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Evidence for direct interactions between the mercuric ion transporter (MerT) and mercuric reductase (MerA) from the Tn*501mer* operon

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Abstract Mercuric ion resistance in bacteria requires transport of mercuric ions (Hg^{2+}) into the cytoplasmic compartment where they are reduced to the less toxic metallic mercury (Hg^0) by mercuric reductase (MR). The long-established model for the resistance mechanism predicts interactions between the inner membrane mercuric ion transporter, MerT, and the N-terminal domain of cytoplasmic MR, but attempts to demonstrate this interaction have thus far been unsuccessful. A recently developed bacterial two-hybrid protein interaction detection system was used to show that the N-terminal region of MR interacts with the cytoplasmic face of MerT. We also show that the cysteine residues on the cytoplasmic face of the MerT protein are required for maximal mercuric ion transport but not for the interaction with mercuric reductase.

Keywords Protein–Protein interactions · Mercuric ion transport · Mercuric ion reductase

Abbreviations
MR Mercuric reductase

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Introduction

Bacterial mercuric ion resistance involves a set of diverse genes, which are widespread in both Gram-positive and Gram-negative bacteria and are located on mobile genetic elements such as plasmids or transposons (Hobman and Brown 1997). These mercuric ion resistance genes are grouped in *mer* operons and encode proteins which detoxify mercury by importing Hg^{2+} ions via the inner membrane protein MerT into the cytosol, where they are reduced to the less toxic and volatile elemental state, Hg^0, by mercuric ion reductase (MR), an NADPH-dependent flavoenzyme (Fox and Walsh 1982).

Functional MR is a dimeric protein, which has mercuric ion binding sites consisting of two pairs of cysteine residues. Cys135 and Cys140 from one subunit make the redox-active site (Distefano et al.

1989), whereas Cys558 and Cys559 from the other subunit are important in Hg(II) removal from large and high-affinity mercury ligands (Engst and Miller 1999; Miller et al. 1989). A crystal structure of MR from *Bacillus* sp. strain RC607 which solved the folding of the protein at the catalytic site (Schiering et al. 1991), implicated a pair of tyrosine residues (which are also conserved in MRs from Gram-negative bacteria) in mercury coordination (Rennex et al. 1993).

Diversity in MRs from different organisms lies in the N-terminal region of the enzyme. Most MRs (such as in the Tn*501* mercury resistance operon) have a N-terminal region of about 100 amino acids, which appears to be a flexible domain in the MR from *Bacillus* sp. RC607 as it did not appear in the crystal structure (Schiering et al. 1991). Some MRs from Gram-positive bacteria have an additional copy of this region (e.g. MR encoded by transposon Tn*5084*) and others lack it (e.g. MR from *Streptomyces* sp.). This MR diversity and the fact that a clipped version of MR, in which the N-terminal region had been removed by chymotrypsin digestion, but was still functional in vitro (Fox and Walsh 1983) suggests that the N-terminal region of MR is not essential for mercury detoxification, but may be required for maximal efficiency. In vivo studies of the Tn*501* MR often show two forms of the enzyme, one full length and the other in which the N-terminus has been proteolytically removed (Moore and Walsh 1989). This has no apparent effect in the mercury reduction capacity of the cell.

In Tn*501*, the N-terminal domain of MR is very similar in amino acid sequence to the small Hg^{2+} binding periplasmic MerP protein (Barkay et al. 2003). It has a cysteine-containing metal binding site, GMTCXXC (Didonato et al. 1997), which implies a metal chaperone function for this region, and which is similar to metal binding sequences of other heavy metal associated (HMA) proteins. Previous studies on the mechanism of bacterial mercury detoxification showed that MerP was acting not only as a periplasmic mercury binding protein (Lund and Brown 1987), but also as a metallochaperone, delivering divalent mercuric ions to the inner-membrane protein MerT (Morby et al. 1995). The hypothesis that the N-terminal domain of MR was acting as a metallochaperone to receive a mercuric ion from the inner-membrane mercury uptake protein (MerT) and transfer it to the catalytic site of the reductase via thiol exchange (Brown 1985) appears quite plausible.

More recently, the N-terminal domain of the *Ralstonia* (now *Cupriavidus*) *metallidurans* CH34 MR (99% identical to MR from Tn*501*) was studied as a single peptide. It had striking similarities in secondary structure to MerP, as well as the ability to bind a single mercuric ion per peptide with the same Kd as MerP (Rossy et al. 2004a). Similarly, the N-terminal domain of MR from Tn*501* (expressed as a peptide) bound mercuric ions in a 1:1 molar ratio and could remove mercuric ions from proteins and deliver them to the catalytic core of MR (Ledwidge et al. 2005). These authors also showed that the role of the N-terminal domain of MR was particularly important under conditions of glutathione depletion. Mercuric ions bound to a peptide mimicking the cytoplasmic loop of MerT were displaced upon addition of a peptide simulating the N-terminal domain of MR from *R. metallidurans* CH34 (Rossy et al. 2004b). Preferential binding of mercuric ions to the N-terminal region of MR suggests that mercuric ions are likely to be transferred from the cytoplasmic loop of MerT to the N-terminal region of it (Rossy et al. 2004b), but evidence of direct interactions between MerT and MR are needed to provide formal confirmation of this model.

In this paper we investigate the hypothesis that a direct interaction occurs between the mercuric ion transporter, MerT, and mercuric reductase from Tn*501*, using a bacterial two hybrid system in *E. coli* (Karimova et al. 1998). We show that the C-terminal region of MerT and the N-terminal region of MR interact weakly and that this interaction is not dependent on cysteine residues or mercury binding. We also show that only cysteine residues in the cytoplasmic loop of MerT, and not in the N-terminal domain of MR, are essential for maximal mercury detoxification.

Methods

Plasmids and strains

Cloning and volatilisation assays were performed in the *E.coli* K-12 strain TG2 (*supE*, *hsdΔ5*, *thi*, *Δ(lac-proAB)*, F′[*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15], Δ(*srl-recA*)306::Tn*10* (tet^r)) and bacterial two hybrid assays were performed in *E.coli* K-12 strain BTH101 (Karimova et al. 1998), a *cya* deficient strain (*F*$^-$, *Cya-854*, *RecA1*, *EndA1*, *gyrA96* (Nal^r),*thi1*, *hsdR17*, *spoT1*, *rfbD1*, *glnV44(AS)*).

The plasmids used in this work are shown in Table 1. Interactions between target proteins were studied with two expression vectors, pKT25 and pUT18 expressing complementary domains of adenylate cyclase. The plasmids pUT18-Zip and pKT25-Zip express the two adenylate cyclase domains fused to leucine zippers as a positive control for protein-protein interaction (Karimova et al. 1998). The *merT* and *merA* genes were amplified by PCR from pBRmerBS2, a pBR322 derivative containing the *mer* operon from Tn*501* (Lund and Brown 1987), and were cloned into pKT25 and pUT18 for protein interaction studies, as detailed below.

Plasmids used in ^{203}Hg volatilisation assays were pBRmerBS2 derivatives in which cysteine codons in *merT* and *merA* within a complete *mer* operon had been mutated to serine codons using the method of Stanssens et al. (1989). Plasmid pBRmerBS2 was used as the mercury resistant control.

Construction of MerT and MR cysteine mutants

Cysteine mutants of MerT at position 76 and 82 were constructed as described previously (Morby et al. 1995). All other cysteine mutants of MerT and MR were constructed by the gapped duplex mutagenesis method of Stanssens et al. (1989), using the mutagenic oligonucleotides listed in Table 2.

Construction of the bacterial two-hybrid plasmids

The *merT* gene was amplified by PCR using different sets of primers (MerT-3, MerT-9 and MerT-14, each with MerT-R) to allow construction of three different fusions with the T25 domain of adenylate cyclase (Table 2). When cloned in pKT25 using *Pst*I and *Kpn*I, the MerT protein was expressed fused to T25 with a 3 amino acid spacer. When *Xba*I and *Kpn*I were used, MerT was separated from T25 by 9 amino

Table 1 Description of plasmids used in this study

Plasmid	Description	Reference
pUT18	Ap^r; encodes the T18 fragment of CyaA and allows a fusion with the C-terminal of the protein of interest	(Karimova et al. 1998)
pUT18-Zip	Ap^r; encodes the T18 fragment of CyaA fused in frame with the leucine zipper of GCN4	(Karimova et al. 1998)
pUT18-MR-N term	Ap^r; encodes the T18 fragment of CyaA fused to the C-terminus of the first 88 aa of MerA from Tn*501*	This work
pUT18- MR-Clipped	Ap^r; encodes the T18 fragment of CyaA fused to the C-terminus of the 91-561 last aa of MerA from Tn*501*	This work
PUT18- MR	Ap^r; encodes the T18 fragment of CyaA fused to the C-terminus of full length MerA from Tn*501*	This work
pUT18-Cxx	Ap^r; encodes the T18 fragment of CyaA fused to the C-terminus of the N terminal domain of CyaA which cysteine residues had been mutated to serine	This work
pKT25	Kan^r; encodes the T25 fragment of CyaA and allows a fusion with the C-terminal of the protein of interest	(Karimova et al. 1998)
pKT25-Zip	Kan^r; encodes the T25 fragment of CyaA fused in frame with the leucine zipper of GCN4	(Karimova et al. 1998)
PKT25-T3, T9 and T14	Kan^r; encodes the full-length MerT from Tn*501* fused to the C-terminus of the T25 CyaA domain respectively with a 3, 9 or 14 amino acids spacer	This work
pKT25-Cxx	Kan^r; encodes the full-length CxxS cysteine mutants of MerT fused to the C-terminus of the T25 CyaA domain with a 9 amino acids spacer	This work
pBRmerBS2	Ap^r ; Hg^r The Tn*501* mer operon cloned into pBR322 (*Eco*RI/*Nru*I)	(Wilson et al. 2000)
pBRmerA CxxS	Ap^r; Hg^r; pBRmerBS2 with a mutation in *merA* to change cysteines at position xx into Serine	This work
pBRmerT CxxS	Ap^r; Hg^r; pBRmer BS2 with a mutation in *merT* to change cysteines at position xx into Serine	This work
pBRmer CxxS,CxxS	Ap^r; Hg^r; pBRmer BS2 with a mutation in *merT* and in *merA* to change respectively cysteines at position xx of MerT and xx of MerA into Serines	This work
pBR322	Ap^r, Tc^r	(Watson 1988)

Table 2 Sequence and description of the primers used to amplify the *mer* genes and for DNA sequencing

Name	Sequence[a]	Restriction site	Purpose
MerT-3	ATT CGA A*CT GCA G*AG CGC **ATG**	*Pst*I	Amplification of *merT* to allow a 3aa fusion to T25
MerT-9	A ATT CG*T CTA GA*C AAG CGC **ATG** TC	*Xba*I	Amplification of *merT* to allow a 9aa fusion to T25
MerT-14	CCA ATT TC*G GAT CC*A AAG GAC	*Bam*HI	Amplification of *merT* to allow a 14aa fusion to T25
MerT-R	CAT GGA G*GG TAC C*GA TCA GTA GAA	*Kpn*I	Reverse primer for amplification of *merT*
MerAN-F	AAA G*AA GCT T*TT GCA TGA CCC ATC	*Xba*I	Amplification of residues 1 to 88 of MerA
MerAN-R	CTT T*TC TAG A*GC GGC CAT CCA TCC	*Hin*dIII	Amplification of residues 1 to 88 of MerA
MerAGlu-F	GA*A AGC TT*C AGT GGC AAC GAG	*Xba*I	Amplification of residues 91 to 561 of MerA
MerA-R	ACC TCC TTT T*TC TAG A*CC GGC	*Hin*dIII	Reverse primer for amplification of MerA
MerT cys 76	GCA GCGGCC AGC AAA CCG GG		MerT mutagenesis TGC (cys) changed to AGC (ser)
MerT cys 82	T GAG GTC AGC GCG ATT CCC C		MerT mutagenesis TGC (cys) changed to AGC (ser)
MerA cys 10	GGC ATG ACT AGC GAC TCG		MerA mutagenesis TGC (cys) changed to AGC (ser)
MerA cys 13	CT TGC GAC TCG AGC GCG GCG C		MerA mutagenesis TGC (cys) changed to AGC (ser)

[a] Italic sequences represent introduced restriction sites, underlined sequences represent changes to the original sequence and bold sequences show start codons

acids. Finally, using *Bam*HI and *Kpn*I cloning, MerT was fused to T25 with a 14 amino acid spacer. These different constructs allowed some flexibility at the junction between the transmembrane regions of MerT and the T25 domain of the adenylate cyclase (Fig. 1).

Regions of *merA* were amplified by PCR using different primers (Table 2) to investigate the possible location for any interaction of MR with MerT (Fig. 2). The N-terminal region of MR was amplified with MerAN-F and MerAN-R, and fused in-frame with the T18 domain of adenylate cyclase when cloned as a *Hin*dIII-*Xba*I fragment. MerAGlu-F and MerA-R primers were used to amplify the glutathione reductase-like region of MR (lacking the N-terminal region) and subsequent *Hin*dIII-*Xba*I cloning allowed an in-frame fusion with T18. Amplification of the full-length *merA* gene was performed using MerAN-F and MerA-R and fusion with T18 was achieved by *Hin*dIII-*Xba*I cloning.

MerT cysteine mutants were amplified using MerT-9 and MerT-R and cloned into pKT25 using *Xba*I and *Kpn*I, which generated fusions of the resulting mutants with the T25 domain of adenylate cyclase. Cysteine mutants in the N-terminal region of MR were amplified using the MerAN-F and MerAN-R primers and fused in frame to the T18 domain of the adenylate cyclase when cloned in pUT18 by *Hin*dIII/*Xba*I cloning. The identity of each clone was verified by DNA sequencing.

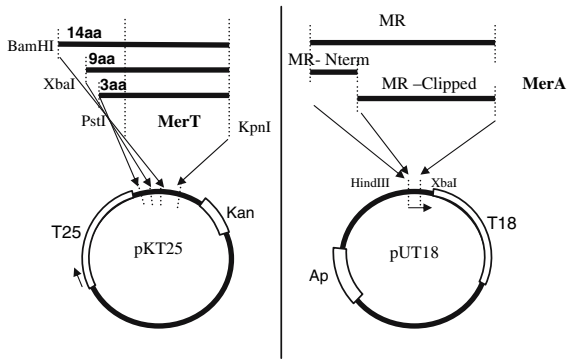

Fig. 1 Gene fusions of *merT* and *merA* constructed in the pKT25 and pUT18 plasmids

Confirming expression of the recombinant proteins

The protocol used for ''maxicell'' ^{35}S-labelling and autoradiographic visualisation of plasmid-encoded proteins was adapted from Sancar et al. (1979) as described previously (Hobman and Brown 1996).

The bacterial two hybrid assay

E.coli BTH101 cells were plated on L agar plates (Sambrook et al. 1989) containing 40 μg Xgal ml^{-1}and 0.5 mM IPTG. A single white *E.coli* BTH101 colony (*lacZ*$^{-}$ phenotype) was inoculated into L broth (Sambrook et al. 1989) and grown overnight at 37°C. This ensured that the adenylate cyclase mutation had not reverted to a wild type phenotype. Cells were

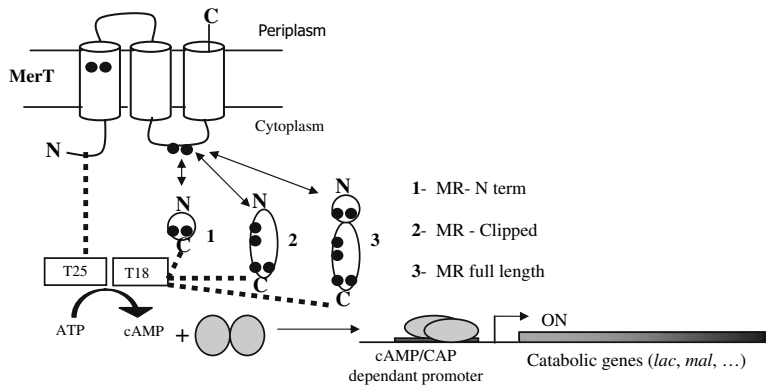

Fig. 2 Use of the bacterial two-hybrid system to study interactions between MerT and MerA (MR). MerT was fused to the C-terminal part of the T25 domain of the adenylate cyclase. Different regions of MerA were fused to the N-terminal part of the T18 domain of the same enzyme (**1–3**). Interactions between tested proteins reconstitute the adenylate cyclase enzymatic activity which leads to activation of catabolic genes (*lac* and *mal* operons). Black dots represent cysteine residues present in MerT and MerA

diluted (1:100) into 5 ml of the same medium and growth was continued until the $OD_{600} = 0.5$. Cells were then harvested at 6,000 *g* for 30 min at 4°C and washed three times in ice cold 10% glycerol. These electrocompetent cells were then resuspended in 2 ml of 10% ice cold glycerol.

Electrocompetent cells were mixed with approximately 10 ng of each of the pKT25 and pUT18 plasmid derivatives and placed on ice in a 2 mm gap electroporation cuvette (Geneflow, UK). Electroporation was performed at 2,500 V in an Equibio Easyject electroporator and cells were immediately transferred into 940 µl of L broth. After 1 h of incubation at 37°C, cells were harvested at 20,000 *g* for 5 min, resuspended in 200 µl L broth and transferred to a sterile 96-well plate. Using a replica plating device, cells were plated onto L agar plates containing 40 µg Xgal ml^{-1} and 0.5 mM IPTG and onto McConkey agar plates containing 1% w/v maltose. Cells were selected on 200 µg carbenicillin ml^{-1} and 50 µg kanamycin ml^{-1}. Interactions were also tested on plates containing mercuric chloride at the sub-lethal concentration of 1 µg ml^{-1}. Plates were incubated at 37°C overnight and at room temperature for an additional 2 days.

LacZ assays:

A single colony of *E.coli* BTH101 transformed with the two plasmids was inoculated into 5 ml of M9 medium supplemented with 0.08% w/v of casamino acids, 1% w/v of maltose, 1 mM $MgSO_4$ and 0.0002% w/v of thiamine, and grown for 3 days at 37°C. *β*-galactosidase assays were performed as described previously (Miller 1972).

Volatilisation assays

Mercury volatilisation assays were performed as described previously (Wilson et al. 2000) using $^{203}HgCl_2$ kindly provided by Delon Barfuss (Georgia State University, USA).

Results and discussion

The N terminus of MR interacts with MerT

The results shown in Fig. 3a suggest that only the N-terminal domain of MR interacts with the inner-membrane protein MerT. *E.coli* BTH101 cells transformed with pKT25-MerT (with three different length fusions) and pUT18-MR-Nterm turned red on MacConkey agar plates containing maltose after 3 days of incubation. Other cells remained yellow/pink, indicating they were unable to metabolize maltose and therefore lacked adenylate cyclase activity. Both positive (pKT25-ZIP and pUT18-ZIP) and negative (pKT25 and pUT18) controls displayed the expected phenotype. The results were confirmed by *β*-galactosidase assays (Table 3). Interactions between MerT and MR appeared to be relatively weak, since cells showing the interaction gave approximately 30% of the

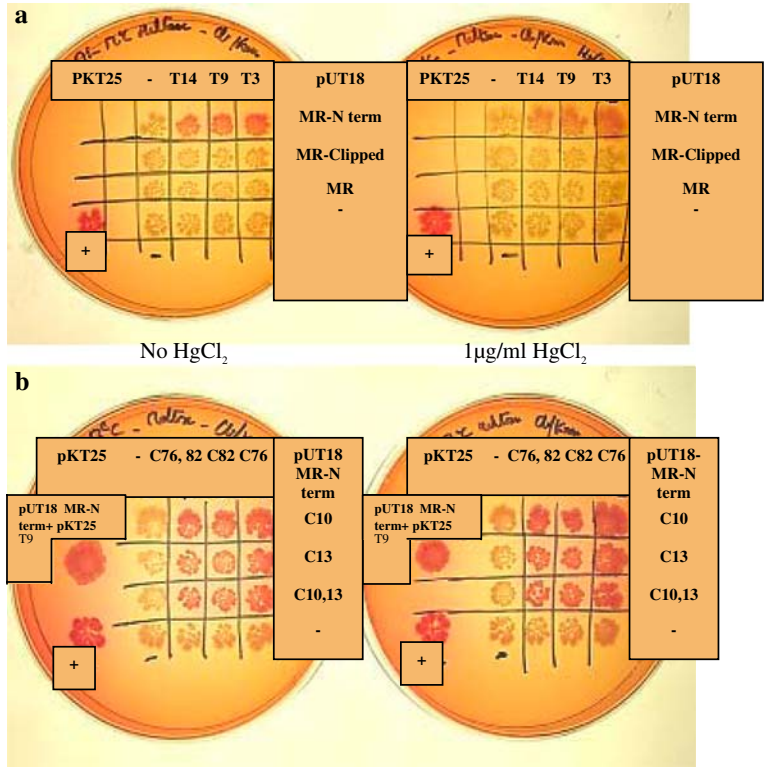

Fig. 3 Activation of *mal* operon in *E. coli* upon interaction between MerT (wild type and cysteine mutants) and different domains of MerA (wild type and cysteine mutants). **+** represents the positive control pKT25-Zip and pUT18-Zip; **–** represents the negative controls either pKT25 or pUT18; **MR-Nterm** represents the N-terminal 88 amino acids of MerA fused to T18; **MR-Clipped** represents amino acids 91-561 of MerA (the glutathione reductase-like region) fused to T18; **MR** represents full-length MerA fused to T18. **T3, T9 and T14** indicate fusions of the whole MerT to the T25 domain of adenylate cyclase respectively with 3, 9 and 14 amino acids spacers in pKT25. **pKT25 c76, c82, c76,82** represent the pKT25- Cxx constructs and **pUT18- c10, c13, c10,13** represent the pUT18- Cxx constructs (Table 1)

β-galactosidase activity observed in the positive control. In a system where mercuric ions are transported from MerT to the catalytic site of mercuric reductase, interactions between the transporter and the enzyme may be transient and weak.

The fact that native MR did not show any interaction with MerT was initially surprising. However, it is known that the N-terminal domain of MR can be proteolytically removed from the catalytic core of the protein in vitro at position 85 by chymotrypsin digestion, and removal of the N-terminus of MR occurs in vivo (Fox and Walsh 1982, 1983; Moore and Walsh 1989). When expression of the fused proteins was checked by radiolabelling, the MR-T18 fusion appeared as two protein bands of approximately 78 kDa and 72 kDa. These bands correspond to the two forms of MR in which the N-terminal region has been either cleaved or is still present, as seen previously in vivo (Moore and Walsh 1989). In this case, the N-terminal region of MR may be present without being fused to the T18 domain of the adenylate cyclase because it had been proteolytically removed from the fusion. It is also possible that the complete MR protein fused to the T18 domain did not allow steric interaction between the two adenylate cyclase domains, even if interaction between MR and MerT did take place.

The glutathione reductase-like region of MR (MR lacking the N-terminal domain) was fused to the T18 domain with no evidence of proteolytic cleavage, and no interaction with MerT could be detected. Such an interaction was not predicted, but expression of the fusion of MR-Clipped with T18 was very low (data not shown). Previous studies on MR also reported a very weak expression of the enzyme from a truncated *merA* gene in which the N-terminal 85 amino acids

were not encoded (Moore and Walsh 1989). From this set of data, weak and transient interactions were shown to occur between the cytoplasmic loop of MerT and the N-terminal domain of MR.

Interactions between MerT and MR were also studied in the presence of mercuric ions to assess whether the interactions were mercury dependent or increased by formation of a ternary complex between MerT, MR and Hg^{2+}. When detected on plates, interactions appeared to be slightly weakened by the presence of mercuric chloride (Fig. 3a). Quantitation of the interactions confirmed this, with significant decrease of β-galactosidase activities, when measured in the presence of 1 μg ml^{-1} mercuric chloride Table 3. Although mercuric chloride was used at a sublethal concentration that did not affect cell growth (Lund and Brown 1987), it may have interfered with the β-galactosidase assay since activities detected in the postitive control were lower in the presence of mercury (30% decrease). When comparing relative activities (in percentage of positive controls), we found that interactions between MerT and the N-terminal domain of MerA were in fact decreased by less than 10%. In the context of a transfer model of mercuric ions from MerT to the N-terminal region of MerA, the recipient peptide is expected to be mercury free in order to accept mercuric ions. In our study, addition of mercuric ions probably resulted in both fused proteins being bound to mercuric ions. Changes in both conformations of interacting domains may be an explanation for the observed decrease in the interaction level.

Altogether, these data show that interactions between the cytoplasmic loop of MerT and the N-terminal domain of MerA do take place in vivo and that they are not dependent on mercury, but are weakened in the presence of mercuric ions.

Cysteine residues in the interacting domains of MerT and MerA are not required for interaction

The observation that mercuric ions did not enhance interactions between regions of MerT and MR was further tested by interaction studies in which fusion proteins carrying mutations in the cysteine residues of the MerT cytoplasmic loop and the MR N-terminal domain were used. The results of these experiments are shown in Fig. 3b. Neither MerT cytoplasmic nor MR N-terminal cysteine residues were found to affect the interactions between the proteins. In all combinations of interactions between the cysteine mutants of MerT and MR, *E. coli* BTH101 cells turned red on MacConkey medium supplemented with maltose after 3 days incubation at room temperature. Similar levels of β-galactosidase activity resulted from interactions between native proteins or with the mutated proteins (data not shown). This suggests that the weak and transient interaction observed between the C-terminal region of MerT and the N-terminal region of MR is dependent on the secondary structure of the proteins, that the cysteine residues are not essential for the correct folding of the proteins, and that Hg(II) binding is not necessary for the interaction. Previous studies on MerT cytoplasmic cysteine mutants (Cys76 and Cys82) showed they were not essential for mercury resistance but, when replaced by serine residues, the mercury resistance level decreased by about 20 % (Morby et al. 1995). Since mercury transport is the rate-limiting step in the detoxification process (Lund and Brown 1987), this decrease in resistance could be the result of less efficient mercuric ion transfer from MerT to MR.

Table 3 β-galactosidase activity measured in *E. coli* BTH101 cells[a]

Plasmids expressed in *E. coli* BTH101	β-galactosidase activity[b] without mercury	β-galactosidase activity[b] with 1 μg/ml $HgCl_2$
pUT18 and pKT25	88 ± 1	79 ± 2
pUT18-MR-Nterm and pKT25-T14	977 ± 27	486 ± 9
pUT18-MR-Nterm and pKT25-T9	1035 ± 34	483 ± 33
pUT18-MR-Nterm and pKT25-T3	1154 ± 16	504 ± 21
pUT18-ZIP and pKT25-ZIP	2968 ± 24	2068 ± 46

[a] Results are the mean of three independent triplicate measurements

[b] Activity expressed in Miller units ($=OD_{414nm}/(T_{min}*V_{ml}*OD_{600nm}) * 1000$)

Maximal mercury transport requires cysteine residues in the cytoplasmic loop of MerT but not in the N-terminal region of MerA

Since transport is the rate-limiting step in mercury detoxification, the rate of mercury volatilisation can be used as a measure of the rate of mercury transport from the periplasm to the cytoplasm. Using in vivo ^{203}Hg volatilisation assays, we found that only the cysteine residues located in the cytoplasmic loop of MerT (Cys76 and Cys82), and not those in the N-terminal domain of MR (Cys10 and Cys13), are important for optimal mercury transport (Fig. 4). Mutagenesis of the cytoplasmic cysteine residues of MerT (Cys76Ser and Cys82Ser) considerably reduced the rate of mercury volatilisation (Fig. 4a),

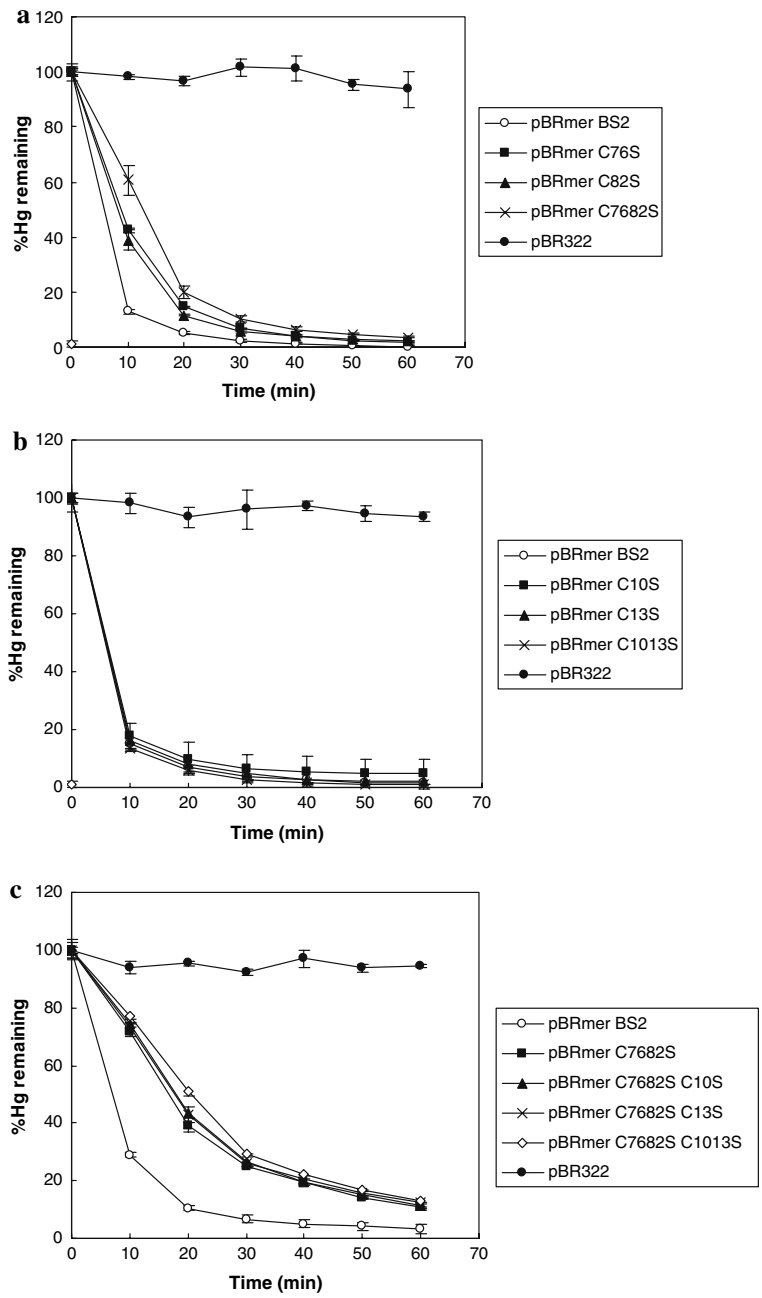

Fig. 4 Mercury Volatilisation Assays on *E. coli* TG2 (pBR*mer*BS2) mutant strains. Cys to Ser changes: (**a**) on the cytoplasmic face of MerT; (**b**) in MR-Nterm; (**c**) in both MerT cytoplasmic face and MR- Nterm. Error bars show one standard deviation (σ_{n-1} of triplicate readings)

showing that they are an integral part of mercuric ion transport across the cytoplasmic membrane. However, cysteines in the N-terminus of MR do not appear essential for in vivo mercury detoxification. When these residues (Cys10, Cys13) were mutated to serine, no change in mercuric ion volatilisation rate was observed (Fig. 4b), suggesting Hg^{2+} is transferred in a different way. It has been recently postulated that other cytoplasmic thiol groups, such as glutathione, which is present at high concentration, could participate in mercury transfer from MerT to MR (Barkay et al. 2003; Ledwidge et al. 2005). Mercuric chloride hypersensitive *E. coli* cells (expressing MerT and MerP without MR) were found to be slightly more sensitive to mercury in absence of glutathione (in a *gshA*::Tn*10* mutant) suggesting that this peptide can be the target of imported mercuric ions by the MerP/MerT transport system, and may therefore act as an internal chaperone for mercuric ions (Latinwo et al. 1998).

When expressed with the MerT C76S, C82S double mutant (Fig. 4c), the MerA C10S, C13S double mutant showed a decrease in the mercury volatilization rate. This result is consistent with the two-hybrid interaction assays where double cysteine mutants of the cytoplasmic loop of MerT and N-terminal domain of MR were found to interact in vivo. We propose that the interaction between the two mutated proteins that are no longer able to exchange mercuric ions is responsible for the decrease in the rate of mercuric ion transfer.

In vivo interactions between the cytoplasmic loop of MerT and the N-terminal domain of MR imply that mercuric ions are preferentially transferred from the cytoplasmic loop of MerT to the N-terminal domain of MR unless thiol-containing molecules from the cytoplasm, such as glutathione, are abundant enough to ensure mercuric ion transfer. The N-terminal domain of MR can be proteolytically removed from the catalytic core of MR in vivo, and there appears to be little effect on overall rates of mercury reduction. In mercury volatilisation assays, mercuric chloride is usually added at a low concentration to induce the mercury resistance genes but is well below lethal concentrations to bacterial cells. At higher mercury concentrations (where mercury volatilization is essential for cell survival), it may be that reduced glutathione assumes a greater role in transferring mercuric ions to the catalytic core of MR. However, under extreme oxidative stress, glutathione is involved in balancing the bacterial cell redox state and might not be as available for mercury binding in the cytoplasm. In those conditions, the presence of the N-terminal domain of MR may be essential to transfer mercuric ions from MerT to the catalytic core of MR. Nothing is known about the fate of the N-terminal domain following in vivo proteolytic cleavage from MR; it is similar in structure to MerP and it may act as a cytoplasmic metallochaperone.

Conclusions

A bacterial two-hybrid protein interaction assay system showed that interaction between the cytoplasmic loop of the mercuric ion transporter MerT and the N-terminal region of MR occurs in vivo, and is not enhanced in the presence of mercuric ions. This interaction did not require functional cysteine residues in the respective interacting domains. ^{203}Hg volatilisation assays in *E. coli* expressing both MerT and MR cysteine to serine mutants showed that cysteines located in the cytoplasmic loop of MerT were essential for a maximal rate of mercury volatilisation, but that mercury volatilisation occurred in their absence. Cysteines located in the N-terminal region of MR only affected the rate of volatilisation in the case of a cytoplasmic loop cysteine deficient MerT. These results provide the first demonstration in vivo of MerT-MR interaction and strongly support the model for Hg^{2+} transfer from the inner-membrane protein MerT to the catalytic site of mercuric reductase via the MerP-like N-terminal domain. This domain can interact with MerT and the cysteine residues located in the cytoplasmic loop of MerT are important in mercuric ion transport. Interactions between the cytoplasmic loop of MerT and MR are not essential to the mercury detoxification mechanism from Tn*501* but may make the system more efficient.

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