Using recombinant *E. coli* displaying surface heavy metal binding proteins for removal of Pb^{2+} from contaminated water

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ABBREVIATIONS

PbrR	Lead specific operon from *C. metallidurans* CH34
MerR	Group of transcriptional activator
SmtB	Synechoccocus metallothionein B
Ag43	Antigen 43 gene
AT	Ag43 auto transporter (auto translocator)
HME	Heavy metal efflux
ND	Not detected
WHO	World Health Organization
AAS	Atomic absorption spectrophotometer
LPS	Lipopolysaccharide
EP	Extracellular polymer
OmpA	Outer membrane protein A
Lpp	Lipopoly protein
EDTA	Ethylene diamine tetra-acetic acid
MGDA	Methyl glycine di-acetic acid

ABSTRACT

Water pollution remains a serious problem with economic and public health concerns worldwide. Lead (Pb) is one of the dangerous metals related to chronic diseases and is responsible for hundreds of thousands of deaths around the world. Exposure to Pb^{2+} is cumulative over time. High concentrations of Pb^{2+} in the body can cause death or permanent damage to the central nervous system, the brain, and kidney. This damage commonly results in behavior and learning problems (such as hyperactivity), memory and concentration problems, high blood pressure, hearing problems, headaches, retardation of growth, reproductive problems in men and women, digestive problems, muscle and joint pain. Despite the advances in technologies for removal of heavy metals e.g. Pb^{2+} from water, all current techniques have shown some limitations that obstructed their application. Taking advantages of usage of surface-displaying proteins by *E. coli* for improving the removal efficiencies of Pb^{2+}, we engineered recombinant *E. coli* to display metallothionein (SmtB) and lead binding protein (PbrR) onto outer membrane. DNA fragments encoding these proteins were fused to DNA fragment encoding β- domain of antigen 43 (Ag43) for translocation of both heavy metals binding protein

The resultant recombinant *E. coli* exhibited a capability to adsorb Pb^{2+} successfully from water samples containing 100 mg/L of Pb^{2+}, and

concentrations of Pb^{2+} reached to undetectable level after 18 hours. Heat-inactivated *E. coli* displaying PbrR and SmtB on outer membrane showed comparable removal efficiencies to live *E. coli* cells. The present study revealed that *E. coli* cells have the characteristics to remove Pb^{2+} from acidic solution, which is a difficult issue because of the high solubility of Pb^{2+} compounds in acidic condition. Unlike the most common methods for removal of Pb^{2+} from wastewater (chemical precipitation in alkaline solution), this method could be considered for removal of Pb^{2+} at acidic solution. It is believed that removal of heavy metals by bacteria depending on their specific binding activity to metal ions has a major concern toward releasing of recombinant organisms into the environment. However, it is noteworthy to mention that heat-treatment (at 60°C for 60 min) is enough to remove viability of recombinant *E. coli* strains without losing its activity to bind Pb^{2+}, which is an important finding for the possibility of application of this system. These observations suggest that this method can be used as a promising, specific and efficient tool for removal of Pb^{2+} from contaminated water.

I- INTRODUCTION

I-1 Lead pollution

Heavy metals include cadmium, lead, chromium, copper and nickel, which contaminate the soils, ground water, sediments and surface waters are extremely toxic to biological and ecological systems. The heavy metals are released due to the discharge of effluent into the environment by large number of processes such as electroplating, leather tanning, wood preservation, pulp processing, steel manufacturing, etc., and the concentration levels of these heavy metals varies widely in the environment. Heavy metals pose a critical concern to human health and environmental issues due to their high occurrence as a contaminant, low solubility in biota and the classification of several heavy metals (in particular Pb^{2+}) as carcinogenic and mutagenic [1].

Although, existence of some heavy metals like Fe, Mn and Cu is an essential for live organisms in restricted concentrations, however other metals like Pb and Cd is very toxic even in low concentrations. Moreover, the metals cannot be degraded to harmless products and hence persist in the environment indefinitely. Pb^{2+} is an environmental pollutant that exists naturally and contaminates air, food and water. Excessive amounts of Pb^{2+} are released into the environment due to car exhausts, fuel burning and direct release from factories. Pb^{2+} may come into drinking water via corrosion of lead from old pipes or can contaminate water source because of discharging of Pb^{2+} contaminated wastes into rivers or lakes [2-4].

In Egypt, many people are living in areas located close to industrial sites such as petroleum refining, batteries production or chemical industries. Some part of sewage water from these industries had been discarded into Nile River or Ismailia canal without appropriate treatments. In previous studies for the analysis of water quality of Lake El-Manzalah (one of the biggest lakes in Egypt and the main outlet of Ismailia canal), it was recognized that the level of heavy metals especially Pb^{2+} is higher than permissible level (standard level of lead in drinking water is less than 0.05 mg/L). The chemical analysis for heavy metals from Ismailia canal reveals high concentrations of lead, copper, cadmium, chromium, nickel and cobalt (Figure 1) [5, 6]. The uses of wastewater for irrigation and the use of sewage sludge as an organic nutrient contributed to increase the quantity of heavy metals in crop fields.

I-2 Effects of lead pollution on human health

Pb^{2+} is a dangerous element; it is harmful even in small amounts. Pb^{2+} enters the human body in many ways. It can be inhaled in dust from lead paints, or waste gases from leaded gasoline. It is found in trace amounts in various foods, notably fish, which are heavily subject to industrial pollution. Some old homes may have lead water pipes, which can then contaminate drinking water. Most of the Pb^{2+} we take in is removed from our bodies in urine; however, there is still risk of buildup, particularly in children.

Exposure to Pb^{2+} is cumulative over time. High concentrations of Pb^{2+} in the body can cause death or permanent damage to the central nervous system, the brain, and

kidneys [7]. This damage commonly results in behavior and learning problems (such as hyperactivity), memory and concentration problems, high blood pressure, hearing problems, headaches, retardation of growth, reproductive problems in men and women, digestive problems, muscle and joint pain [8, 9].

Humans may be exposed to Pb^{2+} via inhalation of polluted air or ingestion of contaminated food and/or water. World Health Organization (WHO) recognizes Pb^{2+} as one of the top-10 toxic chemicals causing major public health problems around the world [10]. In fact, the Institute for Health Metrics and Evaluation estimated approximately 853,000 deaths annually due to Pb^{2+} poisoning. Children are more susceptible to Pb^{2+} -poisoning because they absorb Pb^{2+} 5-times higher than adults [10]. Pb^{2+} accumulates in the body tissues including brain, liver, kidney, and skeletal system to reach toxic levels associated with organ dysfunction [8, 9]. It was noted that, there is a strong relationship between contaminated drinking water with heavy metals in the contaminated areas and the incidence of chronic diseases such as renal failure, liver cirrhosis, hair loss, and chronic anemia [5, 6].

I-3 Methods of removal of lead from environment

There are variety of methods for the removal of heavy metals from the contaminated water including chemical precipitation, membrane filtration, adsorption, electrolysis and photocatalysis [11]. Selection of the most suitable treatment should be considered according to some basic parameters such as the metal species, initial metal

concentration, pH, environmental impact, as well as the operational costs [12]. Although chemical precipitation of Pb^{2+} is a broadly used method due to its low cost and simplicity, some deficiencies have been reported including sludge generation and poor efficiency with the low concentrations of Pb^{2+} [11, 12]. Bioremediation is the use of live organisms including plants, algae, fungus, and bacteria to degrade environmental contaminants. Bioremediation has been proven to be beneficial for removal of metals from contaminated water through physiological uptake. This relies on accumulating the metal inside the living cells or adsorbing it by cell wall components e.g. extracellular polymers or lipopolysaccharides [13].

I-4 Advantages of using biological methods

However, bio-adsorption processes are more applicable than the bio-accumulative processes in large-scale systems because it doesn't require the addition of nutrients to maintain the microbial activity [14, 15]. Factors including characteristics of the metal ion, biosorbent affinity and selectivity to the metal ions, pH, temperature, ionic strength, contact time and biomass concentration are known to influence the binding ability to heavy metals and efficiency of bio-sorption [2, 16, 17].

Bioremediation has numerous applications, including clean-up of ground water, soils, lagoons, sludges, and process-waste streams. This technology has been used on very large-scale applications, as demonstrated by the shore-line clean-up efforts in Prince William Sound, Alaska, after the Exxon Oil spill. Although the Alaska oil-spill cleanup

represents the most extensive use of bioremediation on any one site, there are many other successful applications on smaller scale [18].

Bioremediation frequently must address multiphasic, heterogeneous environments, such as soils in which the contaminant is present in association with the soil particles, dissolved in soil liquids, and in the soil atmosphere. Because of these complexities, successful bioremediation is dependent on an interdisciplinary approach involving such disciplines as microbiology, engineering, ecology, geology, and chemistry.

Several studies have shown that many organisms, prokaryotes and eukaryotes, have different natural capacities to biosorb toxic heavy metal ions, giving them different degrees of intrinsic resistance, particularly in diluted solutions (between 10 to 20 mg/L) due to their mobility, as well as the solubility and bioavailability capacities of these metal ions [13, 15, 16, 19-21].

Eukaryotes are more sensitive to metal toxicity than bacteria. In the presence of high concentrations of heavy metals, several resistance mechanisms are activated. For example, the production of peptides of the family of metal binding proteins such as metallothioneins (MTs), the regulation of the intracellular concentration of metals with expression of protein transporters of ligand-metal complexes from the cytoplasm to the inside of vacuoles and the efflux of metal ions by ion channels present in the cell wall can be activated [22]. For removal of heavy metals from waste water on an industrial scale, it is important to use low-cost biomaterial to be economically viable [23, 24].

Several studies have been conducted with the purpose of improving the resistance and/or the ability of microorganisms to accumulate heavy metal ions, including a number of studies that follow parameters: pH, temperature; different metal concentrations and biomass, competitiveness of ions of different elements; microorganism-metal contact time, composition of the culture medium; bioaugmentation/biostimulation, resistance to toxicity of heavy metals of Gram positive/Gram-negative bacteria; intracellular/extracellular bioaccumulation; viable /non-viable cells, free /immobilized cells, and biological processes by aerobic/anaerobic microorganisms[13, 15, 25-29].

Numerous studies have documented improving the removal efficiency of Pb^{2+} by bacterial strains using recombinant protein technology. For instances, recombinant *E. coli* cells expressing the β-domain of IgA protease of *Neisseria gonorrhoeae* with metallothionein (MT) from rats [22], mammalian metallothionein with LamB protein [30] and lpp-ompA-various sizes of peptides (EC20) have been used as bio-adsorbents with significant improvement of their efficiencies [19]. Heavy metal efflux (HME) family such as Cus, MerR and heavy metal binding domains like zinc finger and lead binding domains are used for the removal of target metals [16, 17, 31].

Lead specific binding protein (PbrR) from *Cupriavidus metallidurans* CH34 (CmPbrR) is considered as the most specific polypeptide that binds Pb^{2+} (Figure 2), it's a transcription regulator of lead resistance locus *pbr*, that involved in uptake, efflux, and accumulation of Pb^{2+}. The *pbr* lead resistance locus (Figure 3) contains resistance genes;

pbrT (encodes a Pb^{2+} uptake protein), *pbrA* (encodes a P-type Pb^{2+} efflux ATPase), *pbrB* (encodes a predicted integral membrane protein) and *pbrC* (encodes a predicted prolipoprotein signal peptidase), *pbrD* (encoding a Pb^{2+}-binding protein required for Pb^{2+} sequestration) [20, 31-34]. MT (*Synechoccocus* SmtB) is a homodimeric transcriptional repressor belonging to the SmtB/ArsR family, with a winged helix DNA binding protein that represses the expression of SmtA operon in Cyanobacteria and is induced by specifically binding to Zn^{2+} (Figure 4). This protein usually harbors one or both of two structurally distinct metal-binding sites containing three to four conserved metal ligands which can bind to other heavy metals such as Pb^{2+}, Co^{2+} and Cu^{2+} [35, 36].

I-5 Advantages of using auto translocation proteins in heavy metal removal

Surface display of metal binding proteins in genetically modified microbes has proven to be an ideal bioremediation technique for removal of heavy metals from polluted environment. One option for cell surface display in *E. coli* is the type V secretion system, also known as the auto-translocator (AT) system. ATs are multidomain proteins consisting of a signal peptide [30], a passenger domain and a translocation unit (TU). The TU consists of a linker region and a β-barrel domain [37]. The linker region anchors the passenger protein to the cell surface. However, some ATs process the linker region after the passenger protein is translocated across the outer membrane, releasing the passenger protein into the milieu; it can also remain strongly associated with the TU [37,

38]. To display a heterologous protein with the AT system, the DNA sequence of the native passenger protein is replaced by the heterologous sequence [39].

The relative simplicity of the AT system makes it potentially useful for the secretion of saccharolytic enzymes in *E. coli* strains engineered to produce biocommodities [40]. Although the ATs share similar structural characteristics, their capacity for secreting heterologous proteins varies widely depending on the genetic background of the *E. coli* strain that is used [15-18]. Additionally, the performance of the cell surface display can be affected by the composition of the media, the presence of proteases, the temperature and the cultivation technique used, among other factors [15].

Although widespread usage of OmpA protein for surface displaying heterologous proteins outside *E. coli* cell, this system showed some limitation for effective displaying of relative big size proteins. Ag43 (Figure 5) is an autotransporter protein exists in most of *E. coli* strains and has all requirements for membrane translocation and extracellular secretion [40, 41]. It consists of 3 subunits, signal peptide (from 1^{st} aa – 52^{nd} aa, for directing the secretion from cytoplasm to periplasm), α-domain (from 53^{rd} aa to 552^{nd} aa, the secretable or passenger protein, that translocate on the outer-membrane) and β-domain (from 553^{rd} aa to 1038^{th} aa, that form the β-barrel protein in outer-membrane for secreting passenger domain) [26]. Because of relative big size of α-domain of Ag43 (488 a. a.), this system may have the ability for surface displaying relative big size proteins comparing to other surface displaying systems.

I-6 Aims of this work

The present study aims to

1. Testing the effective translocation of metal-binding proteins onto bacterial membrane and its Pb^{2+} removal efficiency.
2. Comparison of the efficiency and selectivity of two metal-binding proteins translocated onto bacterial membrane in their Pb^{2+} removal efficiency.
3. Optimizing the conditions for Pb^{2+} removal using bacterial strain (PbrR-SmtB-AT).
4. Evaluating the practical use of heat inactivated bacterial cells in environmental removal of Pb^{2+}.

II- MATERIALS AND METHODS

II-1 Chemicals

DNA oligomers were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Restriction endonucleases, *Bam*HI (20 U/ul), *Bgl*II (40 U/ul), *Eco*RI (20 U/ul), *Spe*I (10 U/ul), digestion buffers (3.1 buffer and CutSmart) and Antarctic Phosphatase (for dephosphorylation of 5' ends of DNA) were bought from New England Biolabs Japan Inc (Tokyo, Japan), DNA polymerase KOD plus Neo, KOD FX-Neo, and the solution for PCR were provided by TOYOBO Co Ltd (Osaka, Japan). Ligation kit, Mighty Mix, was obtained from TaKaRa BIO INC (Shiga, Japan). Kit for purification of DNA fragments, Wizard Sv Gel and PCR Clean-Up System, were supplied by Promega KK (Tokyo, Japan). BigDye Terminator v3.1 Cycle Sequencing Kit was purchased from Applied Biosystems^{TM}, USA. Luria-Bertani broth was purchased from Difco, MD (USA). KAPA Taq PCR Kit was obtained from KAPA biosystems (Massachusetts, USA). The Kit for preparation of plasmid DNA was bought from Nippon Genetics Co., Ltd. (Tokyo, Japan). Arabinose was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). CBB staining, $Pb(NO_3)_2$, $CdCl_2$, $CuSO_4$ and $PbCl_2$ were purchased from Wako Pure Chemical industries (Osaka, Japan).

II-2 *E. coli* and plasmid DNA

E. coli strain DH5α was used for all experiments in this study. Plasmid DNAs used as a templet in this study were derived from BioBrick registry

(http://partsregistry.org/). BioBrick number of the plasmid DNA encoding wild type Ag43 gene (P_{BAD}-RBS-Ag43-T/pSB1C3) is BBa_K759001, BioBrick number of the plasmid DNA encoding PbrR is BBa_K346004. Amino acid sequence of Cyanobacterial Metallothionein Repressor (SmtB) MMDB ID:27695[36] has been obtained from Gene bank of NCBI website (https://www.ncbi.nlm.nih.gov/Structure/pdb/1R22) and synthesized by Integrated DNA Technologies (IDT, USA). All DNA oligomers used in this study (listed in Table 1) were purchased from Sigma-Aldrich, Japan.

II-3 Preparation of PbrR bio-device

DNA encoding P_{BAD} Promoter was amplified by PCR using primer sets (100 up-F and *Spe*I-PBAD-R) and BioBrick (BBa_K759001) as a DNA templet. The resultant DNA fragment (1,319 bp) digested by *Eco*RI and *Spe*I enzymes was ligated with the *Eco*RI/*Xba*I PbrR/pSB1C3 digested vector to make plasmid expressing PbrR under control of P_{BAD} promoter.

II-4 Construction of translocator targeting outer membrane (AT)

For construction of translocator of fused polypeptide targeting outside of outer-membrane, α-domain (amino acids position from 53 a.a. to 552 a.a.) of wild type Ag43 gene (BioBrick number BBa_K759001) was replaced by *Bgl*II restriction site using PCR with Ag43 β-domain-F primer and Ag43-SP-R primer. The PCR product (5,054 pb) digested with *Bgl*II restriction enzyme, ligated and transformed into 20 µl of *E. coli* DH5α, then the sequence was confirmed.

II-5 Construction of SmtB-AT plasmid DNA

DNA fragment encoding SmtB (124 amino acid) truncated by *Bgl*II restriction sites was synthesized by IDT (USA), after optimizing the codon of DNA sequence to be expressed in *E. coli* cells. DNA fragment (372 bp) digested by *Bgl*II was inserted into *Bgl*II site of auto translocator to make plasmid expressing fusion protein of SmtB with β-domain of Ag43 in its C-terminal (pSB1C3-SmtB-AT (Figure 6)).

II-6 Construction of PbrR-AT plasmid DNA

DNA fragment encoding PbrR (109 amino acid) truncated by *Bam*HI and *Bgl*II restriction sites was amplified by PCR using primer set (*Bam*HI-PbrR-F and *Bgl*II-PbrR-R) and BioBrick (BBa_K346004) as DNA templet. The 340 bp amplified DNA fragment, containing the full-length ORF of PbrR gene flanked by *Bam*HI and *Bgl*II respectively, was digested and inserted into *Bgl*II site of auto-translocator to make plasmid expressing PbrR fused to β-domain of Ag43 in its C-terminal (pSB1C3-PbrR-AT (Figure7)).

II-7 Construction of PbrR-SmtB-AT plasmid DNA

DNA fragment encoding MT (372 bp) digested by *Bgl*II was inserted into *Bgl*II site of PbrR-AT plasmid DNA to make plasmid expressing fusion proteins of PbrR and SmtB with β domain of Ag43 in its C-terminal (pSB1C3-PbrR-SmtB-AT (Figure 8)).

II-8 Size analysis of recombinant proteins by SDS-PAGE

Recombinant *E. coli* cells were cultured in LB containing 34 mg/L Chloramphenicol at 37ºC at 180 rpm agitation, final optical density of the cells at 600 nm

was 0.5 ~ 0.6, L-arabinose was added (final concentration 1.3 mM) to the culture for induction of recombinant proteins, and cells were collected 4 hours after incubation at 30ºC at 180 rpm.

Each culture (200 µl) was centrifuged at 13,500 xg for 2 min and the cell pellets were treated in SDS sample buffer (80 µl) at 98°C for 5 min. After centrifugation (13,500 xg, 1 min) of the samples, 8 µl of each sample was loaded onto SDS-PAGE (12% acrylamide) and electrophoresed at 100 V for 2 hours. Total proteins were visualized by staining the gel in Coomassie Brilliant Blue R-250. Images of proteins were recorded using Canon D550 DSLR camera.

II-9 Analysis of localization of outer membrane translocator

Condition of cell culture for analyses of localization of recombinant protein is as described before. Harvested cells were separated into two fractions (cytosolic and membrane fractions) as described below. *E. coli* cells were collected by centrifugation of culture medium (25 ml) at 4,700xg for 15 min at 4°C and rinsed with 10 mM Tris-HCl (pH 7.5) two times. After adding 0.5 ml of 10 mM Tris-HCl to the precipitate, all suspension was stored at -80°C for 2 hours (or overnight) for weakness of cell wall protein.

Cells in the suspension were lysed in 5 intervals of 15 sec sonication at output level 7. The lysate was centrifuged at 6,700xg for 10 min at 4°C to remove cell debris. The supernatant (500 µl) was centrifuged at 108,000xg for 10 min at 4°C to separate

cytosolic fraction (supernatant) from membrane fraction (precipitate). After rinsing the precipitate with 500 µl of 10 mM Tris-HCl (pH 7.5) two times, the precipitate was suspended by 50 µl of 10 mM Tris-HCl and mixed with 50 µl of 2X SDS sample buffer for solubilization of the protein in membrane fraction.

II-10 Preparation of *E. coli* cells for bio-adsorption

Recombinant *E. coli* cells were cultured in LB medium (60 ml) containing 34 µg/ml chloramphenicol at 37ºC with shaking at 180 rpm. L-Arabinose was added to the culture medium (final concentration 1.3 mM) for induction of recombinant proteins when optical density of the cells at 600 nm reached to 0.5. *E. coli* culture was shaken for 4 hours at 30ºC in 250 ml flask, then transferred into 50 ml centrifugation tube, and centrifuged at 4,700 xg for 15 min at 4ºC. Cells were rinsed with 10 ml of 0.5 mM Tris-HCl (pH 7.0), followed by rinsing with 10 ml of 0.9% NaCl two times. Cells were then resuspended in 0.9% NaCl (final concentration 10 g (wet weight)/L) and stored at 4ºC. Appropriate amount of Pb^{2+} (50, 100 or 200 mg/L) has been incubated with *E. coli* for measuring Pb^{2+} removal efficiency and cells adsorption capacity.

For inactivation of *E. coli* cells, the cells dissolved in 0.9% NaCl solution were heated at 60ºC for 1 hour. Viability of inactivated *E. coli* was confirmed by culturing cells on LB agar plate containing 34 µg/ml chloramphenicol at 37 ºC for 24 hours.

II-11 Measurement of heavy metal concentrations using atomic absorption spectrophotometer (AAS)

E. coli cells were centrifuged at 6700xg for 5 min at 4ºC, then 2 ml from the supernatant was transferred into a plastic tube containing 7.9 ml of DW and 0.1 ml of 10 mM HNO_3 (final concentration of acid was 0.1 M), then the samples were kept at 4ºC till measurement of heavy metal content (copper, cadmium and lead) using atomic absorption spectrophotometer (Hitachi A-2000, Hitachi instruments Co, Tokyo, Japan). Calibrations were performed using standards within a linear calibration range of 0 - 20 ppm and the correlation coefficients for the calibration curves were 0.98 or higher. In order to avoid the environmental contamination by Pb^{2+}, all of bacterial samples and Pb^{2+} samples have been treated in a prober way before discarding into environment.

II-12 Statistical analysis

Statistical significance was evaluated using Tukey–Kramer honestly significant difference tests, with $p < 0.05$ considered as significant (JMP program, SAS Institute, Cary, NC, USA).

III- RESULTS

III-1 Successful expression of proteins, PbrR and PbrR-AT in *E. coli*

*Cm*PbrR from *C. metallidurans* CH34, one of MerR-like proteins, that regulates transcription of Pb^{2+}-resistance genes through its binding to Pb^{2+} on operator in the promoter[20, 33]. Since capability of Pb^{2+}-binding of PbrR in the absence of DNA has been described previously[31], the use of the protein as a tool for capturing Pb^{2+} using *E. coli* cells was tested. Furthermore, another construct of gene encoding PbrR fused to domains from Ag43 was made. Ag43 is a self-recognizing surface adhesion protein existing in most strains of *E. coli*. Expression of Ag43 confers aggregation and fluffing of cells, promotes biofilm formation and is often associated with enhanced resistance to antimicrobial agents [42, 43]. Muñoz *et., al*. revealed that presence of both N-terminal signal peptide and C-terminal ß-domain is sufficient for translocating intervening polypeptide to outer membrane [44].

Taking advantage of the characteristics of the translocator described above, domains required for the translocation were fused to both sides of PbrR as shown in figure 9. Both DNA fragments encoding PbrR and PbrR-AT, fusion protein containing signal polypeptide of Ag43 followed by PbrR and ß-domain of Ag43, were inserted downstream of P_{BAD} promoter, ligated with plasmid vector pSB1C3 (http://parts.igem.org/wiki/index.php/Part:pSB1C3), and introduced into *E. coli* DH5α. Both recombinant *E. coli* strains containing plasmid DNA, pPbrR or pPbrR-AT

expressed PbrR or PbrR-AT in the presence of 1.3 mM arabinose at 30°C. Total proteins from equal amounts of bacterial cells were extracted 4 h post induction and separated by electrophoresis on SDS-PAGE (16%). Induction of PbrR-AT (69.25 kDa) and PbrR (11.71 kDa) by arabinose was observed as shown in figure 10.

III-2 Comparison of the efficiency of the removal of Pb^{2+}

Recombinant *E. coli* cells containing PbrR and PbrR-AT could remove 60% and 69% of Pb^{2+}, respectively, at 4 h after initiation of incubation (Figure 11). Efficiencies of Pb^{2+} removal after 24 hours were 99% and 93% by PbrR-AT and PbrR strains, respectively (76% by strain containing empty vector used as negative control). These observations suggest that fusion of PbrR to the translocator had a slight effect in the elevation of removal efficiency of Pb^{2+} from supernatant.

III-3 Differential localization of PbrR and PbrR-AT in *E. coli* cells

The total protein from *E. coli* cells containing PbrR or PbrR-AT were separated into cytoplasmic protein and membrane protein fractions to analyze differential localization of these two different types of recombinant lead binding proteins. As shown in figures 12, PbrR-AT (69.25 kDa) was successfully induced by arabinose (lane 2) and revealed to localize predominantly in membrane fraction (lane 4), while figure 13 showed induced PbrR (11.71 kDa) was successfully induced (lane 2) and localizes in cytoplasmic fraction (lane 3). Theoretically, the signal polypeptide of Ag43 is known to play a role for secretion of fused polypeptide from cytoplasm to periplasm through the

"sac complex" in inner membrane. After secretion of the whole protein to periplasmic space, the passenger domain is expected to localize outside of outer-membrane by passing through β-barrel structure of β-domain.

III-4 Comparison of the efficiency of removal of Pb^{2+} among *E. coli* strains expressing five different types of recombinant proteins

For constructing two more genes encoding fusion proteins, DNA fragment encoding α-domain of Ag43 was replaced by DNA fragment encoding SmtB from Cyanobacteria [36] or by DNA fragment encoding PbrR and SmtB. Expression and induction of all five recombinant genes (drawn in figure 14) were confirmed by SDS gel electrophoresis. Each induced polypeptide corresponding to wild-type Ag43 (106.87 kDa), auto translocator (AT; 57.31 kDa), SmtB fused to translocator (SmtB-AT; 71.04 kDa), PbrR fused to translocator (PbrR-AT; 69.25 kDa) or PbrR-SmtB fused to translocator (PbrR-SmtB-AT; 82.95 kDa) was indicated by an arrow head in figure 15. Optimum expression of all five recombinant proteins was observed at 4 h after arabinose induction (1.3 mM) at 32°C. The results recorded in figure 16 shows time course of the reductions of Pb^{2+} in supernatant at 6, 12, 18 and 24 h after initiation of incubation. *E. coli* strain PbrR-SmtB-AT was shown to be the most efficient bio-adsorbent of Pb^{2+} among all strains, followed by PbrR-AT and SmtB-AT strains. In particular, Pb^{2+} concentration in supernatant was not detectable at 18 h after initiation of the incubation with PbrR-SmtB-AT strain. We also compared the efficiency in the presence of higher

concentration of Pb^{2+} (200 mg/l), and found that capability of each strain for removal of Pb^{2+} has been kept even in the presence of higher concentration of Pb^{2+} (Figure 17).

III-5 Selectivity of *E. coli* strains for adsorption of heavy metal ion species

To test selectivity of *E. coli* strains for adsorption of heavy metal ion species, mixture of heavy metal ions (Pb^{2+}, Cd^{2+} and Cu^{2+}) with equimolar concentration (480 μM) were mixed with each *E. coli* strain at 37°C for 24 h. Concentrations of each heavy metal ion species were monitored every 3 h after initiation of the incubation as shown in figures 18 ~20. Although nonspecific adsorptions of Pb^{2+} or Cu^{2+} were observed when these heavy metals were incubated with *E. coli* strain AT as negative control, three *E. coli* strains showed efficient adsorption of Pb^{2+} (Figure 18), while they showed inefficient adsorption of Cu^{2+} (Figure 20). The most efficient adsorption of Pb^{2+} was observed when PbrR-SmtB-AT strain was mixed with the heavy metal mixture and followed by PbrR-AT and SmtB-AT (Figure 18). On the other hand, efficient adsorption of Cd^{2+} by all of these *E. coli* strains has not been observed (Figure 19). Relative efficiencies of removal of each heavy metal ion by different recombinant strains (SmtB-AT, PbrR-AT and PbrR-SmtB-AT) were summarized in figure 21 after standardization with AT strain. These observations suggest that adsorption of Pb^{2+} by *E. coli* strains PbrR-AT or PbrR-SmtB-AT can be used as bacterial adsorbent for selective removal of Pb^{2+}.

III-6 Optimal condition of removal of Pb^{2+} by bacterial adsorbent, PbrR-SmtB-AT

To optimize conditions for removal of Pb^{2+} by *E. coli* strains PbrR-SmtB-AT, the bacterial cells were incubated with 100 mg/L of Pb^{2+} in different pH or different temperatures. The highest efficiency of the adsorption was observed at pH 6.0 at 37°C as shown in figures 22 and 23. pH higher than 6.0 was not tested for the evaluation, because Pb^{2+} tends to be precipitated chemically in the higher pH. We also tested effects of increase or decrease of wet-weight of PbrR-SmtB-AT (2.5 g/L, 3.75 g/L or 5.0 g/L) added to the mixture containing 100 mg/L of Pb^{2+} at 37°C as shown in figure 24, or the effect of changing initial metal concentrations (50, 100 or 200 mg/L) on the removal efficiency as shown in figure 25. Efficiency of the removal of Pb^{2+} increased depending on the increase of the bacterial adsorbent added to the mixture. The 2.5 g/L of PbrR-SmtB-AT has the capability of removal of 77 mg/L of Pb^{2+} from 100 mg/L of Pb^{2+} in 12 hours.

III-7 Retaining of the activity as a bacterial adsorbent after heat treatment of PbrR-SmtB-AT

The efficiencies of the removal of Pb^{2+} between PbrR-SmtB-AT and heat-treated PbrR-SmtB-AT were tested and it was found that there is no much difference in capability to adsorb Pb^{2+} between live and dead bacterial cells as shown in figure 26. *E.coli* strain PbrR-SmtB-AT was incubated at 60°C for 1 h with shaking, then the cells

were cooled down to 37°C before adding Pb^{2+}. The removal of live cells was confirmed by measuring number of colonies on LB agar plates containing 34 µg/ml of chloramphenicol. Interestingly, intact and heat-treated bacterial adsorbents did not show any significant differences in their removal efficiency even in solution containing higher concentration of Pb^{2+} (200 mg/L) as shown in figure 27. The current observations suggest that the bacterial adsorbent which was generated can play a significant role for the removal of Pb^{2+} even after heat treatment.

IV- DISCUSSION

Removal of heavy metals by bacteria depending on their biosorption activity and ability of binding metal ions is an environmentally friendly method [15]. Accumulating evidences showed the advantage of metal binding proteins in the removal of heavy metals such as Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+} and Zn^{2+}. Studying of the removal of Pb^{2+} by bacteria is controversy and that might be due to the nature of Pb^{2+} and its tendency to precipitate in most of bacterial cultures, buffer solutions, neutral and alkaline medium [16, 21, 26, 29, 45, 46]. On the other hand using plants as a hyper accumulator of phytoremediation of Pb^{2+} showed un successful results because of the low efficiency and requirements of chelators and acids for solubilizing of Pb^{2+} in addition to recontamination of soil by plant foliage after accumulation [47].

Bearing in mind these facts, we constructed recombinant *E. coli* cells expressing lead-binding proteins (PbrR) from *C. metallidurans* CH34, and metallothionein (SmtB) from *Synechoccocus* as membrane-associated proteins. The resultant recombinant *E. coli* expressing PbrR-AT as membrane-associated exhibited higher efficiency in removal of Pb^{2+} (99%) than cells expressing same protein in the cytoplasm (93%). In a related study, displaying of PbrR in *E. coli* cell surface constructed with two different translocators showed that the adsorption efficiency depends on the type of translocator and the initial metal concentration [33]. On the other hand, localization of metal binding protein influences the removal efficiencies of bacteria as noted that removal efficiency of cells

displaying different kinds of MT is slightly higher (1.6 – 3.5%) than cells expressing MT in the cytoplasm [48].

Therefore, we constructed different recombinant *E. coli* encoding AT, SmtB-AT, and PbrR-AT and PbrR/SmtB-AT for expressing PbrR and SmtB on their surface membranes, and to examine their removal efficiency. The adsorbate capacity of *E. coli* to 40 mg Pb^{2+}/g cells (21.30, 25.29, and 31.78 mg Pb^{2+}/g cells, respectively) seemed to be comparable to the earlier studies used surface displayed recombinant *E. coli*.[26, 33, 47]. These results revealed the successful expression of functional SmtB-AT and PbrR-AT recombinant proteins in *E. coli*. Furthermore, we speculated that improvement of the removal efficiency of *E. coli* might be achieved by fusing PbrR with SmtB-AT. The constructed *E. coli* expressing PbrR/SmtB-AT exhibited remarkable increases in the adsorbate capacities of *E. coli* as compared to other recombinant *E. coli*. Lipopolysaccharide (LPS) and extracellular polymers (EPs), which are the major components of the outer membrane are responsible for binding cations of toxic metals and play a role in Pb^{2+} adsorption. Generally, EP is known to have high content of uronic acids (28.29%), which play an important role in specific binding to Pb^{2+} [20]. However, the capacity of Pb^{2+} adsorption can be increased by expressing the recombinant heavy metal binding proteins on the cell wall [20, 26, 33].

Although using of autotransporter for surface displaying of metallothionein or lead binding protein has been previously studied, it was noticed that surface displaying of

heavy metal binding proteins is greatly affected by the type of surface displayed system. Some studies tried secretion of Mt in the periplasmic space to avoid surface displaying difficulties [26]. Another study compared between usage of OmpA protein and Lpp-OmpA protein and found that Lpp-OmpA protein has better secretion of PbrR protein [33]. In a recent study it was found that decreasing size of PbrR protein may elevate the successful surface displaying of PbrR protein, which indicate that Lpp-OmpA have some limitation for surface displaying of such size of protein (109 a. a.) [49]. Since Ag43 surface displaying system is naturally exist in *E. coli* and has the ability to secret its α-domain (499 a. a.), we expected that this system may secrete whole PbrR without any problem which resulted in 2 times better efficiency comparing to same protein with Lpp-OmpA displaying system [49]. On the other hand the ability of Ag43 for surface-displaying this long size passenger domain allows us to fuse SmtB with PbrR to elevate Pb^{2+} removal efficiency in our recombinant cells.

In our study, recombinant *E. coli* demonstrated highly specific affinity to Pb^{2+} and relatively lowers affinity to other metals such as Cd^{2+}, and Cu^{2+}. The maximum adsorption of Pb^{2+} was observed with PbrR-SmtB-AT *E. coli* cells, which has PbrR that shows specific response to Pb^{2+} 1000-fold over other metals such as Hg^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Cr^{3+} and Ag^{2+} [33, 50]. On the other hand, expressing SmtB in recombinant *E. coli* exhibited higher adsorption capacity than wild type due to the nature of MT containing three to four conserved metal ligands, which are capable of binding heavy

metals including Zn^{2+}, Pb^{2+} and Cu^{2+} [51-53]. Another important finding in our study is that incubation of the recombinant *E. coli* at low temperature resulted in a slight reduction in adsorption capacity. These results suggest the efficiencies of our *E. coli* (PbrR/SmtB-AT) in removal Pb^{2+} at broad range of temperatures.

Giving the importance of pH for the capability of recombinant *E. coli* in removal of Pb^{2+}, different conditions of pH were studied. Our data showed that the removal efficiency of recombinant *E. coli* was pH-dependent and noted to be highest at pH 6. These seem to be in agreement with earlier studies reporting the dependency of Pb^{2+} uptake adsorption on pH conditions. The mechanism of uptake of Pb^{2+} at different pH conditions can be explained by the presence of functional groups of several macromolecules that involves in binding to Pb^{2+}. However, in low pH the H^{+} ions compete with Pb^{2+} for the adsorption sites of negative groups like OH^{-}, S^{2-} and PO_4^{3-} [20, 54-57].

The present study revealed that *E. coli* cells have the characteristics to remove Pb^{2+} from acidic solution, which is a difficult issue because of the high solubility of Pb^{2+} compounds in acidic condition. Unlike the most common methods for removal of Pb^{2+} from wastewater (chemical precipitation in alkaline solution), this method could be considered for removal of Pb^{2+} at acidic solution. It is believed that removal of heavy metals by bacteria depending on their specific binding activity to metal ions has a major concern toward releasing of recombinant organisms into the environment [15]. However,

it is noteworthy to mention that heat-treatment (at 60°C for 60 min) is enough to remove viability of recombinant *E. coli* strains without losing its activity to bind Pb^{2+}, which is an important finding for the possibility of application of this system.

Another interesting point of our recombinant is its capability of removal of Pb^{2+} from contaminated soil better than the other existence methods. Generally hyper accumulator plants are using as a phyto-remediation candidate to remove heavy metals from contaminated water. However all articles showed that phytoremediation of Pb^{2+} is not applicable because of its low absorption rate (1-43 µg/g in plant shoot or 11-41 µg/g in plant root) [58], 30.3 µg/g shoot and 43 µg/g root, these amounts had been increased after adding EDTA (5mmol /kg soil) to be 164 µg/g and 126 µg/g respectively [59]. The highest successful example of phytoremediation of Pb^{2+} were recorded using buckwheat that could adsorb 3.3 mg/g dry root and 4.2 mg/g of dry shoot, these amounts have been increased about 5 times by adding 20 mmol methylglycinediacetic acid trisodium salt (MGDA)/kg soil as a chelator to reach to 18 mg/g dry shoot [60], and *Sesbania drummondii* which could accumulate 7% of 1000 mg/L Pb in soil during 5 days (72 mg/g dry rot) after adding 100 mmol EDTA/kg soil and decreasing the soil pH to 5.8 which affected the growth rate of plants [61]. On contrast, our recombinant cells can be distributed into Pb^{2+} contaminated soil to decrease Pb^{2+} bioavailability, and allow plants, crops to grow up.

V- CONCLUSION

One of dangerous contaminant in water is heavy metals that generated by industrial, agricultural and other human activities and released into rivers, lakes, and underground water. Developing a novel technique for removal of heavy metals from water is an important issue seeking more attention and improvements. Herein, we used surface displaying characteristics of Ag43 gene product to develop a quick, specific and efficient bacterial bio-device capable of removal of Pb^{2+} from water. Recombinant *E. coli* were engineered that can display metallothionein (SmtB) and lead binding protein (PbrR) fused to β-domain of Ag43 onto outer-membrane. The resultant *E. coli* exhibited a capability to remove Pb^{2+} successfully from water samples containing 50, 100 and 200 mg/L of Pb^{2+} in a few hours. Selective adsorption of Pb^{2+} has been observed in the presence of mixture of heavy metals (Pb^{2+}, Cd^{2+} and Cu^{2+}). Heat-inactivated *E. coli* cells showed same efficiencies of removal of Pb^{2+} as live cells. These findings revealed advantage of our method for selective, efficient and safe method for removal of Pb^{2+} from contaminated water.

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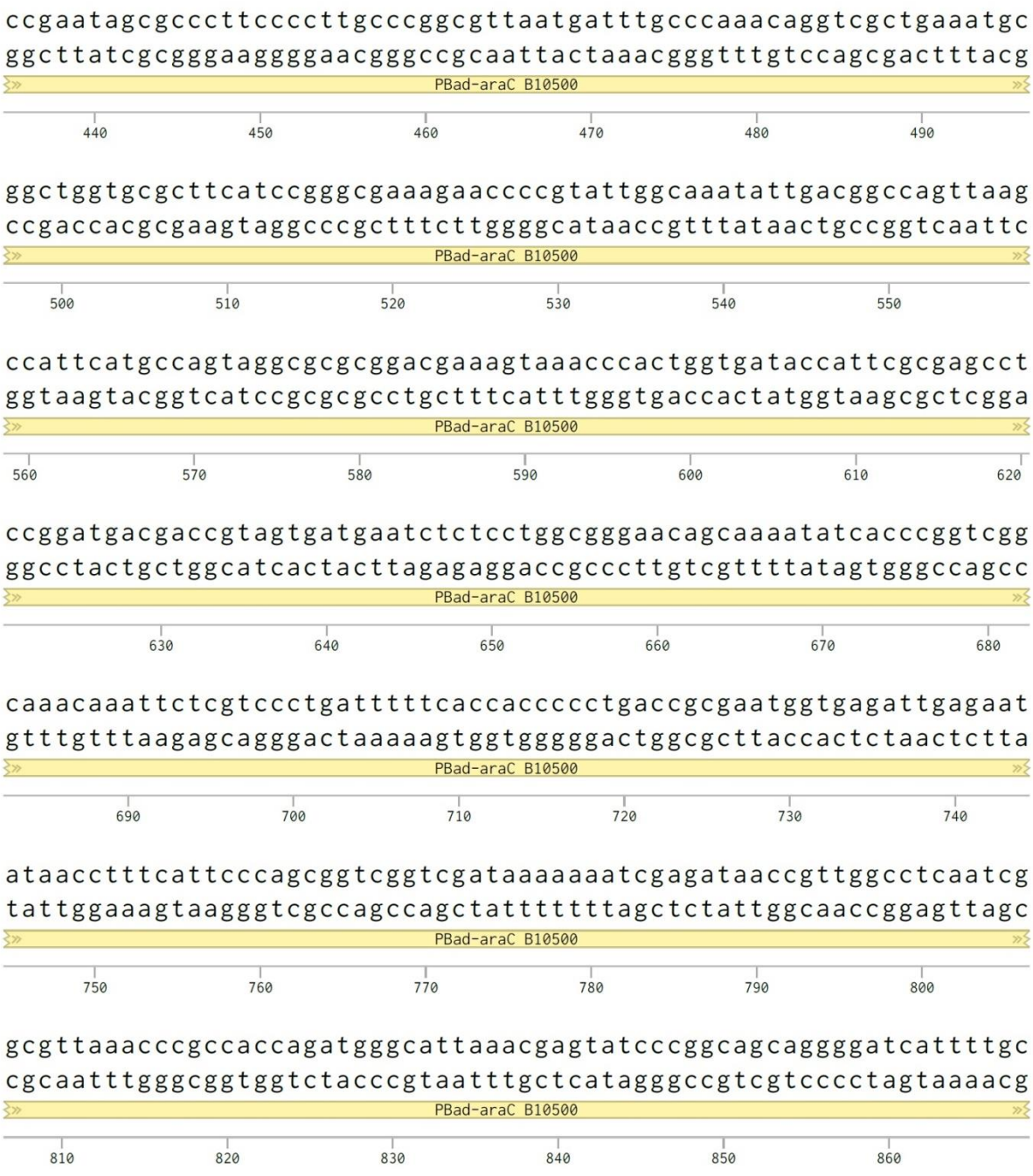
Table 1: Nucleotide sequences of oligo DNAs used in this study.

Primer name	Primer sequence
Ag43-β-domain-F	5’-GGGAGATCTAGGAATGTCACTCTCGCCTC-3’
Ag43-SP-R	5’-CCCAGATCTAGCAGCCAGCACCGGGAG -3’
*Bgl*II-SmtB-F	5’-GAGATCTATGACCAAACCAGTATTGCAGGATGG-3’
*Bgl*II-SmtB-R	5’-GAGATCTGCGAGATTCCTGTAAATGGTCAAGTGC-3’
*Bam*HI-PbrR-F	5’-CCCGGATCCATGCAGGATTGCGGTGAAGTC-3’
*Bgl*II -PbrR-R 2	5’-GGAGATCTCCCGCACGATTGGGCGGGCCTG-3’
*Spe*I-P_{BAD}-R	5’- GGACTAGTGCTAGCCCAAAAAAACGGGTATGGAGA-3’
100 up-F	5’-AACCTATAAAAATAGGCGTATCAC-3’
Pbad-1	5’-ACGAAAGTAAACCCACTGGTG-3’
Pbad-2	5’-CGCAACTCTCTACTGTTTCTC-3’
Ag43-1	5’-ACCATCAATAAAAACGG-3’
Ag43-2	5’-GAATAACGGCGCCATAC-3’
Ag43-3	5’-GCCTTTAACTACTCCCTC-3’
Ag43-4	5’-GGCAGTGCACAACATG-3’
Ag43 200-β-domain-R	5’-CGGGCGTACAGGCAGGCTGATGGTGC-3’
200 dn-R	5’-TCCCCTGATTCTGTGGATAACCGT-3’

(from 1-434 bp)

pPbrR-SmtB-AT (3702 bp)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PBad-araC B10500
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450
460
470
480
490
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510
520
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PBad-araC B10500
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580
590
600
610
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PBad-araC B10500
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660
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PBad-araC B10500
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710
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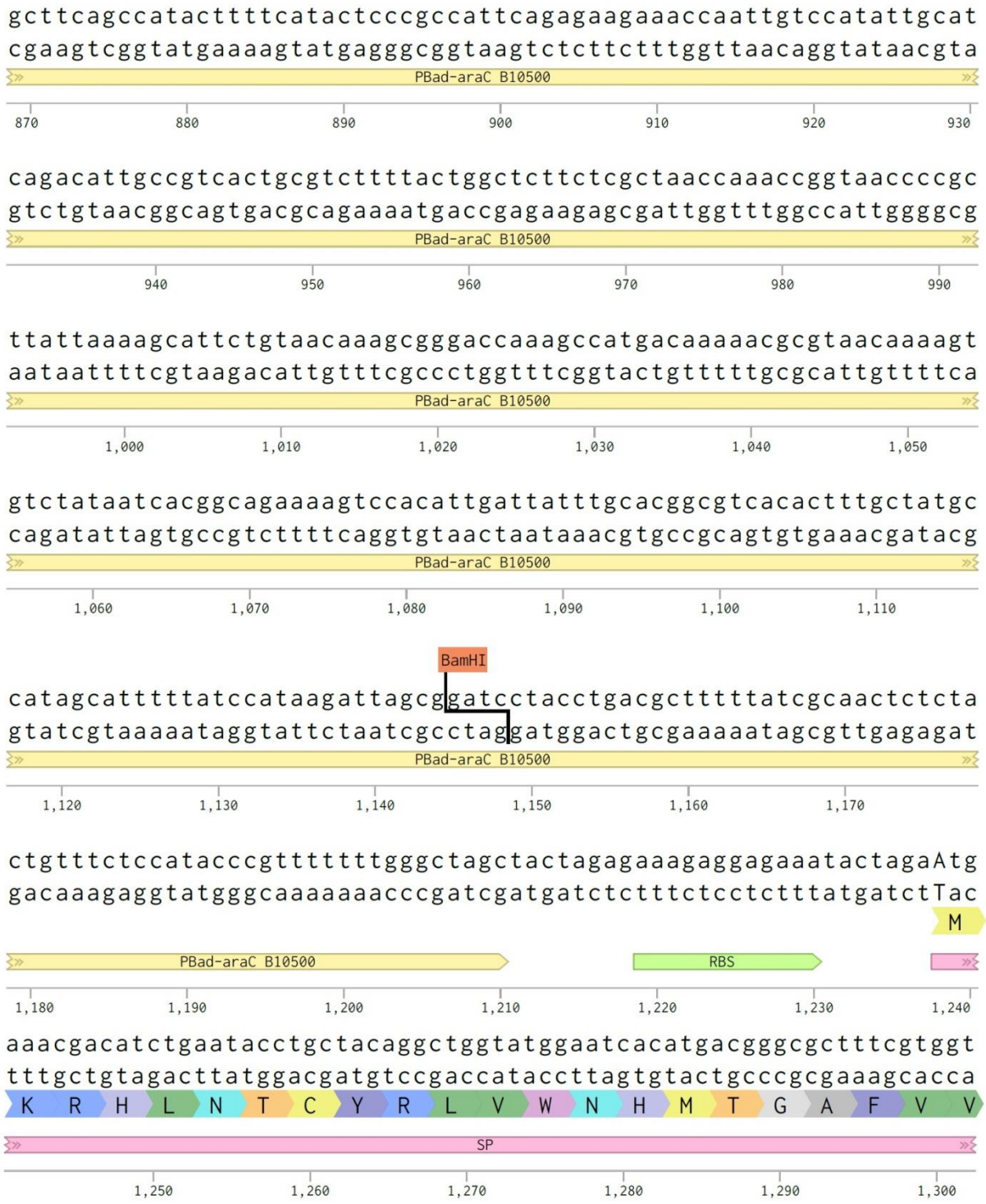
850
860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PBad-araC B10500
870
880
890
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920
930
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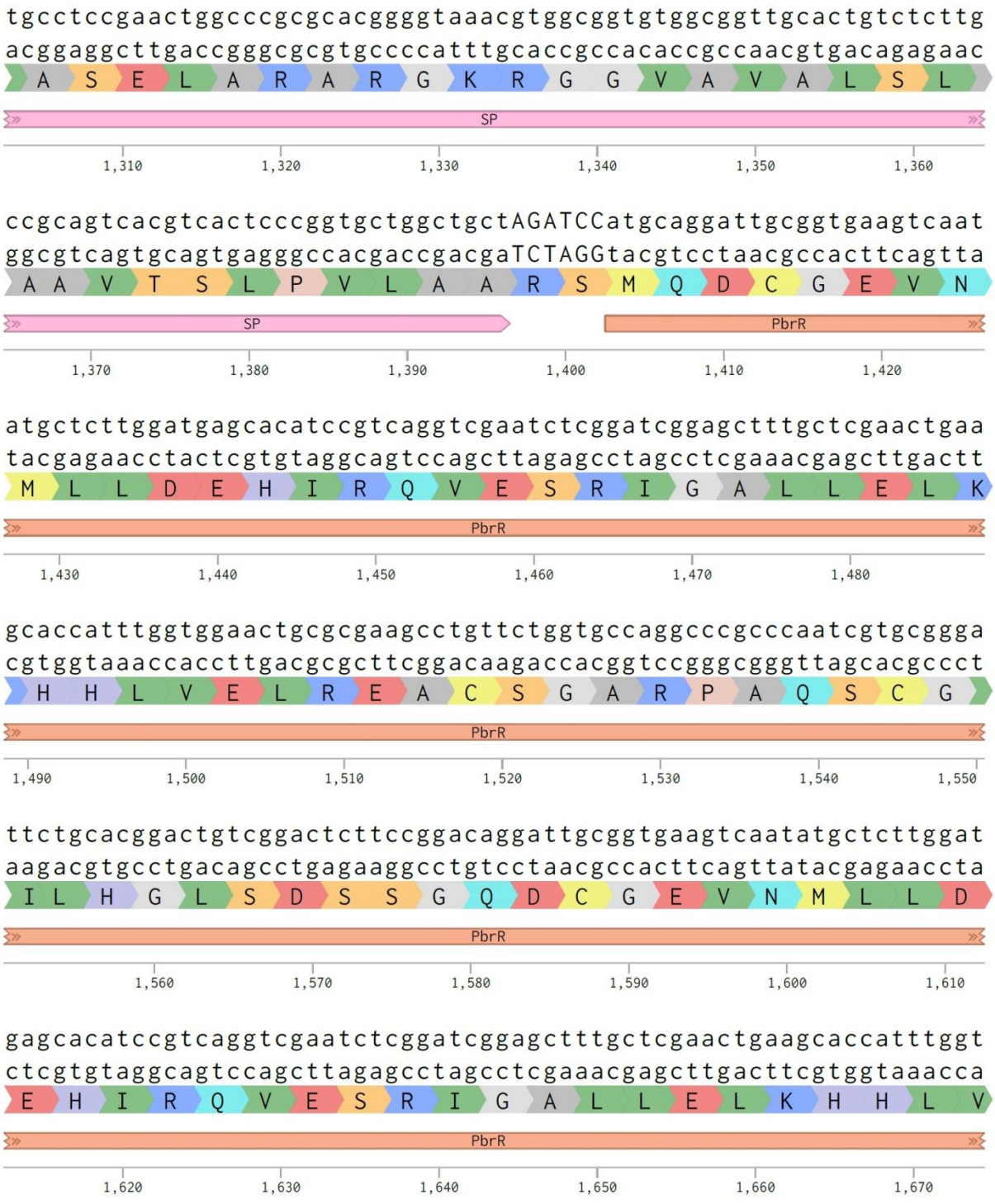
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PbrR
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PbrR
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PbrR
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PbrR
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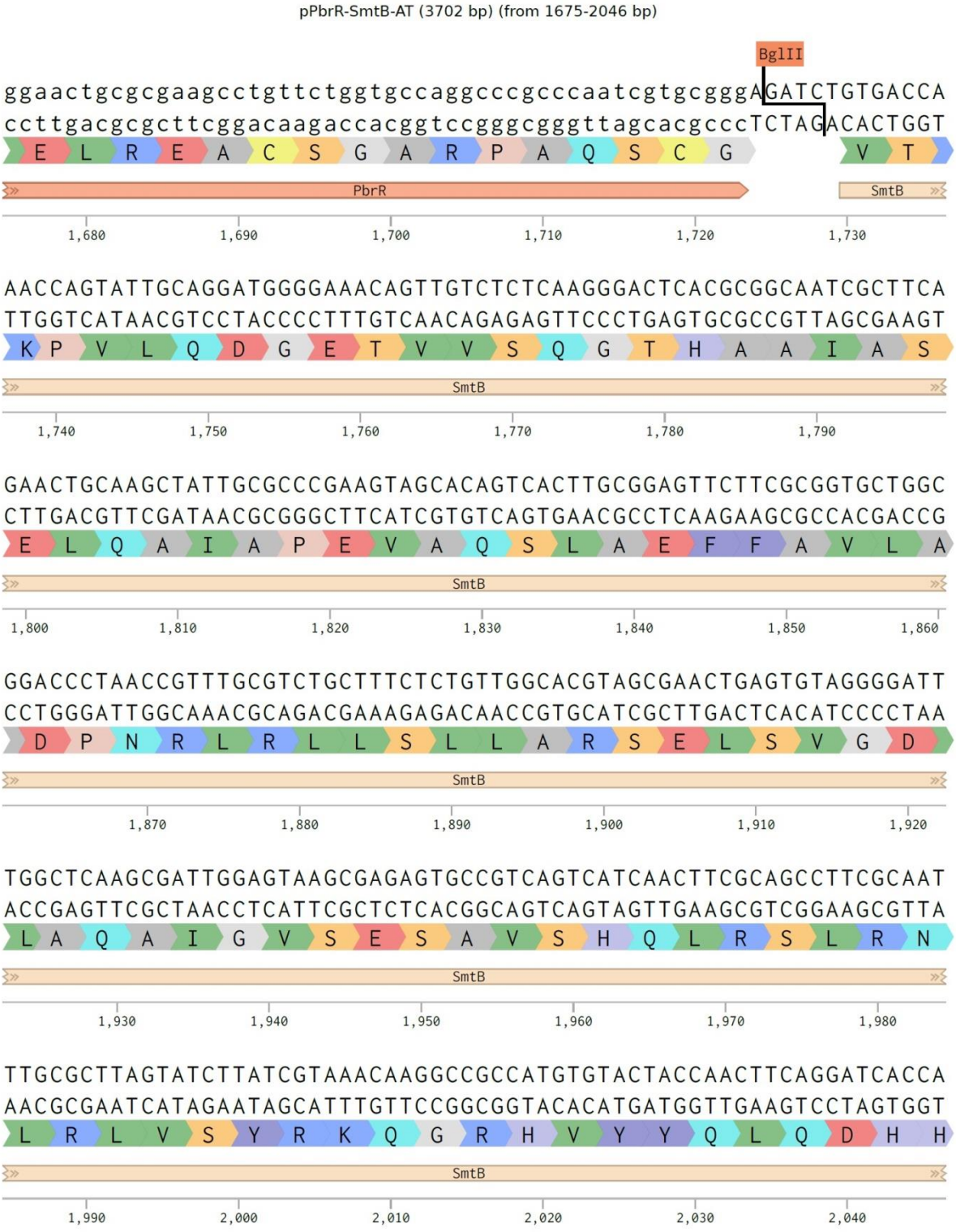
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E L R E A C S G A R P A Q S C G
V T
PbrR
SmtB
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SmtB
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E L Q A I A P E V A Q S L A E F F A V L A
SmtB
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D P N R L R L L S L L A R S E L S V G D
SmtB
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SmtB
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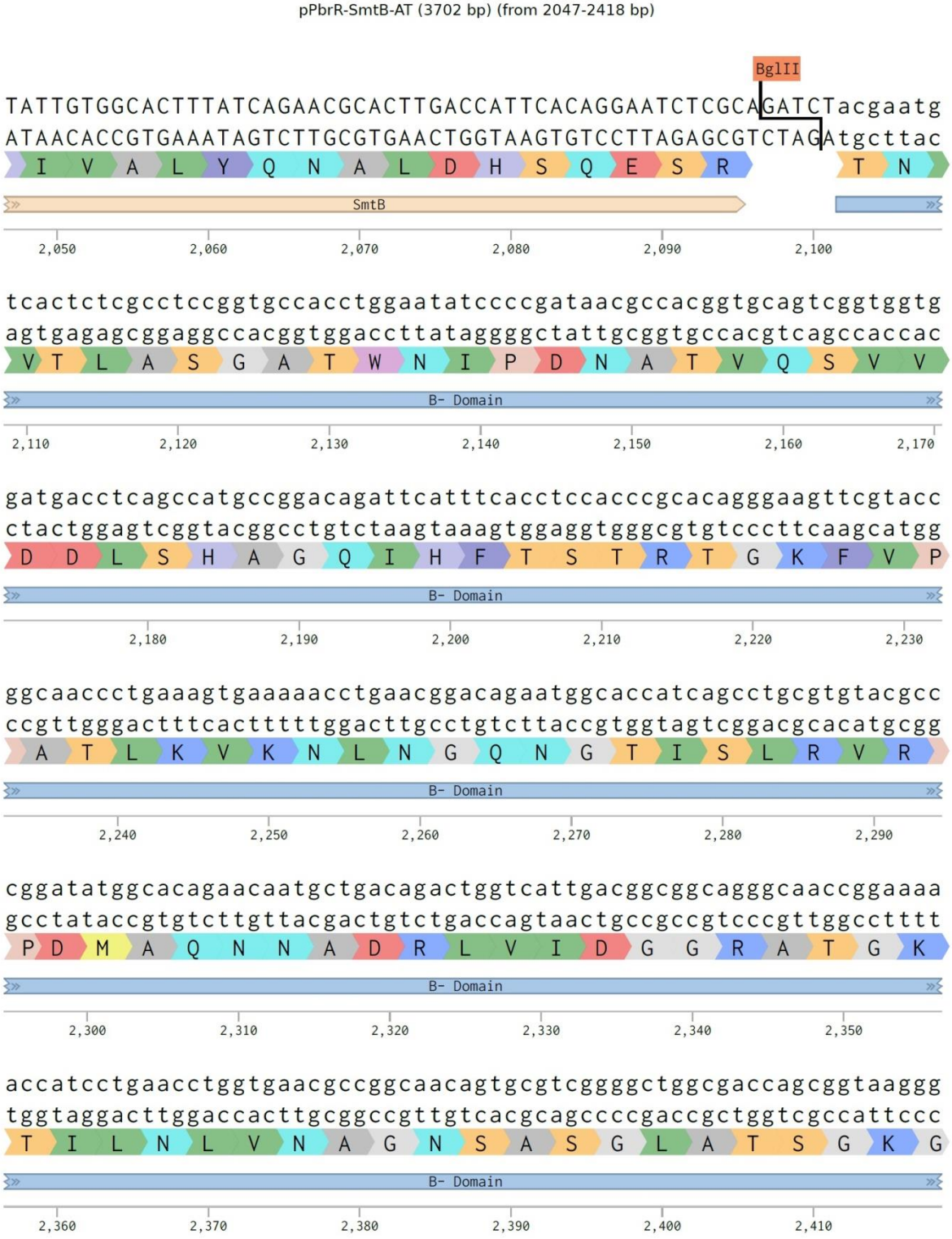
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SmtB
2,050 2,060 2,070 2,080 2,090 2,100
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B- Domain
2,110 2,120 2,130 2,140 2,150 2,160 2,170
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D D L S H A G Q I H F T S T R T G K F V P
B- Domain
2,180 2,190 2,200 2,210 2,220 2,230
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B- Domain
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B- Domain
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B- Domain
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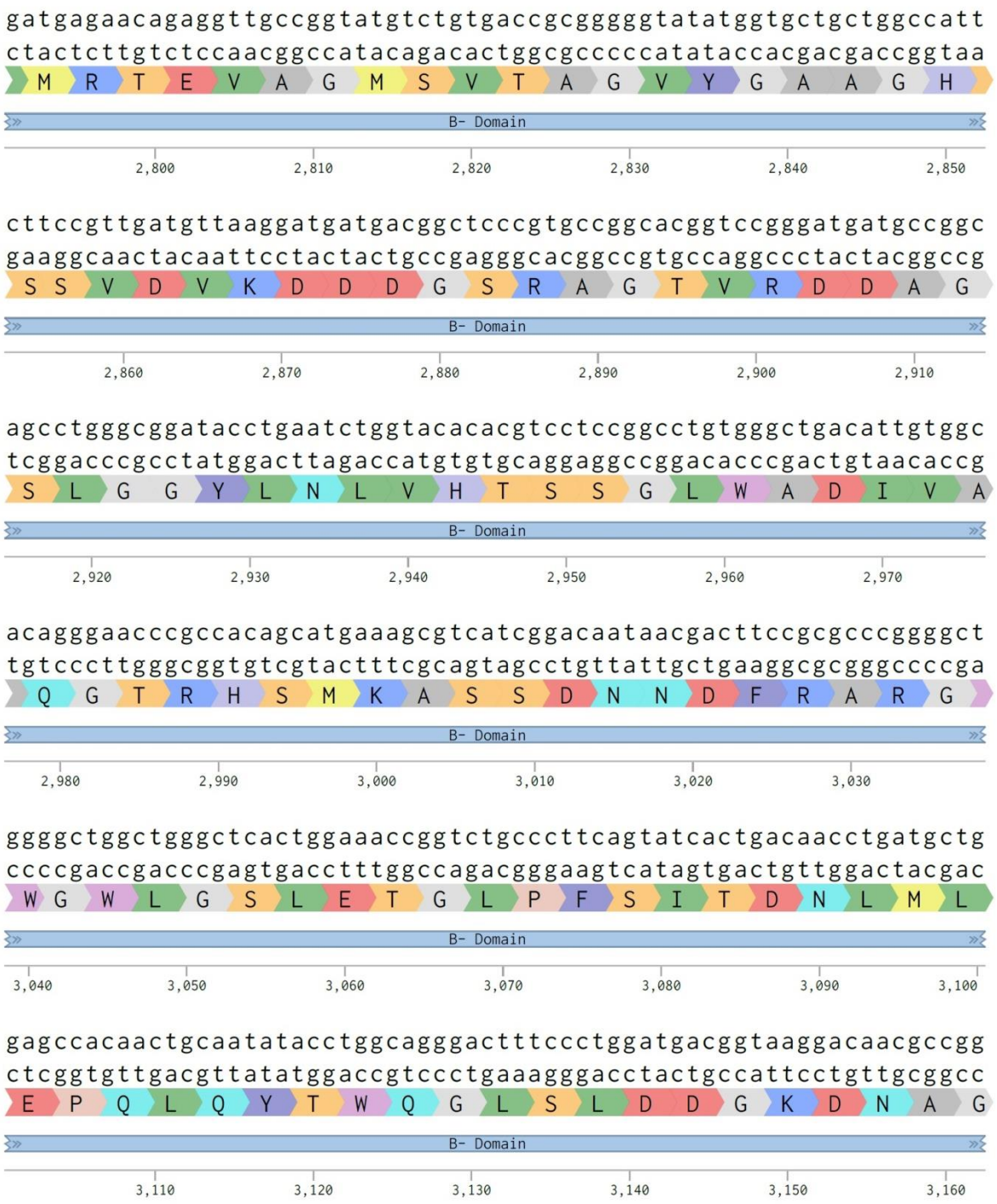
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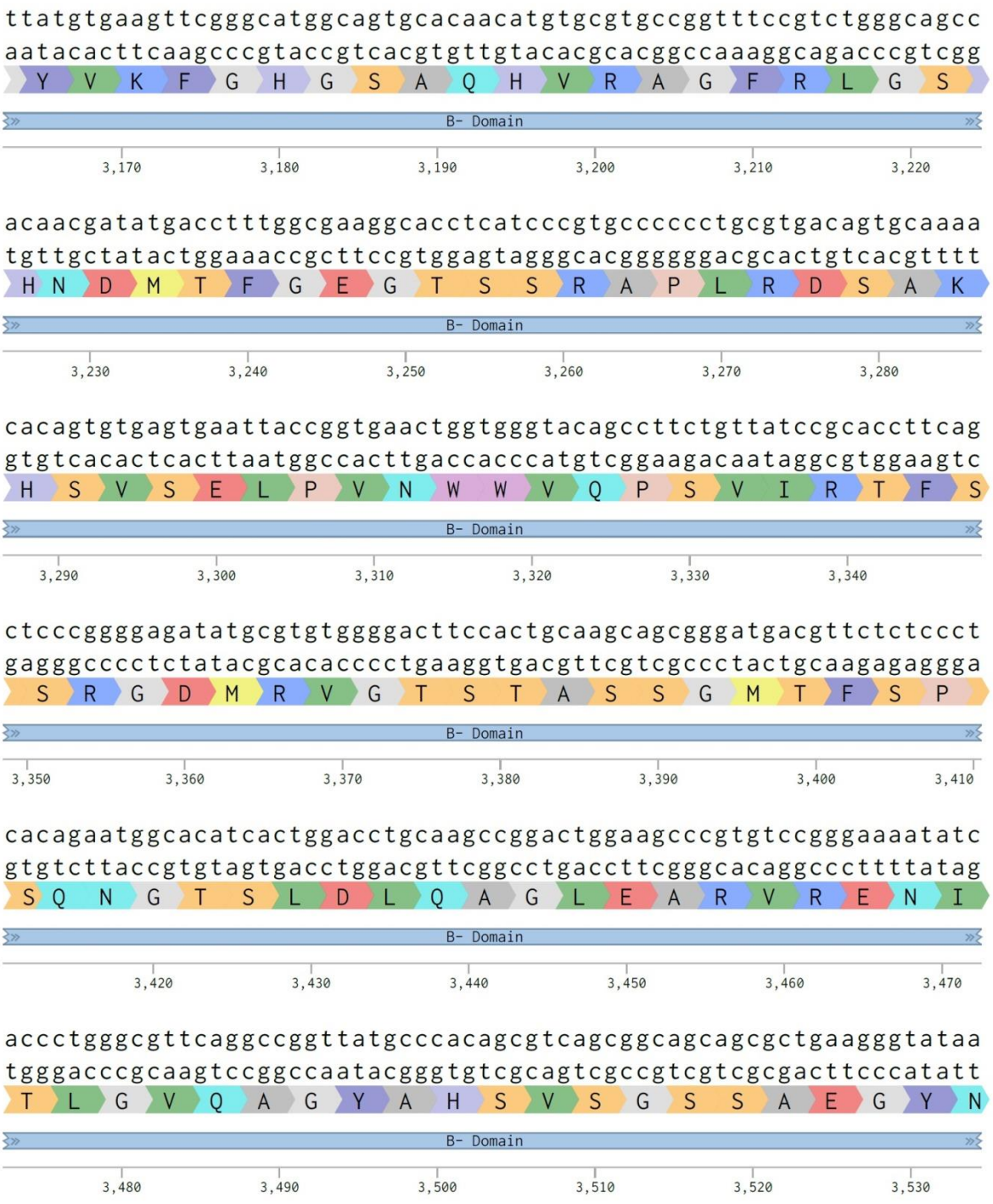
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B- Domain
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B- Domain
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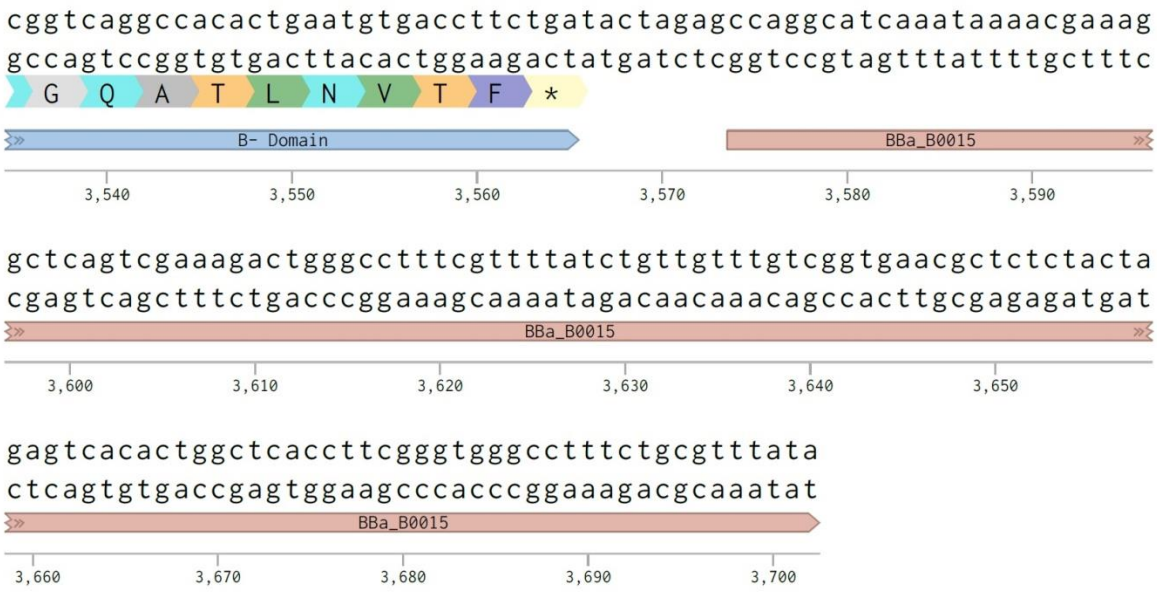
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FIGURES

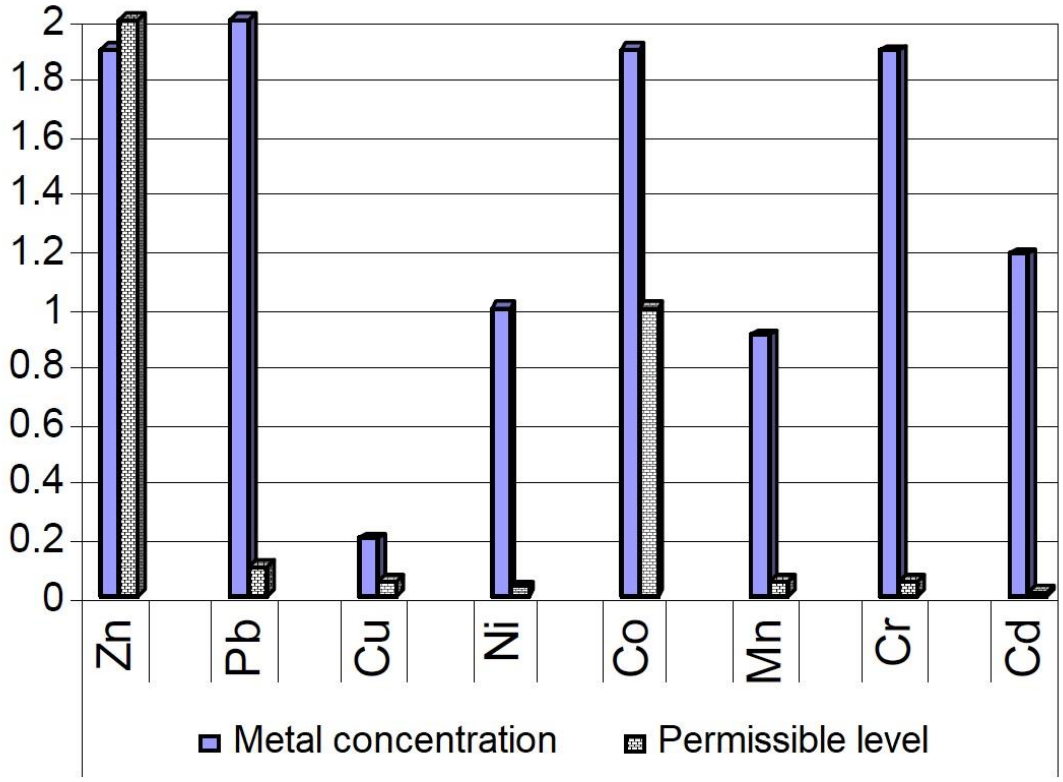

Figure 1: Diagram shows the concentrations of heavy metals in some drainage (Ismailia Canal) in Egypt [6].

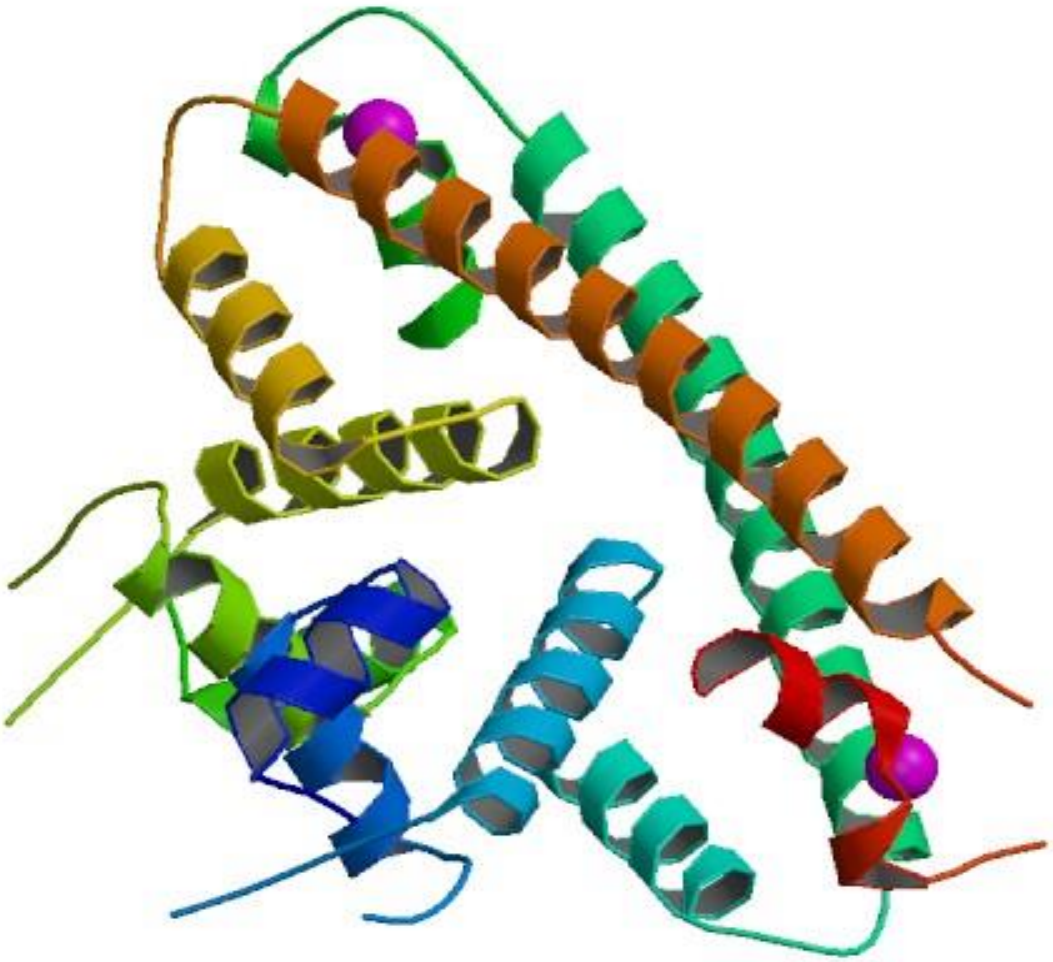

Figure 2: Rhree dimensional structure of PbrR from *C. metallidurans* CH34 in complex with Pb^{2+} showed in violet balls [62]

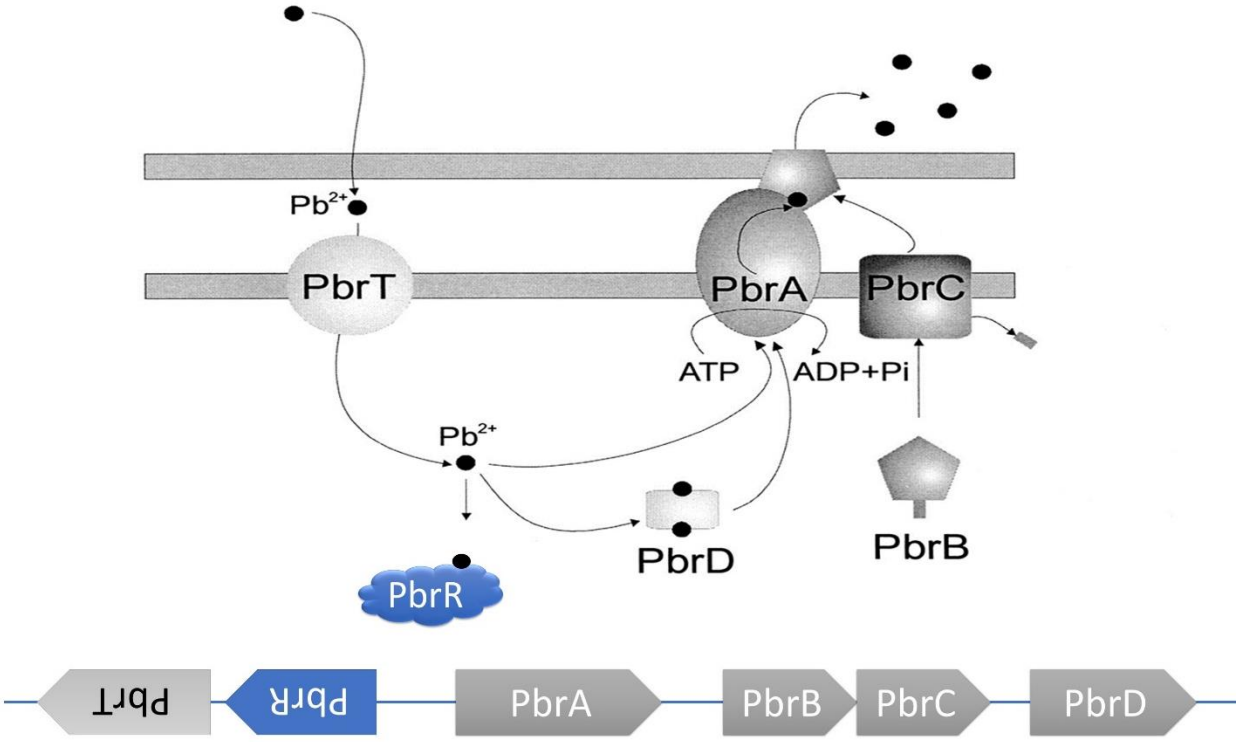

Figure 3: Model for the pbr Pb^{2+} resistance operon-encoded lead resistance of *C. metallidurans CH34*. Edited from [34].

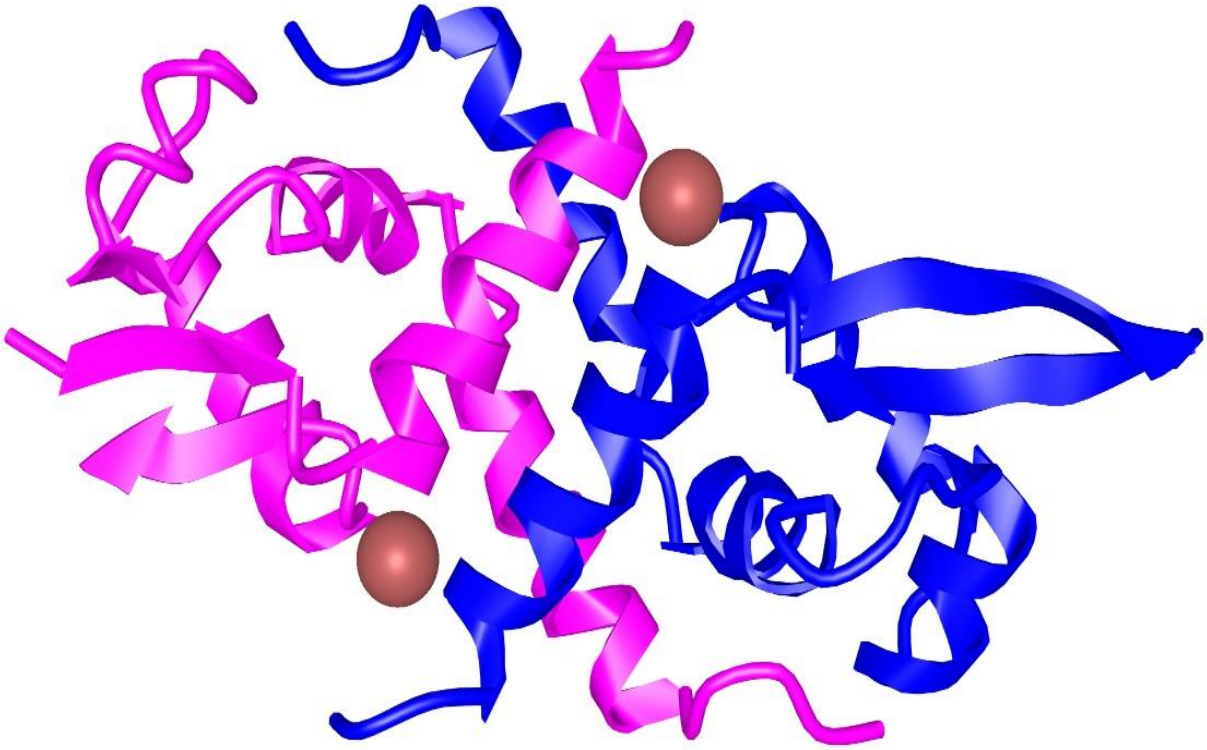

Figure 4: Crystal structure of cyanobacterial metallothionein repressor (SmtB) with Zn^{2+} showed in red balls [36].

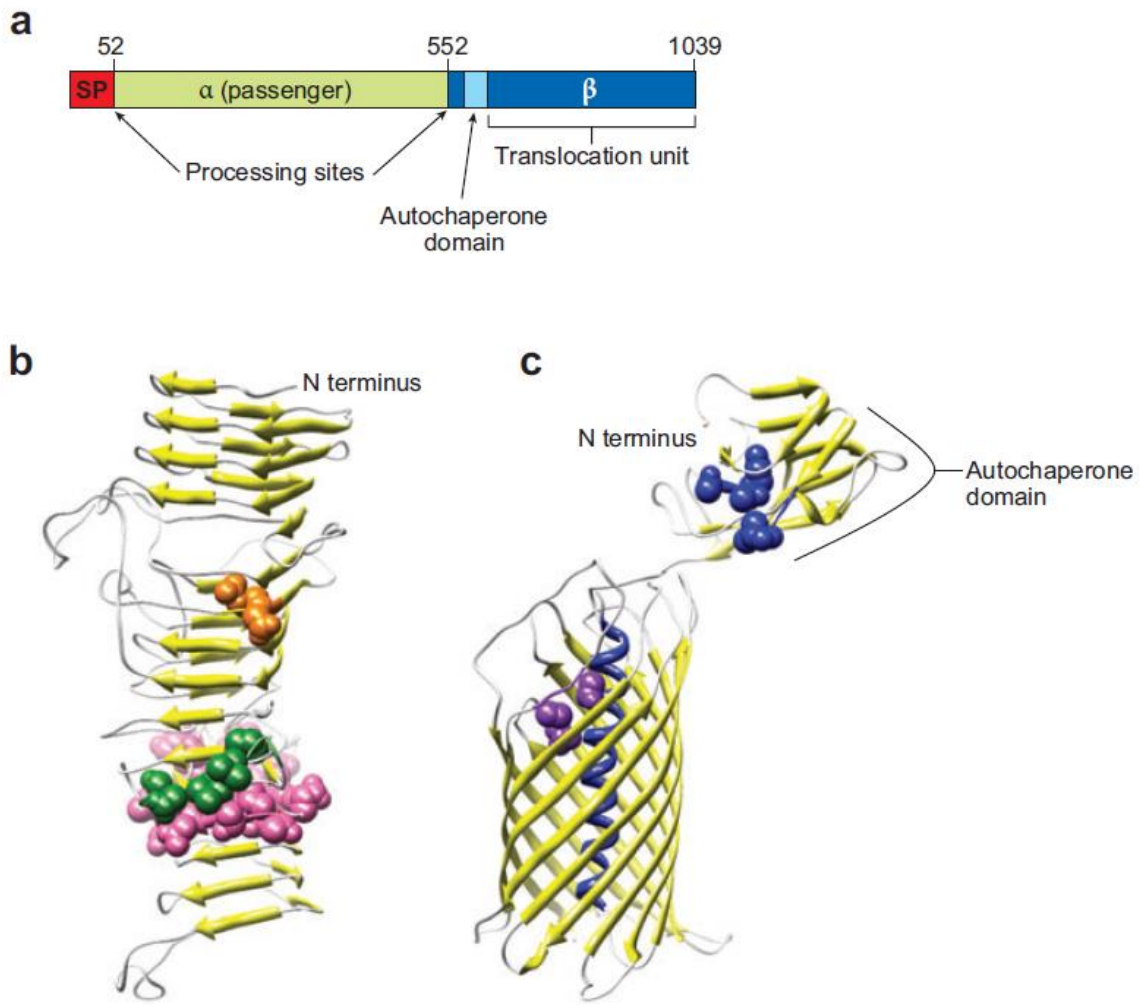

Figure 5: Domain organization of Antigen 43 (Ag43). (**a**) Schematic showing the organization of the protein domains of Ag43. (**b**) α43 and (**c**) β43, shown in ribbon representation. The domain models are depicted as separate molecules. β-strands in each case are colored yellow, loops are gray, and the central α-helix of the translocator domain is blue [43].

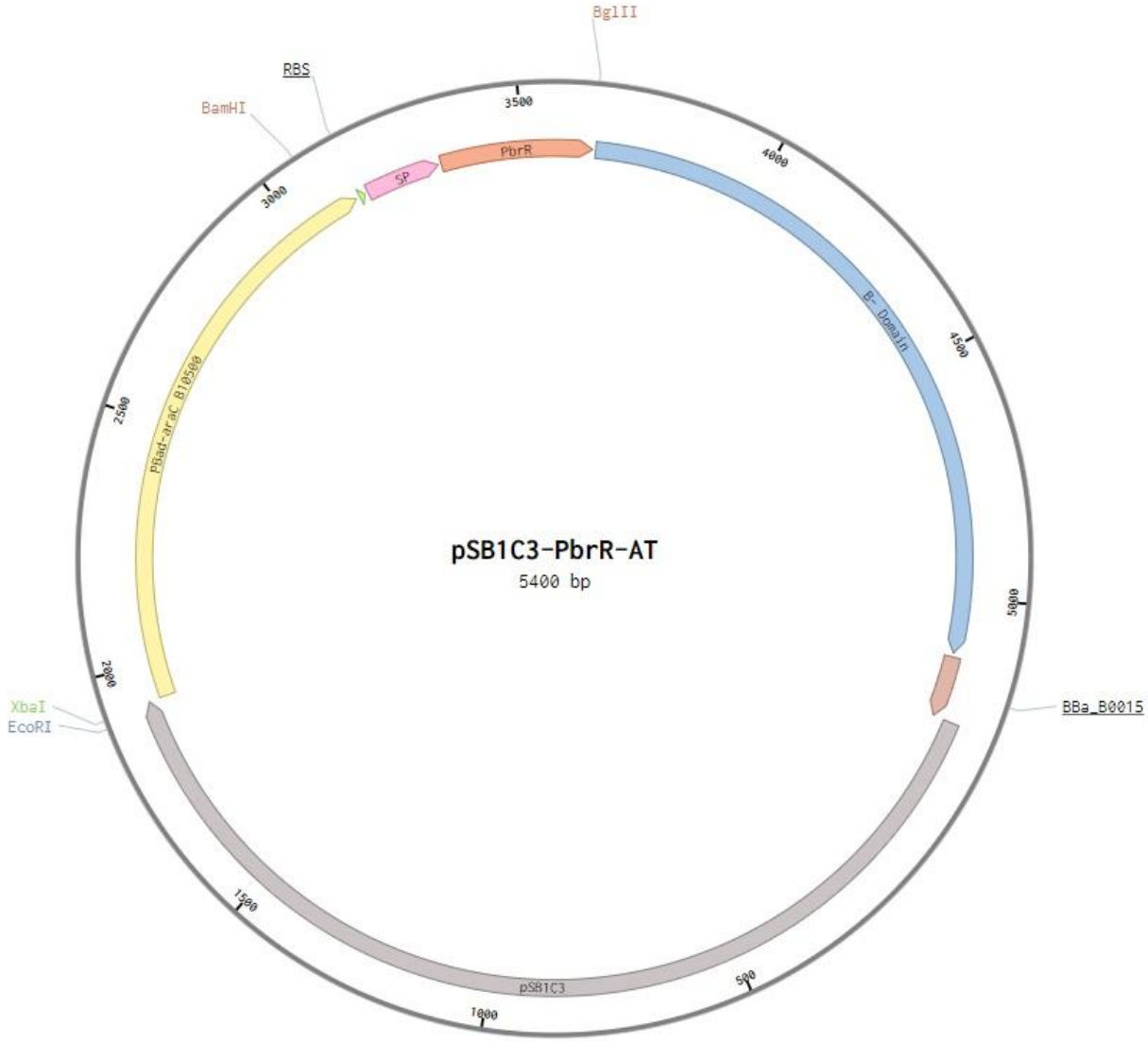

Figure 6: pSB1C3-PbrR-AT plasmid construct

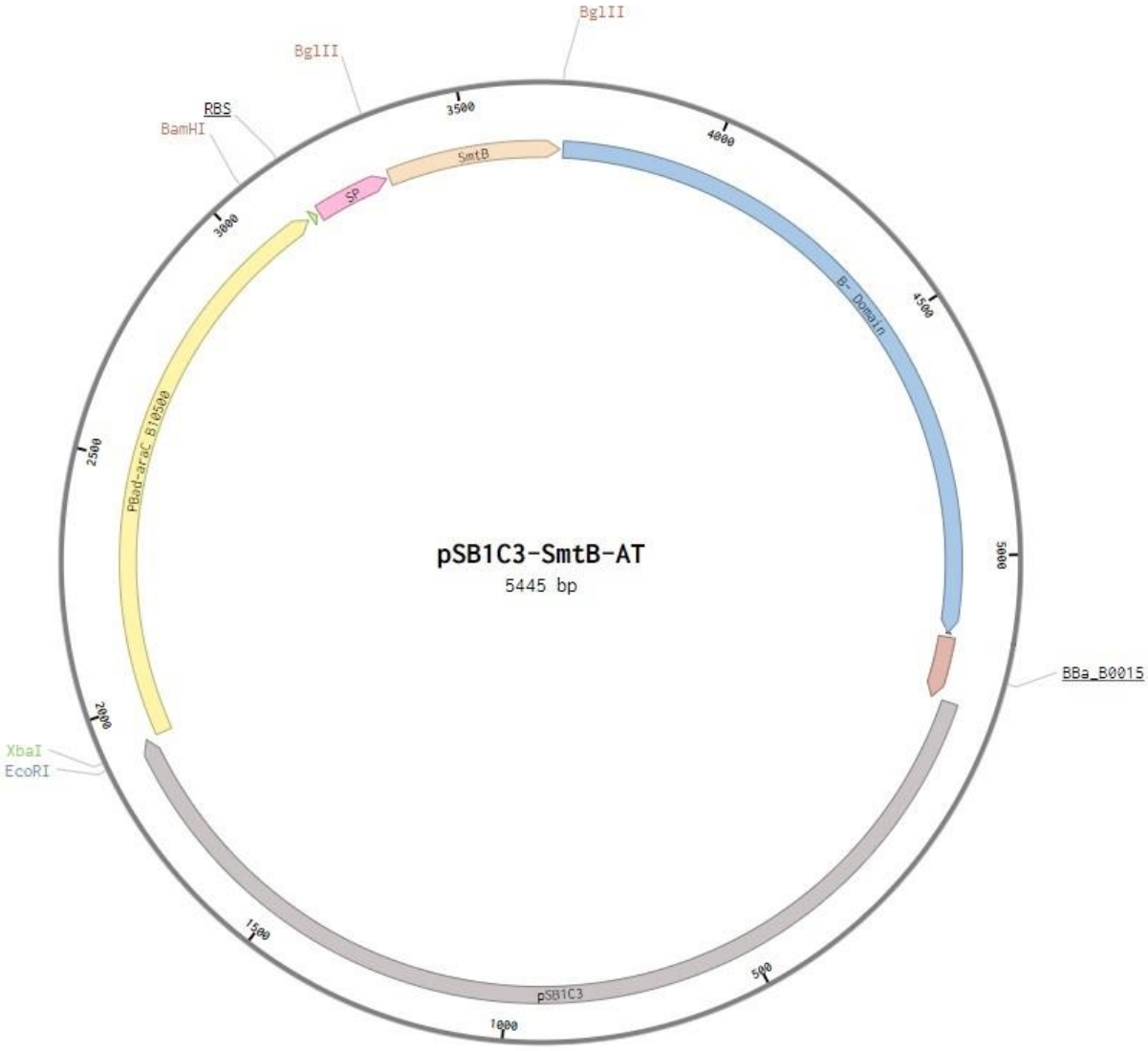

Figure 7: pSB1C3-SmtB-AT plasmid construct

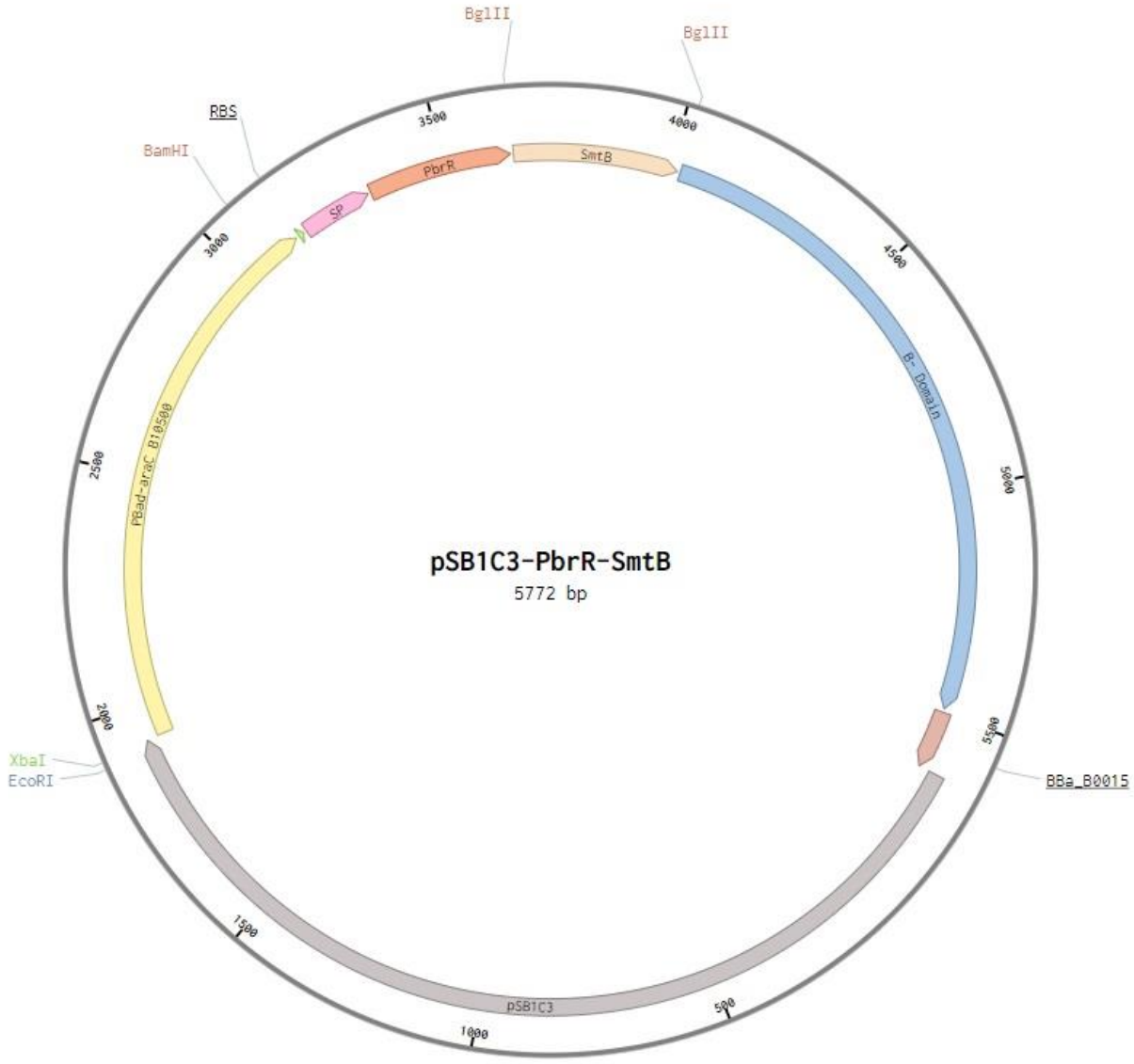

Figure 8: pSB1C3-PbrR-SmtB-AT plasmid construct

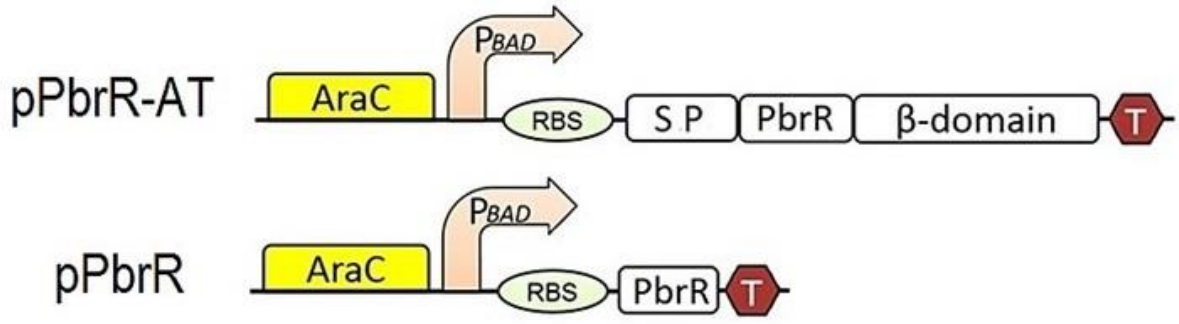

Figure 9: Schematic diagrams of genes for expression of PbrR (pPbrR) and PbrR fused to β-domain of Ag43 (pPbrR-AT) under control of PBAD promoter. AraC: AraC activator expression unit under control of constitutive promoter (BBa_K808000). P_{BAD} : Inducible promoter in the presence of arabinose (BBa_I0500). RBS: Ribosome Binding Site (BBa_B0034), SP: signal peptide (position from 1st a.a. to 52nd a.a.) of Ag43, PbrR: coding region of lead binding operon (BBa_I721002), β-domain: translocation domain (position from 553rd a.a. to 1038th a.a.) of Ag43 which forms β-barrel structure in outer membrane, T: double terminator (BBa_B0015).

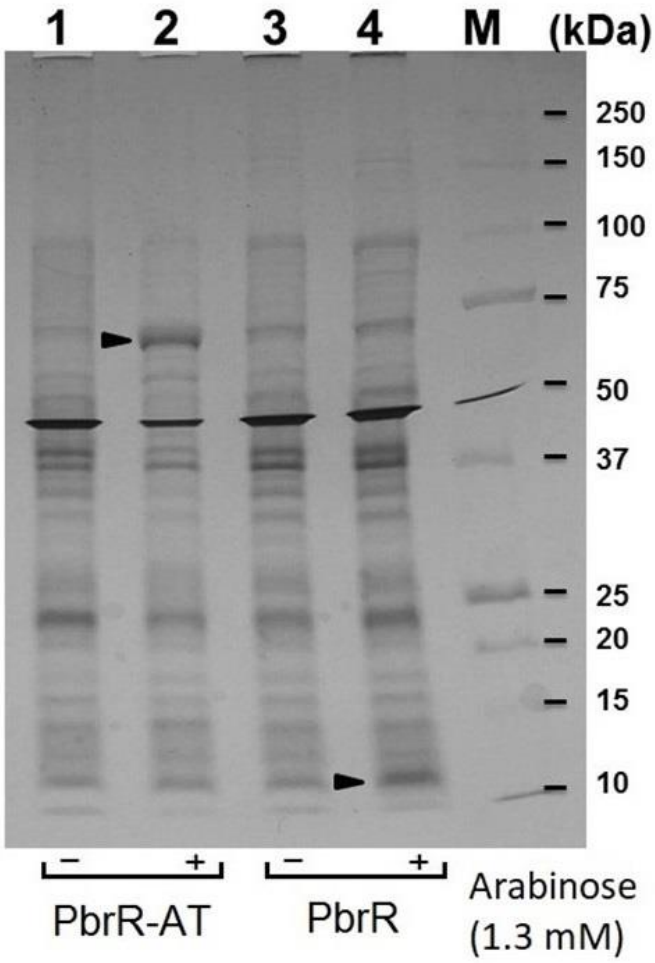

Figure 10: Analysis of induction of PbrR-AT and PbrR by addition of arabinose (1.3 mM) for 4 hours at 30°C. Protein extracted from equal amounts of *E. coli* cells grown in the absence or presence of arabinose were separated on SDS-PAGE (16% acrylamide) and visualized by CBB staining. Each black arrow head indicates the position of induced proteins (69.25 kDa for PbrR-AT and 11.71 kDa for PbrR respectively).

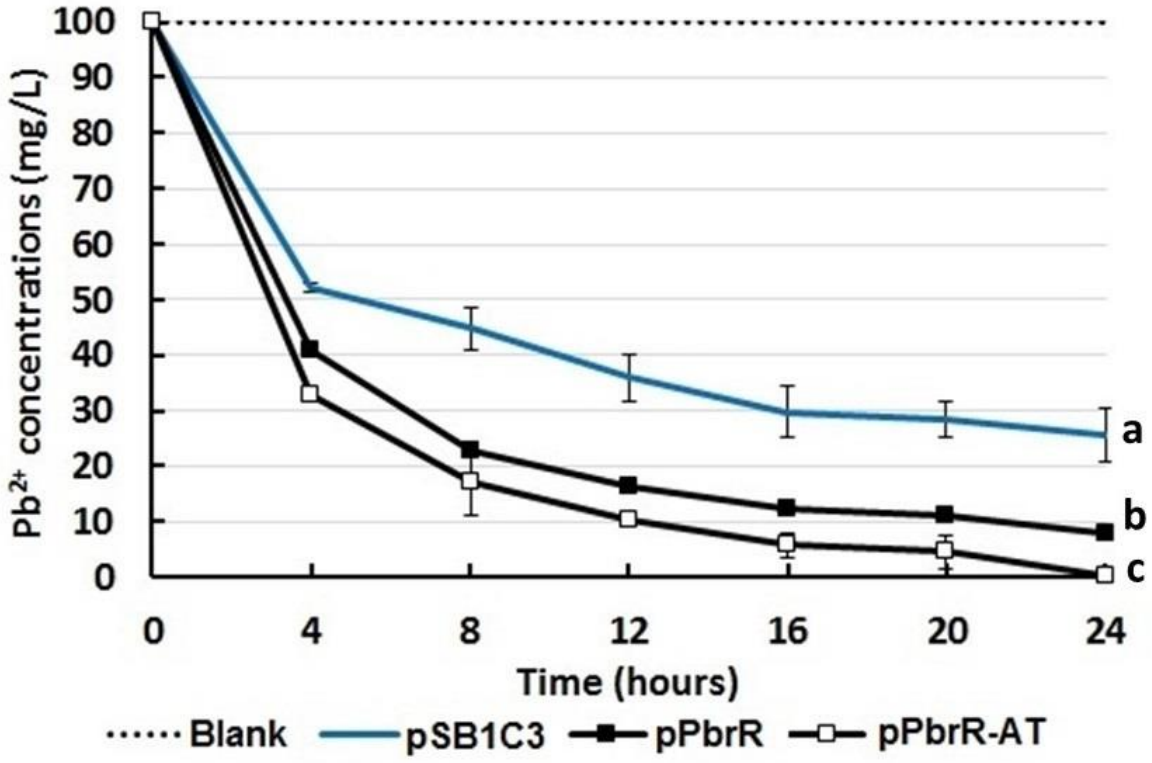

Figure 11: Time course of removal of Pb^{2+} from supernatant by incubating 5.0 g/L of *E. coli* cells (wet weight) in 0.9% NaCl solution containing 100 mg/L of Pb^{2+} (pH 6.0) at 37°C. *E. coli* cells containing pSB1C3 (empty vector control), pPbrR and pPbrRAT were used as bio-sorbents of Pb^{2+}. Change of concentration of Pb^{2+} was monitored in the absence of *E. coli* cells under the same condition (Blank). Each measurement was repeated three times for calculation of averages and standard deviation. Values carrying different super script letters (a, b, c, d) are significantly different at $p<0.05$.

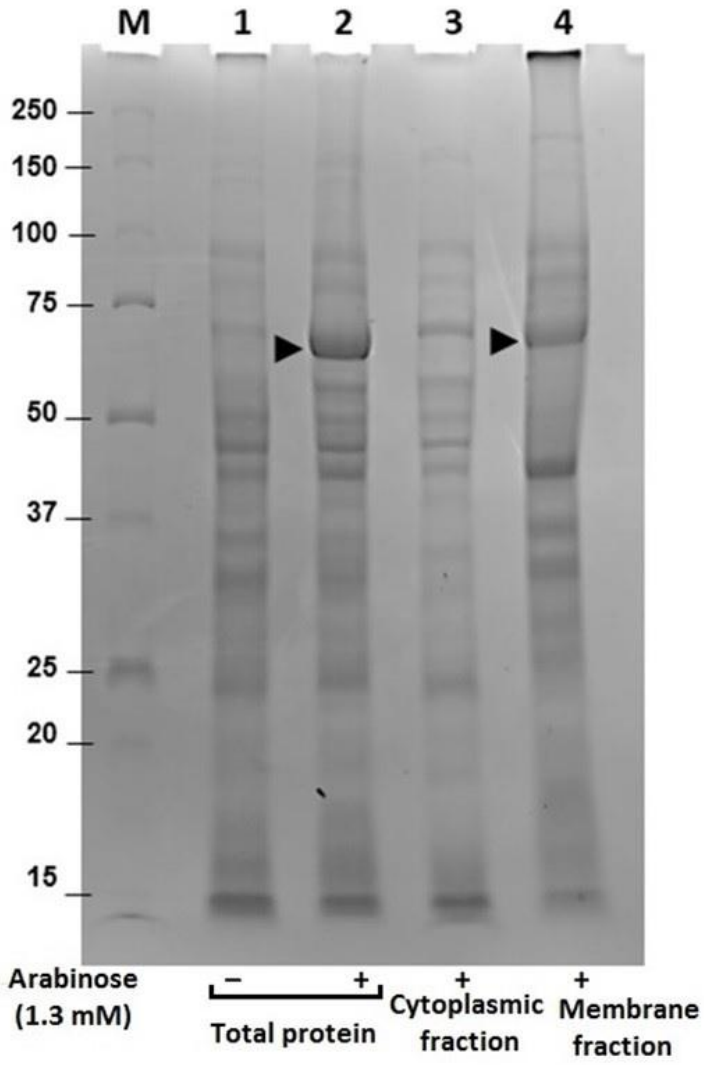

Figure 12: Distribution of total, cytoplasmic and membrane proteins of *E. coli* containing pPbrR-AT after culturing cells in the absence or presence of arabinose (final concentration 1.3 mM) for 4 hours at 30°C. Lane M: polypeptide marker; lane 1: total protein without induction; lane 2: total protein after induction; lane 3: cytoplasmic proteins after induction; lane 4: membrane proteins after induction. Black arrow head indicates the position of the induced PbrR-AT (69.25 kDa).

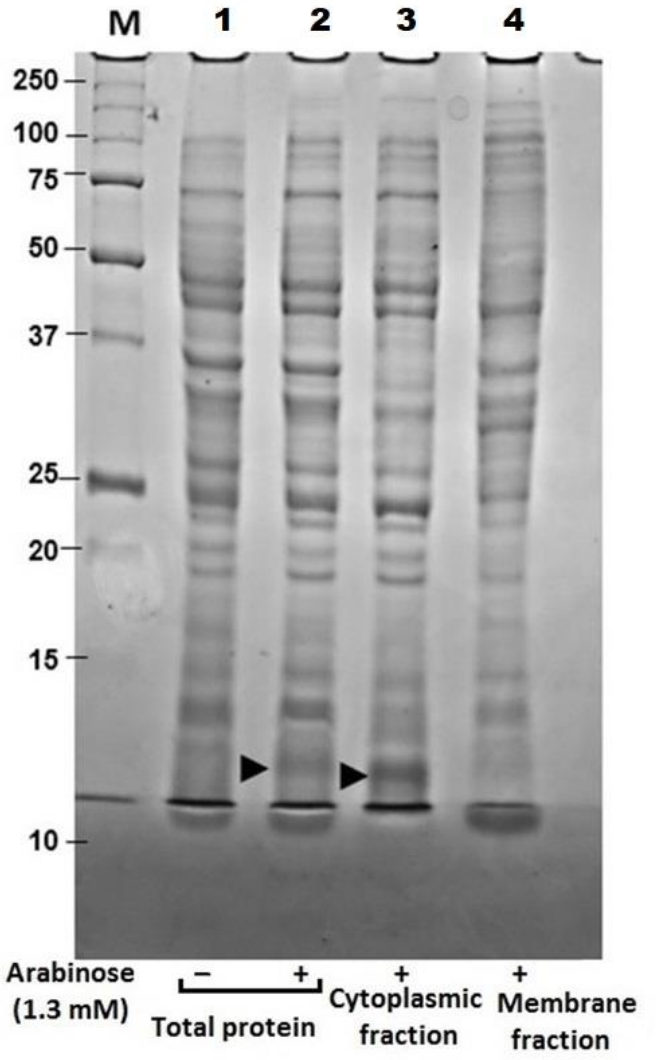

Figure 13: Distribution of total, cytoplasmic and membrane proteins of *E. coli* containing pPbrR after culturing cells in the absence or presence of arabinose (final concentration 1.3 mM) for 4 hours at 30°C. Lane M: polypeptide marker; lane 1: total protein without induction; lane 2: total protein after induction; lane 3: cytoplasmic proteins after induction; lane 4: membrane proteins after induction. Black arrow head indicates the position of the induced PbrR (11.71 kDa).

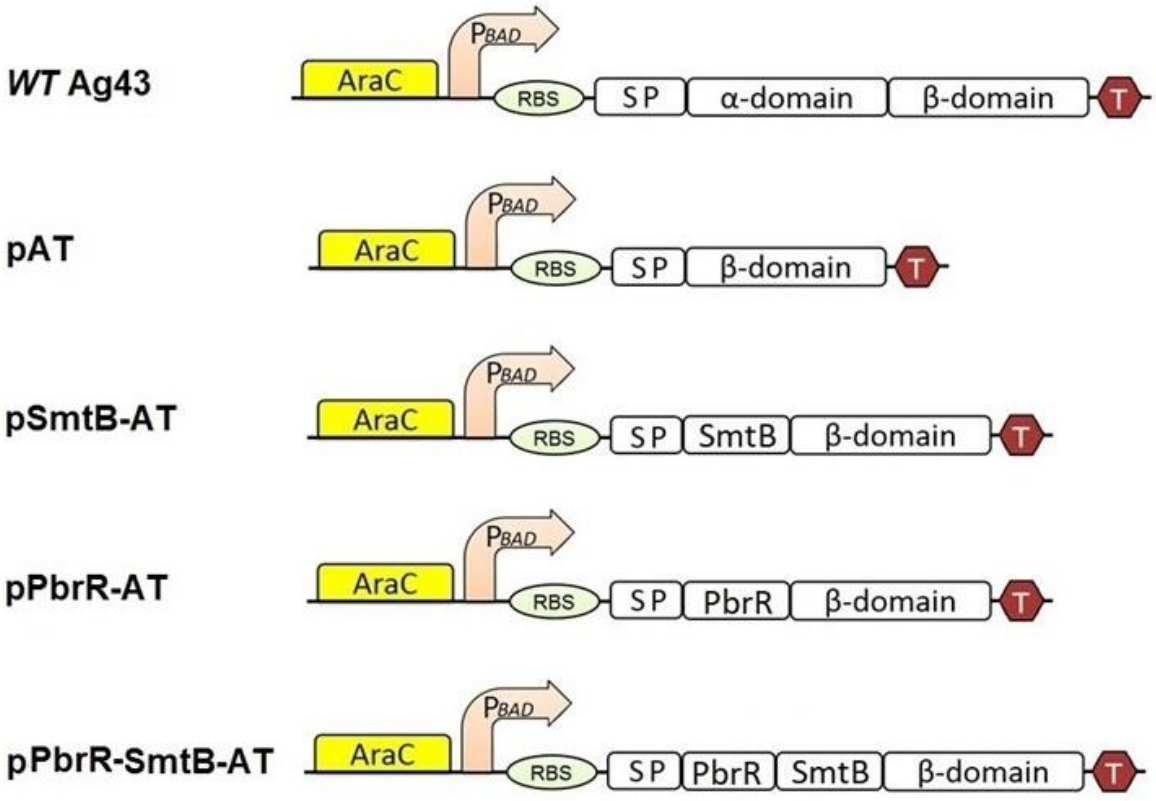

Figure 14: Schematic diagrams of gene encoding wild type Ag43, AT, SmtB-AT, PbrR-AT and PbrR-SmtB-AT fusion proteins under control of P_{BAD} promoter. SmtB: coding region of metallothionein b gene from Cyanobacteria, AraC, P_{BAD} , RBS, SP, PbrR, β-domain and T are as described in the legends for Figure 9.

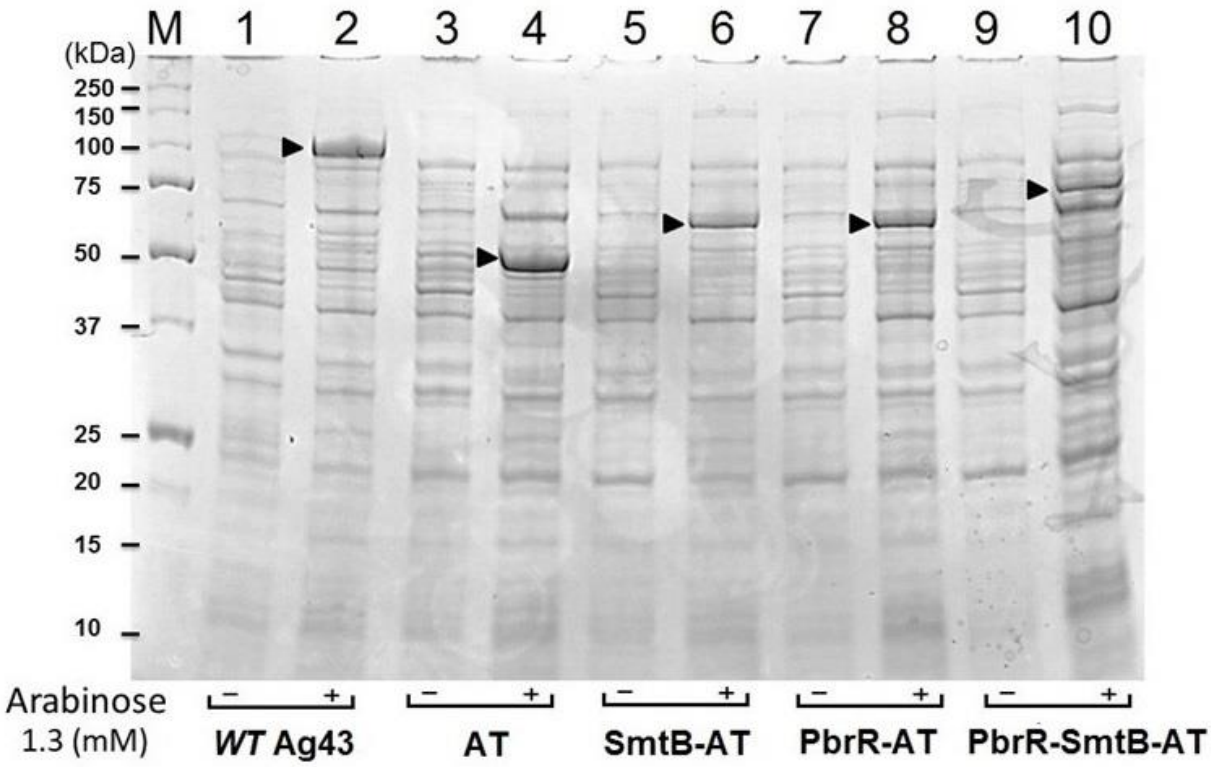

Figure 15: Induction of wild type Ag43 and other recombinant proteins. *E. coli* cells were grown in LB medium containing chloramphenicol (34 μg/mL) with shaking at 37°C overnight. After 1/100 dilution of culture with LB medium, cells were grown at 37°C up to optical density at 600 nm reaching to 0.5 in the absence (lanes 1, 3, 5, 7, 9) or presence (lane 2, 4, 6, 8, 10) of arabinose. Proteins were separated on SDS-PAGE (12% acrylamide) and visualized by CBB staining. Each black arrow head indicates the position of the induced proteins. Size of induced protein from each recombinant gene was as follows: Ag43 (lane 2, 106.87 kDa), AT (lane 4, 57.31 KDa), SmtB-AT (lane 6, 71.04 KDa), PbrR-AT (lane 8, 69.25 KDa) and PbrR-SmtB-AT (lane 10, 82.95 KDa). M: protein size marker.

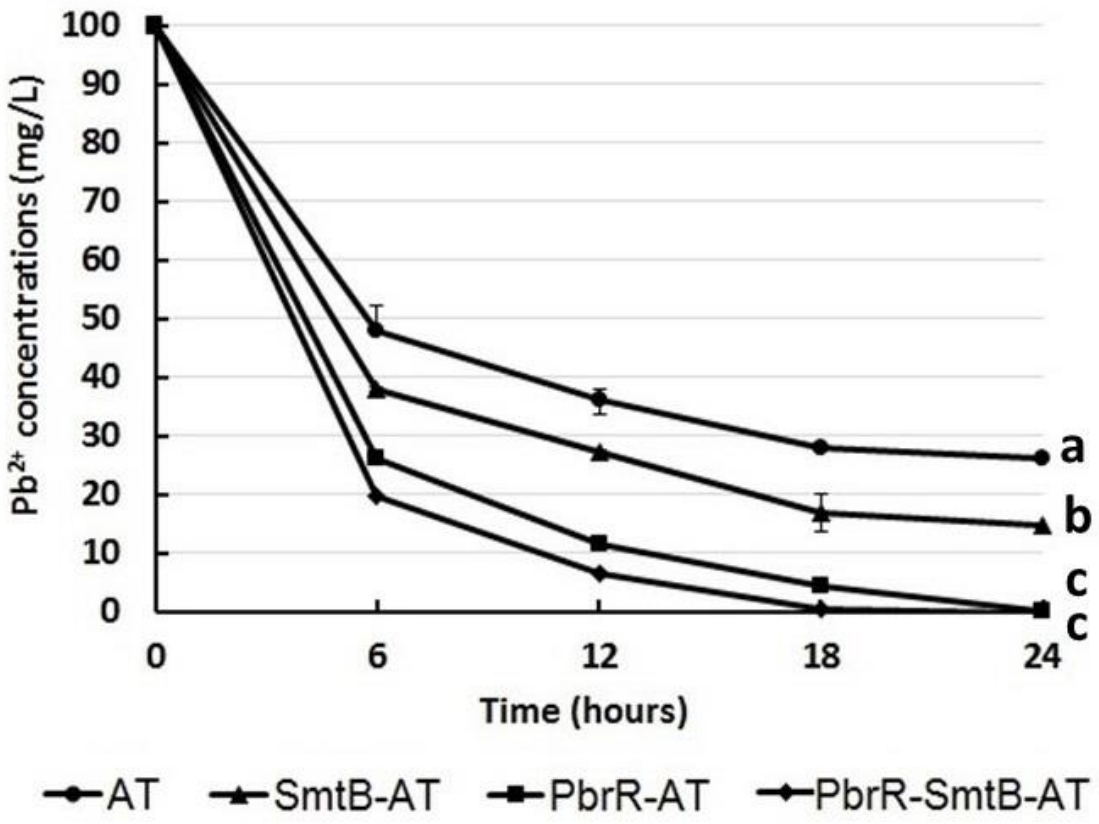

Figure 16: Time course of reduction of Pb^{2+} concentrations in the supernatant after incubating 5 g/L of wet recombinant *E. coli* cells containing pAT, pSmtB-AT, pPbrR-AT and pPbrR-SmtB-AT in 0.9% NaCl solution (pH 6.0) containing 100 mg/L of Pb^{2+} at 37°C with shaking at 180 rpm. Each measurement was repeated three times for calculation of averages and standard deviation. Values carrying different super script letters (a, b, c) are significantly different at $p < 0.05$.

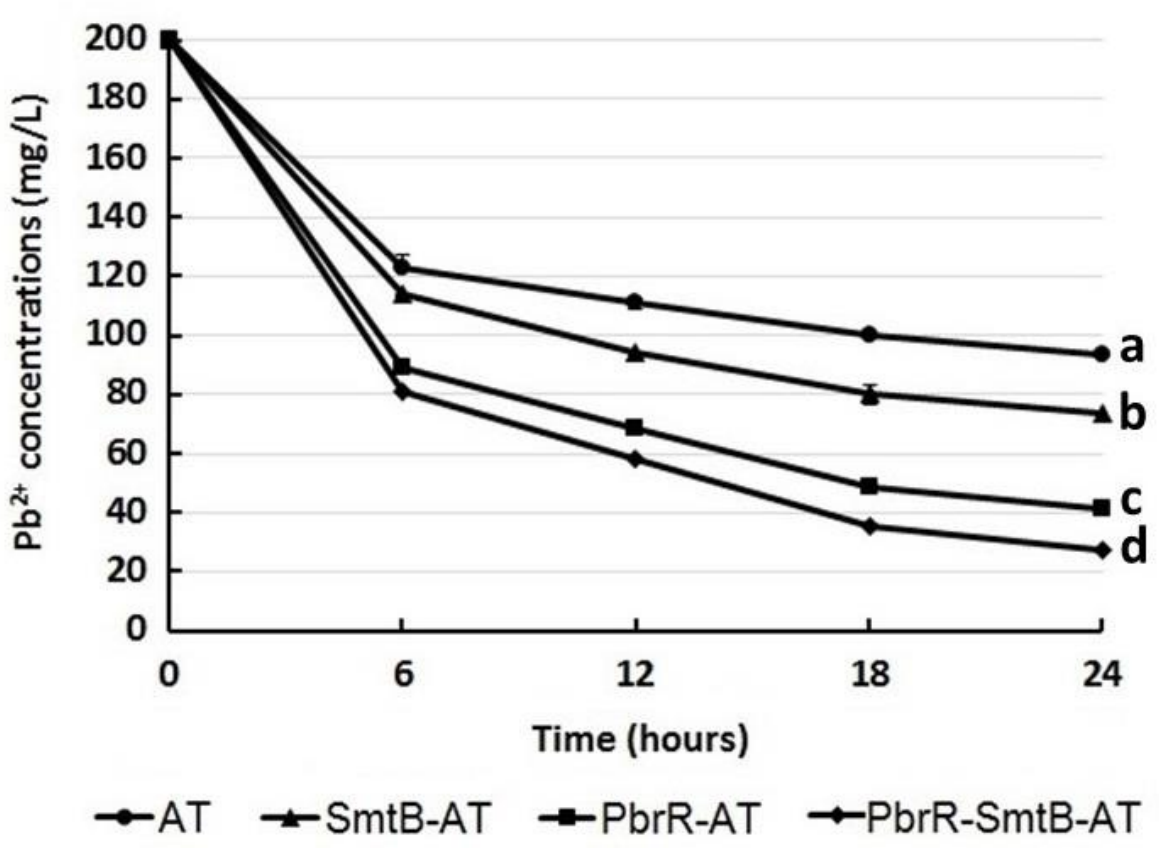

Figure 17: Time course of reduction of Pb^{2+} concentrations in the supernatant after incubating 5 g/L of wet recombinant *E. coli* cells containing pAT, pSmtB-AT, pPbrR-AT and pPbrR-SmtB-AT in 0.9% NaCl solution (pH 6.0) containing 200 mg/L of Pb^{2+} at 37°C with shaking at 180 rpm. Each measurement was repeated three times for calculation of averages and standard deviation. Values carrying different super script letters (a, b, c, d) are significantly different at $p < 0.05$.

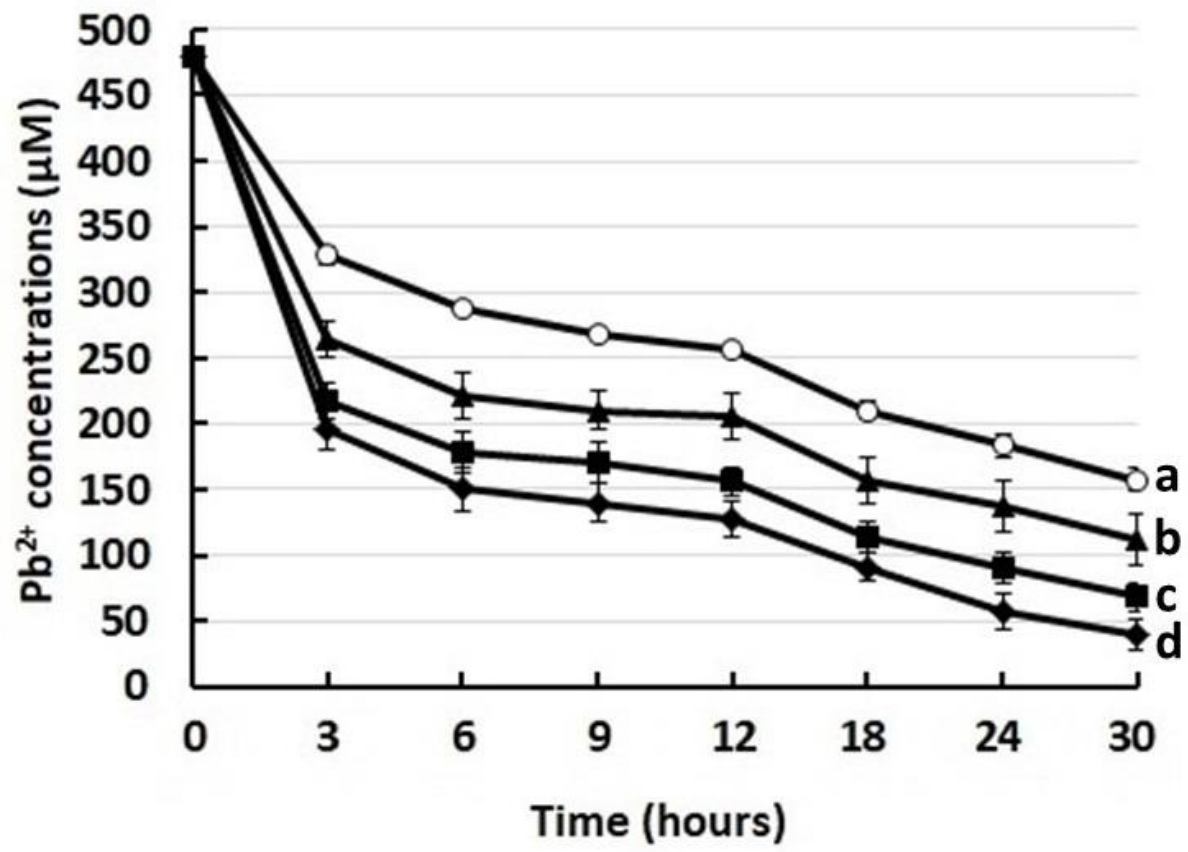

Figure 18: Time course of selective adsorption of Pb^{2+} from mixture solution (pH 6.0) containing 480 µM of Pb^{2+}, Cd^{2+} and Cu^{2+} in 0.9% NaCl using three different types of recombinant *E. coli* cells, AT (⏚), SmtB-AT (⏚), PbrR-AT (⏚) or PbrR-SmtB-AT (⏚).Each measurement was repeated three times for calculation of averages and standard deviation. Values carrying different super script letters (a, b, c, d) are significantly different at $p < 0.05$.

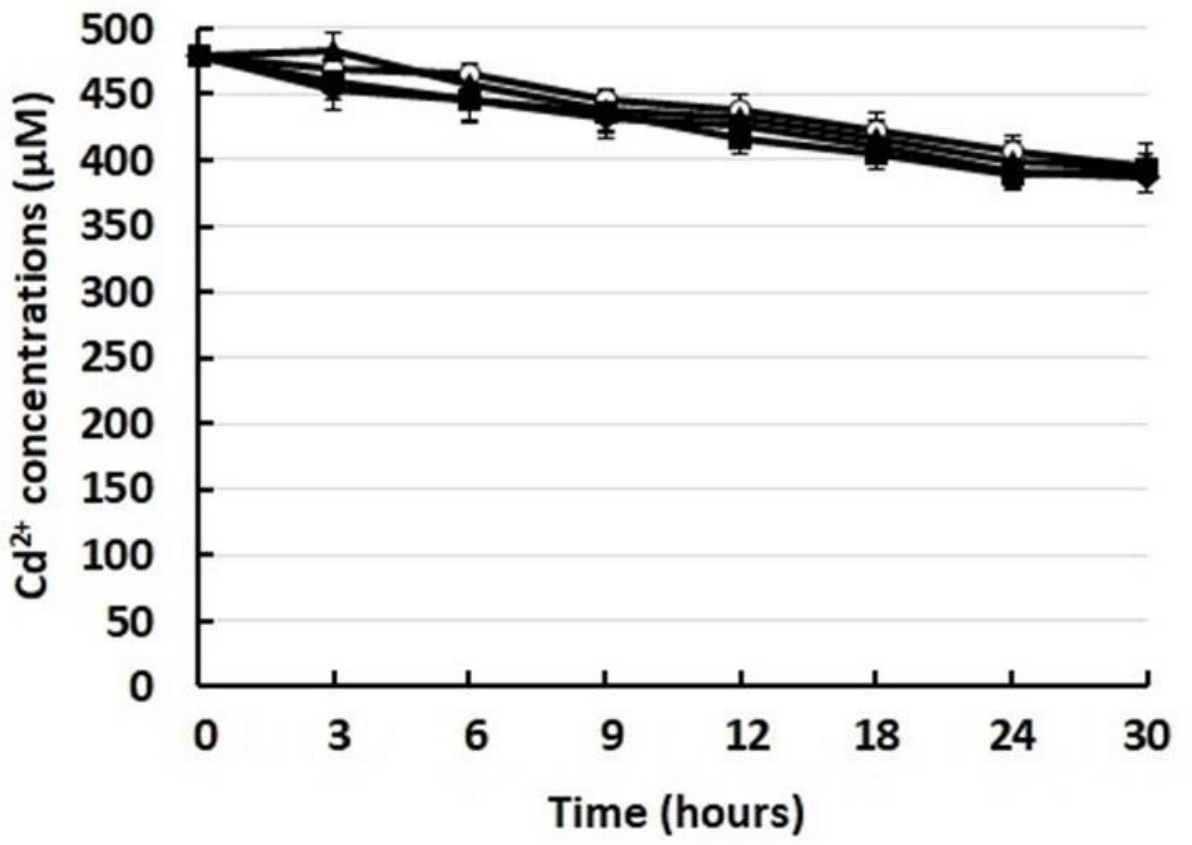

Figure 19: Time course of selective adsorption of Cd^{2+} from mixture of heavy metals solution (pH 6.0) containing 480 µM of Pb^{2+}, Cd^{2+} and Cu^{2+} in 0.9% NaCl using three different types of recombinant *E. coli* cells, AT (○), SmtB-AT (▲), PbrR-AT (■) or PbrR-SmtB-AT (♦). Each measurement was repeated three times for calculation of averages and standard deviation.

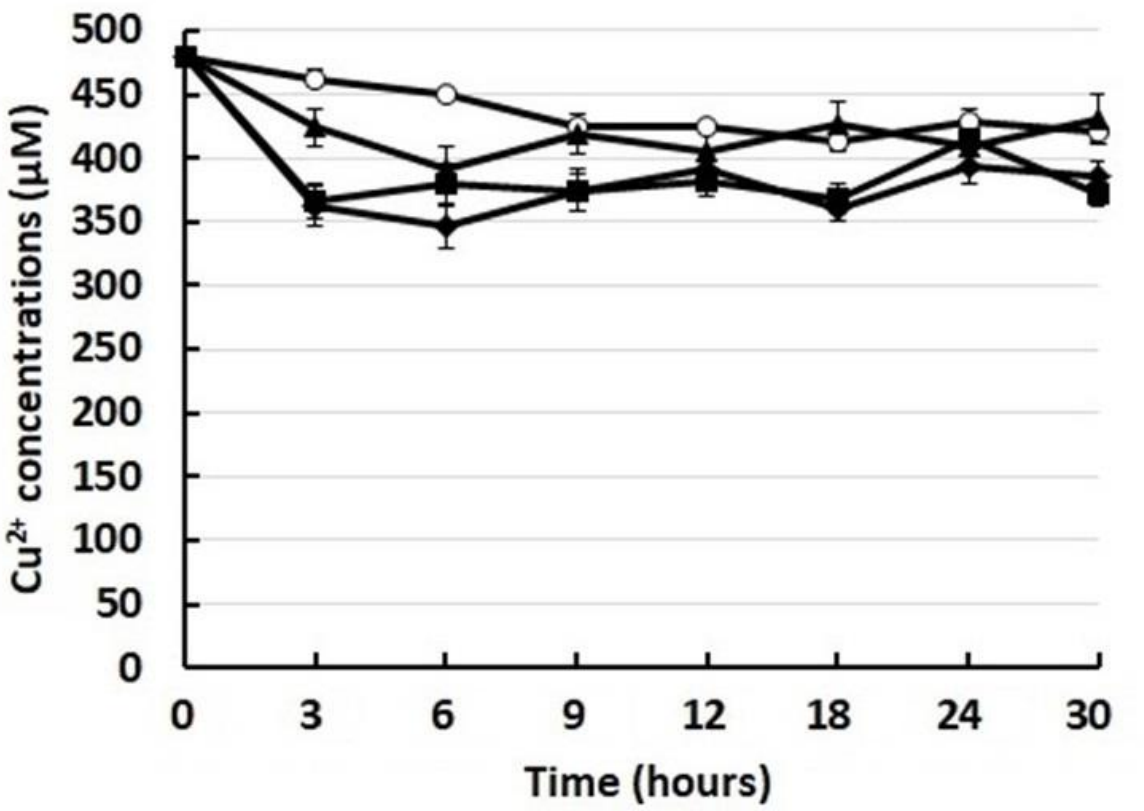

Figure 20: Time course of selective adsorption of Cu^{2+} from mixture of heavy metals solution (pH 6.0) containing 480 µM of Pb^{2+}, Cd^{2+} and Cu^{2+} in 0.9% NaCl, using three different types of recombinant *E. coli* cells, AT (○), SmtB-AT (▲), PbrR-AT (■) or PbrR-SmtB-AT (♦). Each measurement was repeated three times for calculation of averages and standard deviation.

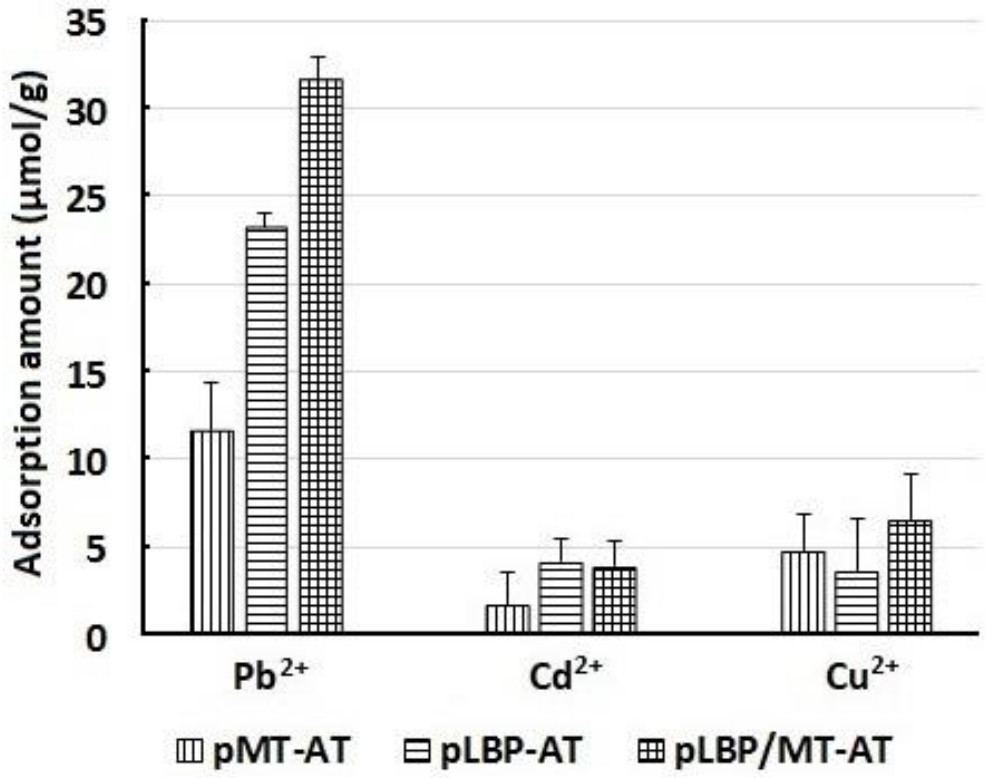

Figure 21: Comparison of selectivity adsorption of *E. coli* strains (SmtB-AT, PbrR-AT or PrbR-SmtB-AT) for removal of Pb^{2+}, Cd^{2+} or Cu^{2+} from mixture of heavy metals solution (pH 6.0) containing 480 μM of Pb^{2+}, Cd^{2+} and Cu^{2+} in 0.9% NaCl. Adsorption values were standardized by removing values of non-specific binding of AT cells.

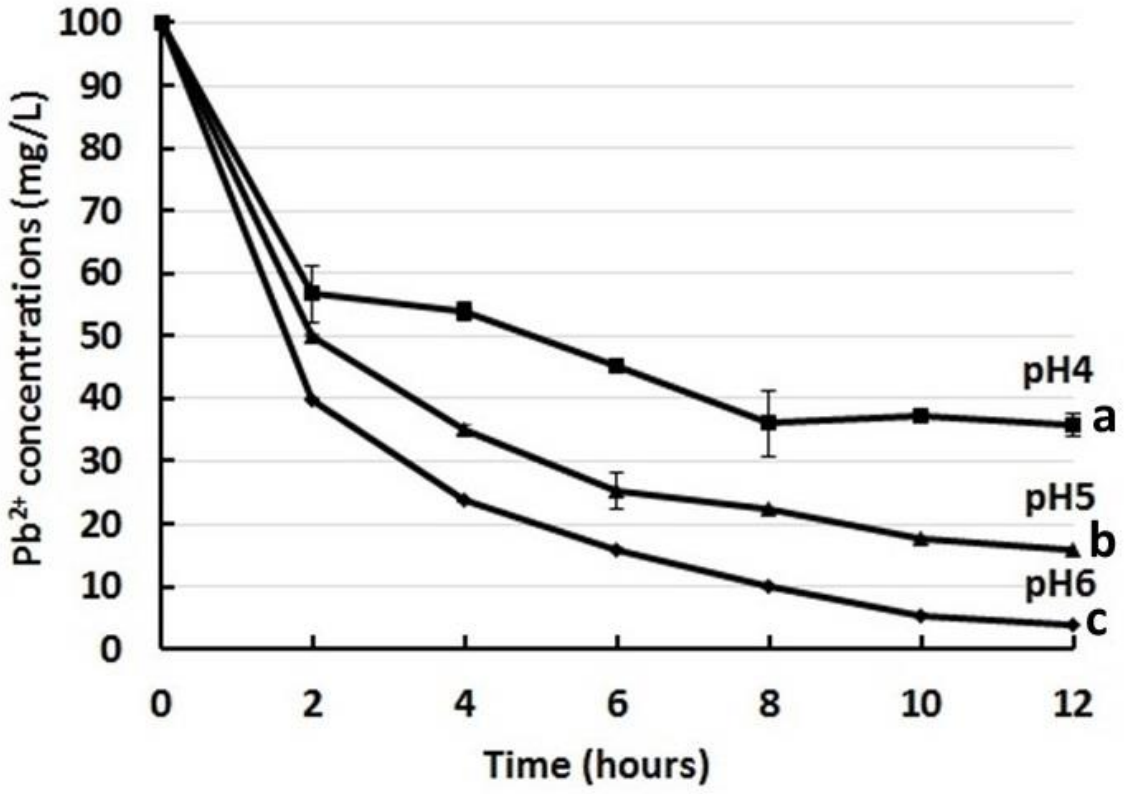

Figure 22: Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution containing 100 mg/L of Pb^{2+} at different pH 4.0, 5.0 or 6.0 at 37°C. Values carrying different super script letters (a, b, c) are significantly different at $p < 0.05$.

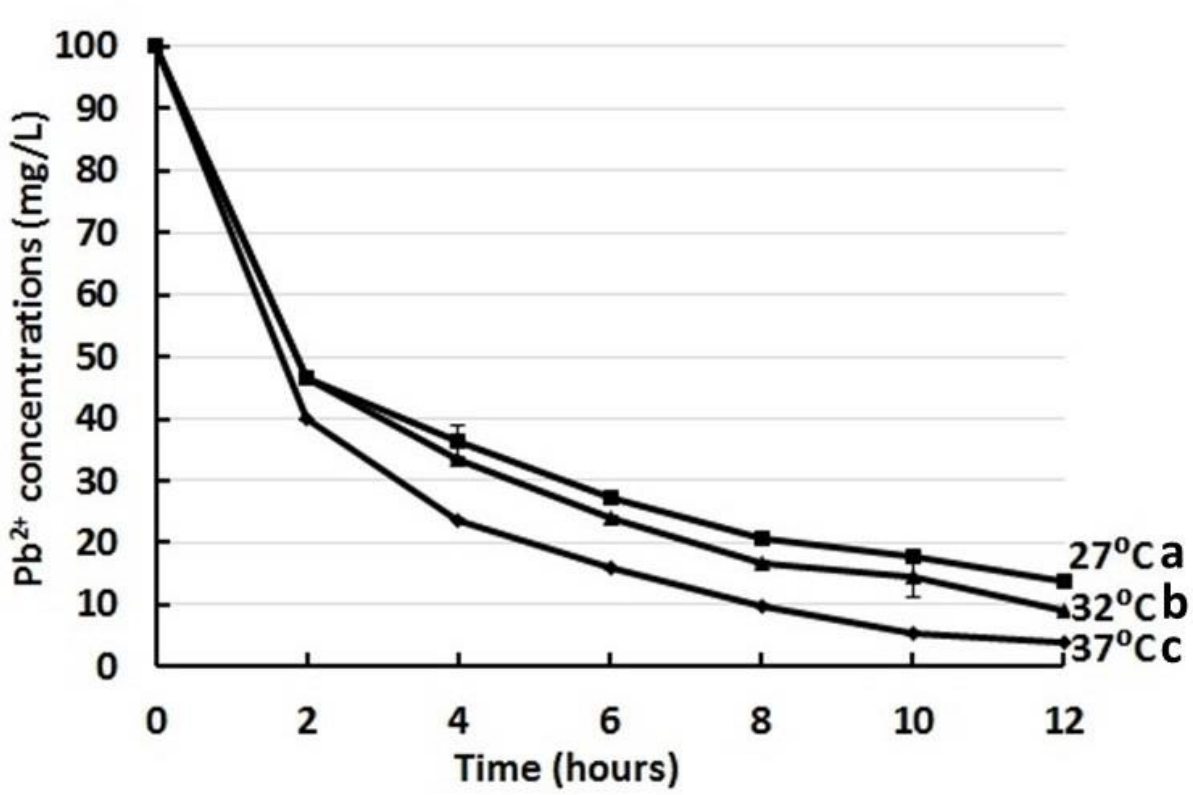

Figure 23: Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution containing 100 mg/L of Pb^{2+} at different temperatures 27, 32 or 37°C at pH 7.0. Values carrying different super script letters (a, b, c) are significantly different at $p < 0.05$.

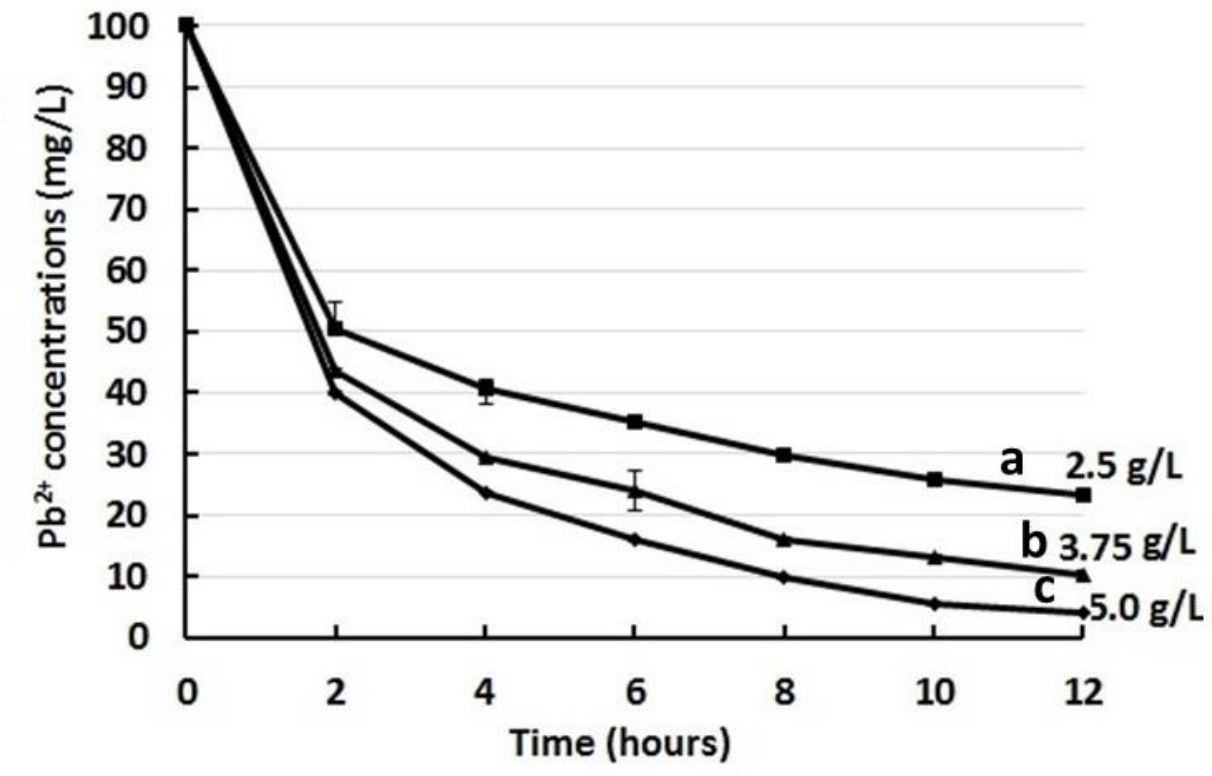

Figure 24: Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 2.5, 3.75 or 5.0 g/L of *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution (pH 6.0) containing 100 mg/L of Pb^{2+} at 37°C. Values carrying different super script letters (a, b, c) are significantly different at $p < 0.05$.

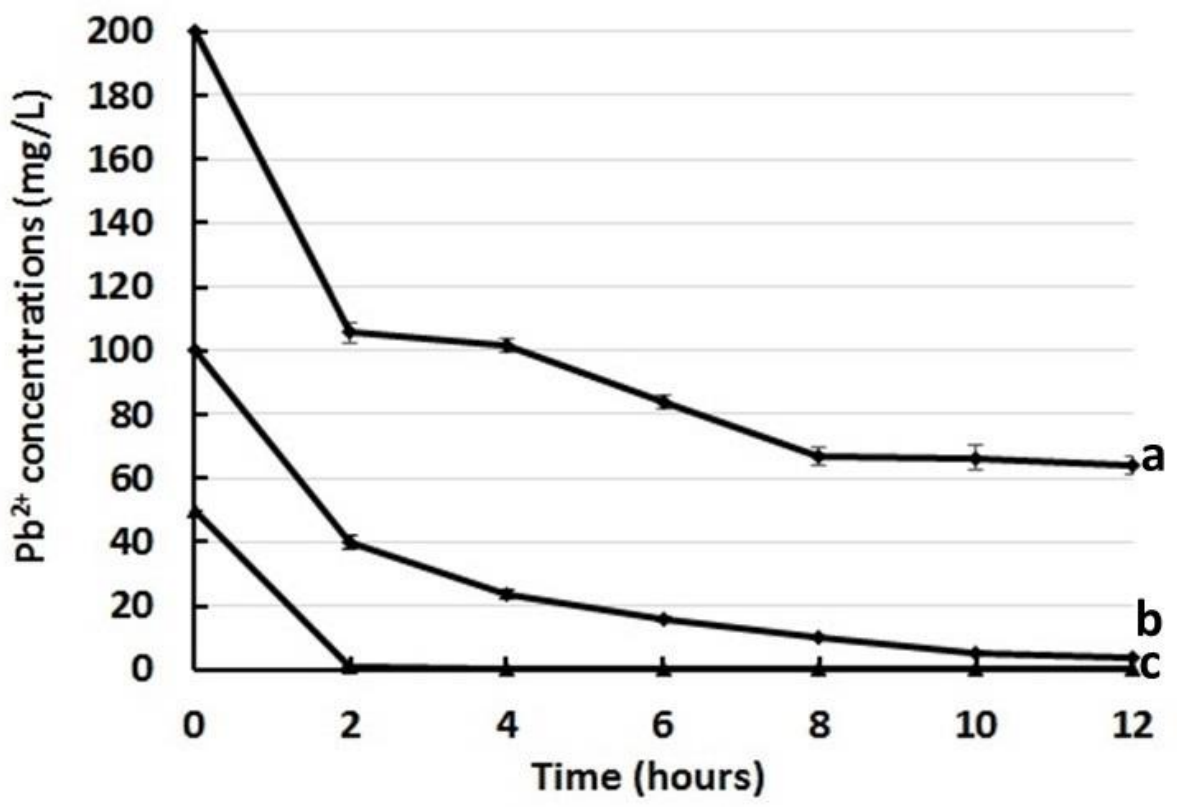

Figure 25: Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution (pH 6.0) containing 50, 100 or 200 mg/L of Pb^{2+} at 37°C. Values carrying different super script letters (a, b, c) are significantly different at $p < 0.05$.

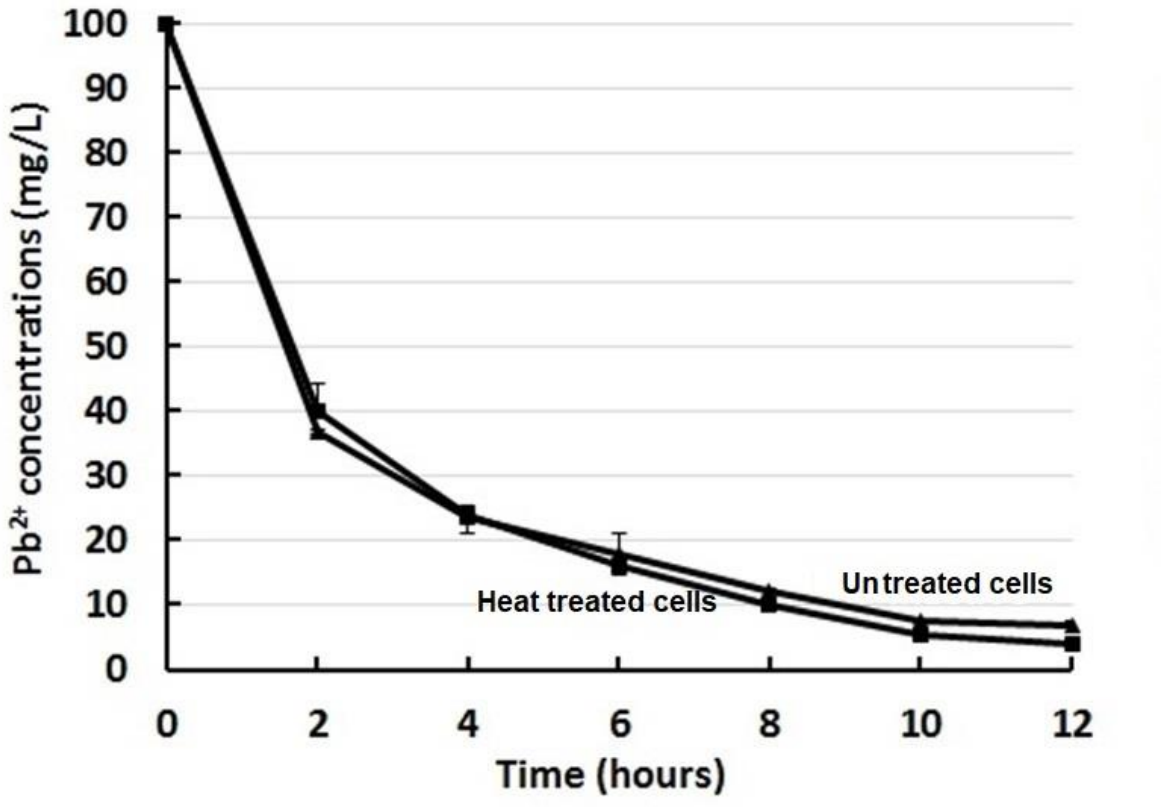

Figure 26: Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of heat-treated or untreated *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution (pH 6.0) containing 100 mg/L of Pb^{2+} at 37°C/180 rpm. For heat treatment process, cells dissolved in 0.9% NaCl solution were incubated at 60°C for 1 hour. Each measurement was repeated three times for calculation of averages and standard deviation.

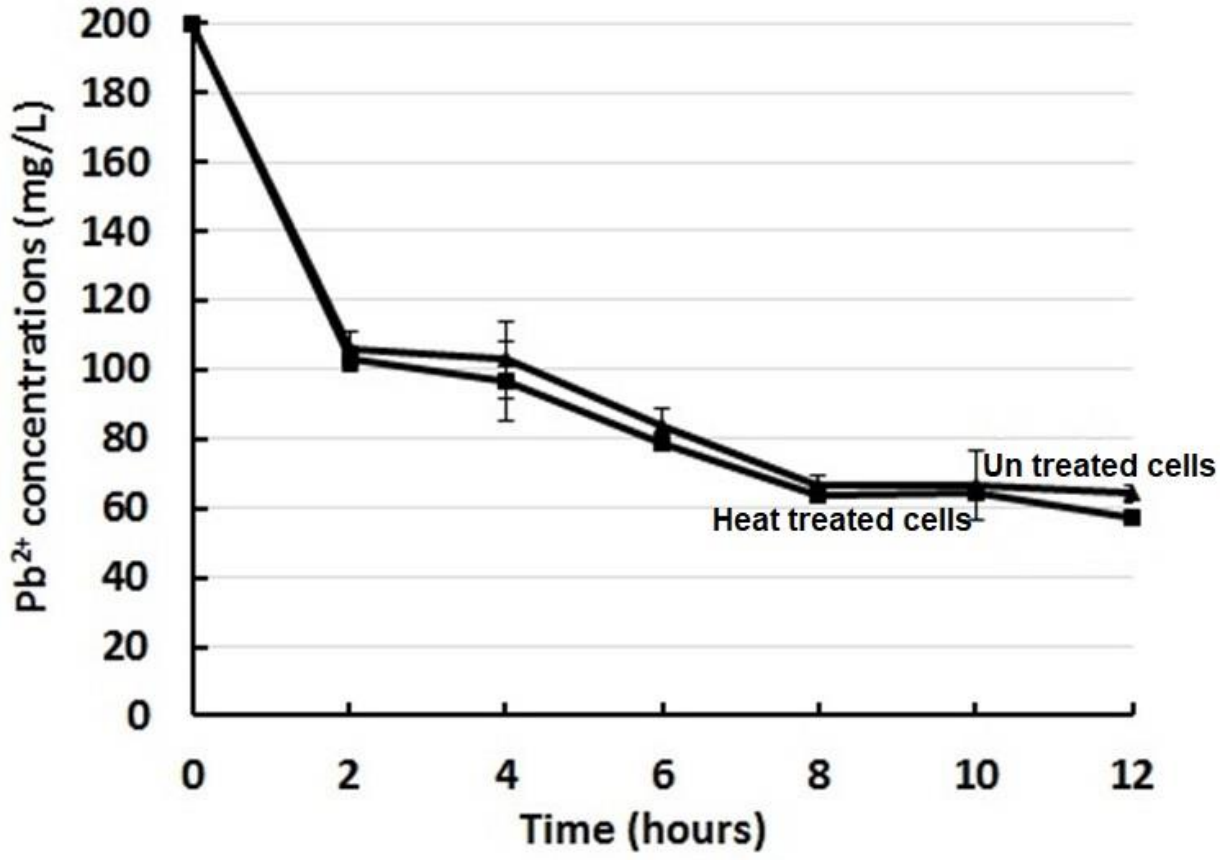

Figure 27: Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of heat-treated or untreated *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution (pH 6.0) containing 200 mg/L of Pb^{2+} at 37°C/180 rpm. For heat treatment process, cells dissolved in 0.9% NaCl solution were incubated at 60°C for 1 hour. Each measurement was repeated three times for calculation of averages and standard deviation.

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Mohamed Shehata

PUBLICATIONS

Shehata M. and Yamazaki K. (2017): Novel method for removal of Pb^{2+} from drinking water using recombinant *E. coli*. Int J Waste Resour 2017, 7:4. Doi: 10.4172/2252-5211-C1-008-33.

Shehata M. and Yamazaki K. (2018): Using Recombinant *E. coli* Displaying Surface Heavy Metal Binding Proteins for Removal of Pb^{2+} from Contaminated Water. J Bioremediat Biodegrad 9: 442. Doi: 10.4172/2155-6199.1000442.