Hg(II) sequestration and protection by the MerR metal-binding domain (MBD)

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MerR, the metalloregulator of the bacterial mercury resistance (*mer*) operon, binds Hg(II) with high affinity. To study the mechanism of metal-induced activation, a small protein was previously engineered embodying in a single polypeptide the metal-binding domain (MBD) ordinarily formed between two monomers of MerR. Here the physiological and biochemical properties of MBD expressed on the cell surface or in the cytosol were examined, to better understand the environments in which specific metal binding can occur with this small derivative. Over 20 000 surface copies of MBD were expressed per *Escherichia coli* cell, with metal stoichiometries of ~1·0 Hg(II) per MBD monomer. Cells expressing MBD on their surface in rich medium bound 6·1-fold more Hg(II) than those not expressing MBD. Although in nature cells use the entire *mer* operon to detoxify mercury, it was interesting to note that cells expressing only MBD survived Hg(II) challenge and recovered more quickly than cells without MBD. Cell-surface-expressed MBD bound Hg(II) preferentially even in the presence of a 22-fold molar excess of Zn(II) and when exposed to equimolar Cd(II) in addition. MBD expressed in the cystosol also afforded improved survival from Hg(II) exposure for *E. coli* and for the completely unrelated bacterium *Deinococcus radiodurans*.

INTRODUCTION

Mercury is the most widely distributed common toxic metal (Goldman & Shannon, 2001; Tchounwou *et al.*, 2003). Presently, the US Environmental Protection Agency (EPA) uses a limit of 0·125 µM as the universal treatment standard (UTS) target end point for mercury in the toxicity characteristic leaching procedure (TCLP) (Fuhrmann *et al.*, 2002). Several physico-chemical procedures are available for mercury remediation (Mulligan *et al.*, 2001), but extensive remediation of toxic metal ions in soil and water is challenging and costly, especially at low metal concentrations. Because of potentially lower costs, higher efficiency and specificity, the use of micro-organisms and plants for sequestering the contaminating metals is currently receiving considerable attention (Chaney *et al.*, 1997; Gadd, 2000; McIntyre, 2003). Several metal-binding proteins and peptides found in some of the bacterial metal resistance or plant and fungal metal tolerance systems have been engineered into plant and bacterial hosts for potential use in bioremediation (Bae *et al.*, 2001, 2002, 2003; Chen & Wilson, 1997a, b; Chen *et al.*, 1998; Deng & Wilson, 2001; Kotrba *et al.*, 1999b; Meagher, 2000; Mejare & Bulow, 2001; Sousa *et al.*, 1996, 1998; Valls *et al.*, 2000a, b; Vieira & Volesky, 2000). Our work extends these observations to include an engineered subdomain of the well-studied metalloregulator, MerR, and contrasts the quantitative performance of this construct with its free form, with its parent protein MerR both free and fused, and with other engineered bacterial metal-sequestering systems.

The mercury resistance (*mer*) operon of the plasmid-borne transposon Tn*21* from *Shigella flexneri* has been extensively described. It consists of five structural genes, *merTPCAD*, encoding the NADPH-dependent flavin oxidoreductase, mercuric reductase (MerA), two inner-membrane proteins (MerT and MerC) and a periplasmic protein (MerP), all three involved in Hg(II) uptake, and the regulatory proteins MerR and MerD (Barkay *et al.*, 2003). The activities of these *mer* proteins reduce soluble ionic Hg(II) to volatile Hg vapour [Hg(0)] which escapes into the atmosphere, a process

ideal for single-cell bacteria but less so for multicellular organisms, the majority of whose cells are far removed from the organism's surface. Thus, multicellular organisms use intracellular metal sequestration by thiol-rich proteins and polypeptides including metallothioneins (in animals and plants) and phytochelatins (in plants). We and others have explored possible uses of engineered versions of various *mer* operon proteins to sequester, rather than to mobilize Hg (see specific references below).

We also study how MerR has evolved to function as a highly specific transcriptional regulator in a cytosolic milieu that contains other d10 transition metal ions such as the essential Zn(II). The transcriptional repressor/activator MerR has the highest affinity of the *mer* proteins for Hg(II) ($\sim 0 \cdot 01$ μM even in the presence of millimolar competing thiols) *in vivo* (Condee & Summers, 1992) and *in vitro* (Zeng *et al.*, 1998). In addition, with *in vivo* (Caguiat *et al.*, 1999; Condee & Summers, 1992) and *in vitro* (Ralston & O'Halloran, 1990) transcription assays, MerR shows high specificity for its metal inducer, preferring Hg(II) by two to three orders of magnitude over related group 12 metal ions, Cd(II) and Zn(II). MerR has three domains: an N-terminal DNA-binding domain, a C-terminal Hg(II)-binding domain and an intervening region of undefined function. MerR's unusual affinity and specificity for Hg(II) have been attributed to the unique trigonal thiolate coordination of Hg(II) in its metal-binding centres (Wright *et al.*, 1990). Trigonal coordination of Hg(II) is much more stable than the dicoordinate arrangement available with the monothiol buffers (glutathione or cysteine) of the typical eubacterial cytoplasm (Fahey, 2001). Three conserved cysteine residues of MerR contribute to this trigonal Hg(II)-coordination site, C117 and C126 from one monomer and C82 from the other (Helmann *et al.*, 1990; Ross *et al.*, 1989). Biochemical (Helmann *et al.*, 1990; Zeng *et al.*, 1998) and genetic (Caguiat *et al.*, 1999; Ross *et al.*, 1989) work predicted that in MerR the metal centres lie exactly at the ends of the coiled helix, and this model was confirmed in the three-dimensional structures of two other MerR family metalloregulators, CueR and ZntR (Changela *et al.*, 2003). To facilitate study of this unusual inter-monomer, antiparallel, coiled-coil metal-binding site, we earlier constructed a small single polypeptide, called the metal-binding domain (MBD) containing a direct tandem duplication of α-helix 5 of MerR (Song *et al.*, 2004). At just 117 amino acids, the MBD is less than half the size of dimeric MerR (288 amino acids). However, in MBD, these direct tandem α-helices fold back on each other into an antiparallel, coiled-coil hairpin (Fig. 1) with *in vivo* and *in vitro* metal-binding properties comparable to those of the full-length MerR dimer (Song *et al.*, 2004).

In this study we fused MBD into the AraC-controlled Lpp-OmpA fusion protein for cell surface expression and examined its metal sequestration properties and its ability to protect cells from Hg(II) exposure even when secreted into the oxidized environment of the outer cell surface. We also examined these latter two properties for MBD expressed in
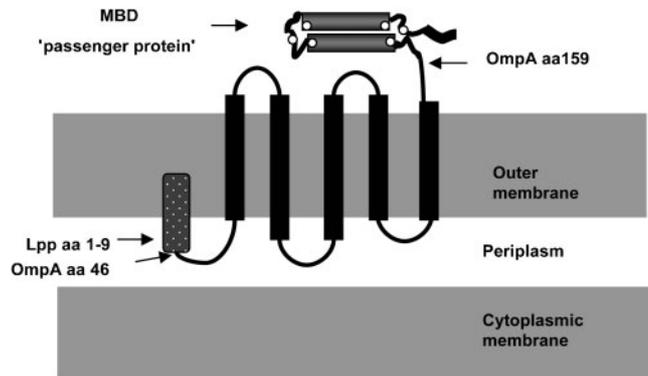

Fig. 1. Incorporation of MBD into the Lpp-OmpA surface-display system (Georgiou *et al.*, 1996). The grey dotted rectangle represents the first nine N-terminal amino acids of the mature *E. coli* prolipoprotein (Lpp). The black rectangles represent membrane-spanning domains of OmpA. MBD is the 'passenger protein' displayed on the outer surface of the outer membrane and 'aa' refers to amino acid(s). In MBD, the tandem copies of MerR α-helix 5 fold back on each other, forming an antiparallel coiled-coil hairpin. The three cysteines (open circles) form trigonal thiolate metal coordination centres at each end of the coiled coil.

the cytosols of *Escherichia coli* and of the completely unrelated, radiation-resistant bacterium *Deinococcus radiodurans*, which can be used in remediation of heavy-metal-contaminated high-level nuclear waste sites (Brim *et al.*, 2000).

METHODS

Bacterial strains. *E. coli* strains XL-1 Blue [*endA1 gyr96 hsdR17 lac recA1 relA1 supE44 thi-1*/F′ *lacI*q Δ(*lacZ*)M15 *proA*+*B*+ Tn*10*(*tetR*)]; MC4100 (*araD lacU169 lac*Δ *thi str-rpsl relA*); SK1592-A (*endA gal hsrR4 sbcB15 thiT1*) were used in construction or assays as detailed below.

The naturally radiation-resistant bacterium *D. radiodurans* expressed MBD via two distinct chromosomal expression systems. The pMD417 system yields low-copy-number integrations (20 copies per cell), each of which is highly expressed. The pS11 system yields high-copy-number integrations (150 copies per cell) (Brim *et al.*, 2000). Plasmid pMD417 is a deletion derivative of pS11 where the integration sequence has been reduced from 11 kb to 4 kb and a constitutive promoter supplied upstream of the cloned gene. *D. radiodurans* strains harbouring just pMD417 or pS11 integrations grow as well on rich medium and are as sensitive to HgCl₂ as the wild-type strain (Brim *et al.*, 2000). Expression constructs are described below.

Construction of plasmids. Construction of the cytosolic MBD- or MerR-expressing plasmid pJC101 was described by Song *et al.* (2004). Construction of the Lpp-OmpA fusion proceeded in two steps to yield the product schematically depicted in Fig. 1. An intermediate plasmid pB30D-Sal [based on the plasmid pB30D (Daugherty *et al.*, 1999), graciously provided by G. Georgiou] was constructed by using PCR to remove part of the scFv fragment of pB30D and to introduce a new *SalI* site on the 3′ side of the Lpp-OmpA module (see Supplementary Fig. S1, available with the online

version of this paper). The primers for the *XmaI-SalI* fragment were 5′-CGAATTCGAGCTCGGTACCCGGGCTAGAG-3′ (upper) and 5′-AACTTCGTCGACTCCGTTGTCCGGACGAGT-3′ (lower).

The pSD-MBD (SD denotes 'surface display'; Fig. S1) was then constructed by substituting the *SalI–SphI* fragment on intermediate plasmid pB30D-SalI with the *SalI/SphI*-digested PCR product of the *mbd* gene from plasmid pJC101(Song *et al.*, 2004). The primers for the *mbd* gene were 5′-AGATAACGAGGGGTCATGACACACTGC-3′ (upper) and 5′-CACTTCACAGGGCATGCTTATTATTTTTCGAAC-3′ (lower). The template was plasmid pASK-MBD (Song *et al.*, 2004). Correct construction was confirmed by sequencing at each stage.

Construction of the *D. radiodurans* chromosomal-integration vectors pS11/MBD and pMD417/MBD involved two stages. First, an *EcoRI* and a *BglII* site were introduced within the unique *XbaI* and *HindIII* sites, respectively, that immediately flank the inserted *mbd* gene in pJC101(Song *et al.*, 2004). The *EcoRI–BglII mbd* fragment was then cloned into the pMD417 *EcoRI–BamHI* duplication insertion vector and into the pMD730 *MfeI–BglII* amplification vector (a derivative of pS11 whose unique *DraI* site was changed to an *MfeI* site). Correct construction was confirmed by sequencing at each stage. It was not possible to construct pS11/MerR in *E. coli*, probably because its high unregulated expression was toxic; since this step is an essential intermediate to engendering insertions in *D. radiodurans*, pS11/MerR insertions into *D. radiodurans* were not made.

Induction of protein expression. *E. coli* cultures were grown in Luria–Bertani (LB) medium at 30 °C to OD_{600} 0·5 and were amended with ampicillin (50 µg ml^{-1}) and/or streptomycin (30 µg ml^{-1}) as appropriate. Cultures producing surface-expressed MBD were then induced with 0·2 % arabinose at 25 °C for 3–4 h. For uninduced cells, 0·2 % glucose was added instead of arabinose. For *E. coli* SK1592(pJC101) producing cytosolic MBD or SK1592(pJC100) producing cytosolic MerR (Song *et al.*, 2004), cultures were grown as above and then induced with 200 µg anhydrotetracycline (AHT) l^{-1} for 3 h.

Fluorescence microscopy. After induction with arabinose at 25 °C for 4 h, 2 ml of MC4100(pSD-MBD) cells or 1 ml of SK1592-A(pSD-MBD) cells were harvested by centrifugation, washed once with 2 ml cold phosphate-buffered saline (PBS), pH 7·2, and resuspended in 1 ml cold PBS. Both of these common K12 strains were used to assure that results were not limited to a single strain. Five microlitres of polyclonal anti-MerR antibody (Kulkarni & Summers, 1999) was added to each cell suspension and the reaction mixture was rocked on a Vari-Mix rocker at 4 °C overnight. The cells were then washed three times with 2 ml cold PBS, rocked on a Vari-Mix rocker at 4 °C for 5 min between each wash, and resuspended in 1 ml cold PBS containing 1:500 fluorescein-conjugated anti-rabbit antibody. After rocking for 1 h at 4 °C, the cells were washed three times with 2 ml cold PBS, resuspended in 30 % Citifluor (Ted Pella, Inc.), and kept in the dark until fluorescence microscopy. A Nikon TE 300 inverted microscope with a black/white digital camera and a filter set for fluorescein was used for fluorescence microscopy. Exposure time was 2·5 s.

Immunoprecipitation. Cells (25 ml) were cultured with aeration (200 r.p.m.) at 30 °C and at OD_{600} ∼0·5 either arabinose (induced) or glucose (uninduced) was added. Incubation continued at 25 °C for 4 h, at which point 5 ml cells were harvested in a Beckman J-17 at 5820 *g* for 15 min at 4 °C. The cell pellets were suspended in 3 ml cold PBS and 20 µl polyclonal anti-MerR antibody was added to each; cell suspensions were then rocked on a Vari-Mix rocker at 4 °C overnight. During this step the anti-MerR antibody attached to MerR epitopes on the cell surface (i.e. those embodied in the MBD protein). Cells were washed three times with 10 ml cold PBS, rocked on a Vari-Mix rocker for 5 min at 4 °C between each wash, and

pelleted in a Beckman J-17 at 5820 *g* for 10 min at 4 °C. This step removed unreacted anti-MerR antibody. Washed cell pellets were resuspended in 40 ml cold TDSET buffer (1 % Triton-100, 0·2 % sodium deoxycholate, 0·1 % SDS, 10 mM tetrasodium EDTA, 10 mM Tris/HCl, pH 7·8) (Balish *et al.*, 2001) and rocked on a Vari-Mix rocker for 1 h at 4 °C. This step released the membrane proteins, including the MBD–anti-MerR antibody complex, from the cell and solubilized them. The cell debris was then removed by centrifugation in a Beckman J-17 at 13 776 *g* for 20 min at 4 °C. Since the anti-MerR antibody had already been washed away, even if the cells lysed during the Triton step, no cytosolic MBD could be complexed with antibody. Fifty microlitres of protein A beads (New England BioLabs), pre-blocked with bovine serum albumin (Sigma), was added to the supernatant (which contained the Triton-solubilized MBD–anti-MerR antibody complex), and the mixtures were rocked for 2 h at 4 °C. During this step the Fc portion of the anti-MerR antibody complexed to MBD was captured by the bead-bound Protein A, which is specific for the constant portion (Fc) of the antibody molecule. The Protein A beads were precipitated in a Beckman J-17 at 5820 *g* at 4 °C for 20 min and washed three times with 5 ml cold TDSET buffer. This step removed adventitiously attached proteins from the beads, leaving only the antibody and whatever it was carrying, in this case the MBD that was present on the cell surface. Finally, the Protein A beads were resuspended in 50 µl TDSET buffer and 100 µl gel loading buffer (Bio-Rad) was added; the mixture was then heated for 10 min at 95 °C to release the fusion protein and antibody from Protein A. The Protein A beads were separated by a magnetic separator, and 45 µl of the supernatant was loaded onto the 4–20 % SDS-PAGE gel (Gradipore) for Western blotting analysis (Song *et al.*, 2004).

Protease sensitivity. Strain SK1593(pSD-MBD) was used for this test because it contains the cytosolic *lacZ* gene as a negative control. Cells were induced with 1 mM IPTG and 0·2 % arabinose for 4 h at 25 °C. Then 1 ml of culture was spun in a microcentrifuge at 14 000 *g* for 2 min at 4 °C. Cells were resuspended in cold PBS with varying concentrations of elastase (0·2, 0·3, 0·5, 1·0, 1·5, 2·0 mg ml^{-1}) and placed on ice for 45 min. Fifty microlitres was removed, mixed with 100 µl loading buffer and heated at 95 °C for 10 min to lyse the cells. Thirty-five microlitres was loaded onto 4–20 % SDS-PAGE gels for Western blotting analysis (Song *et al.*, 2004). Separate gels and blots were used for detection of the MBD fusion protein and LacZ.

Western blotting. *E. coli* cells were inoculated into 25 ml LB medium containing antibiotic as needed and incubated at 30 °C and 200 r.p.m. overnight. Then 25 ml fresh LB medium with antibiotic as needed was inoculated with the fresh overnight culture at 1:100, and incubated at 30 °C and 200 r.p.m. Cells were induced with 0·2 % arabinose at OD_{600} 0·5 for 3 h at 25 °C. The cultures were adjusted to OD_{600} 0·8 and 1 ml of each culture was then centrifuged and the cells were resuspended in 50 µl PBS and lysed by mixing with 100 µl gel loading buffer (Bio-Rad) and heating at 95 °C for 10 min. Lysed cells (35–45 µl) were loaded onto SDS-PAGE gels (4–20 % Gradipore) and electrophoresed for 1·5 h at 100 V. Separated proteins were transferred onto PVDF membranes (Schleicher & Schuell Bio-Science) at 25 V, 4 °C overnight. Western analysis was performed using 1:2000 dilutions of polyclonal anti-MerR (produced in rabbits injected with purified MerR at the Animal Resources Facility of the University of Georgia College of Veterinary Medicine) or monoclonal anti-galactosidase (Rockland Immunochemicals), and 1:1000 dilutions of fluorescein-conjugated secondary antibody (ECF, Amersham Pharmacia Biotech) in TTBS buffer (20 mM Tris/HCl pH 6·7, 137 mM NaCl, 0·1 % Tween-20) (Song *et al.*, 2004). Dried blots were scanned using a Fluorimager 575 (Molecular Dynamics) with a band-pass 570 DF30 emission filter and photomultiplier tube voltage setting at 650 V.

Mercury-binding assay. Cells (25 ml) were cultured with aeration (200 r.p.m.) at 30 °C to OD_{600} 0·5. Both 0·2 % arabinose and 0·8 µM Hg(II) (as $HgCl_2$) were added and incubation continued with shaking for 4 h at 25 °C. Cells were then washed three times with fresh LB, and analysed by wet-ashing followed by inductively coupled plasma-mass spectrometry (ICP-MS; quantification limits for Hg are 10 parts per trillion or 50 picomolar on the Perkin Elmer Elan 6000 at the University of Georgia Laboratory for Environmental Analysis). An aliquot of cells was removed for quantification of the MBD fusion protein by Western blotting with a standard curve of purified protein analysed using ImageQuant software on a Molecular Dynamics Fluoroimager (version 1.2; Molecular Dynamics) (Song et al., 2004). Cell dry weight was determined by harvesting 10 ml of culture in a Beckman J-17 at 5820 g for 10 min. The cells were resuspended in 1 ml Milli-Q deionized water and transferred into a 1·5 ml Eppendorf tube, and pelleted by centrifugation at 14 000 g for 2 min at room temperature. The supernatant was discarded and the cells were dried at 55 °C to a constant weight (approx. 48 h).

Hg(II) challenge and recovery. For E. coli cells with surface-expressed MBD, 25 ml cells were grown with aeration in LB medium containing 50 µg ampicillin ml^{-1} and 30 µg streptomycin ml^{-1} at 30 °C until they reached OD_{600} 0·5. Arabinose (0·2 %) was added to induce protein expression and incubation continued for 3 h at 25 °C (0·2 % glucose was used for the uninduced condition; the final OD_{600} was ~1·3). The cultures were diluted to OD_{600} 0·05 with fresh LB containing 0·2 % arabinose (or 0·2 % glucose for uninduced controls), 50 µg ampicillin ml^{-1} and 30 µg streptomycin ml^{-1}; then Hg(II) (as $HgCl_2$: 0, 60 or 120 µM) was added to challenge the cells. The 20 ml cultures in flasks were shaken at 200 r.p.m., 30 °C, and growth was measured every hour by transferring triplicate 300 µl aliquots of each sample to a 96-well microplate and reading the OD_{600} via a Spectra Max250. After 2 h, 10 ml of challenged cells was removed from the culture vessel and cells were harvested by centrifugation in a Beckman J-17 at room temperature. The supernatant was discarded and cells were resuspended to OD_{600} 0·05 with fresh LB medium containing 50 µg ampicillin ml^{-1} and 30 µg streptomycin ml^{-1} but lacking Hg(II), and cultures were incubated in a 30 °C shaker at 200 r.p.m. Growth was measured hourly by transferring triplicate 300 µl aliquots of each culture to a 96-well microplate and measuring the OD_{600} in a Spectra Max 250.

For E. coli cells with cytosolic expression of MBD, E. coli SK1592-(pJC101) was grown to OD_{600} 0·5, in LB medium containing 100 µg ampicillin ml^{-1} at 37 °C with aeration, and then the culture was split into two aliquots, one of which was induced with 200 µg AHT l^{-1} for 3 h and the other of which was cultivated under same conditions as the uninduced control for 3 h. The induced and uninduced cultures were diluted to OD_{600} 0·1 with LB containing 100 µg ampicillin ml^{-1}, and $HgCl_2$ was added (0, 60 or 120 µM). Each diluted, Hg(II)-treated culture was then immediately pipetted (in quadruplicate) into a sterile 96-well microplate at 200 µl per well. The microplate was shaken for 2·5 h at 300 r.p.m., 37 °C, and OD_{600} was periodically monitored on a SpectraMax 250 spectrophotometer (Molecular Devices). The Hg(II)-treated cultures were then centrifuged at 8000 g for 5 min at room temperature, and the pellet was suspended in fresh LB medium containing 100 µg ampicillin ml^{-1}. The resuspended cells were diluted to OD_{600} ~0·1 and pipetted into a sterile microplate, 200 µl per well. The microplate was shaken at 37 °C, 300 r.p.m. in a New Brunswick model G25 incubator and the OD_{600} was monitored.

For D. radiodurans with constitutive cytosolic expression of MBD, strains containing chromosomal insertions of pS11/MBD or pMD417/MBD were incubated in TGY medium (10 g tryptone, 5 g yeast extract, 1 g glucose in 1 l medium) and 10 µM $HgCl_2$ at 32 °C for at least 9 days. Optical densities were measured at 600 nm.

Competitive binding of other group 12 metals to surface-bound MBD. E. coli cells were cultured at 30 °C to OD_{600} 0·5. Arabinose (0·2 %) and 0·8 µM Hg(II) were added with two different concentrations of $CdCl_2$. Since the concentration of Zn(II) in LB is 19 µM as determined by ICP-MS, no additional Zn(II) was added. The cultures were shaken for 4 h at 25 °C. Cells were then washed three times with fresh LB, and metal content determined by ICP-MS at the Laboratory of Environmental Analysis, University of Georgia School of Forestry. The MBD fusion protein was assayed by quantitative Western blotting as above using ImageQuant software on a Molecular Dynamics Fluoroimager (version 1.2; Molecular Dynamics) (Song et al., 2004). Cell dry weight was determined by harvesting 10 ml of culture in a Beckman J-17 at 5820 g for 10 min. The cells were resuspended in 1 ml Milli-Q deionized water, transferred into a 1·5 ml Eppendorf tube, and pelleted by centrifugation at 14 000 g for 2 min at room temperature. The supernatant was discarded and the cells were dried at 55 °C to a constant weight (approx. 48 h).

RESULTS

Expression of Lpp-OmpA-MBD

The Lpp-OmpA fusion protein system consists of the signal sequence and first nine N-terminal amino acids of the mature E. coli prolipoprotein and amino acids 46–159 of the outer-membrane protein OmpA (Francisco et al., 1992; Fig. 1). It has been used to display proteins as large as 263 amino acids on the surface of E. coli via the tightly controllable arabinose operon promoter (Daugherty et al., 1999; Lobell & Schleif, 1990), which is catabolite-repressible by glucose to avoid potential harmful effects of the fusion protein on the host (Daugherty et al., 1999; Francisco et al., 1992). Induced cells of several distinct clones (Fig. 2a, lanes 8–11) expressed an identical arabinose-inducible protein of ~27 kDa that reacted with anti-MerR specific polyclonal antibody (Song et al., 2004); the predicted size of the Lpp-OmpA-MBD fusion is 26·8 kDa. There was no leaky expression of the fusion protein when cells were grown in 0·2 % glucose (Fig. 2a, lanes 4–7). Thus, Lpp-OmpA-MBD expression can be tightly controlled with the pBAD promoter. Clone pSD-MBD30 was used in all subsequent work.

The MBD fusion is displayed on the surface of E. coli

Fluorescence microscopy of wet mounts of cells reacted with anti-MerR antibody and then with a fluorescein-conjugated secondary antibody (Fig. 2b) detected MerR-derived epitopes on the intact cells (panels C and D) of two different arabinose-induced E. coli strains, SK1592(pSD-MBD) and MC4100(pSD-MBD), but not on the corresponding uninduced cells of each strain (Fig. 2b, panels A and B). Two distinct strains were used to assure that the results were not limited to a single host background.

A second test of surface availability of MBD employed reaction with MerR antibody and capture by Protein A beads. The cells used were intact and fresh, so only MBD epitope on the outer surface should have been available to react with the MerR antibody. After unreacted MerR antibody was washed away, only cells retaining antigen
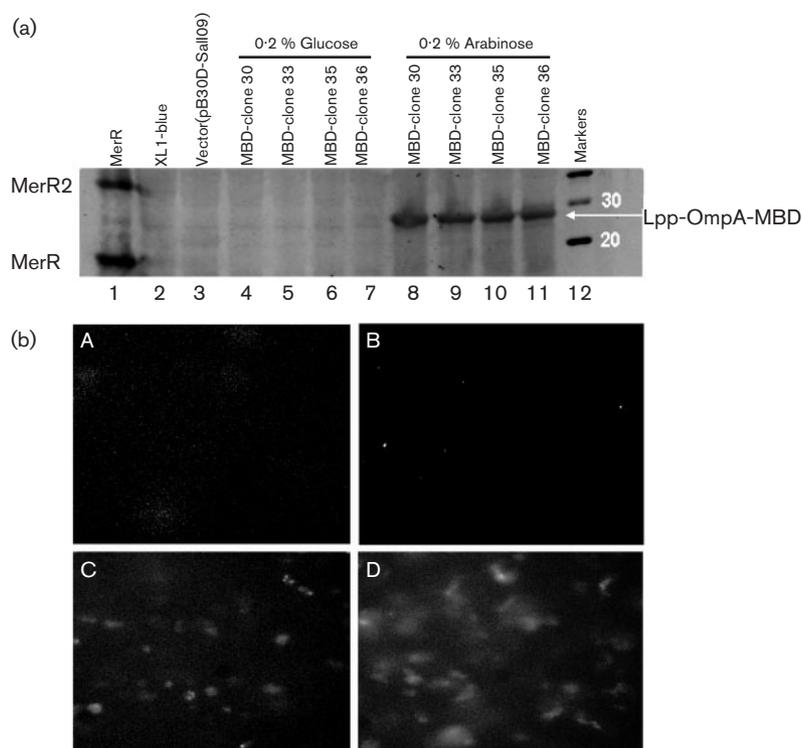
Fig. 2. (a) Lpp-OmpA-MBD is expressed under AraC control. Protein detection was by Western blotting as described in Methods (Song *et al.*, 2004). Pure MerR protein is the positive control (lane 1) and runs as a monomer (MerR) and an oxidized dimer. The host XL-1 Blue and XL-1 Blue with vector pB30D-Sall are negative controls (lanes 2, 3). Lanes 8, 9, 10 and 11 are distinct clones of XL-1 Blue (pSD-MBD) induced with arabinose. Lanes 4, 5, 6 and 7 are the same clones of XL-1 Blue(pSD-MBD) uninduced. Lane 12 is the protein molecular mass standard. (b) Detection of MBD on the intact cell surface by immunofluorescence microscopy. A, Uninduced SK1592(pSD-MBD); B, uninduced MC4100(pSD-MBD); C, induced SK1592(pSD-MBD); D, induced MC4100(pSD-MBD).

(Lpp-OmpA-MBD)–antibody (anti-MerR) complexes should be captured by Protein A beads. SDS-PAGE of material eluted from the Protein A beads showed a strong MerR-antibody-reactive band of the expected size for the Lpp-OmpA-MBD fusion (Fig. 3a, lanes 4 and 7). The MBD fusion also produces a stable disulfide dimer ($\sim$54 kDa, lanes 4 and 7); multiple, thiol-stable, electrophoretic forms of MerR have been noted previously (Kulkarni & Summers, 1999). Note that there was some carryover from lane 7 into lane 8. Faint bands of $\sim$50 kDa and 25 kDa (lanes 2 and 5) result from residual anti-MerA antibody that reacts with the secondary antibody used in Western development. The faint band of $\sim$27 kDa (lanes 3 and 6) may arise from adventitious binding of the coiled-coil Lpp-OMP-MBD to Protein A, which itself consists of multiple, interacting, α-helices (Graille *et al.*, 2000).

Lastly, to the degree the MBD fusion is displayed on the outer cell surface, it will be more sensitive to protease than a cytosolic protein such as *β*-galactosidase (LacZ). We used strain SK1592 to express the Lpp-OmpA-MBD fusion because this strain naturally contains the IPTG-inducible, chromosomally encoded cytosolic LacZ for comparison. Cells induced with both arabinose and IPTG were treated with elastase, which cuts at Ala-X and Gly-X. The band corresponding to *β*-galactosidase did not diminish when the intact cells were treated with increasing concentrations of elastase (Fig. 3b, lanes 2–9). In contrast, the Lpp-OmpA-MBD fusion band faded as the concentration of elastase increased (Fig. 3b, lanes 3–8). Thus, the MBD fusion is more

sensitive to elastase than cytosolic LacZ, consistent with the former being exposed on the cell surface.

These three distinct methods indicate consistently that MBD is displayed on the *E. coli* cell surface in the pSD-MBD30 construct.

Surface-displayed MBD preferentially binds Hg(II)

Quantitative Western analysis indicated that approximately 20 000 copies of MBD were produced by each arabinose-induced cell (Table 1). The stoichiometry of 1·3 Hg(II) ions per MBD monomer was similar to that seen *in vitro* with purified MBD (Song *et al.*, 2004) or full-length MerR (Song *et al.*, 2004; Zeng *et al.*, 1998). Thus, outer-membrane-expressed MBD provides an approximately sixfold increase in Hg(II) binding compared to uninduced cells. This affinity for Hg(II) is the same as measured for the surface-displayed full-length MerR protein in minimal medium (Bae *et al.*, 2003) and is quite striking considering that MBD's binding capacity was measured here in LB, which contains 19 μM Zn(II) (determined by ICP-MS; data not shown) and also has competing thiol ligands including cysteine.

For optimum binding, the Hg(II) must be present at the time of induction with arabinose (data not shown). Since treatment with glutathione (10 mM) does not elicit Hg(II) binding in cells induced before being exposed to Hg(II) (data not shown), we infer that the oxidized thiol groups of secreted cysteines cannot readily be reduced by exogenous
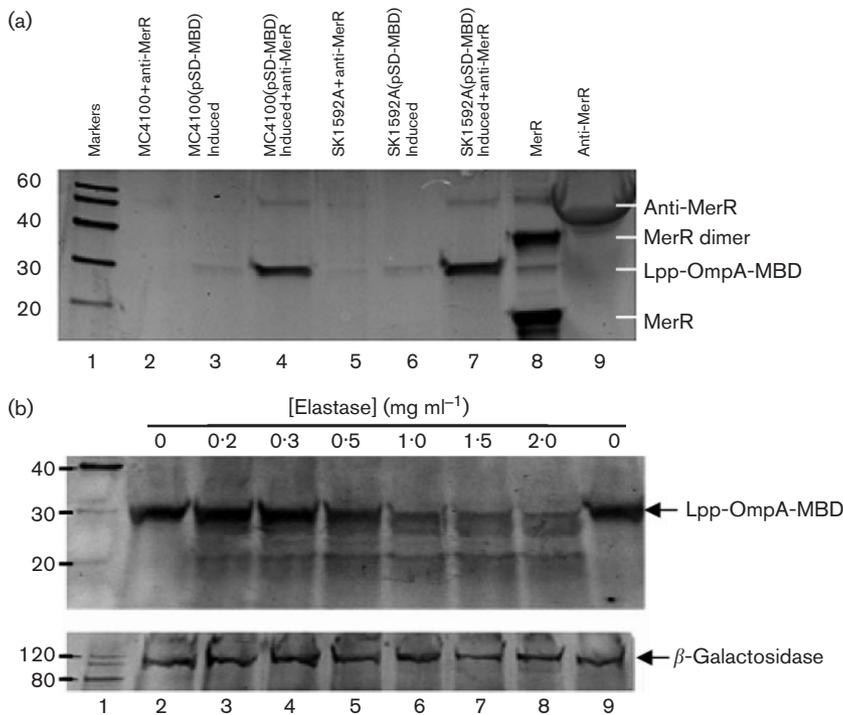
Fig. 3. (a) Immunoprecipitation of MBD from intact cells. The gel image was generated by Western blotting with polyclonal antibody against MerR followed by fluorescein-conjugated goat anti-rabbit secondary antibody. Lane 1, MagicMark molecular mass markers. Samples in lanes 2–7 were all captured on and eluted from Protein A beads. Sample processing, described in detail in Methods, was as follows: lanes 2 and 5, uninduced cells treated with MerR antibody; lanes 3 and 6, induced cells, not treated with MerR antibody; lanes 4 and 7, induced cells treated with MerR antibody. Lane 8 is pure MerR protein and lane 9 is anti-MerR-antibody that reacts with the goat anti-rabbit secondary antibody used in the Western development. (b) Protease sensitivity of MBD in intact cells (0 indicates no elastase). Top, Western blotting using MerR antibody; bottom, Western blotting using LacZ antibody.

glutathione. These observations are consistent with Cd(II) binding by surface-expressed metallothionein (Valls et al., 1998) and suggest that thiol groups on these secreted proteins are quickly and irreversibly oxidized when not bound by metal ions. Although 50-fold more MBD per cell is achievable with cytosolic expression (Table 1), the binding of Hg(II) per molecule of MBD is sixfold lower in the cytosol than on the surface. Two possible causes for this are less effective folding of overexpressed MBD in the crowded cytosol and competition with comparably higher levels of low-molecular-mass thiols such as glutathione, which is approximately 5–6 mM in E. coli (Fahey, 2001).

To examine further the specificity of MBD for Hg(II), we added Cd(II) (as the chloride) to the binding reaction in either equimolar (0·8 μM) or 10-fold excess amounts over Hg(II). The former diminished Hg(II)-binding by MBD by approximately 45 % and the latter large excess of Cd(II) eliminated 96 % of Hg(II) binding (data not shown). These data contrast with those of Bae et al. (2003), who observed in a thiol-free buffer that surface-displayed MerR bound Hg(II) in a 100-fold excess of Cd(II). Possible reasons for this difference will be discussed below.

Surface-displayed and cytosolic MBD protect cells from Hg(II) toxicity

Unlike cells carrying the complete mer operon (Hamlett et al., 1992; Summers et al., 1982), E. coli cells induced for expression of Lpp-OmpA-MBD did not grow in medium containing 60 μM or 120 μM Hg(II) (Fig. 4a). However, when the cells were diluted into fresh medium without Hg(II) (Fig. 4b) those expressing MBD recovered more rapidly than cells not induced for MBD expression. Thus,

Table 1. Hg(II) binding by surface-displayed and cytosolic MBD

Cellular location	Induced MBD molecules per cell*	Hg(II) bound molecules per induced cell	Hg(II) bound molecules per uninduced cell	Mol Hg(II) per mol MBD	Hg(II) per cell Ind/Unind (pmol/pmol)†
Outer membrane‡	$2·31 \times 10^4 \pm 0·13 \times 10^4$	$3·58 \times 10^4 \pm 0·46 \times 10^4$	$0·58 \times 10^4 \pm 0·07 \times 10^4$	$1·29 \pm 0·28$	$6·12 \pm 0·04$
Cytosol§	$1·01 \times 10^6 \pm 2·20 \times 10^5$			$0·21 \pm 0·04$	$1·69 \pm 0·37$

*Determined by quantitative Western blotting (Song et al., 2004) after standard induction.

†Compared to uninduced (outer membrane) or vector (cytosol); determined by ICP-MS (outer membrane) and ^{203}Hg binding (cytosol) as in Song et al. (2004).

‡Four trials of duplicate assays; hosts MC4100 and SK1592.
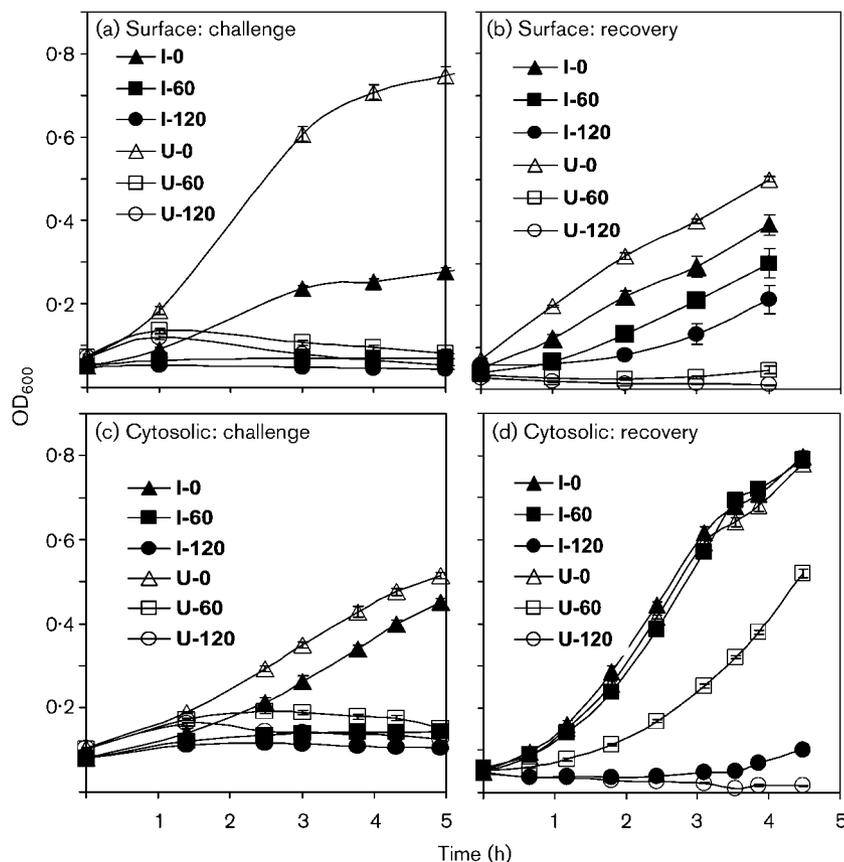
§Two trials of duplicate assays; host XL-1 Blue.

Fig. 4. Recovery of *E. coli* cells expressing MBD after Hg(II) exposure. U (open symbols), uninduced; I (filled symbols), induced for MBD expression; the numbers following U- and I- indicate the concentration of Hg(II) (µM) used for challenge.

surface-displayed MBD allows recovery from a toxic level of Hg(II). This was also the case for *E. coli* cells expressing MBD in the cytosol (Fig. 4c, d). In contrast, high expression of MerR in the cytosol is toxic to *E. coli* cells even in the absence of Hg(II) (Song *et al.*, 2004).

D. radiodurans with pMD417 or pS11 integrations have the same growth rates as the wild-type lacking vector-based insertions, reaching the stationary phase of growth (OD$_{600}$ 1·2) in non-selective, undefined rich medium in less than 24 h at 32 °C (Brim *et al.*, 2000). In the presence of 10 µM HgCl$_2$, wild-type *D. radiodurans*, or strains harbouring pMD417 or pS11 vectors alone that do not encode the MBD, did not grow (Fig. 5). In contrast, *D. radiodurans* expressing MBD on pS11 or on pMD417 reached the stationary phase in TGY + 10 µM HgCl$_2$ in about 14 days (Fig. 5), with final OD$_{600}$ values approaching those of cells grown in the absence of Hg(II). Thus, MBD improves survival of *D. radiodurans* in the presence of Hg(II).

DISCUSSION

The wild-type, full-length MerR dimer has two potential Hg(II)-binding sites, but it only binds one Hg(II) *in vitro*, possibly because an allosteric change upon binding the first metal ion renders the second site unavailable when MerR is not bound to DNA. The stoichiometry of MBD-Hg(II)
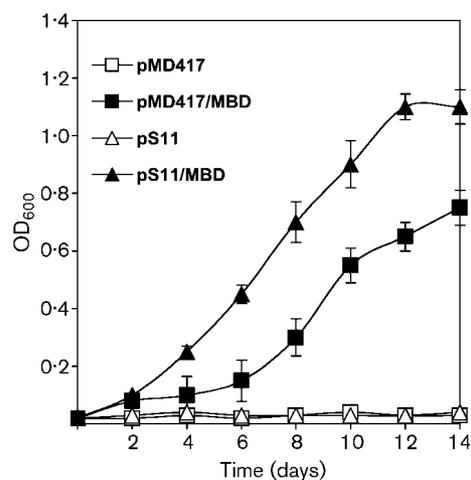

Fig. 5. Tolerance to Hg(II) (10 µM HgCl$_2$) exposure of *D. radiodurans* expressing cytosolic MBD. □, *D. radiodurans* with pMD417; ■, *D. radiodurans* with pMD417/MBD (~10 copies per cell) (Brim *et al.*, 2000); △, *D. radiodurans* with pS11; ▲, *D. radiodurans* with pS11/MBD (~150 copies per cell) (Brim *et al.*, 2000). pMD417 is a derivative of pS11 in which the integration sequence has been reduced from 11 kb to 4 kb (Brim *et al.*, 2000).

binding we observed on the cell surface is consistent with previous *in vitro* observations of full-length and deleted versions of MerR (Zeng *et al.*, 1998), and indicates that MBD has the same mechanism of Hg(II) binding. X-ray absorption fine structure spectroscopy (XAFS) studies (Song *et al.*, 2004) have shown that soluble purified MBD (non-fused) and MerR have nearly identical Hg(II)-binding environments.

Wild-type OmpA is present in the *E. coli* outer membrane at about 100 000 copies per cell (Lugtenberg & Van Alphen, 1983), and Lpp-OmpA passenger protein expression was estimated to have been between 50 000 and 100 000 copies per cell, with the highest copy number seen in overnight cultures (Francisco *et al.*, 1993). The amount of the expressed Lpp-OmpA passenger protein fusion depends on both the inducer concentration and the duration of induction (Daugherty *et al.*, 1999). We found that after only 4 h exposure to 0·2 % arabinose at 25 °C the expression of Lpp-OmpA-MBD was approximately 20 000 copies per cell (Table 1) and we expect the expression level would increase with longer induction time. We chose not to overexpress the protein further to avoid greater stress on the cell also challenged with Hg(II).

The Lpp-OmpA-MBD fusion protein was fully functional on the outer surface of *E. coli*. Even with 19 μM Zn(II) and other competing ligands including low-molecular-mass thiols in rich medium, the Hg(II) occupancy of the secreted protein was comparable to that observed in limiting (i.e. non-equilibrium) dialysis of purified MBD *in vitro* (Song *et al.*, 2004; Zeng *et al.*, 1998). The negative surface charge of *E. coli* (Mangia *et al.*, 1995) binds many divalent cations and the periplasm has cysteine-rich proteins involved in generating disulfide bonds in secreted proteins (Nelson & Creighton, 1994) that will also serve as a sink for thiophilic metal ions such as Hg(II). Nonetheless, under these conditions the amount of Hg(II) bound to the cells expressing MBD increased over sixfold compared to cells not expressing the construct, equivalent to that provided by full-length, surface-displayed MerR in the absence of competing thiols (Bae *et al.*, 2003).

The metal ion specificity of such engineered sequestration systems has been measured in different ways by different investigators. The earliest efforts in this area were those of de Lorenzo and coworkers, who employed surface display of poly-His proteins (Sousa *et al.*, 1996), later of metallothioneins (Sousa *et al.*, 1998; Valls *et al.*, 1998, 2000a) and most recently short peptides (Kotrba *et al.*, 1999a). These several surface-display systems enhanced single metal binding of Cd(II), Cu(II) or Zn(II) in *E. coli* and *Ralstonia eutropha* grown and induced in low-phosphate minimal medium. Wilson's group (Chen & Wilson, 1997b) achieved metal-specific intracellular sequestration of Hg(II) by co-expression of two thiol-rich, cytosolic, nonspecific-metal-binding proteins, metallothionein and glutathione *S*-transferase, concurrently with the Hg(II)-specific *mer* transport system, MerP and MerT. Their work demonstrated for the first time

that these two *mer* transport proteins do not mistake other thiophilic metal ions such as Cd(II) for Hg(II) when exposed to both in a thiol-free phosphate buffer. Their system has been implemented in a hollow-fibre batch reactor (Deng & Wilson, 2001) with fast kinetics and high capacity for Hg(II) removal from dilute, oxidized systems such as urban wastewater. The third major contributors to this area, Wilfred Chen and his coworkers, have compared cytosolic, periplasmic and surface display of synthetic phytochelatins in *E. coli* (Bae *et al.*, 2000, 2001) and *Moraxella* (Bae *et al.*, 2002) for their acquisition of Hg(II) by cells growing and being induced in minimal medium; they found surface expression to be more effective. More recently, Chen's group (Bae *et al.*, 2003) has found that in the absence of competing thiols, cells expressing full-length MerR monomer anchored on the outer membrane by ice nucleation protein can bind sixfold more Hg(II) than cells without this displayed construct. Such cells also bind Hg(II) preferentially in 100-fold excess Cd(II) or Zn(II). All prior work has been reported in terms of mass or moles of metal per gram of cell wet or dry weight. Heretofore, no study has to our knowledge quantified the copies of protein expressed per cell or calculated metal : protein stoichiometry.

We chose to examine the metal-binding specificity of our constructs in a common, inexpensive complex medium as might be employed in a pump-and-treat or chemostat regime. Under these conditions in LB medium there was a default ∼24-fold molar excess of Zn(II) over the 0·8 μM Hg(II) used in these experiments. Nonetheless, the pair of binding sites in surface-displayed MBD molecules was 65 % saturated (Table 1). This is equal to or better than what has been measured for MBD or MerR in non-equilibrium dialysis with a buffer containing no competing metal ions (Song *et al.*, 2004; Zeng *et al.*, 1998). A single additional molar equivalent of Cd(II) excluded all but 45 % of Hg(II) from surface-displayed MBD. The magnitude of Hg(II) displacement by the small increment of Cd(II) in addition to the 19 μM Zn(II) can be explained by two facts. First, Cd(II) is known to be a better inducer for MerR than is Zn(II) (Caguiat *et al.*, 1999; Ralston & O'Halloran, 1990); Cd(II) also has a much higher affinity for MerR than Zn(II) (L. Olliff and others, unpublished). Secondly, while the Hg–S bond is thermodynamically stable, it is kinetically labile (McAuliffe, 1977). Thus, in the presence of low-molecular-mass thiols as in rich media and many biological systems, Hg(II) can be displaced from protein ligands by bis-coordination with buffer thiols, allowing opportunity for Cd(II) to occupy the MBD-binding site, which it does with MBD and MerR *in vitro* and in the cytosol (Song *et al.*, 2004). In thiol-free media there are no ligands sufficiently avid for Hg(II) to displace it from the MerR tri-coordinate site. So Cd(II), even when present in very large excess, has no access to the protein metal-binding site. It would be interesting to see the performance of the surface-displayed MerR monomer (Bae *et al.*, 2003) and the coupled MerP-MerT with cytosolic metallothionein and glutathione *S*-transferase (Chen & Wilson, 1997a, b; Chen *et al.*, 1998) in rich media.

As noted above, MerR has the highest measured affinity for Hg(II) of any of the Mer proteins. In contrast, the transport proteins including MerP and MerT hold Hg(II) only transiently as they shuttle it into the cell for reduction by MerA. Thus, in the high-thiol environment of the cytosol, overexpression of transport proteins and metallochaperones might not provide much protection against Hg(II). Indeed, we have recently reported (Ledwidge *et al.*, 2005) that cytosolic overexpression of the amino-terminal domain of MerA (NmerA), a homologue of periplasmic MerP, alone does not afford any protection against Hg(II). However, since MBD competes effectively for Hg(II) even in a high-thiol milieu we asked whether it could protect cells from Hg(II) exposure. Although *E. coli* containing the wild-type *mer* operon can grow in rich medium with 50 μM Hg(II), *E. coli* with surface-displayed MBD did not grow in LB liquid medium even at 25 μM Hg(II) (data not shown). This is because a strain containing the wild-type *mer* operon reduces Hg(II) to the less toxic monoatomic gas, Hg^0, which evaporates from the culture medium (Barkay *et al.*, 2003). However, a strain expressing MBD on its surface can recover from exposure to even 120 μM Hg(II) when subsequently cultured in Hg(II)-free medium (Fig. 4). Thus, surface-displayed MBD, like other toxic-metal-binding proteins, can sequester Hg(II), preventing, up to a point, its interaction with sensitive cellular targets. So, unlike cells with the *mer* operon, which volatilize Hg^0 into the atmosphere, cells expressing MBD can entrap Hg(II) for subsequent removal by filtration or precipitation. With appropriate safeguards against dissemination of recombinant DNA, cells with surface-displayed MBD might even be employed in treatment of humans or animals exposed to mercury compounds, since the major natural route of mercury elimination is through the intestine.

Surface expression of metal-sequestering proteins has not yet been enabled for bacteria other than the γ-proteobacteria. However, we find that cytosolic expression of MBD in *D. radiodurans* affords increased tolerance of Hg(II) (Fig. 5), consistent with the MBD sequestering cytosolic Hg(II) in this microbe. Future work can be carried out to define and optimize this constitutive sytem for implementation in Hg(II) recovery from high-level radioactive waste environments. The fact that cells of both types can grow for several hours (or days in the case of *D. radiodurans*) while continuously displaying active MBD would be advantageous in either a pump-and-treat or a flow-through regime.

Prior to the year 2000 the Universal Treatment Standard (UTS) limit for mercury was 1 μM for materials subjected to toxicity characteristic leaching procedure (TCLP) as prescribed by EPA regulations 40 CFR 261 (Fuhrmann *et al.*, 2002). However, since 2000 the more stringent UTS limit of 0·125 μM Hg has been the target end point (Fuhrmann *et al.*, 2002). This eightfold more stringent criterion increases the challenge especially for low-level mercury remediation, and the consequent need for novel high-affinity techniques for removal of Hg(II) from ground water. The fact that MBD-expressing cells can sequester Hg(II) while they are growing in complex medium and hold it in a non-toxic form inside or outside the cytoplasm makes such constructs potential candidates for a role in meeting the new standards. In addition, many metalloregulatory proteins function as homodimers and the metal-binding sites are contributed by the two identical monomers as in CueR and ZntR (Changela *et al.*, 2003), SmtB, CzrA (Eicken *et al.*, 2003) and CadC (Wong *et al.*, 2002). Our data show that engineering both binding sites into a single peptide can contribute to understanding the properties of the metal-binding domain itself as well as to making application of it for removing the metal from the environment.

ACKNOWLEDGEMENTS

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