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Tungsten accumulation by highly tolerant marine hydrothermal *Sulfitobacter dubius* strains carrying a *tupBCA* cluster☆

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ABSTRACT

Tungsten (W) has industrial and economic importance, and is in the European Union list of metals with a high supply risk. It is used by living organisms, which transport it into the cell, in the form of tungstate ion (WO_4^{2-}), using three different ABC-type transporters from the specific W-uptake system coded by *tupABC* gene cluster. In this study, strains from a collection recovered from deep-sea hydrothermal sediments were selected according to their ability to tolerate metals and to possess the *tup* genetic determinants. Three multimetal-tolerant strains, *Sulfitobacter dubius* NA4, As(V)4 and Sb5, were chosen. The strains were able to grow in the presence of high tungsten concentrations and their growth was unaffected by 1 mM tungsten. Moreover, strain Sb5 was able to accumulate up to 52 μg W mg^{-1} protein. Their tup genes were shown to be organized as *tupBCA*, which is not the most usual gene arrangement. All three strains had the classical TupA conserved motif TTTS, comprising a first Thr replaced by a Val, which seems to be a common feature of the genus Sulfitobacter. This study was an important first step in the exploration of new biological strategies for recovering tungsten from natural or anthropogenic W-impacted environments.

Introduction

Tungsten (W) has the highest melting point of all metals in its pure form, and is considered to be a critical metal [28]. Tungsten is one of the most important metals used in many key industries and has been included in a group of 14 of the 41 materials identified with a high supply risk and high economic importance [9]. Many of the world's technologies based on high-tech economy LCD panels, TV tubes, laser printers and window heating wires are under threat, due to tungsten's looming scarcity and soaring prices, together with other critical metals essential for their production [9]. It is anticipated that the global demand for tungsten will continue to increase in the future because of a growing global population. Therefore, the development of more effective and environmentally friendly technologies for the recovery of tungsten is essential for ensuring a sustainable supply of this heavy metal.

Tungsten is the chemical element with the highest atomic number used by living organisms, which means it is the only element in the third transition row that exhibits biological activity in enzymes [6,17]. For some microorganisms belonging to the *Bacteria* and *Archaea* domains, it plays an essential biological role in their life process and has therefore been reported to be an essential trace element [2,6,31]. Tungsten shows biological activity after incorporation into cofactors, designated as tungstopterins [2], which serve as an anchor providing efficient control of the redox properties of the metal [30]. Such cofactors are observed in enzymes catalyzing oxygen atom transfer reactions, such as formate dehydrogenases (FDH), aldehyde:ferrodoxin-oxydoreductases (AOR), formaldehyde:ferrodoxin-oxydoreductases and acetylene hydratases [3,5,6,21,30]. Therefore, bacteria carrying enzymes dependent on tungsten for biological functions need to incorporate tungsten into their cells. In general, the cellular uptake of metals from the environment can occur by: (i) a selective substrate-specific uptake system that consumes high energy (ATP), and (ii) a substrate-non-specific uptake system that uses the chemiosmotic gradient [32]. Intracellular accumulation (i.e. bioaccumulation) is an energy-driven process dependent on active metabolism that normally occurs when a portion of a respective metal is retained by an organism [4]. On the other hand, biosorption is a rapid phenomenon of passive metal sequestration by the non-growing biomass/adsorbents. The uptake of metal ions

☆ **Nucleotide sequences**: Sequences of *tup* gene clusters from *Sulfitobacter dubius* strains Sb5 (BankIt1938735 tupABC_Sb5), As(V)4 (BankIt1938735 tupABC_As(V)4) and NA4 (BankIt1938735 tupABC_NA4) were deposited in Genebank database under accession numbers KX809894, KX809895 and KX809896, respectively.

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from the environment, through binding of the adsorbent to the adsorbate, occurs until the solid-bound adsorbate species and the portion remaining in the solution reach an equilibrium [1]. In fact, many studies have shown relevant microbial biosorption of several metal ions, including tungsten. However, biosorption is not as selective a process as bioaccumulation and, in specific cases, the selective recovery of tungsten from multicomponent metal ions in an aqueous solution is essential.

Tungsten forms compounds that have valence states from 2 to 6. However, the dilute aqueous speciation of tungsten in water is presumed to be completely dominated by the oxyanion tungstate (WO_4^{2-}), the most stable oxidation state in aqueous environments [12,17,31]. This metal is found in hydrothermal vents at approximately 1000 times the level found in seawater (0.1 $\mu g\,L^{-1}$) [30]. Therefore, it is predictable that tungsten-dependent bacteria will be found in hydrothermal environments due to its availability in high concentrations.

Moreover, tungsten is known to be transported into the bacterial cells in the tungstate ion form (WO_4^{2-}). Tungsten can be taken up by three different ABC-type transporters, including the tungstate uptake protein (TupABC) that is highly specific for WO_4^{2-} [22], the W-transport protein (WtpABC) that transports both WO_4^{2-} and molybdate (MoO_4^{2-}), [6] and the molybdate transporter (ModABC) that is highly specific for MoO_4^{2-}, although it can also carry WO_4^{2-} [2].

The specific W-uptake system results from the *tupABC* gene cluster that includes the genetic determinants coding for proteins involved in W-binding and membrane W-transport into the cell. The *tupA* gene codes for a protein responsible for binding WO_4^{2-} highly specifically. TupB forms the transmembrane pore that allows W-transport, which is facilitated by the ATP hydrolyzing activity of TupC on the inner surface of the membrane.

The first objective of this current study was to find and characterize bacterial strains from deep-sea hydrothermal sediments that shared the ability to tolerate several metals and also possessed the *tup* genetic determinants. From among the most promising bacteria, three strains were chosen according to their low metal susceptibility abilities in order to determine their tungsten accumulation capacities and to compare their TupABC systems. This study was essential for exploring the use of these strains or their *tup* genetic determinants as a strategy to recover the highly valuable tungsten metal from natural W-rich or anthropogenic W-impacted environments.

Materials and methods

Bacterial strain collection

Sediment samples were collected from the Lucky Strike vent field (37°18.5N, 32°16.5W), located 600 km south of the Azores. The Lucky Strike vent field is one of the largest hydrothermal areas known to date with twenty-one active chimney sites distributed over approximately 150.000 km^2 at an approximate depth of 1700 m.

In order to create a collection of aerobic heterotrophic bacterial strains from the Lucky Strike vent site, aliquots of sediment samples were inoculated on Reasoner's 2A (R2A) agar (Merck), prepared with a seawater filtrate (SWF) collected from coastal seawater. Each dilution was incubated at 15 °C for up to 1 month. During incubation, all the colonies that successively appeared with different colony morphologies were selected, purified by repeated streaking, and preserved at −80 °C in Luria–Bertani (LB) medium (10 $g\,L^{-1}$ tryptone, 5 $g\,L^{-1}$ yeast extract and 5 $g\,L^{-1}$ NaCl) containing 15% glycerol. Isolates were also recovered from the same media with SWF in the presence of the following metals: 5 mM AsO_4^{2-}, 0.5 mM $NaSb(OH)_4$, 1 mM $CuSO_4$, 0.5 mM Na_2CrO_4 and 1 mM $ZnSO_4$ (Sigma-Aldrich, USA), as well as the antibiotics 12 $\mu g\,mL^{-1}$ tetracycline, 20 $\mu g\,mL^{-1}$ ampicillin G, 20 $\mu g\,mL^{-1}$ kanamycin, 24 $\mu g\,mL^{-1}$ nalidixic acid and 16 $\mu g\,mL^{-1}$ gentamicin. The heavy metal resistance determination of the isolates for copper(II), arsenic(V), arsenic(III), cobalt(II), uranium(IV), zinc(II), chromium(VI) and cadmium(II), and antibiotic resistance testing was performed as described previously [10].

The present study included 23 selected isolates from the strain collection created. The bacteria were selected based on multiple metal, metalloid and antibiotic pattern resistances, with the strains being resistant to at least three inhibitory agents. The study included three strains already characterized for their resistance to arsenic and antimonite [10]: *Sulfitobacter dubius* As(V)4, *Dockdonia donghaensis* As(III)6 and *S. dubius* Sb5.

Minimum inhibitory concentration (MIC) assay for tungsten

All bacterial strains were tested for tungsten tolerance on solid media. First, 0.1 mL cell suspensions with an optical density of 600 nm ($OD_{600\,nm}$) were diluted in NaCl (0.9%). Second, 5 μL of each cell suspension were plated onto solid LB medium with 3% NaCl and increasing concentrations of tungsten (0.5 mM, 1 mM, 10 mM and 20 mM) added as $Na_2WO_4.2H_2O$ (Sigma-Aldrich). Bacterial growth was subsequently evaluated after 5 days incubation at 22 °C. The MIC was defined as the lowest concentration of tungsten that inhibited the growth of bacteria on the solid medium after three experiments.

Screening for tup genetic determinants

All 23 strains were screened for the presence of *tupA* and *tupB* genes from the *tupABC* gene cluster. The bacterial DNA was extracted [24] from cells grown at 22 °C for 48 h in R2A medium prepared with SWF. Screening for *tupA* and *tupB* genes was performed by PCR using degenerate primers (Macrogen Inc.). The primers were designed based on the alignment of sequences (in amino acids) obtained from: (1) a previous sequencing study of two *Sulfitobacter* strains isolated by our group (*S. dubius* As(V)4 and *S. dubius* NA4); (2) several *tup* sequences deposited in the National Center for Biotechnology Information public database [(NCBI), Bethesda, MD, USA] from strains related more to the isolates studied in this study (i.e. marine bacteria). The primers 5′GCGGTSACCACSTCSTTCAAC and 5′ATATTGGTTGAACAYKACRGGATCGCC were used for *tupA*, with a starting position 79 nucleotides downstream from ATG (*tupA* sequence in KX809895.1) and a PCR product length of 547 bp. Amplicons were conducted in 30 μL reactions with 1.5 U μL^{-1} *Taq* DNA Polymerase in 1.7 mM $MgCl_2$, 0.6 mM dNTPs, 10 μM *tup* primers and 4 ng of template. The PCR condition protocol was as follows: initial denaturation at 94 °C for 45 s, followed by 30 cycles of 94 °C (45 s), 48 °C (1 min) and 72 °C (35 s) and a final extension phase at 72 °C for 5 min. The primers 5′ATGGGCTTGCCGCCSGTCGTG and 5′GCGGCCAAAGCCRGCCAG were used for the *tupB* gene, starting with the initial ATG of the gene (*tupB* sequence in KX809895.1) that resulted in a PCR product length of 274 bp. Amplicons were also conducted in 30 μL reactions using the same conditions above. The PCR conditions followed the touchdown protocol: initial denaturation at 94 °C for 45 s, followed by 10 cycles of 94 °C (45 s), 55 °C (1 min) minus 0.5 °C per cycle, 72 °C (15 s), followed by 20 cycles of 94 °C (45 s), 50 °C (1 min), 72 °C (15 s) and a final extension phase at 72 °C for 5 min. The PCR products obtained were analyzed by electrophoresis, then they were purified (E.Z.N.A.® Gel Extraction Kit, VWR) and sequenced with the Sanger sequencing method (StabVida).

Bacterial growth in liquid medium containing tungsten

Three strains able to tolerate tungsten that carried *tupA* and *tupB* genes, *Sulfitobacter dubius* Sb5, NA4 and As(V)4, were selected for further studies. Strains were grown in liquid medium spiked with increasing concentrations of tungsten (1–20 mM), added in the form of $Na_2WO_4.2H_2O$ (Sigma-Aldrich). The growth of the *S. dubius* strains was performed in LB medium with 3% NaCl at 22 °C. Bacterial growth was evaluated by optical density ($OD_{600\,nm}$) for 24 h (strain Sb5) or 48 h (strains As(V)4 and NA4). The growth rates of each strain at each concentration were calculated for the exponential growth phase using triplicates.

Tungsten cell accumulation assay

From the growth curve of each strain of *Sulfitobacter dubius*, bacterial accumulation of tungsten was followed by measuring the amount of tungsten in the cells during batch growth in liquid media (LB with 3% NaCl), in the presence of different tungsten concentrations (the same as above). Cells were removed from each culture at three different times during bacterial growth: the beginning, middle and end of the exponential growth phase. Cells were collected and centrifuged at 4000 rpm for 20 min at 4 °C. The resultant bacterial pellet was resuspended in 2 mL phosphate buffered saline solution (PBS) (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4, pH 7.4) and centrifuged. This washing procedure was repeated three times. Suspensions were digested by adding 2 mL 10% HNO_3, incubation in a water bath at 50 °C for 30 min and then centrifuged at 4000 rpm for 20 min at 4 °C. The resultant pellet was resuspended with 2 mL PBS and transferred to sample supports, previously prepared with a Mylar thin-film (3.6 μm) filter. Tungsten measurements were performed by placing each support on an Energy Dispersive X-Ray Fluorescence (EDXRF) spectrometer (Epilson 3^{XLE}) and measuring for 60 s with a current of 80 μA at a voltage of 50 kV. Total protein was determined using the Bradford assay [7].

To determine the maximum tungsten accumulation by each strain, bacteria were grown in the same conditions as described above in the presence of increasing tungsten concentrations (1, 10 and 20 mM) but, in this case, cells were only recovered in the growth phase period that had previously shown the highest tungsten accumulation. The experiments for tungsten measurement were performed in triplicate.

Genetic organization of the tup gene cluster

In a previous study [10], a plasmid from *Sulfitobacter dubius* As(V)4 was isolated and sequenced as described. The plasmid content of the highly metal and antibiotic resistant *S. dubius* strain NA4 was also determined at the same time, and both plasmids were sequenced as described by Farias et al. [10], although the results were not published. The analysis of the plasmids showed the presence of the *tupABC* genes related to tungsten resistance in both strains. In the present study, in order to complete these previous sequencing results and to reveal the gene organization of the *tup* gene cluster in strain *S. dubius* Sb5, two sets of primers were designed. The first set was designed to obtain an amplicon by PCR that included the *tupBC* fragment using forward primer 5′CCGCGAGATGGTCGATAT and reverse primer 5′GCGCCGATCATCCAATGT. The forward primer started 7 nucleotides upstream of the initial *tupB* gene (*tupB* sequence in KX809895.1) and the reverse primer at 19 nucleotides downstream of the stop codon of the *tupC* gene (*tupC* sequence in KX809895.1), and had a length of 1441 bp. The PCR conditions followed the protocol: initial extension at 94 °C for 45 s, followed by 30 cycles of 94 °C (45 s), 49 °C (1 min), 72 °C (2 min) and a final extension phase at 72 °C for 10 min. The second primer set was designed to obtain an amplicon of the *tupCA* genes using forward primer 5′CGGTGGTGACGCAACTT and reverse primer 5′GCGCGGATGGTCTCTAAC. The forward primer started 5 nucleotides upstream of the *tupC* gene (*tupC* sequence in KX809895.1) and the reverse primer at 23 nucleotides downstream from the *tupA* stop codon (*tupA* sequence in KX809895.1), and had a length of 1,551 bp. The PCR conditions followed the protocol: initial extension at 94 °C for 5 min, followed by 30 cycles at 94 °C (45 s), 51 °C (1 min), 72 °C (2 min), and a final extension phase at 72 °C for 10 min. Amplicons from both primer sets were conducted in 30 μL reactions, as mentioned above. The PCR products obtained were analyzed by electrophoresis, then they were purified and sequenced by the Sanger sequencing method.

Results

Heterotrophic aerobic bacteria isolated from the deep-sea site

A total of 23 bacterial strains able to grow in the presence of heavy metals and antibiotics were obtained from deep-sea samples at the Lucky Strike site. The majority of the isolates belonged to *Alphaproteobacteria* (60.8%), and the rest belonged to *Flavobacteria* (26%). The genus *Sulfitobacter* included the majority of the isolates, with seven strains from the species *S. dubius* [arsenic(V), chromium(VI), nalidixic acid and gentamicin resistant strains], one strain identified as *S. litoralis* [copper(II) resistant strain] and one strain identified as *S. pontiacus* [ampicillin resistant strain] (Table 1).

Tungsten-tolerant isolates

The 23 bacterial strains selected were able to grow in LB medium containing 3% NaCl with the exception of *Sulfitobacter dubius* Gm5, *Dokdonia donghaensis* As(III)6, and *Erythrobacter citreus* strains Kan3, Gm3, Gm6 and NA16. Consequently, since these strains did not grow, they were not included in the MIC assay, although they were used for *tupA* and *tupB* gene screening.

The highest levels of tolerance (20 mM) were observed for *S. dubius* strains Cr2, Sb5, NA4 and As(V)4, *Aurantimonas litoralis* As(V)2, *Halomonas meridian* Cr1, *Staphylococcus saprophyticus* Cu4 and *E. citreus* NA6. High tolerance of up to 10 mM tungsten was also obtained for *S. pontiacus* Amp1, and *Mezoflavibacter zeaxanthinifaciens* strains Te13 and Gm11. The remaining six strains were only able to grow at between 0.5–1 mM tungsten (Table 1).

tupA and tupB gene screening

All 23 selected strains were screened for the presence of the *tupA* and *tupB* genes. Amplification of the *tupA* fragment was obtained for six bacteria (Table 1): *Sulfitobacter dubius* strains NA4, As(V)4 and Sb5, *Erythrobacter citreus* NA16, *Dokdonia donghaensis* As(III)6 and *S. litoralis* Cu7. The identification of the amplified fragments using Blastx (NCBI) showed they all had greater than 77% similarity for extracellular tungsten binding protein/ABC tungstate transporter permease, substrate-binding protein. All strains screened for *tupA* were also screened for the presence of the genetic determinant *tupB*, which codes for the membrane transport permease. All strains containing the *tupA* gene, except *S. litoralis* Cu7, showed the presence of the *tupB* genetic determinant (Table 1).

The strains of *S. dubius* NA4, As(V)4 and Sb5, which revealed high tungsten tolerance and positive identification for *tupA* and *tupB* genes, were selected for further experiments.

Table 1
Phenotypic and genotypic screening of strains isolated from the Lucky Strike Mid-Atlantic Ridge hydrothermal vent field. Tungsten resistance on LB solid medium and screening for the presence of tungsten uptake related genetic determinants (*tupA*, *tupB*).

Bacterial strains		Heavy metal resistance	W tolerance (mM)				Genetic determinants	
			0.5	1	10	20	*tupA*	*tupB*
Aurantimonas litoralis	As(V)2	Zn, Cu[a]	+	+	+	+	–	–
Halomonas meridiana	Cr1	Cr, U	+	+	+	+	–	–
	Te14	Cr, U	+	(+)	–	–	–	–
Staphylococcus saprophyticus	Cu4	Cr, U, Cu, Sb, Cd	+	+	+	+	–	–
Sulfitobacter dubius	Cr2	Cr, Cu, Sb, Cd	+	+	+	+	–	–
	Sb5	Cr, Cu, Sb, Cd	+	+	+	+	+	+
	As(V)4	Cr, Cu, Sb, Cd[a]	+	+	+	(+)	+	+
	NA4	As(V), Cr, Cu, Sb, Cd	+	+	+	(+)	+	+
Sulfitobacter pontiacus	Amp1	As(V), Cu, Sb, Cd	+	+	+	–	–	–
Sulfitobacter litoralis	Cu7	Cu, Sb, Cd	+	+	–	–	+	–
Maribacter dokdonensis	Te2	As(V), Zn, Cu	+	+	–	–	–	–
Mesoflavibacter	Te13	Zn, Cu, Sb, Cd	+	+	+	–	–	–
zeaxanthinifaciens	Gm11	Zn, Cu, Sb, Cd	+	(+)	(+)	–	–	–
	Kan1	Zn, Cu, Sb, Cd	+	(+)	–	–	–	–
Maribacter forsetti	Te4	As(V), Cu	+	–	–	–	–	–
Dokdonia donghaensis	As(III)6	Zn, Cu, Sb, Cd[a]	NG	NG	NG	NG	+	+
Erythrobacter citreus	NA6	As(V), Zn, Cd	+	+	+	+	–	–
	Zn1	As(V), Cd	(+)	(+)	–	–	–	–
	NA16	–	NG	NG	NG	NG	+	+

Minimum growth inhibition and the concentration: Arsenate – As(V) (5 mM); Chromate – Cr(VI) (0.5 mM); Uranium – U(IV) (1 mM); Zinc – Zn(II) (1 mM); Cobalt – Co(II) (0.5 mM); Copper – Cu(II) (1 mM); Antimonite – Sb(III) (0.5 mM); Cadmium – Cd(II) (0.2 M). Strains Gm5, Kan3, Gm3, Gm6 did not grow in LB and they tested negative for *tupA* and *tupB* genes.
+, positive result; (+), weakly positive result; –, negative result; NG, no growth.
[a] Data from Farias et al. [10].

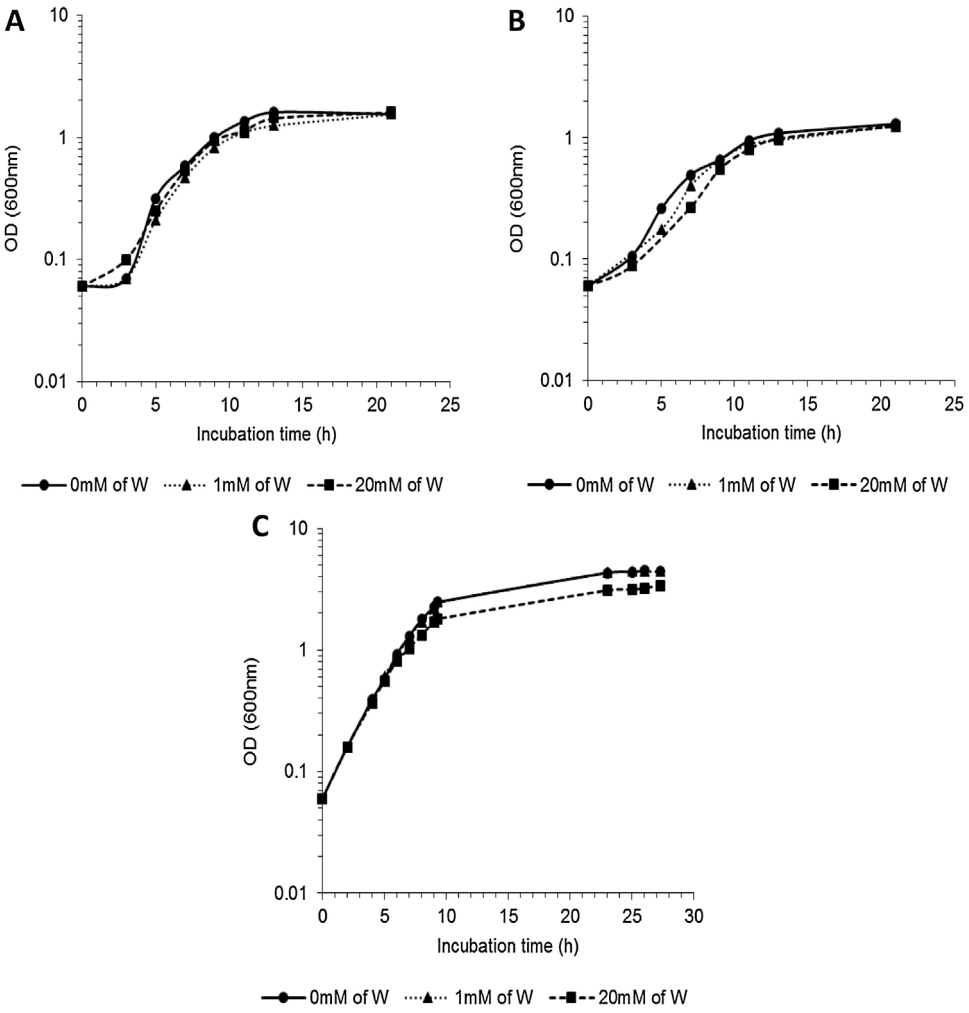

Fig. 1. Growth curves of *Sulfitobacter dubius* strains As(V)4 (A), NA4 (B) and Sb5 (C). Strains were grown in LB with 3% NaCl and different concentrations of tungsten (0 mM, 1 mM and 20 mM).

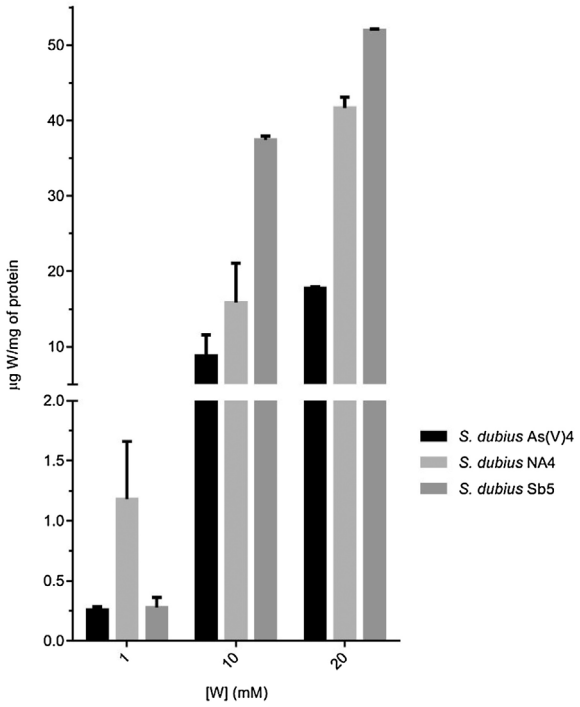

Fig. 2. Maximum uptake of tungsten by *Sulfitobacter dubius* strains. Strains were exposed to 1 mM, 10 mM and 20 mM tungsten. Data shown are the mean values (±standard deviations) obtained from three independent experiments.

Growth rates and tungsten accumulation

Sulfitobacter dubius strains As(V)4, NA4 and Sb5 were tested for their growth in the presence of increasing concentrations of tungsten. When comparing the growth rates of each strain, they were not affected greatly by the presence of up to 20 mM tungsten in the growth medium (Fig. 1). The growth rates at tungsten concentrations of 0, 1 and 20 mM for *S. dubius* As(V)4 were 0.19 ± 0.01, 0.15 ± 0.01 and 0.17 ± 0.01 OD h^{-1} and for strain NA4 they were 0.10 ± 0.01, 0.12 ± 0.01 and 0.13 ± 0.01 OD h^{-1}, respectively. These growth rates were significantly different from those of strain Sb5 (0.48 ± 0.03, 0.45 ± 0.02 and 0.33 ± 0.02 OD h^{-1}) for the same concentrations. Independently of the presence of tungsten in the growth medium, *S. dubius* strains Sb5 and NA4 were able to achieve the maximum OD under these growth conditions, although *S. dubius* NA4 showed a delayed exponential phase.

Tungsten accumulation for each *S. dubius* strain tested was first evaluated at the early exponential, mid exponential and early stationary growth phases (Table 2). All *S. dubius* strains tested accumulated lower amounts of tungsten at low medium concentrations than at high concentrations. While both *S. dubius* strains As(V)4 and NA4 showed a maximum accumulation capacity at the end of the exponential growth phase, strain *S. dubius* Sb5 accumulated more tungsten in the middle of the exponential growth phase. The point corresponding to the maximum accumulation by each strain was used to determine the maximum accumulation for each concentration of tungsten (Fig. 2).

S. dubius As(V)4 exhibited a very similar accumulation pattern to *S. dubius* NA4 but *S. dubius* As(V)4 accumulated a lower amount of tungsten in all assays. In both strains, the tungsten accumulation rose with increasing concentrations of tungsten in the growth media. Moreover, at 1 mM tungsten, *S. dubius* Sb5 accumulated only 0.28 µg W mg^{-1} protein, while *S. dubius* NA4 accumulated 1.18 µg W mg^{-1} protein, which was the highest tungsten accumulation compared to the other strains at this tungsten concentration. On the other hand, at the other concentrations tested, *S. dubius* Sb5 accumulated five times more at 10 mM (37.44 µg W mg^{-1} protein) and three times more at 20 mM tungsten (51.99 µg W mg^{-1} protein) than the other strains.

Sequencing of the tup gene cluster

The information from sequenced plasmids of *S. dubius* strains NA4 and As(V)4, together with degenerate oligonucleotide primers, was used to reveal the sequence of the gene cluster related to the tungsten uptake system in unsequenced strain Sb5. Blast analysis of *tup* systems revealed that they were composed of three open reading frames (ORFs) encoding the proteins TupA, TupB and TupC previously determined to be involved in tungsten transport. Using the primers mentioned above, the organization of the genes in the operon was shown to be *tupBCA*. The first gene of the cluster, *tupB*, codes for a transmembrane protein of 234 amino acids (TupB) referred to as a permease responsible for translocation of tungstate across the membrane. The TMHMM server v.2 and TopPred 1.10 software [15] predicted similar protein topology for the three TupB analyzed, with five transmembrane helices, the N-terminal to the outside and the C-terminal to the inside. Analysis of TupB proteins from other reported organisms, such as *Geobacter sulfurreducens*, *Campylobacter jejuni* and *Eubacterium acidaminophilum*, using the TMHMM server v.2 and TopPred software also revealed a similar structure and predicted the protein topology among the TupB proteins of these organisms and our *S. dubius* strains. The second gene, *tupC*, was identified as encoding an ABC transporter ATP-binding protein comprising 236, 238 and 238 amino acids for strains Sb5, As(V)4 and NA4, respectively. These three TupC sequences, as well as the reported ATPase component of the tungstate ABC transporter of *E. acidaminophilum*, showed the same conserved signatures, such as the ATP binding site and ABC transporter signature motif. The third gene of the cluster, *tupA*, encodes a secretory protein, with 266, 270 and 271 amino acids in strains NA4, As(V)4 and Sb5, respectively, and they shared high homology with the bacterial TupA responsible for binding tungstate, as well as high homology (99%) with the sulfate ABC transporter substrate-binding protein in *Oceanibulbus* and *Sulfitobacter* species. The SignalP prediction software [29] predicted a similar cleavage site located between Ala20 and Ala21 for both *S. dubius* strains As(V)4 and Sb5, but in the case of strain NA4 the cleavage site was identified between Ala19 and Glu20. Despite the general high homology among the protein sequences of these three *S. dubius* strains, the sequence alignments revealed that all Tup protein sequences of strain As(V)4 had higher identity to Sb5 than to NA4 (Table 3).

Alignments of the TupA sequences from *S. dubius*, together with four more TupA sequences already reported in other studies, revealed conserved residues (Fig. 3). All three TupA from the *S. dubius* strains, as well as the others, had Arg158 and Thr165, two putative residues involved in the coordination of tungstate. The alignment identified a motif comprising a VTTS sequence, although this motif showed an unusual first residue valine in place of the traditional threonine found in the signature tungstate binding motif on the TupA of other organisms [27]. The presence of this valine instead of threonine was not exclusive to the TupA from *S. dubius* strains, since it was also common to similar proteins in different species, such as *S. pontiacus* or *O. indolifex* (Fig. 3).

Discussion

In this study, previous isolates from the Lucky Strike hydrothermal vent field were studied in order to find the best organisms able to tolerate and accumulate tungsten. The original niche of these strains represents an environment that has a salinity of up to 3.2% NaCl [11] with temperatures varying from 30 °C to 300 °C

Table 2
Accumulation of tungsten during growth of *Sulfitobacter dubius* strains As(V)4, NA4 and Sb5 at three different times for the exponential phase (beginning, middle and end) in the presence of different concentrations of tungsten and the corresponding $OD_{600\,nm}$.

		W concentration					
		1 mM		10 mM		20 mM	
S. dubius strain	Time (h)	OD	μg W mg^{-1} protein	OD	μg W mg^{-1} protein	OD	μg W mg^{-1} protein
As(V)4	3	0.07	0.00 ± 0.00	0.07	4.12 ± 0.04	0.10	8.96 ± 0.14
	7	0.58	0.24 ± 0.05	0.47	5.06 ± 0.01	0.54	9.62 ± 0.08
	11	1.61	0.25 ± 0.02	1.25	8.49 ± 0.46	1.44	17.09 ± 0.05
NA4	3	0.11	0.56 ± 1.57	0.10	3.55 ± 1.23	0.09	22.52 ± 0.76
	7	0.49	0.15 ± 1.44	0.40	12.00 ± 3.56	0.27	32.45 ± 0.51
	11	1.09	1.14 ± 2.66	0.96	15.25 ± 5.03	1.00	40.06 ± 0.08
Sb5	4	0.39	0.10 ± 1.44	0.37	7.32 ± 9.13	0.37	11.46 ± 0.19
	8	1.78	0.27 ± 0.25	1.70	37.43 ± 5.50	1.33	49.99 ± 0.15
	11	2.90	0.20 ± 0.19	2.98	31.72 ± 1.41	2.89	23.21 ± 1.49

Table 3
Closest relationship (similarity and identity) of the Tup products between each *Sulfitobacter dubius* strain tested.

	Relationship between strains	Identity (%)	Similarity (%)
TupA	*S. dubius* As(V)4 – *S. dubius* NA4	74	83
	S. dubius As(V)4 – *S. dubius* Sb5	91	94
	S. dubius NA4 – *S. dubius* Sb5	72	80
TupB	*S. dubius* As(V)4 – *S. dubius* NA4	74	85
	S. dubius As(V)4 – *S. dubius* Sb5	97	98
	S. dubius NA4 – *S. dubius* Sb5	77	86
TupC	*S. dubius* As(V)4 – *S. dubius* NA4	58	72
	S. dubius As(V)4 – *S. dubius* Sb5	90	95
	S. dubius NA4 – *S. dubius* Sb5	57	73

The identity and similarity values are based on amino acid sequence data.

[8], which, depending on geological events, can be close to 0 °C for long non-eventful periods, in addition to enrichment with high quantities of dissolved material, namely hydrogen sulfide (H_2S), various sulfide minerals, CO_2, methane and several metals [23]. Temperatures of the Lucky Strike field study site vary from 170 °C to 324 °C, which decrease with increasing distance from the vent crater because of the mixture of hot seawater and cold fluids characteristic of these depths (close to 4 °C) [10]. Among the metals present in marine water samples, tungsten exists naturally in trace amounts, generally at levels below 1 ng kg^{-1} [20]. However, the tungsten content for deep hydrothermal vents and marine sediments has been reported as several orders of magnitude above this standard value [19]. Therefore, there is a high probability of recovering strains that are almost certainly well adapted to a particular marine environment containing tungsten. In fact, most of the strains tested showed low susceptibility to tungstate, and were even able to grow at higher concentrations than the tungstate concentration limit (1840 mg L^{-1}) reported for growth of *Bacillus* sp. GT-83, which is one of the most characterized bacteria [12].

From the group of isolates tested, we focused on three strains of *Sulfitobacter dubius*, NA4, As(V)4 and Sb5. *S. dubius* is characterized as having a requirement for Na^+ or seawater for growth, and can grow in media containing 1–12% NaCl [18]. To our knowledge, the tolerance of *S. dubius* species to tungsten has never been evaluated. However, this study showed that all three strains were able to grow in the presence of high concentrations of tungsten and their growth rates were not affected by the addition of 1 mM of this metal, since they were able to achieve the same OD as the non-metal growth control. A decrease in the bacterial growth of *S. dubius* Sb5 was visible only for the very high metal concentration (20 mM). These results were remarkable since experiments using *Bacillus subtilis* and *Pseudomonas fluorescens* ATCC 13525 exhibited a significant decrease of microbial biomass for increasing concentrations of tungsten, with a biomass production reduction of 15% and 38% for bacterial growth in the presence of only 137 and 486 μM tungstate, respectively [34].

The capacity of organisms to recover metals by accumulation and the importance of tolerance are certainly very relevant in this context. The three strains belonging to the species *S. dubius* exhibited an excellent ability to accumulate tungsten in their cells. From all strains tested, *S. dubius* Sb5 was the strain capable of accumulating the highest tungsten concentration in cells, up to 52 μg W mg^{-1} protein, which was higher than *Campylobacter jejuni* NCTC 11168 wild-type cells that accumulated approximately 3.3 μg W mg^{-1} protein [33]. Although there is very limited work concerning the interaction of this metal with bacteria, the *S. dubius* Sb5 strain was many orders of magnitude (10×) more efficient at internalizing tungsten from an aqueous medium. Studies with *Bacillus* sp. GT-83 and *Escherichia coli* evaluated the biosorption of tungstate by cells, which showed levels as high as 194.5 μg W mg^{-1} protein and 183 μg W mg^{-1} protein, respectively [12,26]. Despite these high values, biosorption of metals compared with bioaccumulation cannot be considered as a very selective process. However, this selectivity is essential when application of these microorganism is foreseen in the recovery of W from complex chemical mixtures [25].

It is well established that bacteria can acquire tungsten ions by three different transport systems, Mod, Wtp and Tup, as reviewed by Aguilar-Barajas et al. [2]. The Tup system is considered the most specific for tungstate and, therefore, its presence was determined in the isolates used in this study. The *tupA* and *tupB* genes were found in several strains, however, the work was mainly focused on the analysis of genetic determinants of the most tolerant and W-accumulator *S. dubius* strains NA4, As(V)4 and Sb5.

The studied strains of *S. dubius* had the *tup* genes organized as *tupBCA*, which was a different order when compared with the well-characterized *tupABC* of *Eubacterium acidaminophilum* [22]. However, it is not clear whether this difference could have relevant implications in cellular tungsten uptake. Nevertheless, a large number of sequences of similar *tup* genes deposited in the available databases show different genetic organizations (*tupABC*, *tupBCA* or *tupACB*) but *tupABC* is only reported in a few studies and its

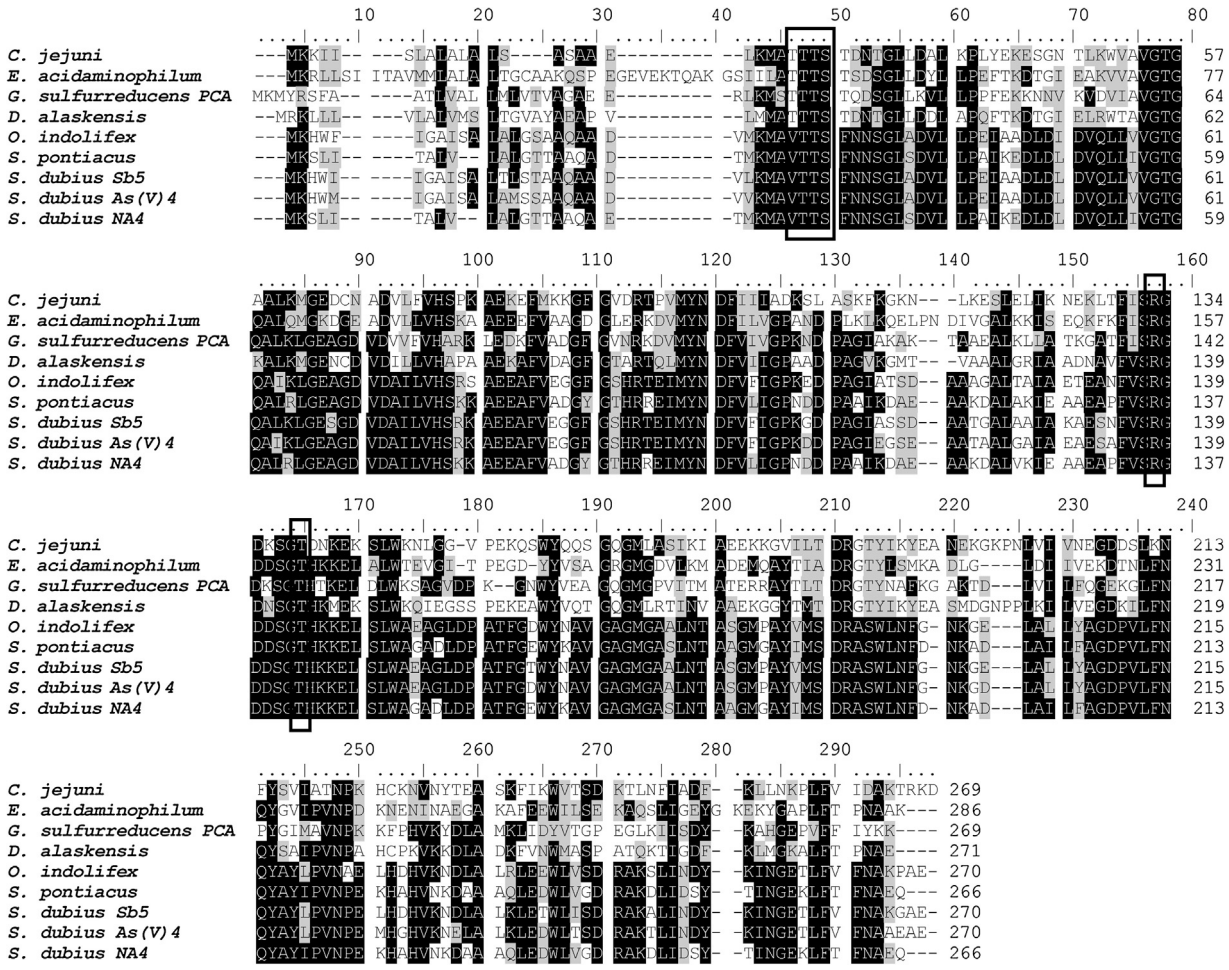

Fig. 3. Alignment of TupA proteins from different organisms. Amino acid sequences (obtained from NCBI) were aligned via CLUSTAL W [13]. Highly conserved residues in TupA homologues (black shading), similar residues in TupA homologues (grey shading) and the putative conserved motif and residues involved in tungsten binding (in the box) are depicted. The additional strains are *Campylobacter jejuni*, ALF94174.1; *Eubacterium acidaminophilum*, Q93KD6.1; *Geobacter sulfurreducens* PCA, NC_002939.5; *Desulfovibrio alaskensis*, WP_011366386.1; *Oceanibulbus indolifex*, HEL-45, EDQ03330; *Sulfitobacter pontiacus*, WP_037965326.

gene products examined [25,38]. This study showed that similar strains belonging to the same species, carrying the same *tup* genetic organization and also having high homology among their genes, exhibited distinct profiles and efficiencies for W-uptake. This finding could not be easily explained because the general W-uptake mechanism is not completely understood and there is not enough information concerning this bacterial species. However, uptake of toxic metals can be controlled and modulated by siderophores produced by resistant bacteria that can, by complexing tungsten in specific situations, allow bacterial growth in the presence of a high concentration of tungstate [36].

One study has described the overexpression of the TupA protein of *E. acidaminophilum* in *E. coli*, demonstrating the specificity of tungstate-binding by TupA [22], and more recently this selectivity was confirmed by the study of TupA in *C. jejuni* [35]. The sequence analysis of TupA from *S. dubius* strains suggested that the proteins were able to bind tungstate ions. The multiple sequence alignments showed that *S. dubius* proteins contained a motif at the N-terminal region that is a typical signature indicating selective binding of tungstate. The classical conserved motif consists of a TTTS amino acid arrangement, where the second threonine and the serine have been shown to form hydrogen bonds with oxyanions [14]. In the case of TupA from *S. dubius*, both residues were also present but the first Thr was replaced by Val. This finding is a common feature of the genus *Sulfitobacter*, as revealed by the TupA amino acid sequence alignment of the different species. Tungstate-protein binding in the three strains also seemed to be supported by the presence of conserved Thr165 and the positively charged Arg158, which have been proposed as important residues in metal coordination [14,27].

In summary, the three *S. dubius* strains tested were highly adapted for survival in environments contaminated with tungsten. However, as they could grow easily in the absence of tungsten, their enzymes had no obligatory requirement for this metal, which meant that they were not dependent on its presence. Despite the fact that tungsten was non-essential, all three strains carried the complete *tup* gene cluster, which may be the system responsible for bacterial tungsten uptake. The data from this study could open up a new avenue in the development of an alternative strategy for recovering tungsten from natural or anthropogenic tungsten-impacted environments.

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References

[1] Abbas, S., Ismail, I., Mostafa, T., Sulaymon, A. (2014) Biosorption of heavy metals: a review. J. Chem. Sci. Technol. 3, 74–102.
[2] Aguilar-Barajas, E., Díaz-Pérez, C., Ramírez-Díaz, M.I., Riveros-Rosas, H., Cervantes, C. (2011) Bacterial transport of sulfate, molybdate, and related oxyanions. BioMetals 24, 687–707.
[3] Andreesen, J.R., Makdessi, K. (2008) Tungsten, the surprisingly positively acting heavy metal element for prokaryotes. Ann. N. Y. Acad. Sci. 1125, 215–229.
[4] Arunakumara, K.K.I.U., Xuecheng, Z. (2008) Heavy metal bioaccumulation and toxicity with special reference to microalgae. J. Ocean Univ. China 7, 60–64.
[5] Bevers, L.E., Hagedoorn, P.-L., Hagen, W.R. (2009) The bioinorganic chemistry of tungsten. Coord. Chem. Rev. 253, 269–290.
[6] Bevers, L.E., Hagedoorn, P.-L., Krijger, G.C., Hagen, W.R. (2006) Tungsten transport protein A (WtpA) in *Pyrococcus furiosus*: the first member of a new class of tungstate and molybdate transporters. J. Bacteriol. 188, 6498–6505.
[7] Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 254, 248–254.
[8] Byrne, N., Strous, M., Crépeau, V., Kartal, B., Birrien, J.-L., Schmid, M., Lesongeur, F., Schouten, S., Jaeschke, A., Jetten, M., Prieur, D., Godfroy, A. (2009) Presence and activity of anaerobic ammonium-oxidizing bacteria at deep-sea hydrothermal vents. ISME J. 3, 117–123.
[9] European Commission, 2014 Report on critical raw materials for the EU, Report of the ad hoc Working Group on defining critical raw materials.
[10] Farias, P., Santo, C.E., Branco, R., Francisco, R., Santos, S., Hansen, L., Sorensen, S., Morais, P.V. (2015) Natural hot spots for gain of multiple resistances: arsenic and antibiotic resistances in heterotrophic, aerobic bacteria from marine hydrothermal vent fields. Appl. Environ. Microb. 81, 2534–2543.
[11] Fontaine, F.J., Wilcock, W.S.D., Foustoukos, D.E., Butterfield, D.A. (2009) A Si-Cl geothermobarometer for the reaction zone of high-temperature, basaltic-hosted mid-ocean ridge hydrothermal systems. Geochem. Geophys. 10, 1–9.
[12] Ghazvini, P.T.M., Mashkani, S.G. (2009) Screening of bacterial cells for biosorption of oxyanions: application of micro-PIXE for measurement of biosorption. Hydrometallurgy 96, 246–252.
[13] Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., Lopez, R. (2010) A new bioinformatics analysis tools framework at EMBL—EBI. Nucleic Acids Res. 38, 695–699.
[14] Hagen, W. (2011) Cellular uptake of molybdenum and tungsten. Coord. Chem. Rev. 255, 1117–1128.
[15] von Heijne, G. (1992) Membrane protein structure prediction. J. Mol. Biol. 225, 487–494.
[17] Hur, H., Reeder, R.J. (2016) Tungstate sorption mechanisms on boehmite: systematic uptake studies and X-ray absorption spectroscopy analysis. J. Colloid Interface Sci. 461, 249–260.
[18] Ivanova, E.P., Gorshkova, N.M., Sawabe, T., Zhukova, N.V., Hayashi, K., Kurilenko, V.V., Alexeeva, Y., Buljan, V., Nicolau, D.V., Mikhailov, V.V., Christen, R. (2004) *Sulfitobacter delicatus* sp nov. and *Sulfitobacter dubius* sp. nov., respectively from a starfish (*Stellaster equestris*) and sea grass (*Zostera marina*). Int. J. Syst. Evol. Microbiol. 54, 475–480.
[19] Kletzin, A., Adams, M.W.W. (1996) Tungsten in biological systems. FEMS Microbiol. Rev. 18, 5–63.
[20] Koutsospyros, A., Braida, W., Christodoulatos, C., Dermatas, D., Strigul, N. (2006) A review of tungsten: from environmental obscurity to scrutiny. J. Hazard. Mater. 136, 1–19.
[21] L'vov, N.P., Nosikov, A.N., Antipov, A.N. (2002) Tungsten-containing enzymes. Biochemistry-Moscow 67, 196–200.
[22] Makdessi, K., Andreesen, J.R., Pich, A. (2001) Tungstate uptake by a highly specific ABC transporter in *Eubacterium acidaminophilum*. J. Biol. Chem. 276, 24557–24564.
[23] Marques, A.F.A., Scott, S.D., Gorton, M.P., Barriga, F.J.A.S., Fouquet, Y. (2009) Pre-eruption history of enriched MORB from the Menez Gwen (37°50′N) and Lucky Strike (37°17′N) hydrothermal systems, Mid-Atlantic Ridge. Lithos 112, 18–39.
[24] Nielsen, P., Fritze, D., Priest, F.G. (1995) Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. Microbiology 141, 1745–1761.
[25] Ogi, T., Makino, T., Okuyama, K., Stark, W.J., Iskandar, F. (2016) Selective biosorption and recovery of tungsten from an urban mine and feasibility evaluation. Ind. Eng. Chem. Res. 55, 2903–2910.
[26] Ogi, T., Sakamoto, Y., Nandiyanto, A.B.D., Okuyama, K. (2013) Biosorption of tungsten by *Escherichia coli* for an environmentally friendly recycling system. Ind. Eng. Chem. Res. 52, 14441–14448.
[27] Otrelo-Cardoso, A.R., Nair, R.R., Correia, M.A.S., Rivas, M.G., Santos-Silva, T. (2014) TupA: a tungstate binding protein in the periplasm of *Desulfovibrio alaskensis* G20. Int. J. Mol. Sci. 15, 11783–11798.
[28] Paulino, J.F., Afonso, J.C., Mantovano, J.L., Vianna, C.A., Cunha, J.W.S.D. (2012) Isolamento do Tungsténio da Volframita da Mina de Igarapé Manteiga (Rondónia – Brasil) por Lixiviação Ácida. Quim. Nova 35, 1854–1857.
[29] Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.
[30] Schwarz, G., Hagedoorn, P.-L., Fischer, K. (2007) Molybdate and tungstate: uptake, homeostasis, cofactors, and enzymes. Minerva Gastroenterol. Dietol. 43, 189–196.
[31] Shanware, A., Priya, P. (2014) Tungsten toxicity in soil and biological role of tungsten in bacteria. Indian J. Sci. 8, 6–15.
[32] Singh, S.N., Tripathi, R.D. 2007 Environmental bioremediation technologies, pp. , 1–518.
[33] Smart, J.P., Cliff, M.J., Kelly, D.J. (2009) A role for tungsten in the biology of *Campylobacter jejuni*: tungstate stimulates formate dehydrogenase activity and is transported via an ultra-high affinity ABC system distinct from the molybdate transporter. Mol. Microbiol. 74, 742–757.
[34] Strigul, N., Koutsospyros, A., Arienti, P., Christodoulatos, C., Dermatas, D., Braida, W. (2005) Effects of tungsten on environmental systems. Chemosphere 61, 248–258.
[35] Taveirne, M.E., Sikes, M.L., Olson, J.W. (2009) Molybdenum and tungsten in *Campylobacter jejuni*: their physiological role and identification of separate transporters regulated by a single ModE-like protein. Mol. Microbiol. 74, 758–771.
[36] Wichard, T., Bellenger, J.-P., Loison, A., Kraepiel, A.M.L. (2008) Catechol siderophores control tungsten uptake and toxicity in the nitrogen-fixing bacterium *Azotobacter vinelandii*. Environ. Sci. Technol. 42, 2408–2413.