Identification and Characterization of the Transcriptional Regulator ChrB in the Chromate Resistance Determinant of *Ochrobactrum tritici* 5bvl1

Rita Branco¹,², Paula V. Morais¹,³*

¹ IMAR-CMA-Marine and Environmental Research Centre, Coimbra, Portugal, ² Interdisciplinary Research Institute, University of Coimbra, Coimbra, Portugal, ³ Department of Life Sciences, FCTUC, University of Coimbra, Coimbra, Portugal

**Abstract**

*Ochrobactrum tritici* 5bvl1 is able to resist to high concentrations of chromate through the expression of an inducible chromate-resistant determinant, found in a mobile element (TnOtChr), which carries the genes, *chrB*, *chrA*, *chrC* and *chrF*. The regulation of *chr* operon present in TnOtChr, which is controlled by a transcriptional regulator, ChrB, was characterized in the current work. Fusions of *chr* promoter, or *chr* promoter and *chrB* gene, upstream of a *gfp* reporter gene, identified the most probable promoter sequence within the *tnpR-chrB* intergenic region. This region contains an AT-rich imperfect inverted repeat sequence, which overlaps a part of the −10 sequence. The results of the *in vitro* DNA-binding assays with purified ChrB (His- or no-tagged) showed that the protein binds directly to the *chr* promoter region. In order to identify the ChrB functional domain for sensing chromate stress and for DNA-binding, site-directed mutagenesis of ChrB was performed. Among several single amino acid mutants, three mutants (R180; R187 and H229) prevented chromate induction without any modification to the protein’s stability. Interestingly, two ChrB mutants (R18 and R23) were constitutively active, regardless of chromate stress conditions, indicating that the residues most probably belong to the protein-DNA binding site. As such, the ChrB was classified as a transcriptional regulator that recognizes a specific DNA sequence, regulating the expression of a chromate resistance determinant.

**Introduction**

Human activities have resulted in the release and introduction into the environment of different chemicals including heavy metals. In general, most metals are essential for microbial cells, as co-factors for different enzymes or structural components of proteins [1]. Nevertheless, many essential metals become toxic at high ion concentrations, while some metal ions are toxic to bacterial cells at any concentration. Therefore, the interest in discovering how bacteria are dealing with hazardous environmental pollutants resulted in numerous and important genetic, biochemical and physiological data, which allowed a deeper understanding of the adaptation capacities of microorganisms. Many bacteria contain genes that encode specific products conferring resistance to heavy metal ions. Some of the proteins that have been characterized are membrane-bound transporters that pump toxic ions out of the cells, cytoplasmic or periplasmic metal transport proteins, metal reductases, metallothioneins and metal-sequestrating proteins [2,3]. In most microorganisms, the expression of such resistance systems is controlled at transcription level by metal sensor proteins, which are known to bind to the promoter regions and are responsible for regulation of metal responsiveness.

The biodegradation capabilities of microorganisms, with the purpose of applying these directly in bioremediation processes, have been looked at with interest [4]. In the past, less attention was endorsed to studies of the regulatory mechanisms, which manage the expression of specific pathways. Nowadays, the particular regulation features of several kinds of expression pathways have attracted interest of numerous researchers [5]. Different groups, that studied microbial resistance mechanisms, also tried to discover which regulatory process is behind it and/or the exact mechanisms of activation and repression of the systems. Moreover, information about the regulation of systems has acquired a special importance, since these regulatory systems have the potential for being used as sensory mechanisms in the construction of bioreporters. These tools have been recognized as useful and very promising instruments in monitoring the quality of many environments, such as water, soil, and air [6–8].

Chromium ion is considered to be an important element on its reduced form [Cr (III)] but becomes toxic on the oxidised state of chromate or dichromate [9]. Therefore, the intracellular amount of chromium ions must be tightly regulated to prevent high chromate concentrations. Several chromate resistance determinants were identified in prokaryotes and among the reported bacterial systems are those of: *Cupriavidus metallidurans* CH34 [10], *Pseudomonas aeruginosa* PA01 [11], *Shewanella* sp. strain ANA-2 [12],...
Materials and Methods

Bacterial Strains, Culture Conditions and DNA Manipulation

The bacterial strains and plasmids utilized in this study are listed in Table S1. In general, bacteria were grown at 37°C in Luria–Bertani (LB) medium with vigorous aeration. When required, antibiotics were added at the following concentrations: 30 mg/ml kanamycin and 15 mg/ml gentamycin. Routine DNA manipulations, including PCR amplifications from genomic DNA templates, were performed as previously described [22]. Preparations of plasmid DNAs were performed using the JetQuick Mini-spin kit (Genomed, Lohne, Germany) and plasmid extraction was performed according to the manufacturer’s instructions.

Identification of the Promoter Region by Construction of Reporter Gene Plasmids

Several DNA sequences shown in Fig. 1 were amplified with specific primer pairs. The PCR-amplified DNA fragments were digested with respective restriction enzymes and ligated into cleaved vector pProbe-NT [23] yielding pchrGFP1; pchrGFP2 and pchrGFP3. The resulting plasmids, which contained the putative promoter sequences upstream of the promoterless gfp gene, were transferred into E. coli DH5α by transformation. Correct gene insertion was confirmed by DNA sequencing, performed by Macrogen (Macrogen Europe Netherlands). The promoter activities were determined by measuring the green fluorescence using a fluorimeter.

Cloning and Purification of ChrB-His6

The chrB coding sequence was also amplified from O. tritici 5bvl1 DNA using specific primers engineered to contain the restriction sites for NdeI and EcoRI (NdechrB1f and EcochrB1r, respectively) but in this approach a stop codon was present into reverse primer to obtain a recombinant protein without a His-tag. The PCR product was digested with these enzymes and cloned into pET30a generating petChrB-His6, which C-terminus recombinant ChrB was fused to a His6-tag. For protein expression, the plasmid was used to transform E. coli BL21 (DE3) cells and the construct was verified by DNA sequencing. To overexpress and purify ChrB-His6, E. coli BL21 (DE3) carrying plasmid petChrB-His6 was grown overnight at 37°C in LB containing kanamycin. The culture was diluted 1:10 into 1L of LB with kanamycin and incubated at 37°C until it reached an optical density at 600 nm of 0.5. Then, isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and incubation was continued overnight at 25°C.

Chemical Crosslinking of ChrB

To determine whether ChrB is in oligomer form, chemical crosslinking assays were performed using glutaraldehyde. Reaction


cytosingenesis elongatus PCC 7942 [13], Lysinibacillus sasiformis ZC1 [14], Bacillus cereus Sj1 [15], Arthrobacter sp. strain FB24 [16] and Ochrobactrum tritici 5bvl1 [17]. Chromate resistance in bacteria is primarily accomplished by a specific efflux system that pumps chromate out of the cell, thereby lowering the intracellular concentration [17–19]. This function is performed by ChrA transporter, which is a chr operon encoded protein. Besides chr, other genes have also been identified in bacterial chr determinants, such as chrB, chrC and other less-studied genes (chrE or chrF). In a previous study, we identified a Tn506chr element of chromate resistant strain O. tritici 5bvl1, carrying the chr operon that comprises chrB, chrA, chrC and chrF genes [20]. The chrB is proposed to play a regulatory role for expression of the ChrA transporter, chrC encodes a putative superoxide dismutase and chrF encodes a protein with uncertain function [21].

In the best of our knowledge no chromate responsive regulators have been well characterized or really studied. In this study, we aimed to identify the chr promoter region and clarify the role of ChrB. Several constructions performed by fusion of putative chr promoter fragments with the reporter gene gfp allowed to limit the most predictable chr promoter sequence. Several approaches, such as transcriptional fusion of chrB with gfp, electrophoretic mobility shift assay (EMSA) and site directed mutagenesis were also performed to characterize the ChrB protein, its binding to the promoter region and the most probable amino acids involved in chromate-protein binding.

Identification of the Promoter Region by Construction of Reporter Gene Plasmids

Several DNA sequences shown in Fig. 1 were amplified with specific primer pairs. The PCR-amplified DNA fragments were digested with respective restriction enzymes and ligated into cleaved vector pProbe-NT [23] yielding pchrGFP1; pchrGFP2 and pchrGFP3. The resulting plasmids, which contained the putative promoter sequences upstream of the promoterless gfp gene, were transferred into E. coli DH5α by transformation. Correct gene insertion was confirmed by DNA sequencing, performed by Macrogen (Macrogen Europe Netherlands). The promoter activities were determined by measuring the green fluorescence using a fluorimeter.

Cloning and Purification of ChrB-His6

The chrB coding sequence was also amplified from O. tritici 5bvl1 DNA using specific primers engineered to contain the restriction sites for NdeI and EcoRI (NdechrB1f and EcochrB1r, respectively) but in this approach a stop codon was present into reverse primer to obtain a recombinant protein without a His-tag. The PCR product was digested with these enzymes and cloned into pET30a generating petChrB-His6, which C-terminus recombinant ChrB was fused to a His6-tag. For protein expression, the plasmid was used to transform E. coli BL21 (DE3) cells and the construct was verified by DNA sequencing. To overexpress and purify ChrB-His6, E. coli BL21 (DE3) carrying plasmid petChrB-His6 was grown overnight at 37°C in LB containing kanamycin. The culture was diluted 1:10 into 1L of LB with kanamycin and incubated at 37°C until it reached an optical density at 600 nm of 0.5. Then, isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and incubation was continued overnight at 25°C.

Chemical Crosslinking of ChrB

To determine whether ChrB is in oligomer form, chemical crosslinking assays were performed using glutaraldehyde. Reaction
mixtures containing 8 μg of purified ChrB or ChrB-His6 in crosslinking buffer (20 mM NaCl, 10 mM KCl, 2 mM DTT in 20 mM Hepes, pH 7.5) were incubated with glutaraldehyde to a final concentration of 0.1%, and the reaction mixture was incubated for 2, 5, 10, 30 and 60 min at 30°C. Crosslinking was terminated by adding SDS-PAGE sample buffer, and the samples were heated at 95°C for 5 min and analyzed by 10% SDS-PAGE.

Construction of Chromate Reporter Plasmids

The chr promoter and chrB gene were amplified from O. tritici 5bvl1 DNA, using the primers XbachrP1f and EcochrB1r, by standard PCR condition. The PCR-amplified DNA fragment (1109 bp) was digested with XbaI and EcoRI and ligated upstream of gfp gene of vector pProbe-NT [23] yielding pChrBGFP. Other constructions were also performed to evaluate reporter plasmids carrying partial chrB sequences. Thus, N-terminal of ChrB was obtained using XbachrP1f and EcochrB2r primers and was cloned upstream of gfp gene resulting pChrBGFPn. To obtain the pChrBGFPc, the ChrB C-terminal sequence, amplified by using BamChrBf and EcoChrB1r primers, was cloned into plasmid pchrGFP1. All these plasmids were introduced into E. coli DH5α and confirmed by DNA sequencing.

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

| Strains and plasmids | Relevant genotype or characteristic(s) | Reference or source |
|----------------------|----------------------------------------|---------------------|
| O. tritici 5bvl1     | ChrVII                                 | 17                  |
| E. coli DH5α         | F- rplD1ΔlacZΔM15 Δ (lacZYA-argF) U169 endA1 recA1 hsdR17 (rK-, mK+) deor thi-1phaA supE44 λ- gyrA96 relA1 | Invitrogen          |
| E. coli BL21 (DE3)   | F- ompT hsdS (rK-, mK-) gal dcm lacY1(DE3) | Novagen             |
| pProbe-NT            | Km', promoterless plasmid, gfp         | 23                  |
| pET30a               | Km', expression plasmid                | Novagen             |
| pchrGFP1             | pProbe-NT containing DNA fragment including chr promoter region between positions −170 and +1 | This study          |
| pchrGFP2             | pProbe-NT containing DNA fragment including chr promoter region between positions −90 and +96 | This study          |
| pchrGFP3             | pProbe-NT containing DNA fragment including chr promoter region between positions −90 and +1 | This study          |
| pChrBGFP             | pchrGFP1 containing the total chrB gene upstream of gfp | This study          |
| pChrBGFPn            | pchrGFP1 containing the partial chrB gene (ChrB N-terminal) upstream of gfp | This study          |
| pChrBGFPc            | pchrGFP1 containing the partial chrB gene (ChrB C-terminal) upstream of gfp | This study          |
| pchrGFPmutP          | pchrGFP1 containing a mutation into the imperfect inverted repeat | This study          |
| pchrGFPmutP          | pChrBGFP containing a mutation into the imperfect inverted repeat | This study          |
| pchrGFPgM            | pchrGFP1 containing a gentamicin resistance gene | This study          |
| pChrBGFP-R18A        | pChrBGFP with Arg18 changed to Ala    | This study          |
| pChrBGFP-R23A        | pChrBGFP with Arg23 changed to Ala    | This study          |
| pChrBGFP-R175A       | pChrBGFP with Arg175 changed to Ala   | This study          |
| pChrBGFP-R180A       | pChrBGFP with Arg180 changed to Ala   | This study          |
| pChrBGFP-R182A       | pChrBGFP with Arg182 changed to Ala   | This study          |
| pChrBGFP-R187A       | pChrBGFP with Arg187 changed to Ala   | This study          |
| pChrBGFP-R195A       | pChrBGFP with Arg195 changed to Ala   | This study          |
| pChrBGFP-R196A       | pChrBGFP with Arg196 changed to Ala   | This study          |
| pChrBGFP-C213A       | pChrBGFP with Cys213 changed to Ala   | This study          |
| pChrBGFP-H229A       | pChrBGFP with His229 changed to Ala   | This study          |
| pChrBGFP-A241R       | pChrBGFP with Ala241 changed to Arg   | This study          |
| pChrBGFP-G244R       | pChrBGFP with Gly244 changed to Arg   | This study          |
| pChrBGFP-H258A       | pChrBGFP with His258 changed to Ala   | This study          |
| petChrBHis6          | pET30a for overproduction of ChrB with an C-terminal hexahistidine tag | This study          |
| petChrB              | pET30a for overproduction of ChrB without any tag | This study          |
| petChrB-R18A         | petChrBHis6 with Arg18 changed to Ala | This study          |
| petChrB-R23A         | petChrBHis6 with Arg23 changed to Ala | This study          |
| petChrB-R180A        | petChrBHis6 with Arg180 changed to Ala| This study          |
| petChrB-R187A        | petChrBHis6 with Arg187 changed to Ala| This study          |
| petChrB-H229A        | petChrBHis6 with Arg229 changed to Ala| This study          |
| petChrB-H258A        | petChrBHis6 with Arg258 changed to Ala| This study          |

doi:10.1371/journal.pone.0077987.t001
Site-directed Mutagenesis

The pChrBGFP and petChrBHis6 plasmids were utilized as PCR templates to induce frequent nucleotide misincorporation into chromate reporter plasmid and recombinant over-expressed ChrB protein, respectively. Mutant variants of the wild-type ChrB protein were generated by overlap extension PCR according to the method of Ho and collaborators [24]. Briefly, the mutagenic reaction mixtures (50 μl) contained: 5 μl of enzyme reaction buffer, 0.2 mM of each dNTP, 150 ng of each oligonucleotide primer, 10 ng of DNA plasmid and 2.5U of Platinum Pfx polymerase (Invitrogen, Carlsbad, CA). The sequence of oligonucleotides used in mutagenesis is described in Table S1, in the supporting information. The reaction was conducted in a thermal cycler (Bio-Rad) for 16 cycles of 94°C for 1 min denaturation, 60°C for 1 min annealing, and 68°C for 16 min extension. PCR reactions were digested with 10 U of DpnI for 2 hours at 37°C and then 5 μl were transformed in 100 μl of competent E. coli DH5α or E. coli BL21. Colonies were selected on kanamycin LB plates and the point mutations were confirmed by DNA sequencing analyses.

Electrophoretic Mobility Shift Assay (EMSA)

The EMSA was conducted as described previously [25]. The promoter sequence DNA probe was generated using the primer pair XbachrP1f and BanchrP1r. The PCR product was gel purified and biotin end labelled by using LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL). In general, DNA binding assay was performed in a 20 μl reaction volume containing binding buffer (10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT), 1 μg poly(dI-dC), 10% glycerol, 1 mM MgCl₂, 30 fmol DNA probe and different concentrations of purified ChrB. To show the effect of chromate in DNA-protein binding assays, the protein was first incubated with chromate (10, 100 or 1000 μM) for 10 min at room temperature and then DNA labelled probe was added. In competition assays, 1 to 5 μg of cold probe was used to challenge the labelled probe. After incubation at 25°C for 20 min, the samples were loaded onto a native 6% polyacrylamide gel and electrophoresed in 1 × Tris-borate EDTA (TBE) buffer for 70 min at 100 mA. Following gel electrophoresis, the complexes were electroblotted to nylon membrane (Roche) and detected using a chemiluminescence based nucleic acid detection kit (Pierce).

Figure 1. Localization of the chr promoter within the tnpR-chrB intergenic region using gfp transcriptional fusions. A) PCR fragments containing the promoter portions (indicated by the boxes) were cloned upstream of a promoterless gfp on pProbe-NT. Sequences are numbered relative to the first nucleotide of the chrB start codon. Primer pairs used in this work are indicated. B) Green fluorescence of E. coli cells harboring the different constructs. The values represent averages and standard deviations of three replicates.

doi:10.1371/journal.pone.0077987.g001
Bacterial Double Plasmid Assays

The previous promoter reporter plasmid pchrGFP1 have been engineered to introduce an additional antibiotic resistance cassette as reported in [26]. In this work, a plasmid pchrGFP1 carrying an additional gentamicin resistance gene was constructed and it was inserted into E. coli DH5α cells yielding pchrGFP2.

Competent E. coli BL21 cells carrying the plasmid petChrBH1s6 or the several mutated plasmids (petChrBR18A; petChrBR23A; petChrBR180A; petChrBR187A; petChrBH229A) were co-transformed with pchrGFP2 and and co-transformants were selected using LB medium that contained kanamycin (30 mg/mL), and gentamicin (15 mg/mL). The co-transformed strains were submitted to chromate and green fluorescence was evaluated.

E. coli Reporter Activity Assay

The E. coli reporter cells were grown overnight, on a shaker (150 rpm) at 30°C in LB medium and used as inoculum. Cultures of these strains were performed in Tris-buffered mineral salts medium [27] supplemented with glucose 0.5% and vitamins [17], designated by TMM. Cells were grown at 37°C up to an OD600nm of about 0.2–0.3, corresponding to cells at exponential phase and were stressed by addition of the indicated concentration of chromate. The stock chromate solution (1 M) was prepared by dissolving the analytical grade metal salt (as K2CrO4) in ultra pure water. The stressed cultures were then incubated at 37°C, for the period of 3 hours. During this period, reaction volumes of 200 μl were transferred onto clear 96-well plates (Thermo Labsystems, Helsinki, Finland) and GFP fluorescence intensity was measured in triplicate.

Spectrofluorometry

Fluorescence intensity was measured with a Gemini EM Fluorescence Microplate Reader (Molecular Devices) with emission, excitation and cutoff wavelengths at 510, 480 and 595 nm, respectively. Relative fluorescence unit (RFU) is defined as the ratio of fluorescence intensity of the culture fluorescence relative to culture biomass at OD 600 nm spectraMax Plus384 Absorbance Microplate Reader (Molecular Devices).

Results

Identification of chr Promoter Region by Construction of Promoter-gfp Fusion Plasmids

In order to identify the shortest chr promoter region, sequences immediately upstream of chrB gene and partial genetic fragments of chrB of TuOChr of O. tritici 5bv1l were analysed. The subfragments indicated in Fig. 1A, created by PCR amplification were introduced upstream of gfp gene to construct the plasmids pchrGFP1, pchrGFP2 and pchrGFP3. These plasmids and pHBeN (negative control) were transformed into E. coli cells, respectively. No green fluorescence was detected with control sample, whereas high GFP activity was detected in cells carrying the other plasmids (Fig. 1B). Similar green fluorescence was obtained when these cells were incubated with different concentrations of chromate (data not shown). These data suggest that chr promoter sequence might be localised inside the last fragment, therefore between bp –90 and +1 with respect to the chrB translational start nucleotide. The high fluorescence signals achieved either in absence or presence of chromate indicate that this chr promoter acted as the constitutive promoter in the absence of the chrB gene.

Identification of Putative Protein Binding Site in chr Promoter through Mutagenesis

A DNA fragment containing the chr promoter and chrB gene was also amplified and cloned upstream of gfp gene resulting in pChrBGFP. E. coli cells were then transformed with this plasmid and showed fluorescence, only when recombinant cells were incubated with chromate. This finding indicates that ChrB works as a regulatory protein and might bind to DNA.

A close examination of the previously identified chr promoter revealed the presence of a 12 bp imperfect inverted repeat separated by a nine base pair linker GTAGATCTATC. This motif was located between bp –37 and –4 with respect to the initial ATG of chrB gene. The relevance of the inverted repeat was tested by nucleotide mutagenesis. Three nucleotides of the motif in constructs pchrGFP1 and pChrBGFP were exchanged resulting pchrGFPmutP and pChrBGFPmutP, respectively (Fig. 2A). Comparing the fluorescence results from mutants with the original recombinant strains, this mutation affected the GFP production. Continuous green fluorescence or chromate-induced fluorescence was detected by pchrGFP1 or pChrBGFP cells respectively, however, respective mutant cells revealed low fluorescence signals either in presence or in absence of chromate (Fig. 2B). These results indicate that the predicted DNA motif is relevant for protein regulation. In order to further confirm the importance of this promoter motif, EMSA experiments using the mutated promoter probe were conducted (data not shown). These assays showed that ChrB is still able to bind to the mutated promoter. Therefore, together, the results seem to indicate that ChrB is able to bind the mutated promoter but does not function as a regulator under these conditions.

ChrB is not Functional as Partial Protein

The effect of the deletion of N-terminal or C-terminal of ChrB was examined for their ability to regulate the green fluorescence expression. When partial genes, with their promoter sequence, were cloned upstream of gfp gene, no regulation of fluorescence emission was observed (Fig. 3). In these experiments, E. coli cells were able to express continuously GFP protein, which did not change by absence or presence of chromate. These results suggest that amino-terminal and carboxyl-terminal are required for functionality of ChrB.

Overexpression and Purification of ChrB and ChrB-His6

In order to obtain the ChrB protein, cloning, gene expression and protein purification experiments were conducted, which resulted in production of large amounts of protein in the soluble form. The protein ChrB-His6 was overexpressed in E. coli and purified by Ni²⁺ affinity chromatography. The apparent molecular mass of purified protein was 35 KDa, which is close to 35.8 KDa calculated for the 312 amino acid protein (Fig. 4A). Moreover, a second strategy of overexpression and purification of ChrB was used to ensure that His-tag did not affect the behaviour or conformation of protein. Thus, the chrB gene including the stop codon was cloned into plasmid pET30a. Figure 4B shows the progress of the purification on SDS-PAGE, where is on evidence a single band corresponding to a molecular mass of 35 KDa, corresponding to ChrB protein. The identity of this protein was determined by Peptide Mass Fingerprinting and corresponded to the predicted sequence of ChrB.

ChrB Exists as Oligomers

To determine whether ChrB existed as monomer or in oligomeric status, chemical crosslinking experiments were per-
formed on purified ChrB-His6 using glutaraldehyde. Analysis of reaction mixtures that contained purified ChrB-His6 with glutaraldehyde resulted in shifting position bands on SDS-PAGE. Figure 5A shows that increasing incubation time of the protein with glutaraldehyde caused the appearance of a strong band on the gel, corresponding to the position of a dimer (approximately 70 KDa) and weak bands with higher molecular weights. These results indicated that ChrB forms oligomers and exists predominantly as dimers. Similar results were obtained when ChrB without His-tag was used (results not shown). Considering that ChrB might interact with DNA and Cr(VI), cross-linking experiments of the protein with DNA promoter sequence and/or Cr(VI) were performed and the reaction products were analysed by using immunoblots probed with anti-His antibody (Fig. 5B). Addition of chromate and/or target DNA sequence had no effect on multidimerization.

ChrB Binds to the Promoter Region of chr Operon

To demonstrate that ChrB binds to the tnpR-chr intergenic region, electrophoretic mobility shift assays were performed, which clearly demonstrated that ChrB bound to the promoter probe DNA and retarded its migration in a concentration-dependent fashion (Fig. 6A). The shifted band became visible at ChrB concentrations of 1 µM and higher concentrations of protein increased the amount of shifted labelled DNA probe. Labelled DNA was challenged with several-fold excess of non-labelled probe DNA to determine the specificity of binding of ChrB to the DNA probe. Prebound ChrB exchanged with non-labelled DNA probe in the presence of a large excess of poly dI-dC (Fig. 6B) demonstrating that ChrB binds specifically to the chr promoter region. Moreover, ChrB did not switch off from DNA independently of the chromate concentrations tested (Fig. 6C).

Design and Construction of ChrB Mutants

Since no crystal structure of the ChrB protein or close relatives have been determined, it was not possible to either predict rigorously the tertiary structure of ChrB or obtain a model for effector interaction with this protein. Despite these difficulties, which impact the prediction of which amino acids in the ChrB could be implied in chromate interaction, the amino acid sequence of ChrB from O. tritici was aligned with a large number of available ChrB sequences. Based on these alignments, a region with a high homology was visible, rich in residues of arginine (Fig. 7). Some of the most conserved basic residues, namely arginines and histidines, were chosen to be mutated in order to identify the most probable amino acids that interact with chromate. A group of mutations were created to validate or refute the hypothesis (Fig. 7). Mutations in this group of basic residues were designed to alter the chemical nature of the residue (charged to non-charged hydrophobic or to non-charged hydrophilic). Therefore, the arginines (R175; R180;
R180; R187; R195; R196) and histidines (H229; H258) selected were changed to alanines and glycines. Additionally, one conserved residue of cysteine (C213) was also chosen for mutation. All mutants were tested in *E. coli* for GFP expression during exponential growth in the presence or absence of chromate. Fig. 8 gives representative GFP induction values after 3 h induction time compared to those of the strain carrying wild type ChrB. The group of mutations which changed the positively charged conserved residues and the conserved cysteine produced the following results (Fig. 8A): 1) mutations R180A, R187A and H229A completely abolished GFP expression upon chromate addition; 2) mutations R175A, R182A, R195A, R196A and C213A did not significantly affect chromate dependent GFP expression; 3) changing His258 to Ala resulted in a very high GFP expression in the absence of chromate when compared to wild-type ChrB activation in *E. coli*. For all of these residues, the Arg, His and Cys substitutions to Gly were created and produced the same effect as indicated for their substitutions to Ala (data not shown). These results suggested that several residues in this conserved area, namely R180, R187 and H229, might affect interaction between chromate and protein.

ChrB is a protein able to bind DNA and since there are no studies indicating which domain is implicated in this assignment, we used two available software tools for identification of the most putative DNA-protein binding domain of ChrB. First, by using the program Network Protein Sequence Analysis [28] for detection of the helix-turn-helix (HTH) domains, a HTH motif was identified with approximately 25% of probability. This motif was emphasized on ChrB alignments shown in Fig. 7. Second, by using the program BindN, for prediction of DNA and RNA binding residues in proteins [29] the residues Arg18 and Arg23 were identified as the residues with the highest score to bind DNA with a confidence.
coefficient of 0.98. In order to determine whether these identified sites were relevant to the DNA binding capacity, two groups of mutations were performed via site-directed mutagenesis. The reporter activities of strains containing mutated ChrB were then assayed. The mutated strains on ChrB residues A241R, G244A, and R252A, included in the predictable HTH motif, exhibited GFP activities when exposed to increasing concentrations of chromate (Fig. 8B). These results might indicate that the selected residues, and most probably this putative motif, are not involved in ChrB-DNA interaction. On the other hand, mutation of the residues R18A or R23A seems to have changed the ability of protein to bind to the DNA. These two mutated ChrB strains expressed the GFP by a constitutive mode and independently of the absence or presence of the inducer (Fig. 8B). The results suggest that these two residues close to the N-terminus of ChrB are implicated in the binding of the protein to the DNA operator, without excluding the involvement of other residues. To assure that the amino acid modifications had not drastically changed or compromised the protein structure, mutant ChrB proteins were purified and their migration on native polyacrylamide gel and multimerization through chemical crosslinking assays were compared with non-mutated ChrB protein. Mutant ChrB proteins and wild-type ChrB (all tagged with His6) were purified by Ni-NTA chromatography. Identical protein expression, gel migration and oligomer production were obtained except for H258A mutant. In the latter, no soluble protein was detected, suggesting a drastic configuration change induced by the mutation, which led to the production of the protein in inclusion bodies.

Therefore, the former results may indicate that mutations, with exception of H258A, did not cause a significant overall change in protein configuration. Consequently, the effects on chromate dependence GFP expression were the end result of a change in a...
critical effector binding region or residue. Likewise, the unregulated reporter activities, exhibited on the previous results, were the result of essential residues modification on protein-DNA binding domain.

Confirmation of ChrB Regulation by Double Plasmid Expression Analysis

To confirm the ChrB regulation of chr operon, a plasmid chr/gfp transcriptional fusion was produced, introduced into E. coli BL21 harboring petChrB, petChrBR18A; petChrBR23A; petChrBR180A; petChrBR187A or petChrBH229A, and the GFP activity was subsequently measured (Fig. 9). The resulting strains and the control strains carrying the empty expression vector pET30a were cultivated in minimal medium with or without the addition of chromate. When E. coli, complemented with a plasmid expressing wild-type ChrB was cultivated in medium with chromate, the strain exhibited green fluorescence dependent on chromate dose. In the absence of ChrB (control strain), GFP expression was high and not dependent on chromate dose, indicating that ChrB exerts a negative regulatory effect. Strains co-transformed with pchrGFP1 and petChrBR18A or petChrBR23A also exhibited high fluorescence signals even in absence of chromate. These data strengthen the previous idea that both arginines (R18 and R23) should be involved in the protein capacity to bind DNA. None of the additional strains co-transformed with pchrGFP1 and the other three petChrB mutants (petChrBR180A, petChrBR187A or petChrBH229A) exhibited GFP activity when incubated with chromate. Thus, these ChrB mutants did not allow transcription of the reporter gene under these conditions, which supported the previous data that these mutated proteins are affected at the chromate interaction site. The combination of presented results confirmed that ChrB regulates the chr operon expression in a strictly chromate-dependent manner.

Discussion

Bacteria must sense and adapt to environmental concentrations of a diversity of metals in order to survive, either low concentrations of essential metals needed as micronutrient or the presence of toxic heavy metal levels. This implies that bacteria must be capable of obtaining or scavenging trace metal ions from their environment to meet basic cellular needs and, on the other hand, must have inducible resistance mechanisms when concentrations exceed physiological needs. This bacterial capacity should require multiple families of metal-responsive transcription factors that discriminate between elements and thereby activate expression of genes responsible for suitable responses. Seven main bacterial families of metal-sensing transcriptional regulators have so far been identified: ArsR–SmtB, MerR, CsoR–RcnR, CopY, DtxR, Fur, and NikR. They span the detection of many transition elements such as Mn, Fe, Co, Ni, Cu, Zn, Ag/Au, Cd/Hg, as well as the p-block elements Pb and As/Sb [30]. These metal-sensor proteins are usually described as specialized allosteric proteins that regulate the transcription of genes linked to transition metal homeostasis, as a result of direct binding of a single metal ion or closely related metal ions [31]. In addition, there are some other metal-sensors belonging to structurally distinct families of regulators, that also include members not involved in metal sensing (e.g. TetR and LysR families) [32].

In contrast to the majority of the metals, only a very small number of transcription factors have been considered for chromate sensing. So far, chrB and chrF of C. metallidurans [20], chrI of Bacillus cereus [15] and chrB of O. tritici 5bvl1 [17] have been reported as the genes which most probably encode for regulatory proteins that control chr operon expression. Some roles for ChrB have been anticipated, including the activity of chromate reductase [16]. However, besides the initial speculation that ChrB acts as a regulator of gene expression, little effort has been made to assign a function to ChrB or to indicate possible mechanisms of action. Therefore, ChrB has remained as an uncharacterized protein. To fill this gap, we used a combined bioinformatics and functional approach to identify and characterize this novel chromate induced transcriptional regulator – ChrB. In contrast to the ArsR and MerR families, whose representative members have evolved to detect a far wider range of metal ions [26], the chromate reporter has been demonstrated to be very specific to this heavy metal [30].

The NCBI database includes ChrB of O. tritici 5bvl1 within the export chromate resistance protein group, and BLAST analyses showed in ChrB a conserved rhodanese domain at the C-terminus. Distinct proteins containing rhodanese-like modules are widely
distributed among diverse proteins, and enable the identification of many subfamilies [33]. Each subfamily harbors conserved motifs in the amino acid sequence of the putative active-site loop. The rhodanese-like domain of ChrB bears weak but significant sequence similarity to the six amino acid active loop [CG(S/T)GVT] of the described 3-mercaptopyruvate sulfurtransferase (MST) proteins. Rhodanese-like domains were also found within regulatory proteins such as the arsenic resistance operon repressor ArsR. The wide variety of amino acids of the putative active-site loop of the rhodanese homology domains has been associated to different substrate specificity. Therefore, the several rhodanese protein subfamilies could be involved in different biological functions [34]. Additionally, the ChrB C-terminal domain comprises the most conserved residues and, among these, the presence of a large number of very conserved arginines was particularly intriguing. This amino acid, as well as other positively-charged residues, has been referred as possibly involved in chromate binding and recognition [15]. Since cysteines are often involved in metal-regulatory protein interactions, a conserved residue of cysteine was also evaluated in this work as a possible key regulatory residue [35,36]. Contrary to what expected, Cys213 cannot function as a site for chromate-binding, but, on the other hand, fluorescence assays revealed that substitution of two arginines (Arg180, Arg187) and one histidine (His229) influence ChrB responsiveness to chromate. The mutagenesis strategy was also used to modify the N-terminal half protein, leading us to form

Figure 7. Alignment of ChrB homologues from different organisms. Amino acid sequences (obtained from NCBI) were aligned via CLUSTAL W. Ochrobactrum tritici 5bvl1 (ABO70326), Pseudomonas aeruginosa (AEQ93502); Cupriavidus metallidurans CH34, ChrB1 and ChrB2 (ABF13062 and ABF10734, respectively); Herminiimonas arsenicoxidans (CALG1077); Burkholderia pseudomallei 1106b (EE21856); Klebsiella pneumoniae 342 (YP_002238121); Jannibacterium sp. Marseille (ABR91486); Azaraus sp. BH72 (CAL95580); Chromobacterium violaceum ATCC 12472 (AAQ58595); Sphingobium japonicum UT265 (BAI96957). Highly conserved residues in ChrB homologues (black shading), similar residues in ChrB homologues (grey shading), conserved residues chosen for mutagenesis approach (arrow) and the putative HTH motif (into the box).
doi:10.1371/journal.pone.0077987.g007
the hypothesis that ChrB could exhibit two functional domains with different roles: the N-terminal being responsible for DNA-binding and the C-terminal for carrying the metal detection motives.

Our experiments revealed that ChrB works as an oligomeric form, mainly as a dimer. Characteristically, the regulatory proteins adopt oligomeric forms, and the dimeric assembly state is the most usual feature among the metal-responsive regulatory proteins. This regulation should also affect the expression of chr operon downstream genes, namely the chrA. This hypothesis is sustained by the fact that coding sequences of genes chrB and chrA are not separated but overlap in O. tritici genome. Transport actions are usually regulated for metalloids and metals such as arsenite and several cations (revised on [35,37]) and therefore so is the export of chromium from cells by the chromate efflux pump, ChrA [38].

The predicted binding site of ChrB to its own promoter DNA is very close to the coding sequence. It suggests that the complex formed by ChrB and the promoter DNA inhibits the binding or sliding of RNA polymerase, causing auto-repression. Another possibility, since the addition of chromate in EMSAs assays did not lead to turning off ChrB-chr promoter DNA, is the complex ChrB-chromate ions glides along the DNA promoter, which may be conducive to initiation or promotion of transcription. This kind of effects of transcriptional regulators was also observed in other regulators [39].

Some evidences showed that ChrB is a chromate responsive DNA-binding regulator of transcription of the chr operator/promoter: (i) Purified ChrB forms complexes with the chr operator/promoter in vitro as shown in EMSA assays; (ii) in absence of chromate, expression of GFP activity from the chr operator/promoter is much more elevated in cells devoid of chrB gene compared with cells containing ChrB; (iii) in cells carrying chr operator/promoter and chrB, expression of fluorescence is elevated in response to chromate concentrations; (iv) mutations of specific residues of ChrB affect GFP performance.

Conclusions

This study increases the knowledge on the cell metal homeostasis regulation mechanisms by studying the protein ChrB, which plays a significant role in chromate resistance in strain O. tritici 5bvl1. This research is an important first step in the characterization of potential regulatory elements of the chr operon, which includes the operator/promoter region and the essential amino acids that control the chr expression system. Further work focused on the structure of ChrB will demonstrate the value of these
predicted elements in the context of chromate sensing and chromate resistance mechanisms. This study confirms that ChrB is a regulatory protein and brings new light on protein-DNA and protein-Cr(VI) interactions.

**Supporting Information**

Table S1 Oligonucleotide sequences.

**References**

1. Waldron KJ, Robinson NJ (2009) How do bacterial cells ensure that metalloproteins get the correct metal? Nat Rev Microbiol 7: 23–35.
2. Busenlehner LS, Penella MA, Giedroc DP (2005) The SmlB/ArR family of metalloregulatory transcriptional repressors: structural insights into prokaryotic metal resistance. FEMS Microbiol Rev 27: 131–141.
3. Singh VK, Xiong A, Usgaard TR, Chakrabarti S, Deora R, et al. (1999) ZntR is an autoregulatory protein and negatively regulates the chromosomal zinc resistance operon znt of *Staphylococcus aureus*. Mol Microbiol 33: 200–207.
4. Dua M, Singh A, Sethunathan N, John AK (2002) Biotechnology and bio-mediation: successes and limitations. Appl Microbiol Biotechnol 59: 143–152.
5. Lin Z, Zhang Y, Wang J (2013) Engineering of transcriptional regulators enhances microbial stress tolerance. Biotechnol Adv. In press.
6. Yagi K (2007) Applications of whole-cell bacterial sensors in biotechnology and environmental science. App Microbiol Biotechnol 73: 1251–1258.
7. Tecon R, van der Meer JR (2008) Bacterial biosensors for measuring availability of environmental pollutants. Sensors 8: 4062–4080.
8. Su I, Jia W, Hou C, Lı Y (2011) Microbial biosensors: A review. Biosens Bioelectron 26: 1788–1799.
9. Zhitkovich A (2011) Chromium in drinking water: sources, metabolism, and cancer risks. Chem Res Toxicol 24: 1617–1629.
10. Nies A, Nies DH, Silver S (1990) Nucleotide sequence and expression of a chromate resistance determinant from *Alcaligenes eutrophus*. J Biol Chem 265: 5648–5653.
11. Cervantes C, Ohtake H, Chu L, Misra TK, Silver S (1990) Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM305. J Bacteriol 172: 287–291.
12. Aguilar-Barajas E, Palacios C, Cervantes C, Renzing C (2008) Expression of chromate resistance genes from *Shewanella* sp. strain ANA-3 in *Escherichia coli*. FEMS Microbiol Lett 285: 97–100.
13. Aguilar-Barajas E, Jerrinmo-Rodrigues P, Ramírez-Díaz MI, Renzing C, Cervantes C (2012) The ChrA homologue from a sulfur-reducing gene cluster in cyanobacterial plasmid pANL confers chromate resistance. World J Microbiol Biotechnol 28: 863–869.
14. He M, Li X, Liu H, Miller SJ, Wang G, et al. (2013) Characterization and genomic analysis of a highly chromate resistant and reducing bacterial strain *Burkholderia phytofirmans* ZC1. J Hazard Mater 193: 682–693.
15. He M, Li X, Guo L, Miller SJ, Renzing C, Wang G (2010) Characterization and genomic analysis of chromate resistant and reducing *Bacillus cereus* strain SJ. BMC Microbiol 10: 221.
16. Henur KL, Nakatsu CH, Thompson DK, Kotopka AE (2009) High-level chromate resistance in *Arthrobacter* sp. strain FB24 requires previously uncharacterized accessory genes. BMC Microbiol 9: 199.
17. Branco R, Chang AP, Johnston T, Gurel V, Morais P, et al. (2008) The chromate-inducible *chrBACF* operon from the transposable element Chr006Gt confers resistance to chromium(VI) and superoxide. J Bacteriol 190: 6996–7005.
18. Ramírez-Díaz MI, Díaz-Pérez C, Vargas E, Riveros-Rosas H, Campos-García J, et al. (2008) Mechanisms of bacterial resistance to chromium compounds. Biometals 21: 321–332.
19. Nies DH, Silver S (1995) Ion efflux systems involved in bacterial metal resistances. J Ind Microbiol 14: 186–199.
20. Juhnke S, Peitzsch N, Hilsenrath N, Große C, Nies DH (2002) New genes involved in chromate resistance in *Ralstonia metallidurans* strain CH34. Arch Microbiol 179: 15–25.
21. Morais PV, Branco R, Francisco R (2011) Chromium resistance strategies and toxicity: what makes *Ochrobactrum tritici* 5bvl1 a strain highly resistant. Biometals 24: 401–410.
22. Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual. 3rd ed. Ed. Cold Spring Harbor New York: Cold Spring Harbor Laboratory Press.
23. Miller WG, Leveau JHJ, Lindow SE (2000) Improved gfp and mcrB broad-host-range promoter-probe. Mol Plant Microbe Interact 13: 1243–1250.
24. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77: 51–59.
25. Yocom S, Veeam J, Park W (2010) Molecular characterization of FinR, a novel redox sensing transcriptional regulator in *Pseudomonas putida* KT2440. Microbiol 156: 1447–1496.
26. Branco R, Cristiante A, Morais PV (2013) Highly sensitive, highly specific whole-cell biosensors for the detection of chromate in environmental samples. PLoS One 8: 1.
27. Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, et al. (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 162: 329–334.
28. Dodd JB, Egan JB (1990) Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. Nucleic Acids Res 18: 5019–5026.
29. Wang L, Brown SJ (2006) BindN: a web-based tool for efficient prediction of DNA and RNA binding sites in amino acid sequences. Nucleic Acids Res 34: W243–W248.
30. Giedroc DP (2001) Metal transporters and metal sensors: metalloregulatory transcriptional regulators for *Staphylococcus aureus*. J Biol Chem 276: 39–42.
31. Guerra AJ, Giedroc DP (2012) The ChrA homologue from a sulfur-reducing gene cluster in cyanobacterial plasmid pANL confers chromate resistance. World J Microbiol Biotechnol 28: 863–869.
32. Ramírez-Díaz MI, Díaz-Pérez C, Vargas E, Riveros-Rosas H, Campos-García J, et al. (2008) Mechanisms of bacterial resistance to chromium compounds. Biometals 21: 321–332.
33. Ma Z, Jacobsen FE, Giedroc DP (2009) Metal transporters and metal sensors: how coordination chemistry controls bacterial metal homeostasis. Chem Rev 109: 4644–4681.
34. Marmo L, Kühlhuth A, Marmo LV (2012) Cysteine oxidases and their role in bacterial extreme environments. IUBMB Life 64: 41–49.
35. Marmo L, Kühlhuth A, Marmo LV (2012) Cysteine oxidases and their role in bacterial extreme environments. IUBMB Life 64: 41–49.
36. Marmo L, Kühlhuth A, Marmo LV (2012) Cysteine oxidases and their role in bacterial extreme environments. IUBMB Life 64: 41–49.
37. Marmo L, Kühlhuth A, Marmo LV (2012) Cysteine oxidases and their role in bacterial extreme environments. IUBMB Life 64: 41–49.