Cysteine coordination of Pb(II) is involved in the PbrR-dependent activation of the lead-resistance promoter, \( PpbrA \), from \textit{Cupriavidus metallidurans} CH34

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**Abstract**

**Background:** The \( pbr \) resistance operon from \textit{Cupriavidus metallidurans} CH34 plasmid pMOL30 confers resistance to Pb(II) salts, and is regulated by the Pb(II) responsive regulator PbrR, which is a MerR family activator. In other metal sensing MerR family regulators, such as MerR, CueR, and ZntR the cognate regulator binds to a promoter with an unusually long spacer between the \( -35 \) and \( -10 \) sequences, and activates transcription of resistance genes as a consequence of binding the appropriate metal. Cysteine residues in these regulators are essential for metal ion coordination and activation of expression from their cognate promoter. In this study we investigated the interaction of PbrR with the promoter for the structural \( pbr \) resistance genes, \( PpbrA \), effects on transcriptional activation of altering the DNA sequence of \( PpbrA \), and effects on Pb(II)-induced activation of \( PpbrA \) when cysteine residues in PbrR were mutated to serine.

**Results:** Gel retardation and footprinting assays using purified PbrR show that it binds to, and protects from DNase I digestion, the \( PpbrA \) promoter, which has a 19 bp spacer between its \( -35 \) and \( -10 \) sites. Using \( \beta \)-galactosidase assays in \textit{C. metallidurans}, we show that when \( PpbrA \) is changed to an 18 bp spacer, there is an increase in transcriptional activation both in the presence and absence of Pb(II) salts up to a maximum induction equivalent to that seen in the fully-induced wild-type promoter. Changes to the \( -10 \) sequence of \( PpbrA \) from TTAAAT to the consensus \( E. coli \) \( -10 \) sequence (TATAAT) increased transcriptional activation from \( PpbrA \), whilst changing the \( -10 \) sequence to that of the Tn501 mer promoter (TAAGGT) also increased the transcriptional response, but only in the presence of Pb(II). Individual PbrR mutants C14S, C55S, C79S, C114S, C123S, C132S and C134S, and a double mutant C132S/C134S, were tested for Pb(II) response from \( PpbrA \), using \( \beta \)-galactosidase assays in \textit{C. metallidurans}. The PbrR C14S, C79S, C134S, and C132S/C134S mutants were defective in Pb(II)-induced activation of \( PpbrA \).

**Conclusions:** These data show that the metal-dependent activation of PbrR occurs by a similar mechanism to that of MerR, but that metal ion coordination is through cysteines which differ from those seen in other MerR family regulators, and that the DNA sequence of the \( -10 \) promoter affects expression levels of the lead resistance genes.

**Keywords:** Metal-resistance, Metal-protein interactions, Metalloregulation, Bacterial gene expression

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Background

Lead (Pb) is a widely distributed, environmentally persistent, toxic metal. Most bacteria that are tolerant or resistant to lead either precipitate Pb in an insoluble form, or actively export it [1]. Although some metal efflux ATPases, such as ZntA from *Escherichia coli*, and CadA from *Staphylococcus aureus* plasmid p258, can export Pb(II) as well as Zn(II) and Cd(II) [2,3], the only characterized bacterial Pb(II) specific resistance system is from *Cupriavidus* (formerly *Wautersia* and *Ralstonia*) *metallidurans* CH34 [4,5] - a Gram-negative, multiply metal-resistant, β-proteobacterium originally isolated from a decantation basin at a Belgian zinc production plant (and originally identified as *Alcaligenes eutrophus* CH34; [6]). Over 150 genes in CH34 are involved in metal resistance, of which at least 70 are carried on the plasmids pMOL28 (171 kb) or pMOL30 (234 kb), and the remainder are carried on the 3.92Mb chromosome or on a 2.58 Mb second chromosome [7]. Plasmid pMOL30 carries the ccc (Cd(II), Zn(II), Co(II)), mer (Hg(II)), sil (Ag(I)), cop (Cu(II)) and pbr Pb(II) resistance operons [4,8].

The pbr lead resistance operon from pMOL30 was originally predicted to contain structural genes which encode PbrT, a putative Pb(II) uptake protein belonging to the ILT (Iron Lead Transporter) family [9], a P-type efflux ATPase (PbrA), a predicted inner-membrane protein (PbrB), a predicted prelipoprotein signal peptidase PbrC and a Pb(II) binding protein, PbrD. The regulator of the pbr operon was shown to be PbrR, which is a MerR family regulator [4,10]. Subsequent work has shown that the pbr operon also contains an interrupted orf, pbrLI upstream of pbrT [11,12] which is predicted to encode a putative inner membrane (Major Facilitator Family MFS1) permease gene, which is probably inactive, but still part of the pbr operon; and that PbrB/PbrC is a fusion protein [11,12], and encodes an inner membrane bound undecaprenyl pyrophosphate (C55-PP) phosphatase [5]. The pbr operon contains a predicted MerR-like promoter from which pbrRTU are transcribed on one DNA strand, and the pbrABCD genes are transcribed as a polycistronic message on the other [4,12]. The most recent work on the mechanism of lead resistance encoded by the pMOL30 pbr operon has proposed a model where Pb^{2+} induces expression of the pMOL30-encoded PbrABC by PbrR, as well as expression of zinc and cadmium efflux ATPase homologs ZntA and CadA which are carried on the chromosome or second chromosome. Each of these three ATPases is involved in exporting Pb^{2+} into the periplasm where inorganic phosphates produced by PbrB are involved in precipitating Pb^{2+} as insoluble lead phosphate. This model finds no role for PbrT, C, and D, yet some reports suggest PbrC may be required for the maturation or activity of phosphatase in the periplasm[5]. PbrR from pMOL30 (Rmet_5946) is related to several other PbrR-like regulators that have been identified in the C. *metallidurans* CH34 chromosome, including pbrR2 (Rmet_2303 also known as pbr691 [13,14] which is believed to regulate a cadA and a pbrC homolog on the chromosome, and pbrR3 (Rmet_3456 also known as pbr710) believed to regulate a zntA homolog on the second chromosome, both of which are believed to be involved in Pb^{2+} export [12]. There is evidence for only very low levels of cross-regulation of the pMOL30 PpbrA promoter by PbrR2 or PbrR3 [15].

Other metal-sensing MerR family members include those responding to cadmium (CadR; [16,17]), copper (CueR; [18-20], ActP; [21], SctR; [22]), zinc (ZntR, [23,24]; ZccR (Zn, Co, Cd), [25]) and gold (GolS, [26]). Metal-sensing MerR family regulators share many common features: they bind to and activate gene expression from promoters with unusually long spacer sequences of 19-20 bp between the −35 and −10 sequences, and contain cysteine and other amino acids that are essential in coordinating metals and activating gene expression [10,16,20,27-29].

The objectives of this study were to 1) Characterize the interaction between PbrR and the pbrA promoter, and study the effects on transcription of shortening the 19 bp spacer between the −35 and −10 sequences, and altering the −10 sequence of PpbrA; and 2) to investigate the importance of cysteine residues in PbrR activation of PpbrA in response to Pb(II) ions. To this end each of the cysteine residues in PbrR (C14, C55, C79, C114, C123, C132 and C134) were individually changed to serine residues and a double mutant (C132S, C134S) was created. The effects of these mutations on *in vivo* transcriptional activation in response to Pb(II) were determined in C. *metallidurans* using β-galactosidase assays.

Methods

Bacterial strains, plasmids and growth media

Bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown in LB broth [30] at 37°C. C. *metallidurans* strains were grown at 30°C in 869 medium, 284 Tris or 284 MOPS medium [4,6]. For β-galactosidase assays of PbrR-regulated PpbrA promoter activity, C. *metallidurans* strains were grown in 284 MOPS medium [4] minimizing any Pb(II) precipitation during growth. C. *metallidurans* strains were grown in SOB medium without MgSO4 [30] prior to electroporation of plasmids, and SOB medium containing MgSO4 after electroporation. Pb(II) induction was achieved by growth in PbNO3 and antibiotics were used at the following concentrations:- for *E. coli*: carbenicillin (Melford laboratories, UK), 200 μg/ml; chloramphenicol 25 μg/ml; kanamycin, 50 μg/ml and trimethoprim lactate 30 μg/ml (all from Sigma Chemical UK); for C. *metallidurans*: trimethoprim lactate 500 μg/ml.
DNA manipulations

DNA manipulations were as described by [30]. Oligonucleotides were synthesized by Alta Bioscience, the University of Birmingham; or MWG Biotech, Germany. The DNA sequence of all mutants and cloned PCR products were confirmed by sequencing using a PE Applied Biosystems Big Dye version 2.0 sequencing kit according to the manufacturer’s protocol, followed by analysis on an ABI 3700 sequencer in the Functional Genomics Laboratory, School of Biosciences, the University of Birmingham. The primers used for sequencing were: pMUforward and pMUreverse, complementary to the sequences flanking the multiple cloning site of pMU2385, and PbrApe for pMapbrR/PbrA clones (Table 2).

PbrR overexpression and purification

The pbrR gene was amplified from pMOL1139 using Vent® DNA polymerase (New England Biolabs) and the primers: pbrRATG (LIC) and pbrRTAG (LIC) (Table 2). The pbrR PCR product was annealed with plasmid pET32-LIC (Novagen), according to manufacturers’ recommendations. DNA sequencing using the primer T7 reverse (Table 2) was used to confirm the nucleotide sequence of the cloned fragment. The thioredoxin-PbrR fusion protein was overexpressed in E. coli BL21 (DE3) pLysS, purified and stored under reducing conditions as described in [23]. The thioredoxin- S tag was cleaved from the fusion protein using enterokinase, according to the manufacturer’s protocol (Novagen) and removed using S-tag affinity agarose. PbrR purity was estimated by PAGE analysis. The concentration of the purified protein was determined by Bradford assay [35].

Gel retardation and DNAse I protection assays of PbrA with PbrR

Gel retardation experiments were as described in [36], with initial experiments to determine PbrR DNA binding using a 1144 bp HindIII/Sall fragment from pMOL1139

Table 1: Bacterial strains and plasmids

| Bacterial strain | Properties or Genotype | Reference or source |
|------------------|------------------------|---------------------|
| E. coli TG2      | supE hisdA5 thiA (lac-proAB) F’ Δ(sel-recA)306: Tn10(Tc’ lacZΔM15 | [31] |
| BL21(DE3)pLysS   | F’ ompT hisdA5 (rps-lac) gal dcm (DE3) pLysS (Cm’)| Novagen |
| C. metallidurans |                         |                     |
| CH34             | Zn, Cd, Co, Pb, Cu, Hg, Ni and Cr resistance | [6] |
| AE104            | Plasmid-cured C. metallidurans strain- sensitive to toxic metals | [6] |
| Plasmid          | Description             | Reference or source |
| pET32LIC        | Ap’ Overexpression plasmid for ligation-independent cloning | Novagen |
| pET32LICpbrR     | Ap’ pbrR cloned into pET32LIC | This study |
| pMa5/8          | Ap’ Cm’ Mutagenesis vector | [32] |
| pMC5/8          | Ap’ Cm’ Mutagenesis vector | [32] |
| pMalpBR/PpbrA   | Ap’ Cm’ Mutagenesis vector with pbrR/PpbrA cloned in to it | This study |
| pMOL1139        | Km’, The pbr operon cloned into plasmid pRK8415 | B. Borremans |
| pMU2385        | Tp’ 13.3 kb low copy number lacZ reporter plasmid | [33] |
| pMUppbrA       | Tp’ pMU2385 containing the PpbrA promoter directing lacZ transcription | This study |
| pMUppbrA-1     | Tp’ pMU2385 containing the PpbrA promoter with a 1 bp deletion | This study |
| pMUppbracon    | Tp’ As pMUppbrA, but −10 sequence changed to E. coli consensus | This study |
| pMUppbrAmer    | Tp’ As pMUppbrA, but −10 sequence changed to mer promoter | This study |
| pMUppbrR/PpbrA  | Tp’, pMU2385 containing pbrR, PpbrA ΔpbrA directing lacZ transcription | This study |
| pMUppbrR145/PpbrA | As pMUppbrR/PpbrA, but Pbr C145 | This study |
| pMUppbrC55S/PpbrA | As pMUppbrR/PpbrA, but Pbr R C55S | This study |
| pMUppbrC79S/PpbrA | As pMUppbrR/PpbrA, but Pbr C79S | This study |
| pMUppbrC1145S/PpbrA | As pMUppbrR/PpbrA, but Pbr C114S | This study |
| pMUppbrC1325S/PpbrA | As pMUppbrR/PpbrA, but Pbr C132S | This study |
| pMUppbrC1345S/PpbrA | As pMUppbrR/PpbrA, but Pbr C134S | This study |
| pMUppbrC132S/C134S/PpbrA | As pMUppbrR/PpbrA, but Pbr C132S/C134S | This study |
| pUC21          | Ap’, high copy number cloning vector; ColE1 replicon | [34] |
| pUK21          | Km’, intermediate copy number cloning vector; p13A replicon | [34] |
| pUK21pbr1      | Km’, HindIII/Sall pbrR/PpbrA/ΔpbrA from pMOL1139 cloned into pUK21 | This study |
Table 2 Oligonucleotides used for site directed mutagenesis, and overexpression

| Oligonucleotide | Sequence | Description or reference |
|-----------------|----------|--------------------------|
| pbrR C14S       | 5’ CCA CCG GGG ATG CGG TGC 3’ | Primer extension/sequencing primer [4] |
| pbrR C55S       | 5’ CCA GAG ACC GGG AGT GAC G 3’ | Primer to amplify PbrR |
| pbrR C79S       | 5’ GAC TTC ACC GGA ATC CTG G 3’ | Primer to amplify PbrR |
| pbrR C114S      | 5’ GCC ACC AGA AGA GGC TTC G 3’ | Primer to amplify PbrR |
| pbrR C123S      | 5’ GGA AAT TTA CCA AGT CA 3’ (primer A) | Primer to amplify PbrR |
| pbrR C132S      | 5’ CTT AAT CCC CAC ATC ACC AG 3’ (primer B) | Primer to amplify PbrR |
| pbrR C134S      | 5’ GGA ATC AGA GCT GCA GTC GCA G 3’ | Primer to amplify PbrR |
| pbrR Ape        | 5’ GCC CCA ACC GTG CTC GGT TCT GGG 3’ | Primer extension/sequencing primer [4] |
| pbrBstEII       | 5’ GCG AAT GGT CAC CAC CCG 3’ | Primer to amplify PbrR |
| pbrNruI         | 5’ GCT TGT CGC GAA TCA GGC 3’ | Primer to amplify PbrR |
| pMU forward     | 5’ GAT TCC CCC CAC ATC ACC AG 3’ | Sequencing primer for pMU2385 |
| pMU reverse     | 5’ TGC CAG CAT TTC ATA ACC AA 3’ | Sequencing primer for pMU2385 |
| M13-F           | 5’ GCC CAG GGT TTT CCC AGT CAC GAC 3’ | Sequencing primer for pUK plasmids |
| M13-R           | 5’ CAG GCA TTA ACA ATG TCA CAC AGG 3’ | Sequencing primer for pUK plasmids |
| compbr:         | 5’ CTAGAGGGTTAATCGGCAAC 3’ | PbrR mutagenesis primer |
| mempr:          | 5’ CTAGAGGGTTAATCGGCAAC 3’ | PbrR mutagenesis primer |
| -1EcoPbr        | 5’ GGG GAA TTC GAA GCT TGC 3’ (3’ primer) | PbrR mutagenesis primer |
| -1CentreBam     | 5’ GCC GAT TTA AAC CAC CTA GT 3’ (primer B) | PbrR mutagenesis primer |
| -1CentreEco     | 5’ CGG CTA AAT TTT GGA GAT CA 3’ (primer A) | PbrR mutagenesis primer |
| -1BamPbr        | 5’ CAG TAT ACC GCA GCT GGC 3’ (5’ primer) | PbrR mutagenesis primer |
| pbrR ATG (LIC)  | 5’ GAC GAC GAC AAG ATG AAT ATC CAG ATC GGC 3’ | PbrR cloning and overexpression primer |
| pbrR TAG (LIC)  | 5’ GAG GAG AGG CCC GGT CTA GTC GCT TGG ATG GGC 3’ | PbrR cloning and overexpression primer |
| T7 terminator   | 5’ CGA TCA ATA AGC AGT CGC C 3’ | Sequencing primer |

Underlined bases highlight alteration from the wild-type sequence.

containing pbrR, PbrR and a truncated pbrA (positions 409 and 1553 on the pbr operon) [4] cloned into pUK21 [34] to make plasmid pUKpbr1. pUKpbr1 was digested with NruI/BstEII and end labelled with [γ^32P]-dATP for gel retardations. Further gel retardation and footprinting experiments used a 296 bp PbrA PCR product, amplified from pMOL139 using the primers pbrBstEII and pbrNruI (Table 2) and labelled using [γ^32P]-dATP. DNAase I protection assays of PbrA with PbrR were as described by [37], using the 296 bp PbrA promoter PCR product detailed above. The DNA sequence of the region was obtained from the 296 bp PbrA PCR product using the pbrApe primer (Table 2) [4] and run alongside the DNAase I footprint (Figure 1B).

Cloning of pbrR-PbrR.Delta pbrA and mutagenesis of the PbrR cysteines

All cloning and mutagenesis work was done in E. coli K-12 TG2. The 1144 bp pbrR-PbrR.Delta pbrA DNA fragment described above was cloned into pMa5/8 [32] from pUK21pbr1 using the flanking EcoRI and BamHI sites to make pMaPbrR/PbrR. Gapped duplex mutagenesis of each of the cysteine residues in pbrR was as previously described [32] using the primers pbrRC14S, pbrRC55S, pbrRC79S, pbrRC114S, pbrRC123S, pbrRC132S, pbrRC134S, or pbrRC132S, C134S (Table 2), and mutants verified by DNA sequencing as described [15]. The wild type and mutant pbrR genes on the 1144 bp pbrR-PbrR.Delta pbrA DNA fragment were individually sub-cloned as EcoRI - BamHI fragments into pMU2385 [33] as described previously [15]. The resulting constructs contained a self-regulating transcriptional unit, with PbrR controlling the transcription of pbrR through PbrR and regulating transcription of lacZ in pMU2385 on the other DNA strand through PbrA. These constructs were the basis of the studies of the regulation of PbrA by PbrR in C. metallidurans AE104.

Cloning and mutagenesis of PbrA

A 266 bp Sphek - NruI fragment containing the PbrA promoter (positions 1062 and 1328 of the pbr operon) was cloned from pMOL139, into the HindIII site of pUK21, by rendering the vector and insert blunt-ended using T4 DNA polymerase. The cloned PbrA DNA fragment was sub-cloned as an EcoRI - BamHI fragment into pMa5/8 for site directed mutagenesis. The –10 sequence of PbrA
was mutated as described above using the primers conpbr and merpbr (Table 2) to change the PpbrA –10 sequence from TTAAAT (wild type) to TATAAT (consensus) or TAAGGT (mer-like). The mutant PpbrA promoters were cloned into pMU2385 using EcoRI and BamHI, creating plasmids pMUPpbrA(con) and pMUPpbrA(mer) in which the pbrA promoter regulates expression of the lacZ gene. After DNA sequencing, the activity of these mutant promoters was assayed in C. metallidurans CH34.

Construction of the PpbrA –1 mutant
Mutagenic PCR [38] of the 1144 bp pbrR-PpbrAΔpbrA DNA fragment from pMapbrR/PpbrA was used to construct the –1 promoter mutant of PpbrA, using the primers A) Gel retardation of PpbrA with PbrR. Each reaction contained the same amount of 32P-end-labelled 296 bp PpbrA PCR product (60 fmol). Lanes 1, 9 and 10 contained no PbrR. PbrR concentrations in lanes 2–8 and 11–17 increase 2-fold from 0.3 to 19.2 pmol. Lanes 10–17 contained 10 μM Pb(II). (b) DNase I protection assay of PbrR bound to the 296 bp PCR product containing the PbrA promoter. Lanes AGCT, DNA sequence of the 296 bp PCR product pbrA promoter, using the pbrApe primer. Lanes 1 and 4, no added pbrR, lane 2 and 3 increasing amounts of added PbrR. (c) Diagram of the PpbrA promoter. The transcript start site is marked in bold and indicated with an arrow [4]. The region of the promoter protected by PbrR from DNAase I digestion is marked with a box. The predicted –35 and –10 sequences are marked in bold, and the dyad symmetrical sequence is marked with arrows.

Figure 1
β-galactosidase assays in *C. Metallidurans*

pMU2385 plasmid constructs were electroporated into *C. metallidurans*, and cultures containing pMU2385 derivatives were assayed for β-galactosidase activity as described in [39] with modifications described by [15].

**Results**

**PbrR binds to the pbrA promoter and pb(II) decreases the binding affinity of PbrR to pbrA in vitro**

PbrR was overexpressed as a thioredoxin-his Tag-S tag-fusion protein using the pET32-LIC expression system, purified and released after enterokinase digestion as untagged, full length PbrR, as described in Materials and Methods. The PbrR preparation was estimated as being >95% pure PbrR by Coomassie Blue staining of standard SDS-PAGE gels (data not shown). We had originally identified a candidate PbrR gel retardation assays on 32P-end-labelled DNA (Figure 2) and no other fragments from the plasmid (data not shown). Addition of PbrR to the end-labelled 296 bp PbrRA PCR product retarded this fragment, and addition of Pb(II) to PbrR and PppbrA increased the amount of PbrR required to retard the PppbrA DNA fragment (Figure 1A) indicating that PbrR-Pb(II) had a lower affinity in vitro with PppbrA than did apo-PbrR did, as is the case with MerR and Hg(II) (reviewed in [10]).

**PbrR protects the pbrA promoter from DNase I digestion in vitro**

The 296 bp PbrRA PCR product described above was also used to determine the PbrR binding site on the promoter by DNase I protection assay. Figure 1B shows the autoradiograph of the PbrR DNase I footprint on PppbrA. The region protected by PbrR on PppbrA includes the −35 and −10 sequences as well as the 19 bp spacer containing an imperfect dyad symmetrical sequence between them, and is consistent with DNase I protection results for MerR, CueR and ZntR [18,20,24,40].

The transcription start site [4], the predicted −35 and −10 sites, and the region of the PppbrA promoter protected by PbrR are shown in Figure 1C. The PppbrA promoter has a −35 sequence (TTGACT) that is identical to those for PmerT from Tn501 and PzntA from E. coli K-12 (Figure 2) and shares 5/6 identity with the consensus E. coli −35 sequence. The predicted PppbrA −10 sequence (TTAAAT) has a 4/6 identity to the consensus E. coli −10 sequence (TATAAT) and the spacing between the −35 and −10 sequences is 19 bp, as is the case with other MerR family regulatory regions except ZntR [20 bp; [23]].

**Promoter DNA mutations alter PbrR activity in C. Metallidurans**

The importance to promoter functionality of the number of nucleotides between the −35 and −10 sequences of the PppbrA promoter, and the effects of altering the DNA

![Figure 2](http://www.biomedcentral.com/1471-2180/12/109)
Figure 3 (See legend on next page.)
sequence of the PbrR binding site or −10 sequence of PpbRA were investigated using pMUPbrR/PpbRA −1 in C. metallidurans AE104. The PpbRA −1 mutant (Figure 3A), in which the spacer between the −35 and −10 sequences was shortened in such a way that the −35 and −10 sequences were not altered, and the dyad symmetrical sequences in the spacer between the −35 and −10 were retained, showed increased promoter activity in the absence of Pb(II) (Figure 3A) compared to the wild type promoter, but no induction beyond the maximum level seen for the wt promoter with 100 μM Pb(II). These results are similar to those seen for the MerR activated promoter PmerT −1 from Tn501 [41], which is constitutively transcriptionally active in both the presence and absence of Hg(II). Changes to the pbrA promoter −10 sequence, so that it more closely resembled the consensus sequence for an E. coli promoter [42], caused up-regulation of PpbRA activity both in the absence and presence of Pb(II). Changes made in PpbRA so that it resembled the Tn501 merT promoter −10 sequence resulted in promoter activity remaining repressed in the absence of Pb(II), but strongly induced in its presence to expression levels 5-fold higher than the wild-type pbrA promoter (Figure 3B). These differences in promoter sequence are likely to alter RNA polymerase binding to the promoter, which could in turn affect the structure of the PbrR-RNA polymerase-DNA ternary complex.

Cysteines 14, 79 and 134 in PbrR are essential for Pb(II) responsive transcription from PpbRA in C. metallidurans AE104

pMUPbrR/PpbRA derivatives carrying PbrR cysteine mutants (C14S, C55S, C79S, C114S, C123S, C132S, C134S, and C132S/C134S) (Table 1) were assayed for Pb(II) −dependent induction of the pbrA promoter in C. metallidurans AE104, which did not carry pMOL28 or pMOL30. These were grown in a sublethal concentration of Pb(II) (20 μM) which was sufficient to activate expression from PpbRA, without affecting growth of the Pb(II) −sensitive AE104 strain. β-galactosidase assays of wild type and cysteine mutant PbrR responses to Pb(II) in C. metallidurans AE104 (Figure 4) showed cysteines C14, C79, and C134 were essential for Pb(II) induced transcriptional activation of PpbRA by PbrR. The double mutant C132S, C134S also lost Pb(II) induced activation of transcription from PpbRA, consistent with the result for the single C134S mutant.

Discussion

PbrR is a member of the MerR family of regulators which sense metals and other environmental stimuli, and activate gene expression in response to these signals. The archetype of the family, MerR, regulates both its own expression and expression of the mercuric ion resistance genes...
in the polycistronic mer operon from a divergent pro-
moter: Pmer. MerR activates expression of the structural
genes at the PmerT operator/promoter (o/p) site, which
has an unusually long spacer of 19 bp between the−35
and−10 sequences of the promoter (compared to the con-
sensus E. coli σ70 promoter spacing of 16-18 bp [10]). The
MerR dimer binds to a dyad-symmetrical DNA sequence
within the spacer, and when three essential cysteine resi-
dues (C89, C117 and C126) in the MerR dimer coordinate
to a mercuric ion in a trigonal coordination [28,29] bridg-
ing between each MerR homodimer; this change in MerR
homodimer interaction is transmitted to the promoter,
causing an allosteric underwinding of ∼33° of the DNA at
the o/p site, which realigns the−35 and−10 sequences of
the promoter so that σ70 RNA polymerase can contact the
promoter sequences forming the transcription open com-
plex [43,44].

PbrR from C. metallidurans CH34 plasmid pMOL30
binds to and protects from DNAase I digestion the pre-
picted PpbRA operator/promoter (Figure 1) (4). PpbRA
has striking similarities to other metal ion-responsive
MerR family promoters (Figure 2). Assays of Ppbra
mutants where the spacing between the−10 and−35
sites are shortened to 18 bp, whilst the internal dyad
symmetry is maintained, showed that PbrR-induced ex-
pression from PpbRA is upregulated even in the absence
of Pb(II) (Figure 3). These data are all consistent with
the model of activation for the MerR promoter
[41,43,44]. Change of the DNA sequence of the−10
element of PpbRA to either the consensus E. coli pro-
moter−10 sequence or the Tn501 PmerT promoter−10
sequence also caused up-regulation of promoter activity,
although the PpbRA/Tn501 PmerT-like promoter still
retained Pb(II) repression and induction, rather than a
constitutive up-regulation seen in the−10 consensus
promoter mutant. These data emphasize the importance
of individual nucleotides within the promoter in affect-
ing promoter strength, and indicate that PpbRA is sub-
optimal for maximum induction of the structural pbr
genes. It is possible that this may represent a mechanism
for fine-tuning of expression of the pbr structural genes.

In other metal ion-sensing MerR family regulators,
cysteine residues are essential for metal coordination
and functionality. In vivo assays of the activity of cyst-
eine to serine mutant PbrR proteins in C. metallidurans
AE104 (which lacks pMOL30) have shown that C14,
C79 and C134 are essential for PbrR Pb(II) sensing and
activation of PpbRA (Figure 4). PbrR C14 lies in the turn
of the predicted helix-turn-helix DNA binding domain
of PbrR (Figure 5) and a change of amino acid at this
point could disrupt the binding of PbrR to PpbRA.
Mutants in the second helix of this region of MerR have
lost both activation and repression activity [45,46]. The
loss of Pb(II) response in the PbrR C79S mutant is
consistent with the prediction from a structure-based se-
quence alignment that this residue is essential for dis-
criminating between +1 and +2 charge ions, with a
cysteine being found at this position in regulators that
respond to +2 ions [27]. Mutagenesis studies have all
identified a cysteine residue at this position as being es-
sential for in vivo metal-dependant activation of expres-
sion in MerR, ZntR, and ZccR.

C134 in PbrR (Rmet_5496) is also essential for Pb(II) re-
response and is part of a CVC (CXC) motif which is often
found in PbrR regulators associated with orthologs of
PbrABC, but not in the PbrR homologues PbrR2
(PbrR691 Rmet_2302) and PbrR3 (PbrR710 Rmet_3456),
or CadR (Figure 5). A CVC motif is also found in the
CadC repressor: alterations of either cysteine in this motif
in CadC reduced or abolished sensing of Pb(II), Cd(II) and
Zn(II) [49] and both cysteines are required for metal co-
ordination [50,51]. Although C79 and C134 of the PbrR
homodimer are essential for Pb(II) induction of PpbRA,
the C132S mutant shows only a slightly reduced, not abol-
ished, response to Pb(II). Pb(II) has been shown to have a
preference for binding to cysteine residues in a tri-
coordinate Pb(II)-thiol conformation [52], and Chen and
coworkers have reported that the PbrR-related PbrR691
(PbrR2, Rmet_2302) regulator from the C. metallidurans
genomic island 1 coordinates Pb(II) via 3 (possibly 4) cyst-
eine coordination [14]. Pb(II) has been shown to coordinate
in biological systems via a distorted trigonal planar
geometry involving S and N coordination in a biomimetic
N2S (alkylthiolate) compound [53], and the Pb(II), Cd(II)
and Zn(II) response of the S. aureus pl258 cadmium re-
sistance repressor CadC is dependent on three cysteine
residues [49,54]. DNA footprinting suggests that like
MerR, PbrR functions as a homodimer. It is possible that
Pb(II) may coordinate to cysteine and histidine (or other
N- side chain amino acid) residues or O-containing side
chain amino-acid residues in the PbrR homodimer and
C79 could provide the ligand for metal bridging between
the homodimers, and in current models is thought to be
necessary to trigger DNA underwinding at the regulated
promoter [27]. There are histidine, glutamine, lysine and
arginine residues in PbrR close to the metal-binding do-
main (Figure 5). In ZntR, each homodimer coordinates
two zinc atoms per metal binding domain (MBD), one via
C114 and C124 of the MBD, and C79 from the other
monomer, whilst the other zinc atom is coordinated to
C115 and H119 of the MBD, and C79 from the other
monomer and both zinc atoms also coordinate to oxygen
from a bridging phosphate [27,54]. Structural studies are
required to understand further how Pb(II) coordinates
to PbrR.

We cannot exclude the possibility that the PbrR C79S
and C134S mutants we have made may have altered
DNA-binding features, which may account for loss of Pb
other metals, arguing that expression of the pbr gene is not influenced by other factors [7,12].

However, transcriptomics experiments indicate that the (II) [10,23,57], as do SmtB/ArsR family repressors [47,54].

spond to a greater or lesser extent to Zn(II), Cd(II) and Pb (II) [15,56] or using FRET (PbrR691, [13]) without any transcriptional response to Zn or Cd, whereas related MerR family regulators that have been tested respond to Pb(II) in plasmid based assays in C. metallidurans: 

Figure 5 ClustalW [47,48] alignment of metal sensing MerR regulators.

The metal-responsive MerR family transcription activators can be classified into groups which sense Hg, or Cu/Ag/ Au, or Zn/Cd/Pb, and several other phylogenetically-related but uncharacterized regulator clusters [55]. PbrR (Rmet_5946) and the related PbrR691 (Rmet_2302) and PbrR710 (Rmet_3456) are unusual amongst the phylogenetic cluster of related Zn (II)/Cd(II)/Pb(II)-sensing MerR family regulators that have been tested for metal specificity, because they exclusively respond to Pb(II) in plasmid based assays in C. metallidurans (PbrR: [15,56]) with the mechanism of transcriptional activation by PbrR appears to be essentially identical to that of MerR family regulators that have been characterized. PbrR contains three cysteine residues that are necessary for Pb(II)-induced transcription from the pbrA promoter. C14 is in the helix-turn-helix DNA binding domain, and may be essential for the regulator/DNA interaction. C79 is essential in all divalent metal ion responsive MerR regulators tested so far, whilst C134 is not found in other characterized MerR regulators. Our data show that PbrR transcription is activated by Pb(II) using different amino acids to other divergent metal ion-activated MerR regulators, but further work is required to determine whether Pb(II) coordinates other residues in PbrR.

Figure 5 ClustalW [47,48] alignment of metal sensing MerR regulators. PbrR (Rmet_5946), PbrR691 (Rmet_2302) and PbrR710 (Rmet_3456) are from the genome of C. metallidurans C34. CadR is from Pseudomonas stutzeri A1501. ZntR, and CueR are from the E. coli K-12 genome, and MerR is from Tn501. The helices of the Helix-Turn-Helix DNA binding domain are boxed. Essential cysteine residues (Cys14, Cys79, and Cys134) are from the genome of C. metallidurans: 

The authors declare that they have no competing interests.

Abbreviations

Tp: Trimethoprim; Ap: Ampicillin; Km: Kanamycin.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

JLH and DJJ carried out the experimental studies. JLH drafted the manuscript. NLB conceived and coordinated the study. All authors read and approved the manuscript.
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