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Efficient bioaccumulation of tungsten by *Escherichia coli* cells expressing the *Sulfitobacter dubius* TupBCA system

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ABSTRACT

Tungsten (W) is a valuable element with considerable industrial and economic importance that belongs to the European Union list of critical metals with a high supply risk. Therefore, the development of effective and new methods for W recovery is essential to ensure a sustainable supply.

In the present study, the *Sulfitobacter dubius* W transport system TupABC was explored in order to demonstrate both its functionality in *Escherichia coli* cells and to construct a bioaccumulator (EcotupW). The complete gene cluster *tupBCA* or partial gene cluster *tupBC* were cloned in an expression vector and transformed into *E. coli*. Metal accumulation was evaluated when each construct strain was grown with three separate metal oxyanions (tungstate, molybdate or chromate). The specificity of the bioaccumulator was determined by competition assays using cells grown with mixed solutions of metal oxyanions (W/Mo and W/Cr). The results showed the relevance of the TupA protein in the TupABC transporter system to W-uptake and also allowed Mo and Cr accumulations, although with amounts 1.7 and 2.9-fold lower than W, respectively. To identify the importance of the valine residue in the accumulation efficiency of the VTTS motif, site-directed mutagenesis of *tupA* was performed. A mutant with a threonine residue, instead of the respective valine, confirmed that W was internalized by nearly double the amount compared to the native form.

The findings indicated that cells carrying the native *S. dubius* TupABC system were great W-bioaccumulators and could be promising tools for W recovery.

Introduction

Tungsten (W) is a very valuable, rare element that is considered to be a critical metal [4,27,30]. Due to its massive use and its valuable role in various fields, the demand for this element will continue to increase in the near future. Therefore, the development of more effective and new methods for recovery of W is essential to ensure a sustainable supply [10,12,20,26,27]. Bioaccumulation and biosorption approaches are considered great strategies in the recovery of soluble metals, since they do not require high quantities of chemical reagents and they minimize the volume of toxic sludge and other waste products. In recent years, many studies have been performed in order to construct bioaccumulators for several metal ions [11,19,36].

Tungsten is the heaviest transition element within the sixth group of the periodic table, which also comprises chromium (Cr) and molybdenum (Mo) [4]. Therefore, these elements show comparable chemical and physical properties in terms of equal atomic and ionic radii, similar electronegativity and coordination characteristics [4,7,18,22,29,32]. The similarities between W and Mo lead to difficulties of discrimination by biological systems that can result in W toxicity [4]. Strategies to differentiate between metals must be present in biological systems to avoid incorrect metal uptake and the possibility of incorrect metal interaction with the active sites of enzymes [29].

In general, the cellular uptake of metals from the environment can occur in two ways: (i) a selective substrate-specific uptake system that consumes high energy, namely ATP; (ii) a substrate-non-specific uptake system that uses the chemiosmotic gradient [34]. Intracellular accumulation (i.e. bioaccumulation) is an energy-driven process dependent on active metabolism that normally occurs when an element is absorbed at a faster rate and then retained by an organism.

In some microorganisms belonging to the *Archaea* and *Bacteria* domains, mainly in some microorganisms from special habitats, such as hot black smokers or sulfide-enriched waters

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[1,2,4,6,10,12,33], tungsten plays an important biological role, for instance, as a cofactor for different enzymes (W-dependent enzymes) [2,6,10] or a facultative bioelement that can replace Mo in specific enzymes [5]. Tungsten and Mo are cofactors in the active centers of enzymes involved in oxygen transfer reactions that require tungstopterin and molybdopterin, respectively [4–6,21,32]. Therefore, selective incorporation of the respective element into cells is required [34].

In aqueous environments, as well as in soils, W occurs mainly in its most stable oxidation state, oxyanion tungstate (WO_4^{2-}) [12,17,31,33]. In the case of Mo, the availability of this element for living cells is in the oxyanion form designated as molybdate (MoO_4^{2-}) [13]. These oxyanions are known to be transported into bacterial cells by ATP-binding cassette (ABC) transport, specifically through three different transporters: the tungstate uptake protein (TupABC, which is considered highly specific for tungstate), the W-transport system (WtpABC, which transports both oxyanions), and the molybdate transporter (ModABC, which is highly specific for molybdate) [2]. These three specific transporters do not only differ in their binding affinity but appear to have different oxyanion coordination chemistry [7,14]. In the species already described in the literature, *Geobacter sulfurreducens* [22] and *Desulfovibrio alaskensis* G20 [29], coordination is carried out by the threonine residue present in the TTTS motif located in the substrate-binding protein TupA, which is assumed to be responsible for tungstate-binding. In *Sulfitobacter* species, a conserved VTTS motif instead of TTTS has been observed. Therefore, it was hypothesized that W-coordination could be maintained by the motif despite the presence of the first valine instead of threonine. Moreover, this difference in the amino acid residue could be involved in better performance of *Sulfitobacter* species for accumulating W [10].

In this present study, in order to obtain cells able to accumulate high quantities of W, the tungstate transport system of strain *Sulfitobacter dubius* NA4 was explored through the overexpression of *tup* genes in *E. coli* DH5α cells. The efficiency and selectivity of the W-binding protein (TupA) in metal uptake efficiency were also evaluated, not only for W but also for the other metal oxyanions of the same group in the periodic table, Mo and Cr. To this end, the heterologous expressions of the complete (*tupBCA*) or partial (*tupBC*) gene clusters were performed in *E. coli* cells and the quantity of the respective metals accumulated in the cells was evaluated. Additionally, site-directed mutagenesis of the TupA VTTS motif was performed to show the importance of the valine residue in enhanced W-accumulation. Consequently, the native TupBCA system of *S. dubius* NA4 was shown to be more efficient than other variants and resulted in an efficient bioaccumulator.

Materials and methods

Strains, media and growth conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 1. *E. coli* strains were grown aerobically at 37 °C on Luria-Bertani (LB) medium containing, per liter, 10 g tryptone, 5 g yeast extract and 5 g NaCl. The antibiotic ampicillin (Amp, 100 μg mL^{-1}) (Nzytech) and the expression inducer isopropyl-D-thiogalactopyranoside (IPTG) (Nzytech) were added to the medium, as required. Bacterial growth was determined by optical density at 600 nm (OD_{600nm}).

For protein expression experiments, *E. coli* cells were grown overnight in liquid medium containing Amp in an orbital shaker at 120 rpm and 37 °C. The cultures were diluted 1:10 in 100 mL fresh LB medium with Amp, and incubated under the same conditions. When OD_{600nm} reached 0.5, 0.5 mM of IPTG was added to the culture and cells continued to grow for 6 h after induction.

Analytical-grade salts of tungsten ($Na_2WO_4{\cdot}2H_2O$) (Sigma-Aldrich), molybdenum ($Na_2MO_4{\cdot}2H_2O$) (BDH) and chromium ($Na_2Cr_2O_7{\cdot}2H_2O$) (Merck) were prepared as 0.5 M stock solutions and sterilized by filtration.

Construction of tupBCA and tupBC containing plasmids

The genomic region containing the complete *tupBCA* gene cluster was amplified from *Sulfitobacter dubius* strain NA4 [11] using specific forward and reverse primers, NcoI_BCAf and BamHI_BCAr (Table 1), respectively. PCR reactions, for a final volume of 50 μL, were performed using 2 U Platinum™ *Taq* DNA Polymerase (Invitrogen), 0.2 mM of each dNTP, 1 × PCR Buffer, 1.5 mM $MgCl_2$, 0.4 μM primers, and 2 ng DNA template. The PCR program involved initial denaturation at 94 °C (5 min), followed by 30 cycles of 94 °C (1 min), 63 °C (1 min) and 72 °C (2.5 min). The genomic region containing *tupB* and *tupC* genes was also amplified from *S. dubius* strain NA4 using specific primers, NcoI_BCAf and BamHI_BCr (Table 1). DNA amplification was performed in reactions as previously described. The PCR program comprised initial denaturation at 94 °C (5 min), followed by 30 cycles of 94 °C (1 min), 64 °C (1 min) and 72 °C (1.5 min).

All PCR-amplified DNA fragments (2220 bp and 1423 bp for *tupBCA* and *tupBC* amplification, respectively), as well as the plasmid pTrc99A, were digested with the restriction enzymes *Nco*I and *Bam*HI. The digested amplified fragments were purified and ligated into the pTrc99A expression vector for 1 h at room temperature using 0.5 U of T4 DNA ligase (Thermo Scientific). The resulting plasmids, represented in Fig. 1, were transformed into competent *E. coli* DH5α cells. The correct construction was confirmed by sequencing the complete DNA fragments cloned into the plasmid (Stabvida).

Site-directed mutagenesis

The pTrc_tupBCA plasmid DNA was used as a PCR template to induce frequent nucleotide misincorporation into this plasmid in order to provide a recombinant mutated TupA protein. The mutant was generated by overlap extension PCR, according to the method of Ho et al. [16], using the oligonucleotide primers V.BCAf and V.BCAr. The mutagenic reaction mixture contained 1x Pfx amplification buffer, 0.15 mM of each dNTP, 150 ng of each oligonucleotide primer, 1 ng DNA plasmid and 2.5 U Platinum Pfx polymerase (Invitrogen), for a final volume of 50 μL. The reaction was conducted for 18 cycles of 94 °C (30 s), 55 °C (1 min) and 68 °C (6.5 min). The PCR reaction was digested with 10 U of DpnI (Takara) for 3 h at 37 °C, and then 10 μL were transformed in 100 μL of *E. coli* DH5α cells, as described above. Colonies were selected on Amp LB plates and the point mutations were confirmed by DNA sequencing analysis.

Evaluation of TupA expression in E. coli *constructs by SDS-PAGE*

E. coli DH5α, Eco_tupBCA, Eco_tupBC and Eco_tupBCA'V26T strains were grown in liquid medium, as mentioned above, and collected approximately at the end of the exponential growth phase, 6 h after IPTG induction. Culture suspensions were centrifuged at 4000 rpm for 20 min at 4 °C. The resultant bacterial pellets were washed with 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). Cells were disrupted by four cycles of sonication (Sonics&Materials Inc. Danbury, Connecticut U.S.A.) at 60 A for 30 s and with 30 s in ice between each sonication cycle. Cell debris was removed by centrifugation at 13,000 rpm for 15 min. The resulting supernatant was transferred to a new tube for total protein quantification using the Bradford method [9]. Protein profiles of each sample comprising 15 μg of total protein were evaluated by electrophoresis on a 0.1%

Table 1
Bacterial strains, plasmids and primers used in this study.

Strain or plasmid or primer	Relevant characteristics	Source or reference
Strains		
Sulfitobacter dubius NA4	*Sulfitobacter* sp. containing the complete *tupBCA* gene cluster	[11]
Escherichia coli DH5α	φ80d*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *relA1 supE44 deoR* (*lacZYA-argF*)*U169*	Promega
Eco_tupBCA	*E. coli* DH5α carrying the pTrc99A plasmid with the complete gene cluster *tupBCA*	This study
Eco_tupBC	*E. coli* DH5α carrying the pTrc99A plasmid with genes *tupB* and *tupC*	This study
Eco_tupBCA'V26T	*E. coli* DH5α carrying the pTrc_tupBCA'V26T plasmid	This study
Plasmids		
pTrc99A	Amp^r; expression vector	Amersham Pharmacia Biotech
pTrc_tupBCA	pTrc99A plasmid modified with the gene cluster *tupBCA* inserted	This study
pTrc_tupBC	pTrc99A plasmid modified with the partial gene cluster *tupBC* inserted	This study
pTrc_tupBCA'V26T	pTrc99A plasmid modified with the complete gene cluster *tupBCA* with the nucleotide change in the *tupA* gene coding the switch of Val for Thr	This study
Primers		
NcoI_BCAf (sense)	5'GGT**CCATGG**CTATGAATGATATCTGGGCGGGC	This study
BamHI_BCAr (antisense)	5'CCT**GGATCC**TCACTGCTCGGCGTTAAAGGT	This study
BamHI_BCr (antisense)	5'TGG**GGATCC**TCATATCAAAATATCTCCATTCAGG	This study
V.BCAf (sense)	5'CCATGAAAATGGCTACCACCACGTCCTTCAAC	This study
V.BCAr (antisense)	5'GTTGAAGGACGTGGTGGTAGCCATTTTCATGG	This study

Enzyme restriction sites are presented in bold and the mutation site is underlined.

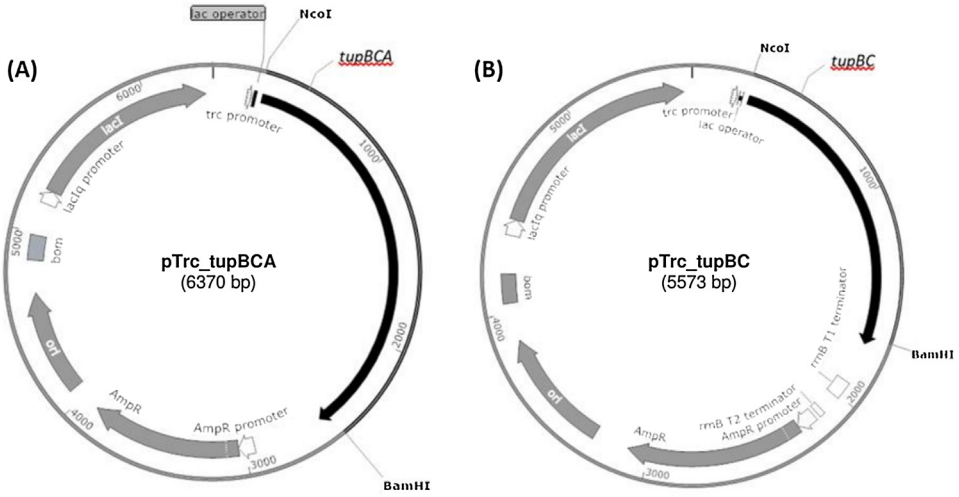

Fig. 1. Schematic diagram of the plasmids engineered for this study. (**A**) pTrc_tupBCA plasmid. (**B**) pTrc_tupBC plasmid. Arrows indicate the direction of transcription. The restriction enzymes indicated were those used in this study. The program used for plasmid design was SnapGene®.

sodium dodecyl sulfate (SDS)-12% polyacrylamide gel, followed by Coomassie blue staining.

Bacterial growth in liquid media with metal oxyanions

E. coli strains were grown separately in liquid medium with the presence or absence of three different metal oxyanions, in the following concentrations: 1 mM and 5 mM tungstate and molybdate, and 100 μM and 200 μM chromate. Culture samples were collected before induction with IPTG, as well as 3 h and 6 h after IPTG induction, and the OD_{600nm} was determined.

Tungsten, molybdenum and chromium cell accumulation assays

E. coli strains were grown in the same conditions as described above in the presence of 1 mM tungstate and molybdate, and 100 μM chromate, separately. Cells were harvested 6 h after IPTG induction and 50 mL of culture suspensions were centrifuged at 4000 rpm for 20 min at 4 °C. The resultant bacterial pellets were washed twice with 50 mL PBS. Cells were digested by adding 0.5 mL 10% HNO_3 with 0.5 mL milliQ water, incubated in a water bath at 50 °C for 1 h, frozen in ice for 30 min and then centrifuged at 4000 rpm for 20 min at 4 °C. For metal quantification in samples containing tungstate, the digested cells (pellets) were resuspended in 5 mL of PBS and stored, since W naturally precipitates with the digested cellular material when submitted to acid treatment. In the other samples containing molybdate and chromate, the supernatant fractions were collected for Mo and Cr determination. Metal measurements were determined by inductively coupled plasma mass spectrometry (ICP-MS) normalized by total protein mass, which was obtained using the Bradford method [8].

Strain growth was also evaluated in the presence of mixed metal oxyanion conditions in order to evaluate preferential metal uptake by the cells. The cells were grown simultaneously in the presence of 1 mM tungstate and 1 mM molybdate or 1 mM tungstate and 5 mM molybdate or 1 mM tungstate and 100 μM chromate or 1 mM tungstate and 200 μM chromate. Since W precipitates during acid treatment, a different methodology to lysate cells was used in these competition assays. Thus, cells were harvested 6 h after IPTG induction, subjected twice to washing steps and then disrupted by sonication, as mentioned in a previous section. After centrifugation at 4000 rpm for 20 min, the supernatants were used for metal quantification by ICP-MS and the pellet fractions were used for protein quantification. All these assays were performed using three replicates.

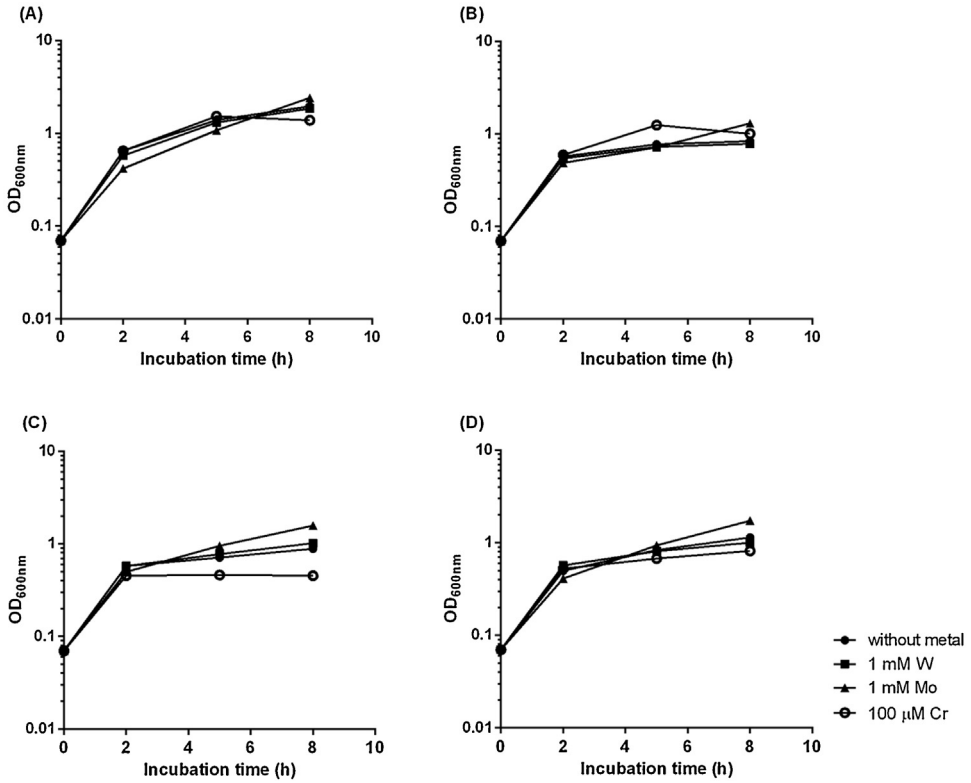

Fig. 2. Growth curves of strains, native *E. coli* DH5α (**A**), Eco_tupBCA (**B**), Eco_tupBC (**C**) and Eco_tupBCA'V26T (**D**) in the absence (black circle) or presence (black square) of W, Mo (black triangle) and Cr (circle). The growth curve experiments were performed with at least two replicates. Each point corresponds to means ± standard deviations. Very small error bars are not visible.

Live/dead membrane integrity assay and viable bacteria counts

The membrane integrity of Eco_tupBCA and Eco_tupBC strains was obtained with Live/Dead® BacLight™ Bacterial Viability Kits (L7007, Invitrogen). The native *E. coli* DH5α was used as a control for the standard curve of the percentage of live bacteria, where 1 mL of the cell suspension was added to each of two tubes (50 mL) containing 20 mL of 0.85% NaCl for live bacteria and 20 mL of isopropanol for killed bacteria. Cells were grown in the presence and absence of 100 μM chromate and, as mentioned before, they were harvested 6 h after IPTG induction and washed with PBS. Washed cells were centrifuged and resuspended in 2 mL 0.85% NaCl. The OD at 670 nm (OD_{670nm}) was measured and then adjusted to an OD_{670nm} of 0.06. To a 96-well flat-bottom microplate, 100 μL of each bacterial suspension were stained with 100 μL of a 2× staining solution (6 μL SYTO 9 and 6 μL propidium iodine with 2 mL filter-sterilized dH_2O) and incubated at room temperature for 15 min in dark conditions. The fluorescence measurement was carried out with an excitation wavelength of 485 nm and fluorescence intensity of 530 nm and 630 nm, for live cells (green) and dead cells (red), respectively.

Viable bacterial cell concentrations were estimated by counting colony-forming units (CFU) after 6 h of IPTG induction. All samples were analyzed by inoculating 0.1 mL of the dilutions of the bacterial suspension on LB plates with Amp. Serial dilutions were performed to obtain countable plates. The plates were incubated at 37 °C for 24 h. The results are presented as the average count per sample expressed as log CFU mL^{-1}. Both assays were performed with two replicates.

Statistical analysis

Statistical analysis of cellular metal accumulation was performed using the software *GraphPad Prism* 6 and values were reported as the means ± SE of three replicates. Statistical analyses of bacterial viability and membrane integrity assays were also performed using the same software, and values were reported as the means ± SE of two replicates. Two-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test were performed for evaluation of differences between treatment means. A *p*-value of 0.05 was considered significant.

Results and discussion

Growth of E. coli *constructs in medium with tungstate, molybdate and chromate*

The effect of the metal oxyanions (tungstate, molybdate and chromate) on the growth of constructs Eco_tupBCA, Eco_tupBC, Eco_tupBCA'V26T and the native *E. coli* DH5α strain was evaluated in order to define the metal concentrations to be used in the metal accumulation assays. For these further studies, the objective was to use considerably higher concentrations of metals but with reduced toxicity for cells (e.g. growth OD should not be drastically affected by the metal oxyanions). Therefore, the strains were grown in the absence and presence of 1 mM tungstate and molybdate, and 100 μM chromate (Fig. 2). The growth curves of each strain for the absence and presence of the tested metals showed similar profiles, although 100 μM chromate slightly affected bacterial growth. Metal concentrations of 5 mM for tungstate and molybdate, and 200 μM for chromate were also tested. However, the chromate concentration of 200 μM exhibited high toxicity to the cells (see Supplementary Fig. S1). The negative effect of chromate was not completely unexpected, since it has been reported that chromate exhibits high toxicity to most organisms [2,9]. Studies have shown that high concentrations of this oxyanion, between 100 μM and 1 mM, tend to affect bacterial growth gradually, even

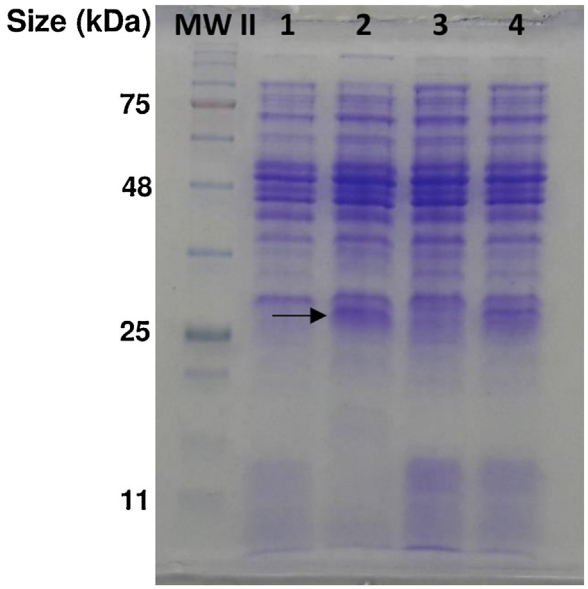

Fig. 3. Protein profiles on SDS-PAGE (12% acrylamide gel) of *Escherichia coli* strains: (1) *E. coli* DH5α, (2) Eco_tupBCA, (3) Eco_tupBC, (4) Eco_tupBCA'V26T. MW II, molecular weight NZYColour Protein Marker II. The arrow indicates the putative TupA protein band.

presenting growth inhibition for *Euglena glacini* cells at the highest concentration [10].

In this study, 1 mM of molybdate or 1 mM of tungstate did not affect bacterial growth, since they were able to achieve the same OD as the non-metal growth controls. This result was different from a study with the *Desulfovibrio alaskensis* G20 strain, which showed that increasing molybdate concentrations up to 500 μM visibly reduced bacterial growth rates [24]. Other studies showed that *Bacillus* sp. GT-83 was able to grow at high concentrations of WO_4^{2-} (up to 1840 mg L^{-1}), but concentrations higher than 184 mg L^{-1} of WO_4^{2-} considerably reduced bacterial growth [12]. Moreover, a sulfur-oxidizing bacterium, *Acidithiobacillus thiooxidans* NB1-3, was inhibited completely by 50 μM tungstate [25].

The relevance of TupA in the accumulation of tungstate, molybdate and chromate

The *tup* genes of strain *Sulfitobacter dubius* NA4 were cloned in an expression vector to evaluate their functionality for metal uptake into *E. coli* cells. The protein expression of the three constructs, namely Eco_tupBCA, Eco_tupBC and Eco_tupBCA'V26T, was analyzed with SDS-PAGE. The presence of a polypeptide produced in a large quantity is shown in Fig. 3 for the fractions Eco_tupBCA and Eco_tupBCA'V26T. This protein, identified by comparison as TupA, exhibited a molecular mass of approximately 30 kDa, which was consistent with the size predicted from the amino acid sequence (28.5 kDa), as well as from the TupA of other reported organisms that have been shown to be 30.9 kDa or 29 kDa [22,29]. Moreover, the identification of this protein band was confirmed by peptide mass fingerprinting and it corresponded to the W-binding protein TupA. Studies performed in native polyacrylamide gels have reported that TupB and TupC proteins possess a molecular weight of 24.5 and 23.6 kDa, respectively [22]. However, in the SDS-PAGE experiments included in the present study, no protein bands related to these proteins were revealed. This may be due to the fact that the separation of integral membrane proteins, like TupB, do not solubilize in non-ionic detergents (usually at low ionic strength), which is problematic in two-dimensional gel electrophoresis [15].

The metal accumulation (W, Mo and Cr) for each *E. coli* strain was tested (Fig. 4). Although the amount of Mo accumulated was always lower than W, Mo exhibited a very similar accumulation pattern to W. The Eco_tupBCA strain accumulated a larger amount of W (1.41 μg W mg^{-1} protein) and Mo (0.84 μg Mo mg^{-1} protein) when compared to the control, native *E. coli* DH5α (0.25 μg W mg^{-1} protein and 0.08 μg Mo mg^{-1} protein). Moreover, the Eco_tupBC strain presented lower quantities of W and Mo (0.16 μg W mg^{-1} protein and 0.05 μg Mo mg^{-1} protein, respectively) relative to the *E. coli* DH5α strain. Thus, Fig. 4 shows that W concentration decreased 8.8-fold in the Eco_tupBC strain compared to the Eco_tupBCA strain (from 1.41 to 0.16 μg W mg^{-1} protein, respectively). In the case of the Eco_tupBCA'V26T strain, the quantities of both metals in the cells, 0.43 μg W mg^{-1} protein and 0.29 μg Mo mg^{-1} protein, were higher than the *E. coli* DH5α strain but significantly lower than the Eco_tupBCA strain. Therefore, mutated strain Eco_tupBCA'V26 T exhibited a reduction of 3.3- and 2.9-fold in the accumulation of W and Mo, respectively, when compared to the non-mutated Eco_tupBCA strain. The results of Cr accumulation showed that all constructs accumulated more Cr than the native control *E. coli* DH5α. Moreover, strains Eco_tupBCA and Eco_tupBCA'V26T were able to accumulate significantly more Cr with 0.49 μg Cr mg^{-1} protein compared to the 0.27 μg Cr mg^{-1} protein accumulated by the control.

It has been well established that bacteria can obtain W and Mo ions by three different transport systems, namely Mod, Tup and Wtp, as reviewed by Aguilar-Barajas et al. [2] and Shanware et al. [33]. In the case of Cr, which is analogous to the element sulfate, it exists mainly in aqueous solutions in the oxyanion form chromate (CrO_4^{2-}) [9] and can be taken up by bacteria through sulfate transport systems [2]. It has been described that *E. coli* has two ABC transporter systems [23], namely CysP and Mod, and it is possible that these systems were responsible for metal accumulation in the control *E. coli* DH5α cells for the metals tested in the present study even if they were residual.

Considering all metals tested, W was accumulated in higher quantities in the Eco_tupBCA strain. However, quantities of Mo and Cr were also incorporated into the cells, although in lower amounts than W ([W] < [Mo] < [Cr]). Studies from other authors, which evaluated the biosorption of W and the bioaccumulation of Cr in *E. coli* cells, exhibited levels of 0.30 μg W mg^{-1} protein [27] and 0.023 μg Cr mg^{-1} protein [3], which were significantly lower than the concentrations determined in the present study. In the case of Mo, the maximum accumulation determined in this study was similar to the levels of 0.88 μg Mo mg^{-1} protein reported in the literature for *D. alaskensis* DaG20 [24].

The current results were remarkable since there is not much information concerning the interaction of W and Mo with bacteria carrying the TupABC transporter system, and there is no data relative to Cr. The Eco_tupBCA strain also had the capability to accumulate Cr compared to the control, in addition to the other metals W and Mo.

The *tupBCA* gene cluster includes the genetic determinants coding for proteins involved in W-binding and membrane transport into the bacterial cell. The protein TupA is responsible for the recognition and binding of a highly specific substrate (WO_4^{2-}). The other two proteins, TupB and TupC, are responsible for the transport of the substrate and for ATP-binding, respectively, thereby facilitating the transport of the respective substrate into the cell. In this present study, by removing the gene responsible for metal binding (*tupA* gene) from the gene cluster, the quantities of W and Mo accumulated decreased drastically compared to Cr, which only showed a slight difference. This result demonstrated how important the TupA protein was to the transporter system for the incorporation of heavy metals into bacterial cells, especially for W and Mo.

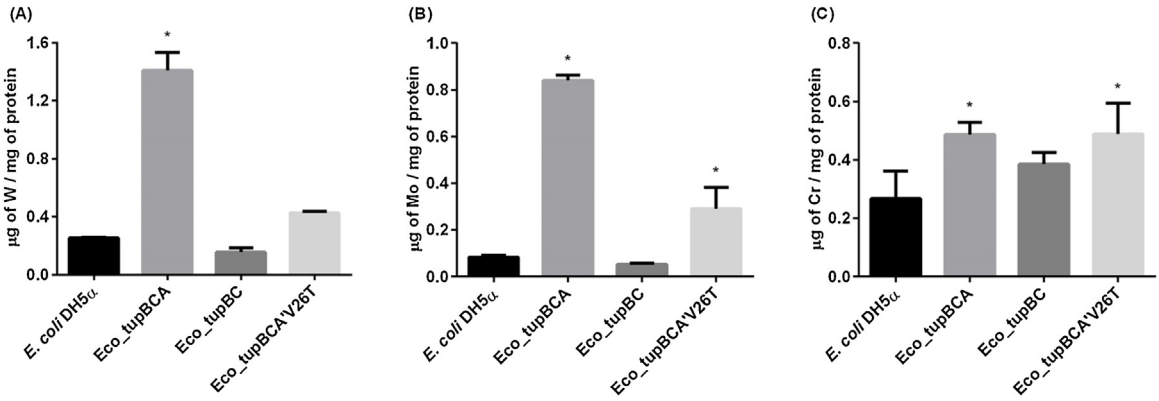

Fig. 4. Accumulation of tungsten, molybdenum, and chromium following 6 h growth after IPTG induction by *Escherichia coli* strains DH5α, Eco_tupBCA, Eco_tupBC, and Eco_tupBCA'V26T. Cells were exposed to 1 mM W (**A**) and Mo (**B**), and 100 μM Cr (**C**). Results are the concentrations of metals after normalization for protein content (means ± standard deviations – error bars – from three independent experiments; * - means significant difference compared to the control *E. coli* DH5α).

It is well known that the TupA protein is classified as component A from the TupABC system because of the presence of a classical conserved motif related to tungstate binding, which is the TTTS motif [22,28,29]. As described in a previous study [10], *Sulfitobacter* sp. also has a conserved motif, differing in the first residue (valine instead of threonine) from other bacteria carrying the TupABC transporter system.

The results relative to the mutated strain indicated that the predicted motif was very relevant for metal-binding, especially for W and Mo, since a high reduction of accumulation was demonstrated compared to its original motif, with valine in the first residue. In the case of Cr, no significant difference was shown in terms of accumulation when the residues threonine or valine were present in the motif. This result demonstrated that, although the TupABC transporter system was able to accumulate the three metals mentioned in this study, it had greater specificity for W and Mo. Therefore, it is very important for *Sulfitobacter* sp. to carry the residue valine in its motif for better accumulation efficiency and it is required for the functionality of the TupA protein in this species.

Molybdenum and chromium act as a competitors with tungsten

To test the specificity of the metal-chelator TupA, *E. coli* strains were grown in the presence of different mixed metal oxyanion solutions. Fig. 5A shows the relationship of the accumulation of W and Mo by the *E. coli* DH5α, Eco_tupBCA and Eco_tupBC strains, at concentrations of 1 mM tungstate, 1 mM tungstate and 1 mM molybdate, and 1 mM tungstate and 5 mM molybdate. The Eco_tupBCA strain showed the capability to accumulate both metal oxyanions when present in the same liquid medium, with 1 mM tungstate and 1 mM molybdate or 1 mM tungstate and 5 mM molybdate, with values ranging from 1.48 to 2.12 $\mu g\,W\,mg^{-1}$ protein and 0.18 to 1.08 $\mu g\,Mo\,mg^{-1}$ protein, respectively. Thus, with the increase of the competitor concentration, the quantities of accumulated Mo increased inside the cells, with the highest concentration being 1.08 $\mu g\,Mo\,mg^{-1}$ protein. In the case of the Eco_tupBC strain, the difference between W and Mo values inside the cells was higher than in the Eco_tupBCA strain, which was more visible when the competitor was present at the higher concentration than W. Thus, in this strain, the competitor showed an increase from 0.36 to 2.72 $\mu g\,Mo\,mg^{-1}$ protein, but the W concentration did not show a high discrepancy (1.44 and 2.08 $\mu g\,W\,mg^{-1}$ protein).

Fig. 5B shows that the accumulation pattern between W and Cr was similar to W and Mo. However, the Eco_tupBCA strain was able to accumulate high quantities of Cr with 1.33–2.72 $\mu g\,Cr\,mg^{-1}$ protein when cells were grown in the presence of 1 mM tungstate and 100 μM chromate or 1 mM tungstate and 200 μM chromate, respectively. In the case of strain Eco_tupBC, W accumulation decreased drastically in the presence of 100 μM of the competitor (0.63 $\mu g\,W\,mg^{-1}$ protein) but the amount of Cr was still higher than W (0.82 $\mu g\,Cr\,mg^{-1}$ protein). Moreover, raising the concentration of Cr to 200 μM led to an increase of the W concentration to 2.01 $\mu g\,W\,mg^{-1}$ protein, whereas the competitor increased to 3.50 $\mu g\,Cr\,mg^{-1}$ protein. The native *E. coli* DH5α strain presented a slight accumulation of Cr, although in quantities much lower than the other tested strains.

To our knowledge, the preference for metals, as well as the importance of the TupABC transporter system to metal-binding protein, has never been well evaluated. However, in this study, both conditions tested (W/Mo and W/Cr) showed that the TupABC transporter system was able to bind and consequently accumulate both metal oxyanions, W being preferably accumulated when in the presence of higher competitor concentrations (Mo or Cr) without changing the protein structure. From the existing group of W/Mo transporter systems (Tup, Mod and Wtp), only Bevers et al. [6] have studied and characterized the WtpABC transporter in the presence of W and Mo using *Pyrococcus furious*, which supports the present experiments. The accumulation of the competitor was higher than W only when the TupA protein was removed and the competitor (Mo or Cr) concentration was greater than W in the liquid medium. This meant that both metal oxyanions were internalized into the cells by the transporter protein, TupB, and since the protein responsible for metal-binding was not present, the selectivity of the channel to the metals ceased to occur. These results are notable since it confirms the high specificity of the TupABC transporter system for the metal W instead of Mo or Cr.

Chromium toxicity

In order to explain Cr accumulation by the cells, an approach with live/dead staining was used to assess membrane damage after chromate treatment. As shown in Fig. 6A, the Eco_tupBC strain in the presence of 100 μM chromate exhibited the highest death rate caused by membrane damage, which decreased approximately 2-fold in live bacteria compared with Eco_tupBC untreated cells (57.8 and 100%, respectively). The presence of Cr did not have a strong effect on the Eco_tupBCA strain, since there was no significant difference in the values of live bacteria in the treated or untreated cells (96.2% and 83.6%, respectively). A second approach for viable bacterial counts was carried out, which in turn supported the previous results (live/dead) (Fig. 6B). The Eco_tupBCA strain had approximately the same colony-forming units (CFU) relative to the strain treated with Cr. However, the Eco_tupBC strain, compared to the Eco_tupBCA strain, showed a lower number of colonies,

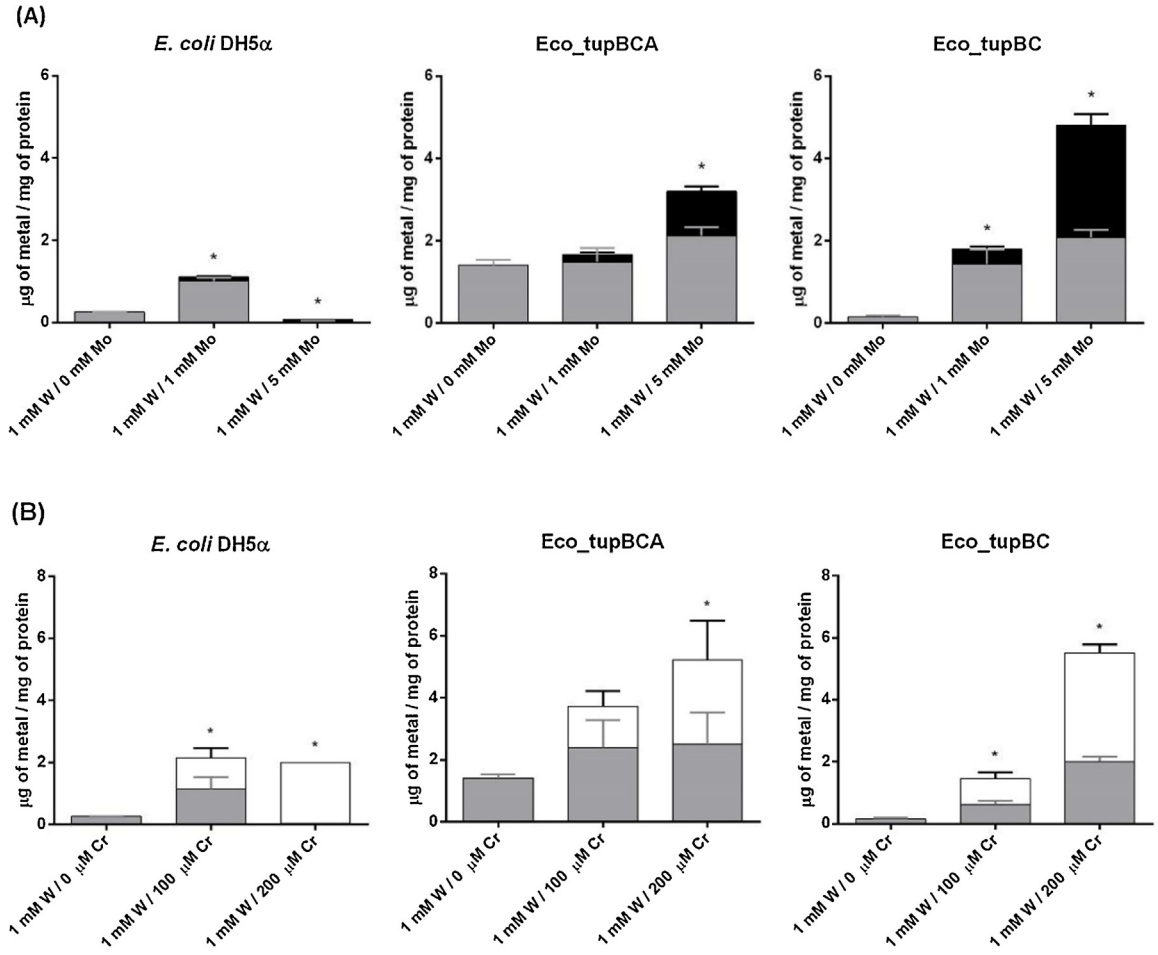

Fig. 5. Accumulation of tungsten (grey bar) and molybdenum (black bar) (**A**), and tungsten and chromium (white bar) (**B**) by *Escherichia coli* strains. Results are the concentrations of metals after normalization for protein content (means ± standard deviations – error bars – from three independent experiments; * - means significant difference compared to the control *E. coli* DH5α).

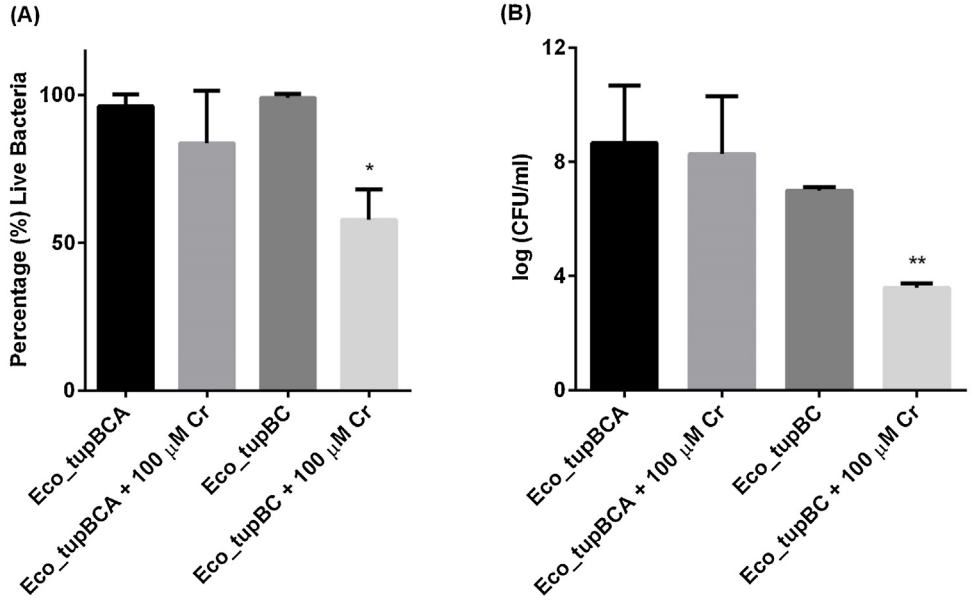

Fig. 6. Chromium toxicity results for Eco_tupBCA and Eco_tupBC strains performed with two methods: direct damage of the bacterial membrane by live/dead staining (**A**), and the number of bacteria evaluated by plate counts, CFU (**B**) (means ± standard deviations – error bars – from two independent experiments; * - means significant difference compared to the untreated cells).

with a viable colony reduction of log (3.4) CFU mL^{-1} compared to the untreated cells. The lower survival rate (shown by the number of viable cells) and the higher cell death (shown by green/red dyes) of strain Eco_tupBC in the presence of chromate could explain the lower levels of chromate accumulated by these cells, since less viable cells are able to accumulate Cr. As chromate is a mutagen and causes reactive oxygen species (ROS) leading to oxidative stress in cells, it can damage the cell membrane resulting in a dysfunctional membrane, as well as leading to protein, DNA and intracellular system damage [35].

Conclusions

In this study, it was shown that expression of the complete *tup* gene cluster from *Sulfitobacter dubius* NA4 in *E. coli* cells (e.g. strain Eco_tupBCA) resulted in a high bacterial capacity to accumulate W. This great W bioaccumulator was also able to take up Mo and Cr. The modified strain containing the plasmid coding for TupA with a VTTS motif from *Sulfitobacter* sp., instead of TTTS, was shown to be more efficient for W uptake. Therefore, this study demonstrated that recombinant cells overexpressing the original TupBCA from strain *S. dubius* NA4 could be explored with the objective of having an alternative strategy to recover W from natural environments or even from anthropogenic W-impacted environments.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.126001.

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