Role of MerH in mercury resistance in the archaeon *Sulfolobus solfataricus*

James Schelert, Deepak Rudrappa, Tyler Johnson and Paul Blum

Sulfolobales includes diverse thermoacidophilic microbes including species that inhabit hot metal-saturated locations (Orell et al., 2010; Simbahan et al., 2005; Wang et al., 2011). Studies on mercury resistance in *Sulfolobus solfataricus* established the existence and critical regulatory features of an archaeal mercury resistance (*mer*) operon (Dixit et al., 2004; Schelert et al., 2004, 2006). The *S. solfataricus* *mer* locus encodes four genes where *merH*, *A* and *I* are arranged in one transcription unit and *merR* is divergently transcribed upstream of *merH*. Protein phylogenetic analysis and gene disruption studies indicated that *merA* encoded a mercuric reductase required for reduction of mercuric ion, Hg(II), to its elemental form, Hg(0) (Schelert et al., 2004), despite its lack of an active tyrosine residue (Simbahan et al., 2005) in its putative active site. *merI* (122 aa) is located 3’ to *merA* and is separated by a 142 nt intergenic region. It is expressed by constitutive transcription (from its own promoter, *merIp*) and by read-through transcription initiating upstream at *merHp*. Gene disruption studies, however, exclude a role for MerI in mercury resistance or *mer* regulation (Schelert et al., 2006). The *S. solfataricus* MerR transcription factor regulates *merHAI* transcription in a metal-dependent fashion, and site-specific mutations in the DNA binding site of *merR* created *in vivo* positioned the binding site immediately 5’ of the predicted *merHp* TATA box (Schelert et al., 2006). Electrophoretic mobility shift assay demonstrated that MerR/merHp DNA complex formation was template specific and dependent on the

INTRODUCTION

Metallochaperones are cytoplasmic proteins that traffic metal ions to other proteins including metalloenzymes and metal resistance systems (Carter et al., 2009; Robinson & Winge, 2010). While most studies have focused on copper-metallochaperones, other metal specificities occur (Grosseohme et al., 2007; Herbst et al., 2010; Okamoto et al., 2010). Much less is known about the trafficking of heavy metals such as mercury. In the case of proteobacteria, the mercury-specific transport protein, MerP, binds and transfers this metal to the integral membrane protein MerT, thereby depleting its concentration in the periplasmic space (Morby et al., 1995; Serre et al., 2004). Cytoplasmic trafficking of mercury has also been reported through transfer between cysteine residues encoded in the N-terminal domain of bacterial mercuric reductase (Hong et al., 2010).

Microbes that inhabit naturally occurring metal-rich niches provide an opportunity to identify and study novel metal resistance mechanisms. The order Sulfolobales includes extremely thermoacidophilic organisms that thrive in geothermal environments dominated by sulfidic ores and heavy metals such as mercury. Mercuric ion, Hg(II), inactivates transcription in the crenarchaeote *Sulfolobus solfataricus* and simultaneously derepresses transcription of a resistance operon, *merHAI*, through interaction with the MerR transcription factor. While mercuric reductase (MerA) is required for metal resistance, the role of MerH, an adjacent small and predicted product of an ORF, has not been explored. Inactivation of MerH either by nonsense mutation or by in-frame deletion diminished Hg(II) resistance of mutant cells. Promoter mapping studies indicated that Hg(II) sensitivity of the *merH* deletion mutant suggested that the requirement for MerH could result from metal trafficking to the MerR transcription factor.
presence of the binding site, but was insensitive to Hg(II) addition as well as site-specific binding site mutations that relieved in vivo merHp repression (Schelert et al., 2006).

While the roles of MerA and MerR have been determined, the function of MerH was unknown. The merH gene was first identified because of its Cys-Xaa19-22-Cys-Xaa3-Cys or Cx CX C motif together with its location immediately adjacent to mercuric reductase (MerA) that suggested a role in trafficking of mercury (Ettema et al., 2003). However, S. solfataricus MerH lacks homology to well characterized bacterial mercury binding proteins, such as periplasmic MerP and the N-terminal domains found in some MerA proteins (Barkay et al., 2003) (but lacking in S. solfataricus), or to a membrane transporter called MerH in Mycobacterium marinum (Schüe et al., 2009). The results presented here suggest that S. solfataricus MerH is a mercury trafficking protein.

**METHODS**

**Archael strains and cultivation.** Archael strains and plasmids are indicated (Table 1). Primer sequences are available upon request. S. solfataricus strain 98/2 and its derivatives were grown at 80 °C with aeration in batch culture as described previously (Allen, 1959; Rolfsmeier & Blum, 1995; Worthington et al., 2003b) at 80 °C in Allen’s basal salts (Allen, 1959) as modified (Brock et al., 1972) at pH 3.0. Liquid media were supplemented with 0.2 % (w/v) sucrose (SM), 0.2% (w/v) lactose or 0.2% (w/v) tryptone (RM) as carbon and energy sources. Growth was monitored at 540 nm using a Cary 50 spectrophotometer (Varian). When investigating the effect of mercuric ion, cells were treated with HgCl2 (Sigma) from a freshly made 10 mM stock.

**Strain construction.** Strain construction procedures were as described previously (Maezato et al., 2011; Sowers et al., 2007) unless otherwise noted. DNA was electroporated into strain PBL2025 (Table 1) and its derivatives. Recombinants were enriched and individuals isolated, screened and processed as described (Maezato et al., 2011; Sowers et al., 2007). PCR, restriction analysis and DNA sequencing were used to genotype alleles. Overlap extension PCR (OLEPCR) (Higuchi et al., 1988) was used to create site-specific mutations and DNA fusions. The merRpTATA (PBL2044) and merHpTATA (PBL2042) mutants were constructed by targeted recombination and markerless exchange as described previously (Maezato et al., 2011; Sowers et al., 2007) with plasmids pBN1050 and pBN1049, respectively. PCR of the merRpTATA and merHpTATA fragments used primers merR-L-BamHI-F and merH-L-BamHI-R (Schelert et al., 2006) following by insertion at the BamHI site of plasmid pBN1053. BosI sites in the TATA boxes of the merR and merH fragment were created by OLEPCR with primers p22 and p23 (merRp) or p18 and p19 (merHp). The merHpTATA/merRptata double mutant (PBL2052) was constructed by markerless exchange using plasmid pBN1056 using primers merR-L-BamHI-F and merH-L-BamHI-R (Schelert et al., 2006). The BosI site located in the merAp TATA box of the merHpTATA/merRpTATA fragment was created by OLEPCR with primers p10 and p11 using plasmid pBN1049 as the template so as to include the merHpTATA mutation. Plasmid pBN1056 was constructed by insertion of a BamHI digested PCR of a merHpTATA/merRptata amplicon into the BamHI site of pBN1035. The merH in-frame deletion mutant (PBL2114) was constructed by OLEPCR and markerless exchange as described previously (Schelert et al., 2006) using pBN1035. Two different PCRs were used to generate fragments identical in sequence to either end of the merH region targeted for deletion using primer p31 combined with p29 and primer p31 combined with p32. These amplicons were annealed at their overlapping region and amplified using OLEPCR to produce a single amplicon. The merH in-frame deleted product was then cloned at the spfI site of pBN1035 and integrated by markerless exchange.

**merA expression plasmids and archael hosts.** Construction of the S. solfataricus mercury-inducible merA expression strain (PBL2045) used markerless exchange (Schelert et al., 2006) and plasmid pBN1052 and pBN1000. Primers p8 and p28 were used to amplify merA from WT cells followed by insertion into Nhel/BamHI sites of plasmid pET28B to make pBN1000. A hexahistidine tag was fused to the N terminus of merA and a 69 nt fragment was added 5’ to the merA start codon derived from pET28b. Primers p13 and merR-L-BamHI-F (Schelert et al., 2006) were used to amplify merR and flanking regions from strain PBL2000 to create a merRH fragment. OLEPCR was used to fuse and extend his6-merA and merRH PCR amplicons. pBN1052 was made using primers p12 and merH-L-BamHI-R to amplify the his6-merA fragment from PBN1000 followed by digestion and insertion into the BamHI site of pBN1035 (Schelert et al., 2006). Construction of the merR::TATA merA expression strain (PBL2048) used markerless exchange (Schelert et al., 2006) and plasmid pBN1053. Primers merR-L-BamHI-F and merHR-L-BamHI-R (Schelert et al., 2006) were used to amplify the merR::TATA::merA expression fragment. The BosI site located in the merR TATA box of the merR::TATA::merA expression fragment was created by OLEPCR with primers p18 and p19 and DNA from strain PBL2045 as template so as to include the N-terminal hexahistidine tag and thrombin site with merA. Plasmid pBN1053 was constructed by insertion of the merR::TATA fragment into the BamHI site of pBN1035 (Schelert et al., 2006). Construction of the merR-L-BamHI-F (Schelert et al., 2006) and p13 were used to amplify the merR-L::lacS merA expression strain (PBL2053) used linear DNA transformation as described previously (Schelert et al., 2006). Primers merR-L-BamHI-F (Schelert et al., 2006) and p13 were used to amplify the merR-L::lacS::merA fragment using plasmid pBN986 (Schelert et al., 2004) as template.

**Single copy complementation analysis.** The S. solfataricus 98/2 pNO88-like plasmid, designated here as p98-2, was isolated from mid-exponential phase WT (PBL2000) cells grown in RM using alkaline lysis extraction (Greve et al., 2004). EcoRI-digested DNA was randomly cloned into pNEB193 and one of the resulting plasmids, pBNClone3, contained a fragment of a trbE conjugal transfer protein homologue (222/422 aa, 52 % identity to trbE from ‘Sulfobolus islandicus’). trbE was amplified from plasmid pBNClone3 using primers p1 and p3 and inserted at the EcoRI site of pNEB193 to form pBN1030 while the trbE BspEI site was created by OLEPCR with primers p2 and p4. An S. solfataricus strain 98/2 genomic BAC library (Amplicon Express) was constructed following EcoRI, BamHI or HindIII digestion in agarose plugs followed by size fractionation by pulse field electrophoresis into the copy control plasmid pCC1BAC (Epigenet). Restriction analysis of representative isolates verified a mean insert size of approximately 108 kb and the resulting 1536 plasmid clones constituted 30-fold coverage. Of 259 BAC clones screened by colony PCR, three trbE containing BACs were found. BAC inserts from pBNSSC004EB, pBNSSC006D3 and pBNSSC007C4 were sequenced using primers p24 and p25 and localized the p98-2 plasmid to a 70 kb region spanning S. solfataricus ORFs SSO0451 (39 1180 nt) and SSO0583 (46 1484 nt). A 14 nt S. solfataricus plasmid integration site (Greve et al., 2004) was identified within this region between ORFs SSO0307 and SSO0508. The identity of
genes flanking trbE was determined by BAC DNA sequencing using primers p27 and p26. Genes flanking trbE were identified as traC (30/98 aa, 30% identity to traC from ‘Sulfobus tengchongensis’ plasmid pTC) and pTC_p16 (28/74 aa, 37% identity to pTC_p16 from ‘S. tengchongensis’ plasmid pTC). Construction of the merH::lacS/merA-N-His (KpnI) mutant (PBL2060) employed transformation by targeted recombination (Schelert et al., 2006) using plasmid pBN1061 and strain PBL2065 using primers p20 and p21. Plasmid pBN1033 was constructed by insertion of a BspEI-digested merHA amplicon into the naturally occurring MfeI site in merA (Schelert et al., 2004) in plasmid pBN1033 using primers lacS-MfeI-F and lacS-MfeI-R (Schelert et al., 2004).

**Reverse transcription-PCR.** Quantitative reverse transcription-PCR (qRT-PCR) using SYBR-I Green and a real-time PCR instrument (Eppendorf Mastercycler) was performed as described by the manufacturer or, as described by Bradford et al. (2005), using a variable range of PCR cycles (Marone et al., 2001; Nakayama et al., 1992) with RNA prepared according to described methods (Bini et al., 2002; Haseltine et al., 1999a). The exponential range of PCR product abundance (Noonan et al., 1990) was determined for all targets and product qualities were verified by examination of melting curves. Parallel RT-PCR amplifications were used to evaluate RNA levels from experimental genes relative to those of the reference gene, tbp, or 7S RNA, as described (Lesur & Campbell, 2004; Schelert et al., 2006). RNA was treated to remove DNA by addition of 1 U DNase I (Fermentas) per μg of total RNA at room temperature for 15 min and then neutralized with 2 μl 25 mM EDTA and incubated at 70 °C for 10 min. cDNA synthesis used 20 pmol of PCR antisense primer, 20 mM dNTPs mix (Invitrogen) and 200 U M-MuLV reverse transcriptase (Fermentas), for 60 min at 37 °C. Synthesized cDNA was subjected to standard PCR and analysed using 2% (w/v) TBE agarose gels. Initial semi-quantitative reverse transcription-PCR (RT-PCR) was performed according to a cDNA amplification protocol (Sambrook & Russell, 2001) using a variable range of PCR cycles (Bradford et al., 2005; Marone et al., 2001; Nakayama et al., 1992) with RNA prepared as described (Bini et al., 2002; Haseltine et al., 1999b) and treated to remove DNA by addition of 1U DNase I (Fermentas) per μg of total RNA at room temperature for 15 min. RNA was denatured by adding 25 mM EDTA and heating for 10 min at 70 °C. cDNA synthesis was primed using 20 pmol of RT-PCR primers p14 (merH), p7 (merA) and p5 (7S RNA), 20 mM dNTPs mix (Fermentas) and 200 U M-MuLV reverse transcriptase (Fermentas), for 60 min at 37 °C followed by standard PCR using primers p15 and p14 (merH), p9 and p7 (merA), and p6 and p5 (7S RNA) and monitored using 2% TBE agarose gels.

Table 1. Microbial strains and plasmids

<table>
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<th>Strain or plasmid</th>
<th>Genotype or sequence</th>
<th>Source or derivation</th>
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<td>WT <em>S. solfataricus</em> strain 98/2</td>
<td>Lab collection</td>
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<tr>
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<td>del(SSO3004-3050)</td>
<td>PBL2000 (Schelert et al., 2004)</td>
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<td>merH-TATA-BsaII</td>
<td>PBL2025 by markerless exchange</td>
</tr>
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<td>merR-TATA-BsaII</td>
<td>PBL2025 by markerless exchange</td>
</tr>
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<td>merA-N-His</td>
<td>PBL2025 by markerless exchange</td>
</tr>
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<td>merR-TATA-BsaII/merA-N-His</td>
<td>PBL2025 by markerless exchange</td>
</tr>
<tr>
<td>PBL2052</td>
<td>merH-TATA-BsaII/merA-TATA-BsaII</td>
<td>PBL2025 by linear recombination</td>
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<td>merR::lac::merA-N-His</td>
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<td>merH-TAG-SpeI</td>
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<td>NE Biolabs</td>
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<td>Novagen</td>
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Identification, purification and characterization of MerA. For detection of MerA in whole-cell extracts, samples were recovered from cultures that had been treated with mercuric chloride (0.3 μM) at a cell density of 10⁶ ml⁻¹ (0.1 OD₅₄₀) and harvested 4 h later. Cell suspensions were prepared using interroment sonication then analysed by 2D SDS-PAGE as described by Hajducz et al. (2005). Spots were sequenced by tandem mass spectrometry (MS/MS) and peptides identified by local BLAST against the S. solfataricus proteome. His-tagged recombinant MerA protein was isolated from the S. solfataricus mer-inducible expression strain PBL2045 and the mer constitutive expression strains PBL2048 and PBL2053. MerA synthesis in PBL2045 was induced by mercuric chloride treatment (0.3 μM) in 500 ml cultures with additional incubation (4 h). Use of PBL2048 and PBL2053 obviated metal treatment and cells could be grown in larger scale (10 l) as described (Schelert et al., 2004; Worthington et al., 2003a) or using 3 l Aplikon bioreactors at 80 °C, pH 3.0, with mixing (200 r.p.m.) and aeration (1 volume of air per volume of medium per min). Cell pellets were stored at −20 °C or resuspended in 10 ml buffer A (50 mM Tris/HCl pH 7.8, 0.5 M NaCl, 10% glycerol and 20 mM β-mercaptoethanol) and then lysed using pulsed sonication or pressure (French pressure cell with passage twice at 12000 p.s.i.). Lysates were clarified by centrifugation for 10 min (5000 g) then processed by repeated nickel-affinity chromatography as described (Novagen). Protein eluted using 0.5 M imidazole was dialysed into buffer B (pH 7.0 (100 mM Tris pH 9.0, 1 mM β-mercaptoethanol). Samples analysed by SDS-PAGE used either 12.5 or 16% polyacrylamide gels. Protein concentrations were determined by BCA assay (Pierce). MerA protein yields were 2.0 mg l⁻¹ of culture, equivalent to 2.5% of total protein. Reductase assays were performed as described by Fox & Walsh (1982) using an Agilent Cary100 UV-Vis spectrophotometer at 65 °C in 100 mM Tris/HCl (pH 9.0) containing 100 μM β-mercaptoethanol, 200 μM NADPH and 100 μM mercuric chloride. Reactions were initiated by NADPH addition and mercury-dependent oxidation of NADPH was monitored by the rate of decrease in absorbance at 340 nm. Control reactions were performed without enzyme and without metal addition and the difference in slopes was subtracted from reactions containing all components. FAD (100 μM) was added to reaction mixtures to monitor its effect on enzymic activity. Efforts to reactivate MerA thiol groups involved sample dialysis into tris(3-hydroxypropyl)phosphine (0.5 mM) followed by enzyme assay. Specific activity was recorded in units (U) representing μmol NADPH oxidized min⁻¹ (mg protein)⁻¹ (Fox & Walsh, 1982). MerA absorbance spectra were examined at a protein concentration of 5 mg ml⁻¹ using an Agilent Cary100 UV-Vis spectrophotometer at room temperature.

Mercury resistance determination. Strains were grown with aeration in a defined minimal medium (SM). At a cell density of 10⁸ cells ml⁻¹, 0.5 μM mercuric chloride unless otherwise indicated was added to each culture. Cultures of strains with no added mercury were included as controls. Growth was monitored by measuring the absorbance at 540 nm and growth curves were plotted. All cultivation experiments were repeated at least three times and representative data are shown from these biological repeats.

Molecular biology methods. DNA cloning, PCR and plasmid transformation of Escherichia coli were performed as described (Haseltine et al., 1999c; Rockabrand et al., 1998). DNA sequencing was as described by Rolfsmeyer et al. (1998). DNA and RNA concentrations were measured using either a DyNA Quant 200 fluorometer (Hoerger) or a UV-visible spectrophotometer Genesys 2 (Spectroscopy). All manipulations of RNA were as described (Bini et al., 2002; Haseltine et al., 1999b). Protein concentrations were measured using the BCA Protein Assay Reagent kit (Pierce). Unless otherwise indicated, all chemicals were obtained from common chemical suppliers.

Inductively coupled plasma-MS analysis. To determine the intracellular concentrations of mercury, cells were grown to an optical density (540 nm) of 0.1, corresponding to approximately 10⁸ cells ml⁻¹ and treated with 0.5 μM mercuric chloride from a freshly made 10 mM stock. Samples (1 ml) were then removed at the indicated time points and cells were harvested by centrifugation at 10,000 g for 5 min followed by two successive washes using distilled water to remove free mercury. Cell pellets were frozen at −20 °C for subsequent analysis. Cell pellets were extracted using 50 μl concentrated nitric acid and the resulting extracts analysed by inductively coupled plasma-mass spectrometry (ICP-MS) using an Agilent ICP-MS 7500cx. A certified mercury reference standard was used for sample normalization. All values are the means of samples from replicate cultures.

RESULTS

Effect of a merH nonsense mutation. To assess the role of MerH in metal detoxification, a premature stop codon was created in the merH gene of the WT strain (Fig. 1a, c). The merH TAG mutant (PBL2054) was created by transforming strain PBL2025 with plasmid pBN1058 using markerless exchange as described (Schelert et al., 2006). To assess the physiological consequence of the merH TAG mutation, the response of the mutant strain to mercuric chloride challenge was compared to that of the otherwise isogenic WT strain and a merA mutant (Fig. 2a). When treated with mercury, growth of the merH TAG mutant was strongly inhibited by metal addition relative to the WT strain and nearly identical to the pattern exhibited by the merA mutant.

Single copy merH complementation analysis. To determine the importance of MerH in the merH TAG mutant’s sensitivity to mercury, a functional copy of merH under the control of its native promoter (merHp) was reintroduced as a single copy chromosomal insertion (Fig. 1b). The inserted DNA encoded both WT merH, an inactivated copy of merA disrupted by lacS thereby providing a selection for DNA integration, and flanking DNA sequences to target integration. Recombinants were obtained by linear DNA recombination as described (Maezato et al., 2011; Schelert et al., 2006) using the merH TAG mutant as the host. The site of insertion was within the trbE gene of an integrated copy of plasmid p98-2 (Greve et al., 2004) present in the chromosome of S. solfataricus strain 98/2. The trbE gene was used because the plasmid genes were unlikely to be involved in metal resistance. As the second copy of merH was inserted within an integrated conjugative pNOB8-like plasmid named p98-2, plasmid segregation was evaluated by enumerating loss of lacS located in the flanking merA region. Segregation within the metal-treated population was not detectable (<0.1%). The response of the merH-complemented strain to mercuric chloride (0.5 μM) challenge was compared to that of the otherwise isogenic WT and parental strain during growth in SM (Fig. 2b). The merodiploid strain...
remained significantly more sensitive to metal challenge than the WT strain but more resistant than the merH\_TAG mutant. This result indicated that MerH complementation could reconstitute at least partial mercury resistance but the contribution of translational polarity on downstream expression of merA remained unclear.

**Polycistron and promoter analysis**

More precise mutant analysis was necessary to determine the in vivo contributions of MerH and MerA on the phenotype of the merH\_TAG mutant. Though previous studies had provided information on the mer regulatory region including the MerR binding site and the merH transcription start site (Schelert et al., 2006), the location of the mer operon promoter(s) had not been determined. As proposed previously, occurrence of a putative promoter located immediately 5' of merA (Schelert et al., 2004) would circumvent a requirement for merHA cotranscription and therefore change the identity of the mer polycistron. To clarify the identity of the primary mer promoter, the predicted sequences were modified by markerless exchange and the resulting mutants characterized. Mutant strains (merP\_TATA and merH\_TATA) were created in which the T/A-rich octameric sequences centred 26 nt upstream of the merR or merH (Schelert et al., 2006) transcription start site, or 33 nt upstream of the merA transcription start (merA\_TATA) (Schelert et al., 2004), were replaced with G/C-rich sequences and the insertion of a BsaI site (Fig. 1f). To assess the physiological consequence of the mutations, the response to mercuric chloride challenge was evaluated relative to controls during growth in defined medium (SM). Higher metal doses were used to accentuate strain differences. While the merHp\_TATA mutant was more sensitive than the WT to metal challenge, it retained significant resistance relative to the merA disruption mutant (Fig. 2d). Because there was a lag associated with the growth of the merHp\_TATA mutant compared to the WT, the TATA box mutation may have negatively affected merHp activity resulting in a reduction or delay in merHA transcript production. Residual resistance of the merHp\_TATA mutant might arise from the activity of an internal promoter located upstream of merA (Schelert et al., 2004). This was tested by insertion of a G/C-rich mutation...
centred 33 nt from the merA transcription start site in the merHpTATA mutant background creating a double mutation (Fig. 1f). The double TATA box mutant had identical sensitivity towards metal challenge relative to its parent (merH) indicating that the proposed merA promoter was not functional. In contrast, the merRpTATA mutant was more resistant to metal challenge than the WT and similar to the merR disruption mutant (Fig. 2e). Similarity between the phenotype of the merRpTATA mutant and the merR disruption mutant suggested that there was constitutive expression of the mer operon in this strain due to loss of MerR production.

MerH in-frame deletion
To further understand the contribution of MerH towards mercury resistance, an in-frame merH deletion mutant containing both merH start and stop codons was constructed (Fig. 1d, e). This strain avoided the complication of polarity inherent to the system described above using the merH-complemented merHTAG mutant. Like the merH merodiploid, the in-frame merH deletion mutant was more resistant to Hg(II) than a merA mutant but more sensitive than the WT (Fig. 2c).

S. solfataricus-derived MerA protein purification and analysis
Despite reduction of MerA levels observed in merHTATA mutant extracts (Fig. 3c), it remained possible that MerH was catalytically required for MerA activity and metal resistance was therefore compromised in the mutant strain. To determine whether or not MerA alone could reduce Hg(II) to Hg(0), it was necessary to obtain purified MerA

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Fig. 2. Response of mer operon mutants to mercuric chloride. All strains were treated with mercuric chloride (arrows) at 0.5 μM unless otherwise indicated. Closed symbols (treated cultures), open symbols (untreated cultures). (a) merHTATA mutant (squares), merA mutant (triangles) or WT (circles). (b) merHTATA (triangles), merHTATA/merH+ (squares) and WT (circles). (c) ΔmerH in-frame deletion mutant (inverted triangles), merA mutant (squares) and WT (circles). RNA was extracted for qRT-PCR analysis of merA at 32 h post-challenge (arrow). (d) merHTATA mutant (triangles), merA disruption mutant (squares) and WT (circles) treated with 0.75 μM mercuric chloride. (e) merRpTATA mutant (squares), merR mutant (triangles) or WT (circles). Cultivation experiments were repeated at least three times.
MerA homodimer and a tag at this end may have interfered with MerA enzymic activity. To assess the physiological effect of the N-terminal hexahistidine tag on MerA activity in vivo, the response of the MerA expression mutant strain (PBL2045) to mercuric chloride challenge was compared to that of the otherwise isogenic WT strain and the merA disruption mutant. The three strains were grown in defined medium and at a cell density of $10^8$ cells ml$^{-1}$, 0.3 μM mercuric chloride was added to each culture. Cultures of all strains with no added mercury were included as controls. Growth of the S. solfataricus MerA expression mutant was similar to the WT strain indicating that the N-terminal hexahistidine tagged MerA was active in vivo. The merA disruption mutant was included as a control and exhibited a significantly longer lag than WT. Mercury induction of the MerA expression mutant resulted in production of MerA (49 kDa) in sufficient abundance to be detected by Coomassie blue staining following 2D SDS-PAGE (data not shown), and one more purification by nickel-affinity chromatography (Fig. 3a). Protein sequencing by MS/MS confirmed the identity of the protein as S. solfataricus MerA. An alternative strain, PBL2048, was then constructed in which merA was expressed constitutively by blocking MerR synthesis due to TATA box inactivation of merRp. To assess the physiological effect of the hexahistidine tag and to verify that MerA remained active, the response of strain PBL2048 was evaluated by metal treatment relative to the WT. The absence of a growth lag for PBL2048 despite metal addition demonstrated that MerA was active and constitutively produced. Purified protein (Fig. 3a, lane 2) had a specific activity of 0.30 U mg$^{-1}$ (± 0.02) for mercury-dependent NADPH oxidation. Spectroscopic analysis of purified protein did not indicate presence of FAD-associated absorption peaks (340, 450) at protein concentrations of 5 mg ml$^{-1}$, while FAD addition (100 μM) had no impact on enzyme activity. Thiol reactivation by dialysis of MerA using tris(3-hydroxypropyl)phosphine also did not affect MerA activity. Since MerH protein was not evident in the purified active MerA protein samples, MerH was not required for MerA catalysis in vitro.

**Effect of merH mutations on merH and merA expression**

qRT-PCR analysis was used to determine the impact of the merHpTATA mutation on transcript abundance and to determine if the merHpTAG mutation influenced merA transcript abundance (Fig. 3b, c). Parallel qRT-PCR amplifications were used to evaluate RNA levels from experimental genes relative to those of the 7S rRNA reference gene as described previously (Lesur & Campbell, 2004; Schelert et al., 2006). Batch cultures grown in defined medium were treated with 0.3 μM mercuric chloride, and samples were removed for analysis at times thereafter. The lower dose of mercuric chloride was used for these experiments because this dose was sufficient to induce expression of the merHA transcript without significantly retarding cell growth. Prior to mercury addition (0 h),
merH and merA RT-PCR products were not detected in the WT, merHpTATA or merHTAG mutants (data not shown). After mercury addition (4 h), merH and merA RT-PCR products were detected in all strains yet their abundance was significantly reduced in both the merHTATA mutant and the merHTAG mutant relative to the WT (Fig. 3c). MerA protein abundance in the merHTAG mutant was also evaluated using 2D SDS-PAGE and extracts from previously constructed merR and merA disruption mutants (Schelert et al., 2004; data not shown). MerA protein abundance was significantly reduced in the merHTAG mutant and likely the cause of the merHTAG mutant phenotype of reduced metal resistance.

**Intracellular concentrations of mercury**

To understand the specific contribution of MerH towards metal resistance, ICP-MS of whole-cell extracts was used to measure intracellular levels of mercury in the WT, merH deletion and merA mutant strains during metal challenge. In the WT strain, intracellular mercury underwent a rapid but transient increase within 5 h of metal challenge, returning to baseline 70 h later and commensurate with resumption of exponential growth (Fig. 4). In the mutants, a similar initial increase in mercury was observed, but both the amount of metal and its rate of return to pre-challenge levels were significantly different as compared to the WT strain. Both mutants accumulated higher levels of metal than the WT. In the case of the merA mutant this was consistent with loss of enzymic mercury reduction. Assuming MerA levels were unaffected by polarity in the merH deletion mutant, these results also indicated that MerH plays an important role in mediating metal resistance in vivo. Interestingly, the post-challenge rate of reduction of cell-associated mercury was slower for the merH deletion mutant than the merA mutant. In an attempt to distinguish between an effect of MerH deficiency on MerA reductase activity versus an effect on derepression of merHA transcription, qRT-PCR analysis was conducted of merA transcript abundance in the WT and merH deletion mutant during metal challenge. Samples were removed at a time (32 h post-challenge; Fig. 4a, arrow) when both merH deletion mutant phenotypes (increased mercury content and reduced growth rate) were apparent. At this time, the abundance of merA mRNA was 5-fold lower in the merH mutant relative to the WT after internal normalization to transcription factor B (tfd) mRNA levels (Fig. 4b). Thus, MerH deficiency reduced the rate of derepression of merHA in response to metal challenge leading to elevated metal content and slow growth. As merHA transcription requires that MerR bind mercury to enable its dissociation from merHp DNA (Schelert et al., 2006), these data suggest that MerH promotes the interaction between MerR and mercury as part of the normal metal regulatory response. These data clarify the identity of the merHA operon and its promoter, showing that the merHTAG mutation was polar on the expression of merA and, therefore, that both MerH and MerA deficiency could underlie mercuric ion mutant sensitivities.

**DISCUSSION**

The data presented here indicate MerH as a likely mercury metallochaperone that plays a critical role mediating heavy metal resistance in the archaeon *S. solfataricus*. MerH trafficking of Hg(II) may have two distinct roles. By analogy to the actions of bacterial MerP (Gambill & Summers, 1992; Morby et al., 1995; Serre et al., 2004) and the N-terminal bacterial MerA motif (Hong et al., 2010; Ledwidge et al., 2005), archaeal MerH could facilitate metal transfer to MerA for reduction followed by metal efflux in its volatile state. However, as described here, MerH can also control derepression of transcription at the merHp promoter indicating its role in trafficking mercury to the MerR transcription factor. Transcription factor interactions of a metal chaperone offer new ways in which these proteins can control metal resistance mechanisms.
Interestingly, MerH has no homology to other proteins outside of a C-terminal domain, called TRASH, suggested previously to be involved in metal sensing and trafficking (Ettema et al., 2003). The constitutively expressed *S. solfataricus* CopR transcription factor (Villafane et al., 2011) (also called CopT; Ettema et al., 2006) also encodes a TRASH domain and modulates copper resistance. These findings implicate the TRASH domain in the metabolism of two distinct metals, copper and mercury. However, the determinants of this domain that confer metal specificity remain to be identified.

In the present study, two genetic approaches were taken to evaluate MerH function. A nonsense mutation located at the 5′ end of *merH* resulted in mercury sensitivity. This mutation had a polar effect on *merA* expression; therefore, reduced metal resistance could reflect deficiencies of both MerA and MerH. Consistent with this observation, the introduction of a second but functional single copy of *merH* into the *merH* nonsense mutant strain but at a separate chromosomal location conferred only partial levels of mercury resistance. This effect was likely because MerA abundance remained low due to continued nonsense polarity of the *merH* nonsense mutation on *merA* expression. A second approach was therefore employed that used an in-frame deletion of *merH* to avoid nonsense polarity. However, in this case the continued reduction in *merA* expression arose from inefficient merHA derepression and not by polarity. The combination of these two genetic approaches indicated that MerH is a required component of mercury resistance.

Analysis of the *merHA* nonsense mutant established that the nonsense mutation compromised metal resistance by reducing both MerA protein and *merA* transcript abundance. Since this polar effect on gene expression is mediated through an effect on transcription, it closely resembles the process of polarity in bacteria. However, the mechanism responsible for polarity in archaea is unclear since homologues of the bacterial termination factor, Rho, are not evident in the genome of *S. solfataricus* or other archaea (Santangelo & Reeve, 2006), and bacterial terminators are largely absent (Ermolaeva et al., 2000; Unniraman et al., 2002). While cotranscription can be inferred by detection of transcribed intergenic sequences using RT-PCR, in the case of *mer* an internal promoter had been proposed that could bypass a requirement for coupled gene expression (Schelert et al., 2004). Thus, an alternative strategy was required and TATA box mutagenesis was used to identify the main *mer* operon promoter. *S. solfataricus* promoters studied *in vitro* are typically T/A-rich octameric sequences while weaker noncanonical promoters are G/C-rich (Bell et al., 1999; Reeve, 2003; Reiter et al., 1990). In the data presented here, *in vivo* replacement of both *merRP*-TATA and *merHP*-TATA with G/C-rich octameric sequences resulted in reduced promoter activity and noticeable physiological effects while similar manipulations had no effect on a putative *merAp*. Because the mercury resistance phenotype of the *merRP*-TATA mutant was similar to the *merR* disruption mutant, MerR was not produced and cells were unable to maintain repression of the *mer* operon. This confirms the identity of the *merRP* TATA box.

Curiously, *merHP* was not fully inactivated despite complete TATA box substitution with a sequence that bears no similarity to canonical crenarchaeotal promoters. This was evident in the *merHP*-TATA mutant since it retained significantly higher resistance to Hg(II) than the *merA* disruption mutant (Fig. 2d). This finding may suggest that *merHP* functions as a TATA-less promoter or another promoter located elsewhere in the region may provide this function. In eukaryotes TATA-less Pol II promoters employ other proteins, notably TAFs, to ensure positioning of TBP/TFIID, proper DNA topology (bending) and Pol II recruitment (Wright et al., 2006). As the *S. solfataricus* MerR protein remains DNA-bound during metal ligand interaction and is positioned immediately adjacent to the TATA box (Schelert et al., 2006), MerR may provide a TAF-like function to ensure proper TBP positioning and RNA Pol II recruitment. MerR and other bacterial-like archaeal transcription factors could constitute the functional archeal TAFs that are also not evident in the genomes of these prokaryotes.

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