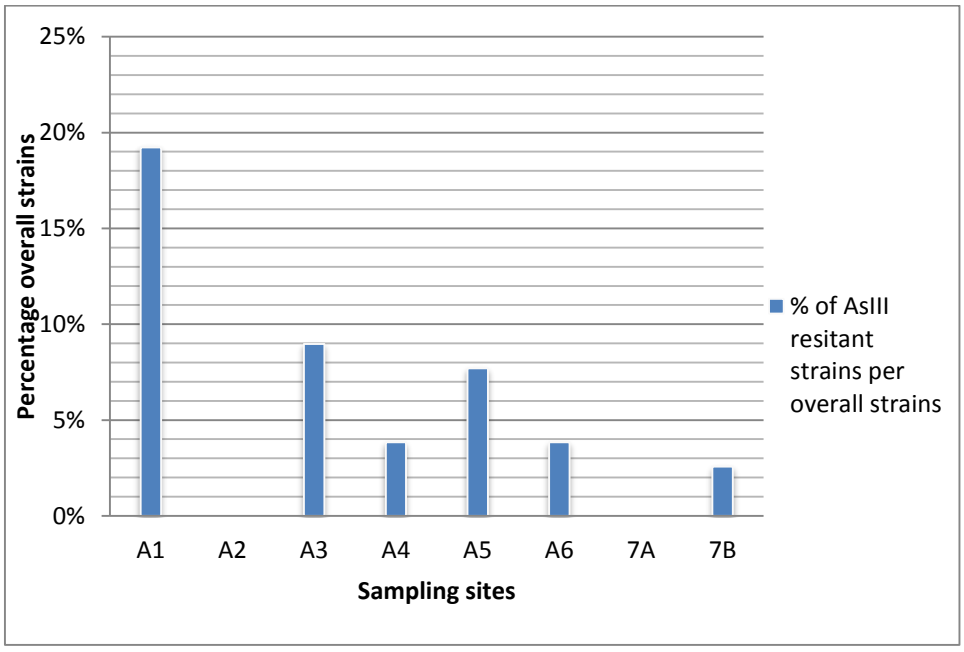

Fig. 9 Percentage of resistant strains in each individual sampling site compared to the total number of isolates. Values are expressed as a percentage where 100% is the total number of isolates retrieved in the experiment.

4.3 Identification of Antimonite resistance isolates

Arsenic resistant isolates were tested for antimonite resistance since anionic pumps involved in arsenite resistance also confer some degree of resistance to antimonite [35]. Resistance was defined as the ability to survive in an LB plate supplemented with 1 mM antimonite (Table 18). Of the isolates from area A1, selected for having arsenic resistant genes, over 70% exhibited resistance to antimonite (Figure 10). All of the selected isolates from areas A3, A4 and A5 exhibited resistance to antimonite. Only one of the selected isolates from area A6 exhibited antimonite resistance which represents a percentage of 33%. All isolates selected from area 7B exhibited resistance to antimonite, although the isolate 7B 832 was only resistant to concentrations of 1 mM.

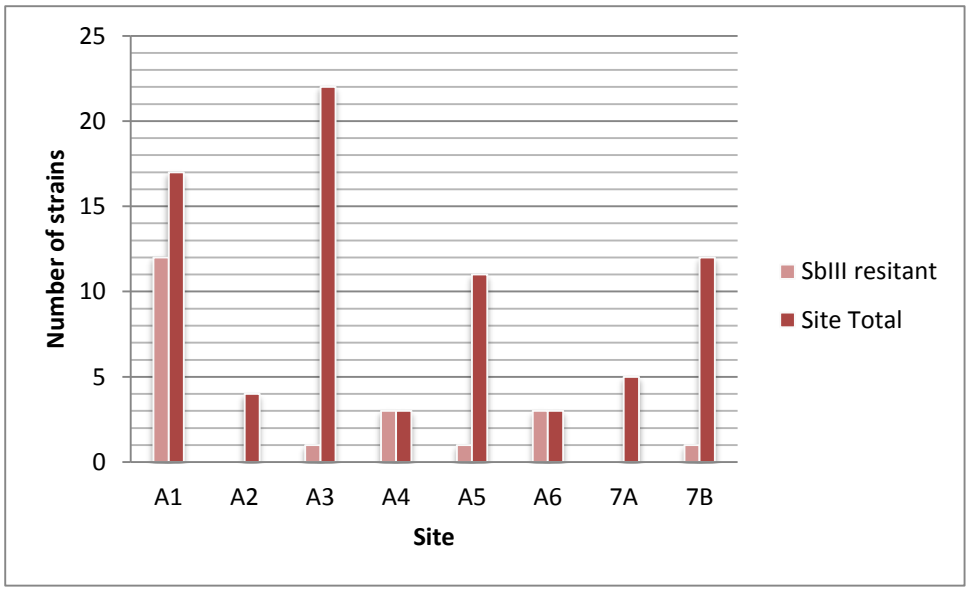

Fig. 10 The number antimonite resistant strains in each sampling site compared to the total number of strains isolated from the site. Strains were considered resistant if significant growth was observed when cultured on agar supplemented with 1mM potassium antimony tartarate.

Table 18 Characteristics of Bacterial isolates with antimonite resistance

Isolates	Closest organism when compared to EzTaxon database	Degree of similarity	ID	7 Days resistance to [SbIII]		
				1mM	2mM	4mM
A1 654	*Chryseobacterium indoltheticum LMG 4025*	98,09%	A1 654	-	-	-
A1 655	*Pseudomonas cedrina P515/12*	100%	A1 655	++	++	+
A1 662	*P. cedrina P515/12*	100%	A1 662	++	++	++
A1 844	*P. cedrina P515/12*	100%	A1 844	++	++	++
A1 845	*P. cedrina P515/12*	100%	A1 845	+	+	+
A1 846	*P. cedrina P515/12*	100%	A1 846	++	++	++
A1 847	*P. cedrina P515/12*	100%	A1 847	++	++	++
A1 890	*P. cedrina P515/12*	100%	A1 890	++	++	++
A1 891	*P. cedrina P515/12*	100%	A1 891	++	++	++
A1 892	*P. cedrina P515/12*	100%	A1 892	+	+	+
A1 893	*P. cedrina P515/12*	100%	A1 893	++	++	++
A1 894	*P. cedrina P515/12*	100%	A1 894	++	++	++
A3 737	*Roseomonas lacus TH-G33*	99,75%	A3 737	++	++	++
A4 873	*Pseudomonas lurida DSM 15838*	99,58%	A4 873	+	+	+
A4 874	*P.s lurida DSM 15838*	99,58%	A4 874	+	+	+
A4 875	*P. arsenicoxydans VC-1*	99,58%	A4 875	+	+	+
A5 852	*Rhodococcus globerulus DSM 4954*	100%	A5 852	++	++	+
A6 856	*R. globerulus DSM 4954*	100%	A6 856	-	-	-
A6 857	*R. globerulus DSM 4954*	100%	A6 857	-	-	-
A6 863	*Acinetobacter indicus A648*	98,29%	A6 863	++	+	-
7B 832	*Bacillus drentensis LMG21831(T)*	97,04%	7B 832	++	++	+

-,+,++, represent the degree of observed growth, absent, average and regular respectively when samples were retrieved after being cultured in antimonite supplemented media. Some samples were absent, we were unable to recover the samples for cultivation.

4.4 Identification of arsenite oxidizing isolates

All isolates exhibiting resistance to arsenite were tested for phenotypical evidence of arsenite oxidation. None of the tested isolates exhibited any oxidation of arsenite in chemically defined medium.

4.5 Arsenite transporter genes *arsB* and *ACR3* from arsenite resistant isolates

For the detection of arsenic transporter genetic traits, *arsB*, *ACR3*(1) and *ACR3*(2) , three sets of degenerate primers were used. For the *ACR3* gene a pair of primers was used, one based on the *E. coli* operon *ACR3* and other based on the Yeast *S. cerevisiae ACR3* to guarantee the ability to detect the presence of the gene regardless of the form of the gene present. As a positive control for the desired sequences *Ochrobactrum tritici* DNA was included in each experiment. Amplification products of the expected size, 1000 bp (*arsB*), and 700 bp (*ACR3(*1) and 2) were obtained and confirmed through comparison to positive controls (data not shown). Of the 78 isolates, 25 strains showed amplification with either one or two of the specific primers designed for this step (Figure 11). The presence of *arsB* genes was detected in 22 isolates: (γ-Proteobacteria (15), α-Proteobacteria (1), Flavobacteria (1), Actinobacteria (1), Firmicutes (4) (Figure 12). The *ACR3* gene was found in 14 isolates belonging to the families: (γ-Proteobacteria (9), β-proteobacteria (2), Flavobacteria (1), Firmicutes (2). Among of these isolated that exhibited the *ACR3* genes, 11 strains also contained the *arsB* gene.

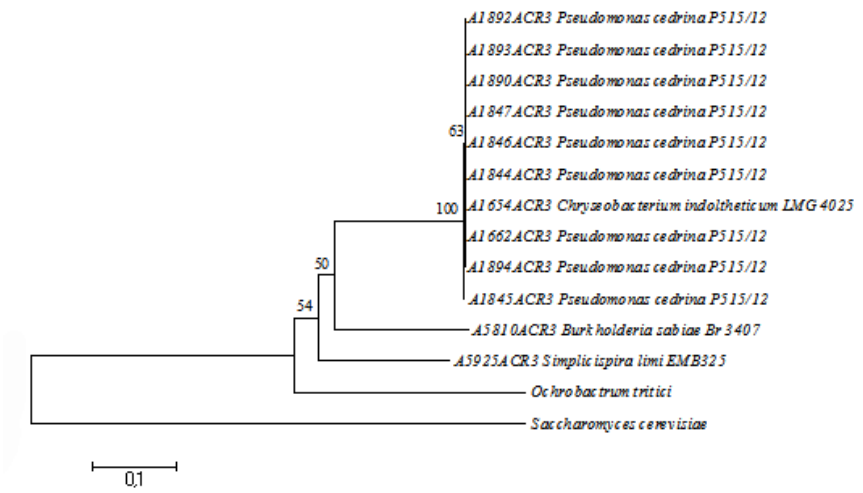

Fig. 11 Phylogeny of the retrieved sequences (700bp) from *ACR3* gene amplifications. Bootstrap values below 50% are not shown. Two reference sequences, *Ochrobactrum tritici* and *Saccharomyces cerevisiae*, were added as reference representing the targeted *ACR3* gene amplified by primer sets *ACR3*1 and *ACR3*2 respectively. Sequences from this study are identified by closest organism according to the EzTaxon database with the study reference number and corresponding gene appearing as prefix

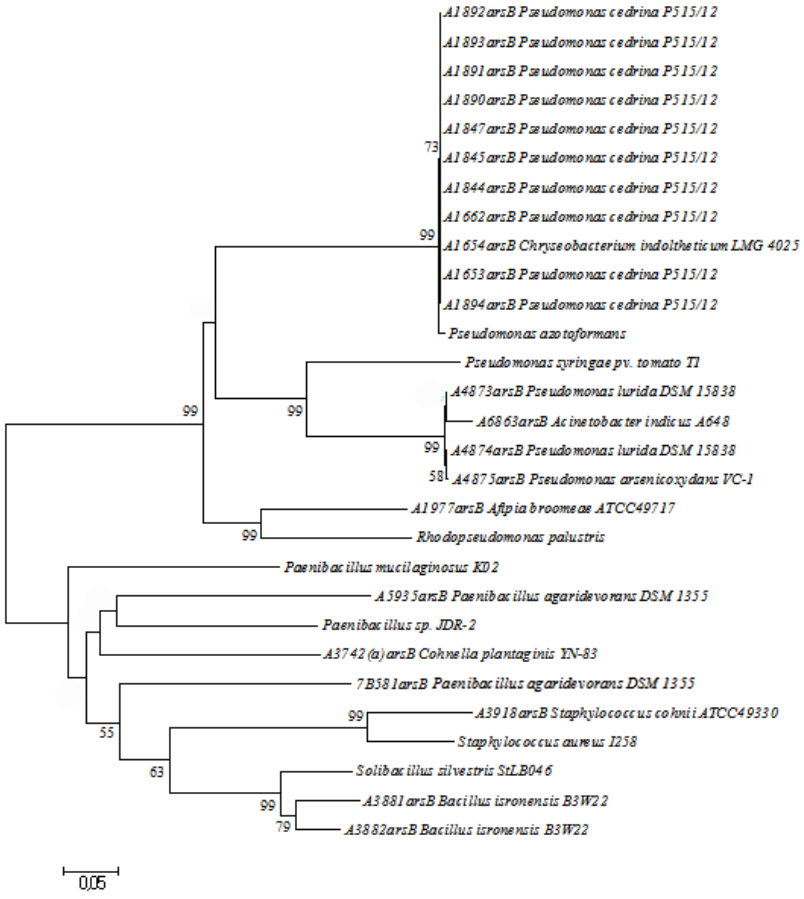

Fig. 12: Phylogeny of retrieved DNA sequences (~950 bp) from *arsB* amplification. Bootstrap values below 50% are not shown. Reference *arsB* sequences obtained through comparison to the BLAST database were used, and are represented by the species name. Sequences from this study are identified by closest organism according to the EzTaxon database with the study reference number appearing as prefix.

4.6 Comparison of phylogenetic relationships between organisms and their arsenic resistance determinants.

After identification and phylogenetic relationship establishment, 16 rRNA phylogenetic trees where compared to arsenic resistance determinant phylogenetic trees in order to identify horizontal gene transfer events among the isolates tested in this work. Regarding *ACR3* determinants we can observe the grouping of the *ACR3* gene from strain A1-654 *Chryseobacterium* in the same cluster as the *ACR3* gene found in strains from the *Pseudomonas* genus (Figure 13). Regarding *arsB* phylogeny, as verified in figure 14, strain A1-654 *Chryseobacterium indoltheticum* had its *arsB* gene clustered with genes detected in *Pseudomonas*. Also of note is the separation of the *arsB* determinants from same genus strains, namely *P. lurida* and *P. arsenoxydans arsB* was on a different branch form *P. cedrina arsB.*

4.7 Arsenate reductase gene *arsC* from arsenite resistant isolates.

For the detection of arsenate reductase traits, *arsC*, one set of degenerate primers was used. As a positive control for the desired sequences *Ochrobactrum tritici* DNA was included in each experiment. There was no verified amplification of the desired fragment in the designed experiment thus the presence of arsenate reductase genes *arsC* was not confirmed in the selected arsenite resistant isolates.

4.8 Arsenite respiratory reductase arrA from arsenite resistant isolates.

For the detection of arsenite respiratory reductase genes from arsenite resistant isolates one set of degenerate primers was used. The protocol was developed according to the work reported in [64]. No positive amplification was detected in any of the samples.

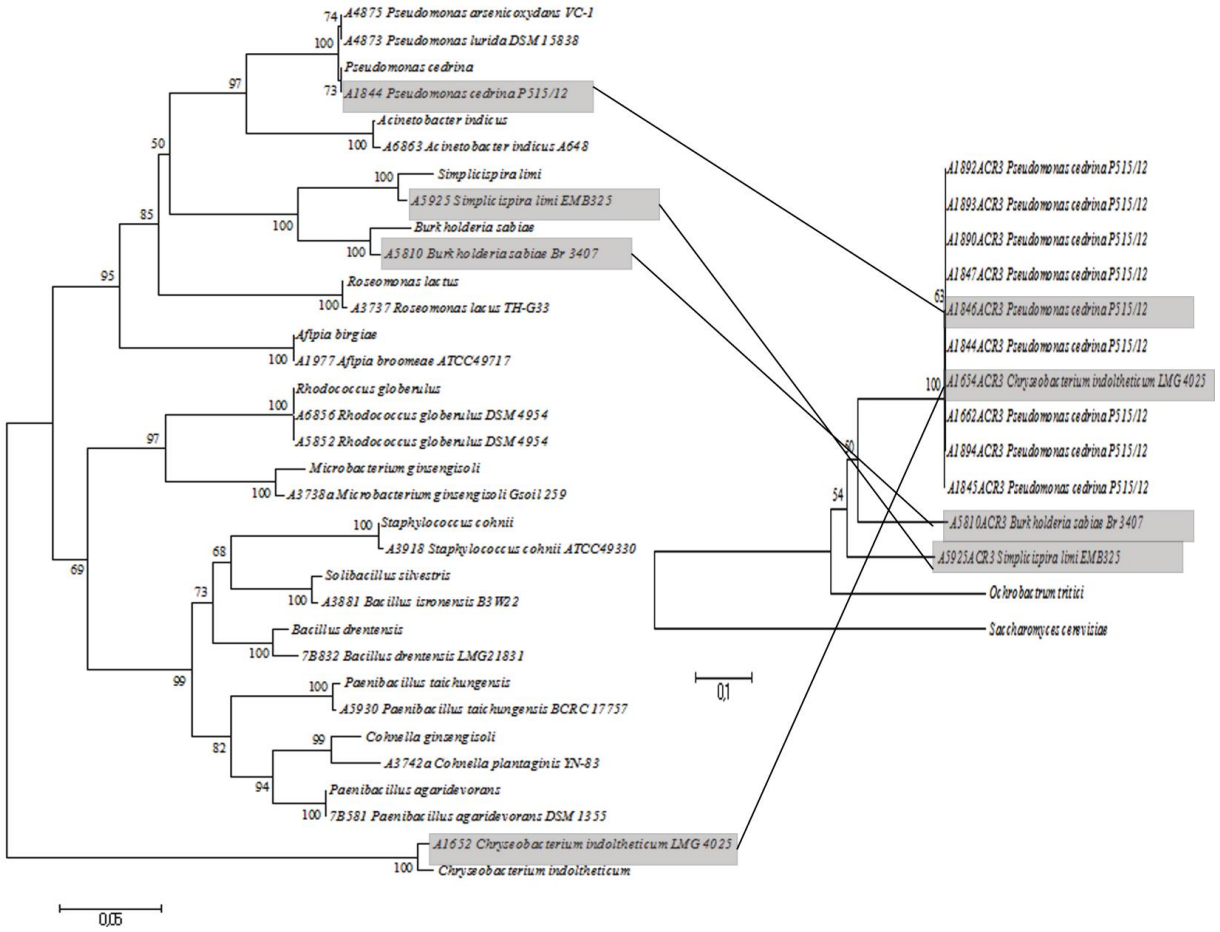

Fig. 13 Neighbor-joining analysis of (left) 16S rRNA and (right) *ACR3* sequences from arsenic resistant samples. Individual *ACR3* genes are linked to the corresponding organism of origin in the 16S tree. Unique entries were shaded for ease of interpretation. Two reference sequences, *Ochrobactrum tritici* and *Saccharomyces cerevisiae*, were added as reference representing the targeted *ACR3* gene amplified by primer sets *ACR3*1 and *ACR3*2 respectively. Sequences from this study are identified by closest organism according to the EzTaxon database with the study reference number and corresponding gene appearing as prefix.

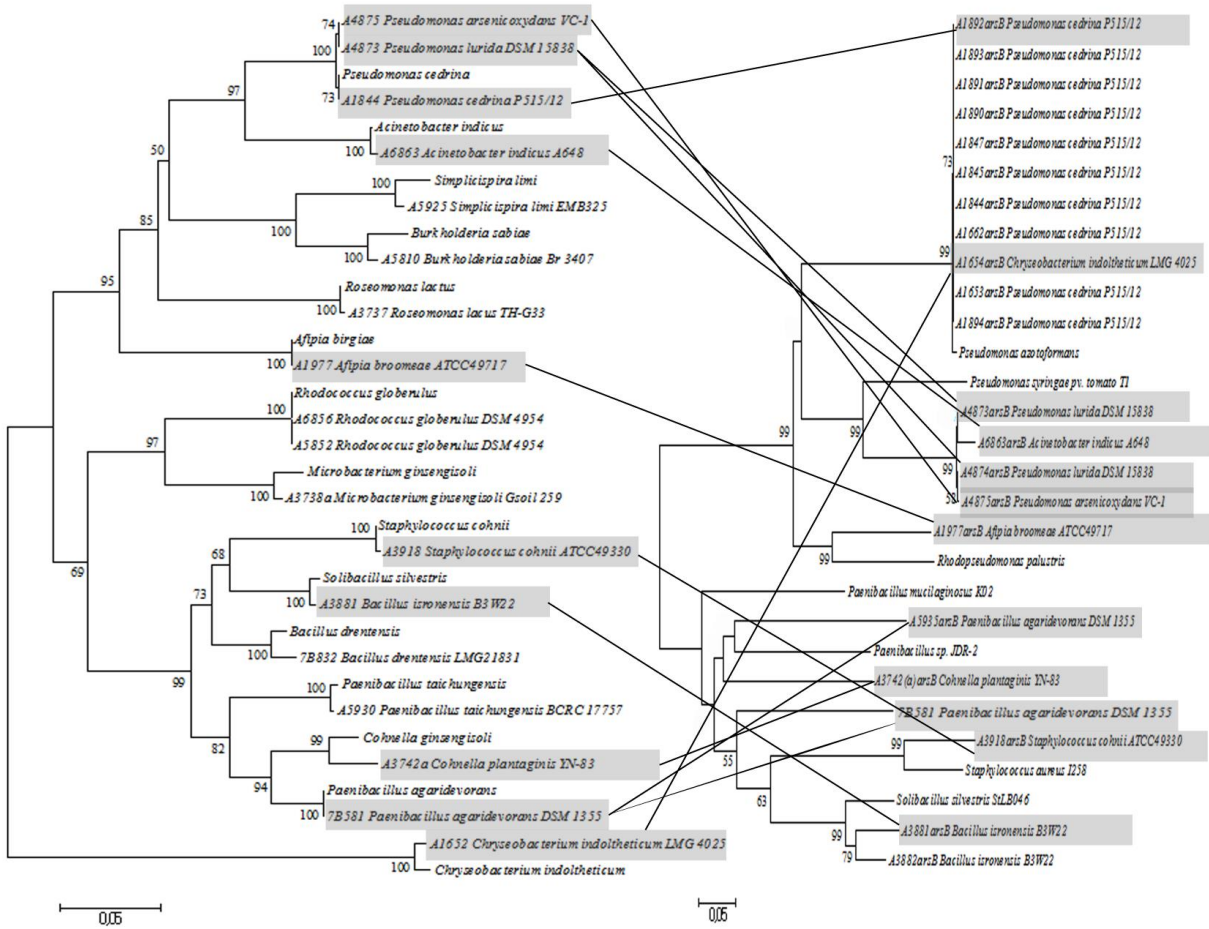

Fig. 14 Neighbor-joining analysis of (left) 16S rRNA and (right) *arsB* sequences from arsenic resistant samples. Individual *arsB* genes are corresponding to organism of origin in the 16S tree. Unique entries were shaded for ease of interpretation. Reference *arsB* sequences obtained through comparison to the BLAST database were used, and are represented by the species name. Sequences from this study are identified by closest organism according to the EzTaxon database with the study reference number and corresponding gene appearing as prefix

4.9 Arsenite oxidase gene *aioB* from arsenite resistant isolates.

For the detection of arsenite oxidase genes from arsenite resistant isolates two sets of degenerate primers were used. The protocol was developed according to the work by Sun *et al* [76]. No positive amplification was achieved in isolated samples.

4.10 Arsenite oxidase gene *aioB* from whole soil samples.

For the detection of arsenite oxidase genes from arsenite resistant isolates two sets of degenerate primers were used in order to increase the spectrum of detected genes. The protocol was developed according to the work by Sun *et al* [76]. In spite of the absence of *aioB* genes in isolated strains, whole DNA from soil samples was also tested. The A1 and A5 areas had positive hits for the mopterin subunit of the aio protein. These sampled amplicons were then cloned in order to be able to recognize different *aioB* genes after sequencing. Identification using a translated nucleotide database, BLAST [77] ensued. All sequences obtained were identified as *Bradyrhizobium sp*. S452 *aioB* alpha subunits, which indicates that either this was the only organism present that carried the gene or that this gene was transmitted horizontally to other individuals in the population..

Chapter 5 Discussion

5 Discussion

The overall diversity of the bacteria found in the 8 sampling sites was high; we analysed 78 bacterial strains belonging to 22 different genera (data not shown); these samples were isolated in a previous study [68]. Arsenic resistance tests were performed in the 78 isolates and among the resistant bacteria there was also high diversity as the 36 resistant bacterial strains found were identified as belonging to 13 genera. Previous studies (Jackson *et* al and Achour *et al*) demonstrate that arsenic resistant bacteria are phylogeneticaly diverse and are highly distributed in the environment so the great diversity found in this study was expected [35, 78]. The highest number of resistant strains was found in site A1 which had no detectable contamination with arsenic or Uranium. The site was treated with barium and calcium, if these contaminants caused stress in the microbial population it is possible that this stress motivated the transfer of mobile genetic elements which could explain the existence of a bacteria belonging to the genus *Chryseobacterium* with an *arsB* like gene very similar to the ones found in most *Pseudomonas* in this site. Most of the resistant strains isolated from site A1 belonged to the *Pseudomonas* genus. This is not uncommon and several strains of *Pseudomonas* have been shown to exhibit arsenic resistance traits [79, 80]. Most of the *Pseudomonas* strains found to be resistant in area A1 carry both arsenic resistance genes for pumping arsenite considered in this work, *arsB* and *ACR3*. At least one of these genes was present in all of the resistant *Pseudomonas* strains from this study which is in accordance with the observed phenotypic resistance results.

In Area A2 the diversity was significantly lower and none of the isolated strains exhibited resistance to arsenic. The site had a moderately acidic pH (5.0) and vestigial quantities of arsenic (<0,03ppm) and uranium (0,212ppm) which were probably insufficient to

generate evolutionary pressure towards arsenic resistant organisms. It is likely that there is a different contaminant exerting stress in this site and which results in the selection of different traits than those being studied in this work. Water from site A1 flows into site A2 yet we were unable to find organisms of the same genera as those found in A1, this may relate to the aforementioned stress conditions found in A2.

Area A3 has a great bacterial diversity; 7 distinct genera were identified among the 23 isolated strains. Interestingly enough, this site had the highest concentration of uranium of all the sampled locations which makes this result a very curious find because of the diversity found in the presence of significant concentrations (381ppm) of uranium. Despite the great number of isolates (the highest number of isolates in all of the areas) only seven strains exhibited variable resistance to arsenic. In this site there were some interesting finds. Bacterial strains from the same genus exhibiting different degrees of resistance. Strain A3-738 and A3-742a exhibited significant growth only in the medium supplemented with 2mM arsenite. Genes *ACR3* were not present in these bacteria but strain A3-742a had gene *arsB*. Gram-staining-positive bacteria, were previously reported in the literature as being resistant to arsenite despite the absence of the genes *arsB* and/or *ACR3*. Strain A3-742a is Gram-staining- positive, this indicates that the density of the cell wall in Gram-positive-stain bacteria may confer resistance to arsenite [35]. The minimum inhibitory concentration of arsenite increased at the least five fold when compared to Gram-staining-positive subjects from the same sampling site which contained arsenic resistance genes, this indicates that despite not being an essential factor in arsenic resistance in Gram positive bacteria, these genes increased the efficiency of the detoxification process enabling the organisms to resist to higher concentrations of contaminants.

All of the strains from A4 were resistant and belonged to the *pseudomonas* genus, which, as described above, has been shown to include strains highly resistant to arsenic contamination. All of the three strains exhibited the gene *arsB.*

From site A5, both resistant strains and non resistant strains were isolated. The diversity was high with representatives from 6 genera present in 11 strains. Strains A5-930 and A5-935 both exhibited the same arsenite minimum inhibitory concentration yet genes *arsB* and *ACR3* were absent from strain A5-930. It would be interesting to further investigate this particular pair of strains in order to fully understand if *arsB* and *ACR3* were being expressed in strain A5-935 or the mechanism for arsenic resistance was similar to the strain A5-930. Furthermore strains A5-810 and A5-852 (belonging to genera *Burkholderia* and *Rhodococcus* respectively) exhibited resistance. Interestingly the minimum inhibitory concentration for strain A5-852 was tenfold higher (20mM) than with strain A5-810. This indicates differences in resistance, in this case, may be related to the different composition of the cell wall since the Gram positive strain exhibits a higher minimum inhibitory concentration than Gram negative.

In site A6, all the strains were highly resistant to arsenite, a comparable situation to the verified results pertaining site 4. The amount of cultured strains in this site was limited to 3 isolates.

In site 7A there were no resistant strains, though arsenic resistance traits are ubiquitous in the environment. Arsenic concentration in the site was very low, the absence of arsenic resistance traits indicates that the stress exerted by the low arsenic concentration in this site was insufficient to exert evolutionary pressure in the direction of arsenic resistance phenotype selection.

In site 7B there were two resistant strains, 7B-581 and 7B-832. The minimum inhibitory concentration in strain 7B-832 was higher than in strain 7B-581 yet the observed growth

under the conditions applied was lower when compared to strain 7B-832. This is in accordance with previous results in this work that indicate that the presence of arsenic resistance genes improves the ability of the bacteria to cope with the contaminant which may explains why strain 7B-581 is able to form larger colonies than 7B-832 when exposed to arsenite for the same amount of time.

Regarding the antimonite resistance tests, all of the tested isolates were resistant (table 19). These results were, for the most part, expected as the mechanisms for antimonite penetration and extrusion in the cell are the same as arsenite. For cell penetration, aquaglyceroporins; and for extrusion from the cell, ARSB proteins [24]. The only curious cases are strains A5-856, A5-857 and A5-854. These strains were resistant to arsenite yet were unable to grow in antimonite supplemented medium. This may happen due to the higher degree of toxicity of antimonite.

Regarding arsenite oxidation, all of the 36 arsenite resistant strains (growth on 2mM supplemented agar) were tested for phenotypic traits of arsenite oxidation. None of the strains exhibited the ability to oxidise arsenite (data not shown). PCR for the amplification of the gene *aioB* was performed in each of the selected arsenite resistant strains, we were unable to observe amplification for the desired gene. A different approach was then taken and DNA was extracted from whole soil samples, the resulting DNA was then scanned for the presence of *aioB* genes. There were several positive amplification results in the PCR with DNA from sites A1 and A5. Those amplicons were cloned. 25 clones from each of those two sites were selected (Xgal blue/white colonies selection approach) and plasmid DNA was extracted. After amplification of the selected clones' plasmid DNA the resulting amplicons were sequenced. The resulting 50 sequences where then compared to the BLAST database where all of the sequences matched a single *aioB* gene belonging to the strain *Bradyrhizobium sp*. S452, this gene was first described in this

organism by M. Sultana *et al* [81]. Besides arsenite oxidation arsenate reduction traits were also investigated with primers for the amplification of arsenate reductase *arrA*. None of the samples exhibited the amplification for the tested gene which indicates that there were no dissimilatory arsenate reducing prokaryotes in the tested samples.

Regarding the phylogeny of the different *ACR3* determinants, as seen on figure 13, we can observe the grouping of the *ACR3* gene from strain A1-654 Chryseobacterium to those of the strains from the *Pseudomonas* genus. This is a strong indicator that horizontal gene transfer occurred as these samples were retrieved from the same site A1. This is interesting since site A1 was treated with barium and calcium but uranium wasn't present which suggests that the stress was caused by the substances used to treat the water rather than uranium. Other than this indicator there was no evidence for horizontal gene transfer on other strains analysed regarding *ACR3* determinants.

Regarding *arsB* phylogeny, as verified in the analysis of *ACR3* phylogeny, strain A1-654 *Chryseobacterium indoltheticum* had its *arsB* gene clustered with *Pseudomonas* genes in the phylogenetic tree, which indicates that the transfer of genetic characteristics may have happened in tandem transferring both *ACR3* and *arsB*. Other interesting find was that in spite of the several species of strains identified pertaining to the genus *Pseudomonas* not all of their resistance genes were clustered in the phylogenetic analysis. A4-873 and A-874 *Pseudomonas lurida* and A4-875*Pseudomonas arsenicoxidans arsB* genes were clustered with the *arsB* gene from another gammaproteobacteria, site A6 *Acinetobacter indicus A648*. This indicates that arsenic resistant organisms are phylogenetically distinct and diverse and that the genes for arsenic resistance are genetically distinct even among phylogenetically close organisms. The fact that the previously mentioned *arsB* genes from strains from A4 samples were clustered together may also indicate that there was effectively horizontal gene transfer of arsenic determinants between both strains since

they both belong to the same sampling site and site A4 had the highest concentration of uranium in all the sampled sites. The remainder of the strains appear to have vertical gene transfers.

Chapter 6 Concluding Remarks

6 Concluding remarks

The results obtained in this work are in line with previous studies [35, 78, 80]. The diversity of arsenic resistant bacteria of the analysed sampling sites in this study was high. Among all the resistant strains, 36, we found representatives for 13 different genera. There were several sites with no uranium or arsenic detected yet arsenic resistance determinants were found in 7 among the 8 sampled sites and all of the sites with no arsenic present. This indicates, in accordance with the literature, that arsenic resistance structures are ubiquitous and the existence of arsenicals in the sampling sites does not necessarily indicate that there are no resistant bacteria present. In order to competently find evidence of the sharing in resistance traits one would need further the study of the resistance determinants. The used protocol, despite highly specific, was limited. It would have been interesting to clone the amplicons of individual strain resistance genes in order to evaluate if more than one allele were present. This would enable us to conclude if there was effectively horizontal gene transfer between A4-873 and A-874 and A4-875. This would also have enabled us to infer whether there was only one copy of the studied structures or several, their origin and if they were present in mobile genetic elements or the genome of the studied strains. This approach would, however, be very time consuming due to the sheer amount of samples to be analysed and would severely extend the period of time needed to execute the analysis. Another limitation was the inability to identify all the contaminants on the sampling sites. It would have been interesting to have the concentration of barium, calcium and other contaminants in area A1 to be able to understand what the main contaminant was and if the proposed horizontal gene transfer between *Pseudomonas* present in the site A1 and the *Chryseobacterium,* also from site

A1 can be related to the existence of the contaminants or happened unrelated to stressing conditions in the environment.

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7 Bibliography

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