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Characterization of Cd(II)/Pb(II) transcriptional regulator *Alcaligenes faecalis* SF-S1-60

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ABSTRACT

Aims: Metal transcriptional regulators controlled the regulation of metal ion homeostasis in bacteria genera. Cd(II)/Pb(II) transcriptional regulator is one of the member of MerR family found in *Alcaligenes faecalis* SF-S1-60 (PbrT-AF).
Methodology and results: The *PbrT-AF* gene with 432 bp open reading frame was successfully isolated from genomic DNA of *A. faecalis* using polymerase chain reaction (PCR) analysis. This gene was phylogenetically grouped with *A. alcaligenes* species using PHYLIP version 3.69 by the neighbor-joining method with 1000 bootstrap replicates. Phylogeny analysis shows that these proteins have distinct amino acids compared to Cd(II)/Pb(II) regulators from different species. The structure of PbrT-AF shows similar conformation with other members of MerR family using MODELLERv9.17. We also demonstrated that the expression of *Pbrt-AF* in *Escherichia coli* BL21 were able to increase the bacteria tolerance towards Pb up to 1000 ppm.
Conclusion, significance and impact of study: This result suggests that *PbrT-AF* promotes cell adaptation and tolerance towards Pb toxicity.

Keywords: Cd(II)/Pb(II) transcriptional regulator, MerR family, *Alcaligenes faecalis*, plumbum resistance

INTRODUCTION

Metals with atomic density larger than 5 g/cm^3 are classified as heavy metals (Nies, 1999). Heavy metals are not biodegradable; therefore, it can be accumulated in environment, human, plants, animal and microorganisms (Bhattacharyya *et al*., 2008; Issazadeh, 2013) and are group into essential and non-essential. Essential heavy metals such as zinc, nickel, manganese and cobalt are requisite for organisms' development in a trace quantity (Filali *et al*., 2000). Non-essential heavy metals including lead, mercury, cadmium and chromium do not have biological roles and small amount of these substances can cause damage to organisms and environment (Ghosh and Singh, 2005). Heavy metals that are concern for the ecosystem and human are categorized into three groups i.e toxic metals (mercury, chromium, lead, zinc, copper, stannum, nickel, cadmium, cobalt, arsenic), precious metals (aurum, palladium, platinum, argentum, ruthenium) and radionuclides (uranium, americium, thorium, radium) (Järup, 2003; Wang and Chen, 2006). Lead (Pb) together with cadmium (Cd) is the prime pollutants in the environment and these two heavy metals can cause severe damage to cell membranes, enzymes and the structure of deoxyribonucleic acid (DNA) (Olaniran *et al*., 2013). In Malaysia, these pollutants lead to serious deterioration of human health status due to unhealthy effects through consumption of contaminated fish (Nor Hasyimah *et al.*, 2011).

Strategies such as filtration, reverse osmosis, chemical precipitation, ion exchange and evaporation recovery electrochemical have been demonstrated to be able to remove heavy metals from the environment (Keng *et al*., 2014). Nonetheless, most of these techniques are expensive, less efficient and labor-demanding operational (Jaishankar *et al*., 2014). Alternative strategy using microorganism has been suggested as a number of microbes have evolved and gain the ability to tolerate metals (Nanda *et al*., 2011). These microorganisms use the heavy metals as their source of energy by breaking down the contaminants to a less toxic form (Alluri *et al*., 2007).

The ability to regulate metal ion is a vital key for bacterial cell survivality (Smith *et al*., 2009). The first metal ion transcriptional regulator, mercury resistance transcriptional factor regulator was discovered 30 years ago (Brown *et al*., 1983). Since then, a series of regulators that are sensitive to various metal ions and

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small organic molecules have been identified and characterized in a wide range of bacterial genera. Currently, there are seven known metal ion transcriptional regulators: ArsR-SmtB, MerR, CsoR-RcnR, CopY, DtxR, Fur and NikR (Pennella and Giedroc, 2005; Osman and Cavet, 2010). The metal-responsive MerR protein family are inclusive of protein sensors for mercury MerR (Chang *et al*., 2015), copper CueR (Changela *et al*., 2003), zinc ZntR (Smith *et al*., 2009), cadmium CadR (Wu *et al.*, 2009), plumbum PbrR (Borremans *et al*., 2001), aurum GolS (Checa *et al*., 2007), oxidative stress responder SoxR (Watanabe *et al*., 2008) and multidrug transporter (Heldwein *et al*., 2001; Raju and Sharma, 2017).

All these regulators share high sequence similarity, especially in the N-terminal DNA-binding domain whilst the varied C-terminal domains are responsible for the specific effector recognition. The Cd(II)/Pb(II) transcription regulator belongs to the MerR family and this protein has a combined function of uptake, efflux and accumulation of Pb(II).

In the present study, the Cd(II)/Pb(II) transcription regulator from *A. faecalis* SF-S1-60 designated as PbrT-AF was characterized. Sequence analysis showed that *PbrT-AF* has high sequence similarity with other members in MerR family. The secondary structure of PbTr was also found to be conserved among the homologs. It was also demonstrated that with the addition of *PbrT-AF* gene in *E. coli*, it enhances the uptake of lead up to 1000 ppm.

MATERIALS AND METHODS

Bacterial strains and media

Alcaligenes faecalis was isolated from the crude oil-contaminated soil of Kerteh, Terengganu, Malaysia. Meanwhile, *E. coli* BL21 was purchased from New England Biolabs (NEB, Ipswich, MA). These bacteria were grown in nutrient agar and nutrient broth at 37 °C for 24 h. For the Pb(II) bioremediation analysis, the cells were grown in Mineral Salts Medium (MSM) (Zajic and Supplisson, 1972). The MSM was prepared by dissolving 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4{\cdot}7H_2O$ in 1 L distilled water. The pH of the medium was adjusted to 7.0 and autoclaved at 121 °C for 15 min. The medium was supplemented with 0.1% trace element ($MnSO_4{\cdot}7H_2O$ 0.1 g/L, $CuCl_2$ 0.025 g/L, $NaB_4O_7{\cdot}10H_2O$ 0.025 g/L, $Co(NO_3)_2{\cdot}6H_2O$ 0.025 g/L, $ZnCl_2$ 0.025 g/L, NH_4NO_3 0.01 g/L and $(NH_4)O_7O_{24}{\cdot}H_2O$ 0.025 g/L) (Bouchez *et al*., 1995). After the MSM was autoclaved, $Pb(NO_3)_2$ was added at the required concentrations (0-1500 ppm).

Nucleic acid extraction

Alcaligenes faecalis genomic DNA was extracted using boiling method (Holmes and Quigley, 1981). Briefly, the bacteria were grown on nutrient agar at 37 °C overnight. A single colony was picked and added to 40 µL of 10 mM Tris-HCL pH 7.4, 1 mM EDTA, pH 8.0. The bacterial suspension was heated at 100 °C for 10 min and centrifuged for 4 min at 4 °C. The supernatant was collected, and agarose gel electrophoresis was conducted to detect the extracted *A. faecalis* DNA.

Amplification of full length *Pbrt-AF* gene

The *PbrT-AF* gene was amplified by polymerase chain reaction (PCR) using primers PbTr_F 5'-ATGAAAATTGGTGAGTTGGCG-3' (forward) and PbTr_R 5'-TTAGCCCAGATGAGTGCTGGG-3' (reverse). The PCR mixture contain 5 × PCR buffer, 25 mM $MgCl_2$, 10 mM DNTP, 2.5 U *Taq* DNA polymerase, 10 mM PbTr_F and PbTr_R. All the PCR components were from *GoTaq*® *Flexi DNA Polymerase* kit supplied by Promega, USA. Each reaction was performed to a final volume of 25 µL.

PCR amplification was performed as follows: melting of DNA at 95 °C for 3 min, followed by 30 cycles of amplification at 95 °C for 30 sec, 48 °C for 45 sec and 72 °C for 45 sec. A final step was performed at 72 °C for 10 min. The PCR product was visualized on a 1% agarose gel.

Construction of recombinant plasmid vector

The PCR product with the expected size was cloned into pJET1.2 cloning vector (Thermo Fischer, USA). The constructed recombinant plasmid was confirmed by PCR plasmid and further analysis via DNA sequencing was performed to validate that the correct gene has been ligated into the cloning vector. A 432 bp gene fragment was isolated from the cloning plasmid and ligated into the expression plasmid, pET-28a (Novagen, Germany). The recombinant expression vector carries *PbrT-AF* gene was transformed into *E. coli* BL21 (DE3). Bacterial cells with the recombinant plasmid were designated as *E. coli*_PbrT-AF.

Sequence and phylogeny analysis

The sequence was compared with other CdR/PbR genes from bacteria using NCBI BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for their pairwise identities. The GeneBank accession number of each sequence was as follows: WP_021446839.1 (*Alcaligenes* sp.), WP_094196082.1 (*A. faecalis*), WP_012806076.1 (*Candidatus Accumulibacter phosphatis*), WP_009664870.1 (*Oxalobacteraceae bacterium* IMCC9480), WP_041224358.1 (*Dechloromonas aromatica*), WP_089083338.1 (*Aquitalea magnusonii*), WP_062787683.1 (*Aquitalea pelogenes*), WP_061175432.1 (*Burkholderia pedi*), WP_033537018.1 (*Caballeronia zhejiangensis*), WP_010107182.1 (*B. oklahomensis*), WP_038791806.1 (*B. pseudomallei*), WP_009906678.1 (*B. thailandensis*), WP_017923564.1 (*B. glumae*), WP_035482057.1 (*Paraburkholderia phenoliruptrix*), WP_027799112.1 (*P. dilworthii*) and WP_074301137.1 (*P. phenazinium*). Multiple sequence alignment of these sequences was carried out by Jalview. Phylogenetic tree was built using PHYLIP version 3.69 by

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the neighbor-joining method with 1000 bootstrap replicates.

Homology modeling of PbrT-AF protein

The structure of PbrT-AF was modeled based on the structure of *Ralstonia metallidurans* CH34 lead transcription regulator (PDB code 5GPE). The template searched was done using HHpred (http://toolkit.tuebingen.mpg.de/hhpred; Biegert *et al*., 2006) and 5GPE has the highest sequence similarity (51%) with PbrT-AF. The structure was constructed using MODELLERv9.17 (Webb and Sali, 2014). Evaluation of the structure was done via RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage2.php). The structure was viewed using PyMOL (v.13; Schrodinger).

Analysis of *PbrT-AF* effect on *E. coli* growth

A single colony of *E. coli* cells that contain expression vector carrying *PbrT-AF* were grown overnight in 50 mL sterile nutrient broth. The cultures were incubated at 37 °C with agitation of 150 rpm. Thereafter, the cells were harvested by centrifugation at 4000 rpm at 4 °C for 15 min. The pellet was resuspended in 0.85% saline until the optical density reached a value of approximately 0.5 at 550 nm wavelength.

A 10% of the resuspended cells were mixed into 20 mL sterile MSM containing different concentrations of Pb(II) (50, 100, 500, 1000 and 1500 ppm). These Pb concentrations were modification from the previous study by Garhwal *et al*. (2014). The experiment was also conducted in MSM without $Pb(NO_3)_2$ as a negative control. The culture was diluted up to seven times and 100 µL of the mixture was spread onto NA plate followed by incubation overnight at 37 °C. The colonies formed were analysed by calculating the colony forming unit (CFU/mL). All the experiments were carried out in three replicates.

RESULTS AND DISCUSSION

Sequence analysis *of PbrT-AF*

The *PbrT-AF* gene was successfully isolated from genomic DNA of *A. faecalis* (Figure 1). Based on the sequence analysis, the full length open reading frame of *PbrT-AF* gene was 432 bp encoding 144 amino acids (Figure 2) with a theoretical mass of 16.4 kDa. As revealed by the sequence alignment analysis with other protein in MerR family, PbrT-AF protein contains three cysteine residues (Cys77, Cys 112 and Cys 121) which may be involved in the Pb(II) binding (Figure 3).

The amino acid encoded by *PbrT-AF* open reading frame showed striking similarity to members of MerR family that involved in metal ion regulation. The gene exhibited close relationship with *Alcaligenes* sp. (WP_021446839) with 99% sequence similarity. Sequence analysis also revealed that the PbrT-AF protein has conserved residues (Lys2, lle3, Arg18, Asn35, Tyr36

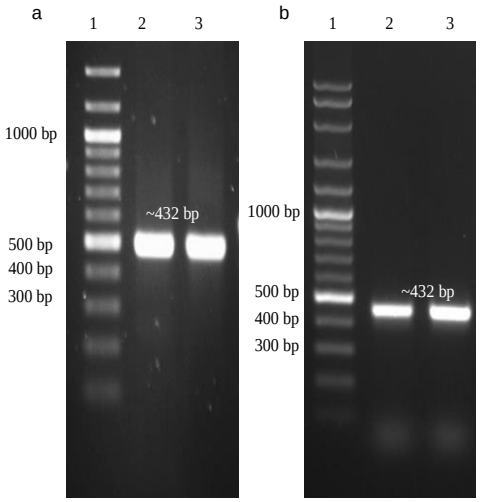

Figure 1: PCR of the (a) genomic DNA of A. *faecalis* and (b) *E. coli* colony shows the presence of *PbrT-AF* gene fragment. Lane 1: DNA marker 100 bp (Vivantis), Lane 2 and 3: PCR products.

```
Atgaaaattggtgagttggcggaaaaaagccactgcaacacggaaaccatccgctattac
 M  K  I  G  E  L  A  E  K  S  H  C  N  T  E  T  I  R  Y  Y
gagaaggtcggactgctgcccgagccggaacgcagccgagggcaactatcgcctgtaccgc
 E  K  V  G  L  L  P  E  P  E  R  S  E  G  N  Y  R  L  Y  R
aatatccacttggaacggctacgttttattcgcaactgccgcagcctggatatgacacac
 N  I  H  L  E  R  L  R  F  I  R  N  C  R  S  L  D  M  T  H
gaagaaatccgcggcctgctggcatttatggacggcccggcgcaagactgcgccccggtg
 E  E  I  R  G  L  L  A  F  M  D  G  P  A  Q  D  C  A  P  V
aatgatttgctggatgagcatatcgagcatgtagccgtgcgcatcgcggaactggaacac
 N  D  L  L  D  E  H  I  E  H  V  A  V  R  I  A  E  L  E  H
ctgcaaaaacaactgattgaattgcgccatcgctgcgggcaggccggtggcctggagcat
 L  Q  K  Q  L  I  E  L  R  H  R  C  G  Q  A  G  G  L  E  H
tgcggcatcttgcaaggtttggtgaatatggagccagatcaagtcccggctcccagcact
 C  G  I  L  Q  G  L  V  N  M  E  P  D  Q  V  P  A  P  S  T
catctgggctaa
 H  L  G  -
```

Figure 2: Nucleotide and the deduced amino acid sequences of *PbTr-AF* of *A. faecalis*. Bold letters indicated the start and stop codons.

and Arg37) that may function as DNA binding domain at the N-terminal region. This region was predicted to exhibit a helix-turn-helix motif (Brown *et al*., 2003). An alignment of amino acid sequence and phylogenetic tree was constructed to determine the similarity between PbrT-AF protein and other known MerR regulators.

Phylogenetic analysis

PbrT-AF was aligned with 16 members of MerR family and a phylogenetic tree was constructed (Figure 4). The *A. faecalis* SF-S1-60 *PbrT-AF* was phylogenetically grouped with *A. alcaligenes* species indicating that these proteins have distinct amino acids compared to Cd(II)/Pb(II) regulators from different species.

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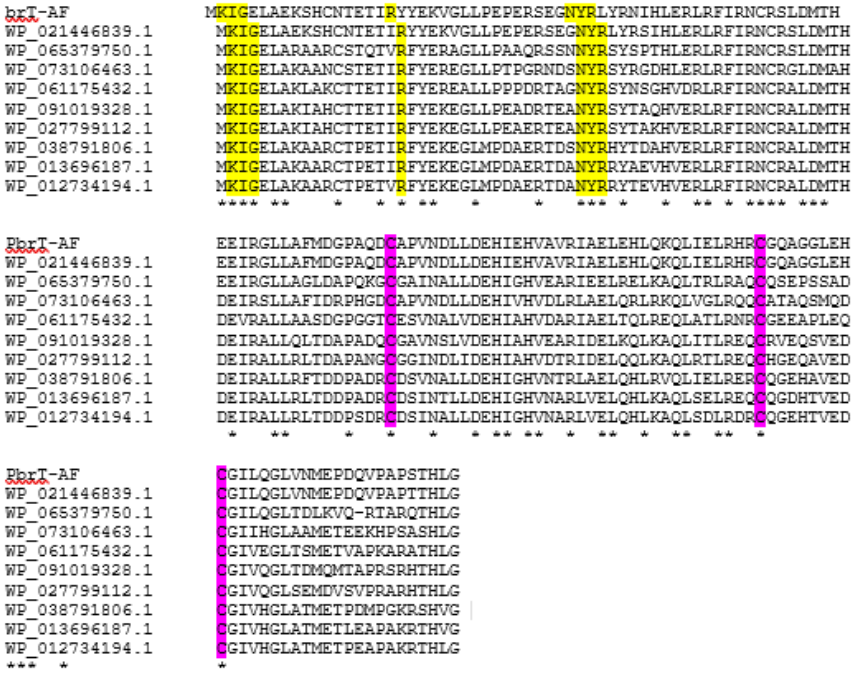

Figure 3: Protein sequence alignment of PbrT-AF with 9 Cd(II)/Pb(II)-responsive transcriptional regulators from different organisms. Conserved residues were labeled with star below the sequences, nucleic acid binding residues were highlighted with yellow background and metal binding site was highlighted with purple background. PbTr-AF was aligned with WP_021446839.1 (*Alcaligenes* sp.), WP_065379750.1 (*C. Glomeribacter gigasporarum*), WP_073106463.1 (*C. bauzanensis*), WP_061175432.1 (*B. pedi*), WP_091019328.1 (*P. megapolitana*), WP_027799112.1 (*P. dilworthii*), WP_038791806.1 (*B. pseudomallei*), WP_013696187.1 (*B. gladioli*) and WP_012734194.1 (*B. glumae*).

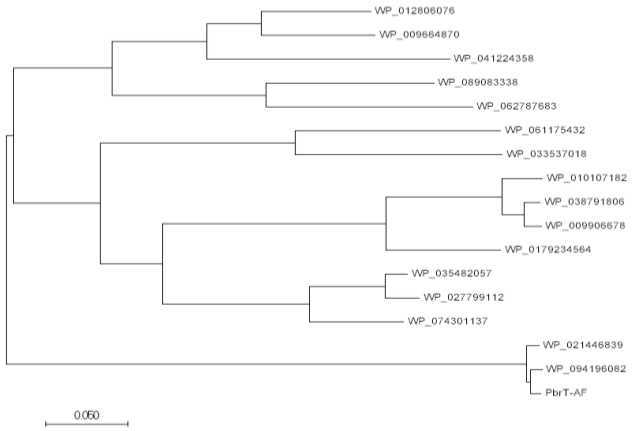

Figure 4: Phylogenetic tree to indicated the relationship between *PbrT-AF* and 16 other Cd(II)/Pb(II) transcription regulator genes from different organisms. GenBank accession numbers are as follows: WP_012806076 (*Candidatus Accumulibacter phosphatis*), WP_009664870 (*Oxalobacteraceae bacterium* IMCC9480), WP_041224358 (*Dechloromonas aromatic*), WP_089083338 (*Aquitalea magnusonii*), WP_062787683 (*Aquitalea pelogenes*), WP_061175432 (*Burkholderia pedi*), WP_033537018 (*Caballeronia zhejiangensis*), WP_010107182 (*Burkholderia oklahomensis*), WP_038791806 (*Burkholderia pseudomallei*), WP_009906678 (*Burkholderia thailandensis*), WP_035482057 (*Paraburkholderia phenoliruptrix*), WP_027799112 (*Paraburkholderia dilworthii*), WP_074301137 (*Paraburkholderia phenazinium*), WP_021446839 (*Alcaligenes* sp.) and WP_094196082 (*Alcaligenes faecalis*).

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Overall structure of PbrT-AF protein

Based on the previous solved structures of PbrT-AF, the protein exists as a homodimer. Structure of PbrT-AF protein was constructed using MODELLERv9.17 (Webb and Sali, 2014) to provide the molecular insight of this regulator. PbrT-AF protein structure was modeled based on the structure of lead transcription regulator from *R. metallidurans* CH34 (PDB code 5GPE) (Huang *et al*., 2016). The structure shows similar conformation with other members of MerR family, which N-terminal was characterized with helix-turn-helix DNA binding region whilst the C-terminal domain consist of a long coiled-coil architecture that act as the dimer interface (Figure 5). The superimposed of 5GPE and PbrT-AF structure gave an rmsd of 0.41 based on 128 residues in Cα position. By using RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage2.php), analysis of Ramachandran plot revealed that most of the amino acids are in preferred region (96%) whereas the other residues (4%) are in allowed region.

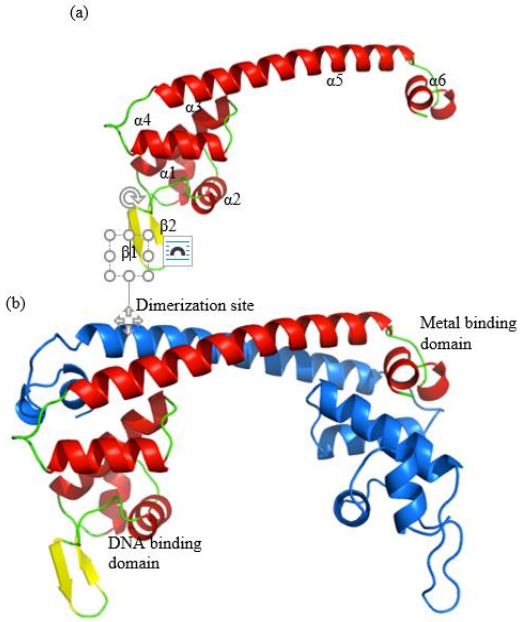

Figure 5: Model structure of PbrT-AF protein using 5GPE as a template. (a) monomer structure of PbTr-AF. The α-helices were shown in red, β-strands in yellow and coils in green. The α-helices and β-strands were designated as α1- α6 and β1- β2, respectively. (b) Homodimer structure of PbTr-AF.

PbrT-AF monomer is formed by six α-helices (Ile3-Ser10, Thr14-Val23, Asn41-Leu56, His60-Asp71, Ala78-Arg111 and Leu118-Leu127) and two β strands (Glu30-Glu33 and Tyr36-Leu38). Residues Phe49, Asn52, Leu56, Asp57, Ala78, Asn81, Leu84, Ile88, Arg94, Leu101, Gln102, Arg109 and Ile123) from helices (α3, α4 and α5) of both monomers form the dimerization sites. The metal binding site (Cys77, Cys 112 and Cys 121) has a distinct trigonal-pyramid conformation that recognizes lead according to the structural study of PbrR transcriptional regulator from *R. metallidurans* CH34 by Huang *et al*. (2016). Based on the analysis by Hobman *et al*. (2012), PbTr of *Cupriavidus metallidurans* AE104 lost its activity when mutagenesis study was carried out on the conserved cysteine residues. According to Pennella and Giedroc (2005), all MerR family members (MerR, CueR, ZntR and PbrR) shows the same motifs.

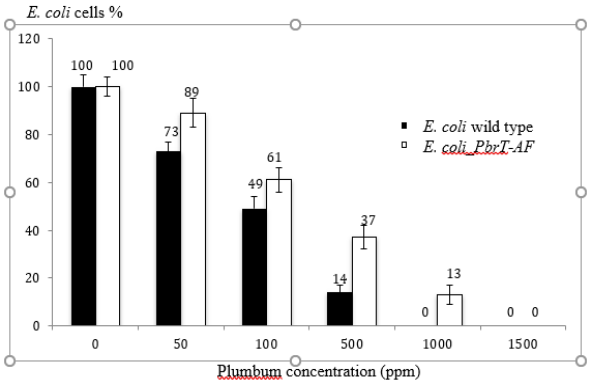

Figure 6: Profile of *E. coli* with and without *PbrT-AF* growth in media supplemented with different concentration of Pb(II). Vertical bars represent the means ± SD (n= 3).

Metal tolerance in *E. coli* harboring PbrT-AF

This analysis was done to assess the function of PbrT-AF in regulating the growth of *E. coli* in the presence or absence of lead. Different lead concentrations (50, 100, 500, 1000 and 1500 ppm) were tested in order to determine PbrT-AF effect. For each concentration, three replicates were performed.

From the analysis, it shows that *E. coli* cells with PbrT-AF protein demonstrated higher growth of colony compared to cells without PbrT-AF in media containing lead (Figure 6). It was also shows that, with the addition of PbrT-AF, the cells were able to absorb Pb(II) up to 1000 ppm in contrast to cells destitute of the transcriptional regulator from *A. faecalis*. As shown in Figure 6, the survival of *E. coli* cells with PbrT-AF was 89% whilst the cells without PbrT-AF have a growth of 73% in 50 ppm lead. At 500 ppm, *E. coli*_PbrT-AF cells showed 37% viability whereas *E. coli* without PbrT-AF only retained 14% cells growth. In addition, at 1000 ppm, *E. coli*_PbrT-AF cells have 13% cell viability whereas control *E. coli* showed a total cell loss. This result implicates that the survival rate of *E. coli* containing PbrT-AF was higher than *E. coli* without PbrT-AF in medium supplemented with lead. This outcome also revealed that with the addition of PbrT-AF, *E. coli* was able to enhance the metal ion regulation and uptake of the cells.

Previous studies on MerR family member shows that when the protein was expressed heterologously in *E. coli*, the cells increased its tolerance towards metal ion

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(Blencowe *et al*., 1997; Ladomersky and Petris, 2015). Higher concentrations of heavy metals affect the growth of microorganisms by inhibiting the cells growth that eventually lead to cell death (Jaishankar *et al*., 2014). However, according to Olaniran *et al*. (2013), microbes that capable of regulating and activating the heavy metals resistance gene has a better adsorption and able to survive the metal toxicity. According to Gaur and Adholeya (2004), heavy metals absorbed by microorganisms were converted into non-toxic substances. This action was the results of proteins modulated by a group of regulators that recognizes metals and activates the gene expression. Earlier studies revealed that all MerR proteins bind to specific operator/promoter at DNA sequences. When metals were detected in the cells, MerR proteins activate the DNA hence trigger the resistance genes expression (Hobman *et al*., 2012).

CONCLUSION

We have successfully isolated a Cd(II)/Pb(II) transcriptional regulator gene from *A. faecalis* and designated as *PbrT-AF*. The expression of *PbrT-AF* in *E. coli* proved that the gene was important for the bacterial survival in the presence of a very high concentration of Pb(II). We therefore concluded that the gene *PbrT-AF* plays a very significant role for bacterial cell adaptation towards Pb(II) toxicity. The ability of *PbrT-AF* to regulate the absorption of Pb(II) was up to 1000 ppm.

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