

2014

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McCarthy, Samuel; Al, Chenbing; Wheaton, Garrett; Tevatla, Rahul; Eckrich, Valerie; Kelly, Robert; and Blum, Paul H., "Role of an Archaeal PitA Transporter in the Copper and Arsenic Resistance of *Metallosphaera sedula*, an Extreme Thermoacidophile" (2014).  
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# Role of an Archaeal PitA Transporter in the Copper and Arsenic Resistance of *Metallosphaera sedula*, an Extreme Thermoacidophile

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Thermoacidophilic archaea, such as *Metallosphaera sedula*, are lithoautotrophs that occupy metal-rich environments. In previous studies, an *M. sedula* mutant lacking the primary copper efflux transporter, CopA, became copper sensitive. In contrast, the basis for supranormal copper resistance remained unclear in the spontaneous *M. sedula* mutant, CuR1. Here, transcriptomic analysis of copper-shocked cultures indicated that CuR1 had a unique regulatory response to metal challenge corresponding to the upregulation of 55 genes. Genome resequencing identified 17 confirmed mutations unique to CuR1 that were likely to change gene function. Of these, 12 mapped to genes with annotated function associated with transcription, metabolism, or transport. These mutations included 7 nonsynonymous substitutions, 4 insertions, and 1 deletion. One of the insertion mutations mapped to pseudogene Msed\_1517 and extended its reading frame an additional 209 amino acids. The extended mutant allele was identified as a homolog of Pho4, a family of phosphate symporters that includes the bacterial PitA proteins. Orthologs of this allele were apparent in related extremely thermoacidophilic species, suggesting *M. sedula* naturally lacked this gene. Phosphate transport studies combined with physiologic analysis demonstrated *M. sedula* PitA was a low-affinity, high-velocity secondary transporter implicated in copper resistance and arsenate sensitivity. Genetic analysis demonstrated that spontaneous arsenate-resistant mutants derived from CuR1 all underwent mutation in *pitA* and nonselectively became copper sensitive. Taken together, these results point to archaeal PitA as a key requirement for the increased metal resistance of strain CuR1 and its accelerated capacity for copper bioleaching.

Thermoacidophilic archaea include taxa that are lithoautotrophs with high levels of metal resistance, such as *Sulfolobus*, *Metallosphaera*, and *Acidianus* (1, 2). They occur in environments that are sulfur rich and pyritic, including mining-related heaps of extracted ores. These organisms are of particular importance in the extraction of base and precious metals through the bioleaching process (3). Metal bioleaching has been proposed to use both direct and indirect mechanisms that convert metals to soluble forms (4, 5). Metal release may occur via biooxidation of the sulfur and iron component of minerals, thereby releasing complexed metals, or by direct metal oxidation involving cell contact with metal surfaces (6). Advances in understanding this type of lithoautotrophic metabolism are coming from genome sequencing of biomining organisms (7–10), transcriptomic analyses (10–12), and metagenomic investigations (13).

Copper resistance mechanisms among the archaea are understood primarily from genome sequencing, where the distribution of CopA (P-type ATPase) copper efflux transporters is broad (1, 3). Biochemical studies have been conducted on CopA from the sulfate reducer *Archaeoglobus fulgidus* (14, 15) and CopB from *Sulfolobus solfataricus* (16, 17). Multiple mechanisms of copper resistance also have been examined using genetic and molecular approaches in *S. solfataricus*, including *copA* and *copB* (P-type ATPase efflux pumps), *copR* (transcriptional regulator), and *copT* (a putative metallochaperone) (16, 18–20). An inorganic polyphosphate symport system with importance in copper resistance also has been identified in *Sulfolobus metallicus* (21). Intracellular polyphosphate hydrolysis catalyzed by exopolyphosphatase (Ppx) could provide a counter ion for metal complexation whose subsequent symport through a phosphate efflux system would reduce metal burden. Interestingly, polyphosphate is produced by both chemoheterotrophic and lithoautotrophic thermoacidophilic archaea, but only

the latter organisms accumulate significant levels, perhaps in concert with increased levels of metal resistance (21, 22).

*Metallosphaera sedula* is a lithoautotrophic archaeon that grows optimally at 75°C over a pH range of 2.0 to 4.5 (23). It differs greatly from its relatives, *S. solfataricus* and *S. acidocaldarius*, because these organisms are strict chemoheterotrophs (1, 24). Autotrophy in *M. sedula* uses a modified 3-hydroxypropionate cycle for carbon fixation (25, 26). *M. sedula* also is more resistant to metals than chemoheterotrophic *Sulfolobus* species, perhaps as a consequence of its metal-rich habitat (19, 23, 27). The first genetic system for *M. sedula* was developed recently to investigate parameters that limit bioleaching of chalcopyrite (CuFeS<sub>2</sub>) (27). Cross-species complementation using a *S. solfataricus* *copR* mutant (19) demonstrated the *M. sedula* *copRTA* operon was active and that gene expression signals are conserved between these genera. Inactivation of the *M. sedula* copper efflux protein, *copA*, using targeted recombination based on the *pyrE* genetic marker compromised metal resistance and eliminated copper bioleaching from chalcopyrite. In contrast, a spontaneous *M. sedula* strain, called CuR1, that had supranormal metal resistance leached copper from chalcopyrite at an accelerated rate. Taken together, these data showed that metal resistance was integral to copper bioleaching. While CuR1 exhibited normal growth rates and cell yields under chemoheterotrophic conditions, transcriptomic analysis

Received 1 April 2014 Accepted 28 July 2014

Published ahead of print 4 August 2014

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doi:10.1128/JB.01707-14

identified changes in the expression of 22 proteins by 3-fold or more and 13 proteins by 5-fold or more 15 min after copper shock than the wild type grown under the same conditions. These included putative transporter-encoding AAA-ATPase motifs. In this study, transcriptomics, genome resequencing, solute transport, and classical genetics were used to identify regulatory and genetic differences resulting in extreme copper resistance of this cell line, including a specific role for phosphate transport.

## MATERIALS AND METHODS

**Archaeal strains, cultivation, and microscopy.** Cell lines used in this study included *Metallosphaera sedula* (DSM 5348T) (wild type) and the CuR1 copper-resistant derivative (27). They were grown in a basal salts medium (BSM) (28) adjusted to pH 2.0 using sulfuric acid. The cultures were incubated at 75°C in either glass screw-cap flasks with aeration in orbital baths or in glass screw-cap test tubes, which were placed in rotary drum agitators that were mounted in incubators that had external DC motors. Chemoheterotrophic growth used tryptone at 0.05% (wt/vol). Cultures were frozen at −80°C as described for other thermoacidophiles (29). Prior to inoculation into liquid medium, cultures were streaked on a solid complex medium prepared using BSM mixed with 0.6% (wt/vol) gelrite (Kelco) and supplemented with 0.05% (wt/vol) tryptone. Planktonic growth was monitored by light adsorption at a wavelength of 540 nm. Cultures were supplemented with different concentrations of potassium arsenate (Sigma) to determine its effect on growth in both strains. Arsenate-adapted derivatives of CuR1 were isolated by growing the cells in 15 mM potassium arsenate for two growth cycles and then subculturing into 20 mM arsenate followed by 25 mM arsenate. To isolate arsenate-resistant mutants of CuR1, CuR1 cultures that grew in 25 mM arsenate were plated on solid complex medium (without arsenate), and colonies were screened for the ability to grow in the presence of 25 mM arsenate upon inoculation back into liquid medium. The Msed\_1517 allele of five of these arsenate-resistant isolates were determined.

**Transcriptomics methods.** For transcriptomic experiments, *M. sedula* (DSM 5348T) (wild type) and the CuR1 copper-resistant derivative were cultured aerobically in DSM88 medium adjusted to pH 2.0 using sulfuric acid, supplemented with 0.05% (wt/vol) tryptone and 25 mg/liter uracil, and incubated at 70°C in an oil bath with a shaking rate of 100 rpm. The first global transcriptional response experiment was designed to compare wild-type and CuR1 strains under normal growth conditions (i.e., no copper challenge). The second was aimed at comparing the response of wild-type and CuR1 strains under subinhibitory copper challenge (2.0 mM copper sulfate). For the normal condition comparison, cells were grown to a cell density of approximately  $1.0 \times 10^8$  cells/ml (optical density at 540 nm [ $OD_{540}$ ], 0.1) and harvested, while for the copper challenge comparison, cells were grown to a similar density shocked by addition of 2.0 mM copper and harvested 15 and 60 min postshock. For both experiments, cells were harvested by quickly chilling, centrifuging at  $6,080 \times g$  for 10 min, and washing the cell pellet with Tris-EDTA buffer to remove residual media. Epifluorescence microscopy, using acridine orange stain, was used for determination of cell densities.

RNA was extracted by phase separation with TRIzol reagent (Invitrogen), purified using an RNeasy minikit (Qiagen), reverse transcribed with Superscript III (Invitrogen), repurified, labeled with either Cy3 or Cy5 dye (GE Healthcare), and hybridized onto the *M. sedula* array, constructed as previously described (7, 12, 30). RNA from biological replicates was pooled prior to the reverse transcription to cDNA. Slides were scanned using a Genetix 4000B scanner, and average intensities for all spots were normalized using an analysis of variance (ANOVA) mixed-effects model and analyzed using JMP Genomics (SAS Institute, Cary, NC). Bonferroni's correction was utilized to determine the statistical significance of differential transcription, and it was 5.00 for the normal condition and 5.53 for the copper challenge.

**Genome sequencing and bioinformatics.** High-molecular-weight genomic DNA was prepared from clonal cultures of *M. sedula* strains as

described previously (7, 29), and its integrity and purity were confirmed by agarose gel electrophoresis and spectroscopy. The genomic DNA samples were dissolved in 10 mM Tris-Cl, pH 8.5, and sonicated using a Misonix ultrasonic liquid processor (model 4000) at a setting of 7 for 30 s with 1-min intervals at 20% power output. The sheared DNA ranged in size from 100 bp to 800 bp, with a peak area of 250 to 500 bp, and was used to prepare DNA libraries. DNA was sequenced using an Illumina GAIIX to obtain 75-bp single-end reads using Illumina FC-102-1001 sequence kits, a TruSeq SR cluster generation kit, v5-SCS (v2.8 protocol), and two 36-cycle TruSeq sequencing kits, v5 (v8 protocol). *M. sedula* wild-type strain sequencing yielded 32 million 75-bp reads (2.3 Gb), while the mutant strain, CuR1, had 25 million 75-bp reads (1.8 Gb). The genomes of both strains then were assembled by *de novo* assembly. Raw sequence data files were compiled into single FASTQ files for each *M. sedula* strain. Initial contigs were assembled from these files using VELVET (version 1.2.07), a sequence assembler for very short reads (31), while *k*-mer length settings were manually set to 49. The outputs from Velvet were 70 contigs ranging from <100 to nearly 250,000 bp for each genome. Full genomes were assembled from the contigs using the CODON CODE ALIGNER genome assembly program (CodonCode). Mutations that were located within open reading frames and identified through sequence comparisons were analyzed in more detail by cross-referencing the genome coordinates and type of mutation at each position to these same positions in the NCBI gene database to determine their codon positions and the effects they would have on protein sequence. The coordinates of each mutation also were cross referenced to locations of the known domains in each protein to verify whether or not any mutations occurred within important functional domains. Nonsynonymous changes that were found then were verified by PCR and sequencing, followed by comparison to the genome resequencing results of the same region.

In addition, a guided assembly was conducted using both sets of genome sequence reads and were aligned uniquely via bowtie2 (version 2.0.0-beta6) to the reference *Metallosphaera sedula* DSM 5348 genome (RefSeq accession no. NC\_009440.1). Matching reads were extracted via SAMtools (version 0.1.18) and converted into regions via bedtools (version 2.16.1), and FASTA sequences of these regions were extracted from NC\_009440.1 to serve as the reference regions for the genome assembly. Velvet (version 1.2.07) (31) was used again for the genome assemblies with a *k*-mer value of 57, the extracted reference in the case of the guided assembly, and automatic determination of expected coverage and cutoffs, as these were very similar to manual cutoffs determined by visual inspection (wild type, expected coverage of 291 and cutoff of 145; CuR1, expected coverage of 219 and cutoff of 109). The resulting contigs for each assembly were reordered against NC\_009440.1 via the progressive Mauve tool (version 2.0), and a draft genome compiled from the contigs was interspersed with lowercase N nucleotides to the estimated size of any gaps in the genome compared to the reference (in-house Perl script).

**Phosphate transport assays.** Cells were grown to mid-log phase in BSM containing 0.05% tryptone and then washed twice and resuspended in uptake media (BSM lacking phosphate). After a 5-min incubation at 75°C,  $10 \mu\text{Ci}$  of  $^{32}\text{PO}_4^{3-}$  (specific activity, 900 to 1,100 mCi/mmol; PerkinElmer) was added to the suspension, and the suspension was sampled in 0.5-ml amounts every 15 s over a period of 1 min. The samples were applied to 0.45- $\mu\text{m}$  filters (Whatman Nuclepore track-etch membrane) supported on a filtration manifold. The filters containing the labeled cells then were washed with 3 ml of 100 mM LiCl, dried, and placed in scintillation vials with 5 ml of scintillation fluid (EcoLite). Radioactivity was measured using a Packard liquid scintillation counter. Gramicidin (32) was added to the cells to a final concentration of 12.5  $\mu\text{M}$  immediately prior to the 5-min preincubation period and before  $^{32}\text{PO}_4^{3-}$  addition. Phosphate transport rates were determined as described previously (33–35) and were determined over a range of 0 to 200  $\mu\text{M}$  external phosphate. Phosphate transport rates also were determined over a range of 0 to 200  $\mu\text{M}$  potassium arsenate at a constant phosphate concentration of 200  $\mu\text{M}$ .  $K_m$  values for phosphate and  $K_i$  values for arsenate for both strains

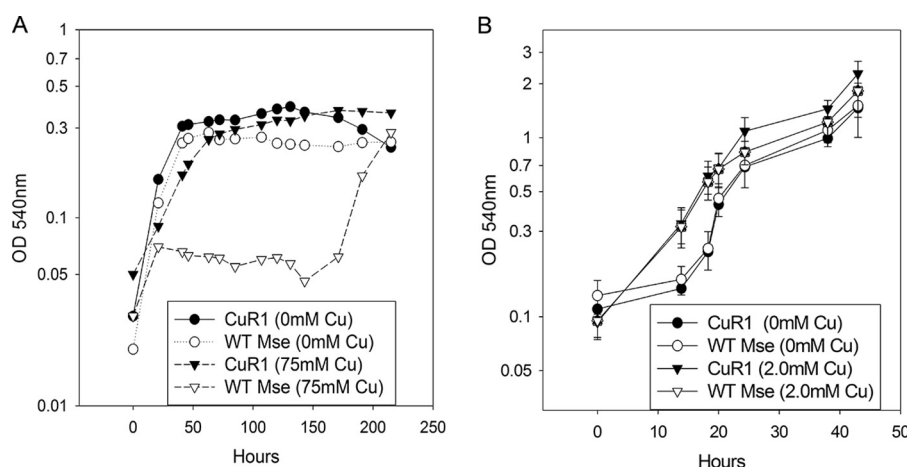


FIG 1 Response of *M. sedula* and CuR1 to copper challenge. (A) Effect of 75 mM copper [Cu(II)]. WT Mse, wild-type *M. sedula*. (B) Effect of 2.0 mM copper shock.

were extrapolated from a Michaelis-Menten plot. All rates were verified in replicate.

#### Nucleotide sequence and transcriptomic data accession numbers.

The *M. sedula* strain CuR1 genome sequence is available at GenBank under the accession number [CP008822](#). The transcriptomic data for wild-type *M. sedula* and CuR1 are available at the Gene Expression Omnibus under the following accession numbers: [GSM1431885](#), [GSM1431886](#), [GSM1431887](#), [GSM1431888](#), and [GSM1431889](#).

## RESULTS

**Transcriptomics of copper shock.** *M. sedula* strain CuR1 has increased resistance to copper relative to that of the wild-type strain (27). When an exponentially growing culture of this strain was challenged by addition of 75 mM copper [cupric ion, or Cu(II)], there was no apparent change in growth rate. In contrast, when a wild-type culture was challenged at the same metal dose, there was a prolonged lag phase eventually followed by the resumption of growth (Fig. 1A). This demonstrated that increased copper resistance in strain CuR1 does not depend on a period of adaptation but instead occurs in a constitutive manner. The immediate demonstration of resistance suggested genes involved in this trait will be evident through transcriptomic analysis without prior metal challenge. Transcriptomic analysis was conducted initially using RNA from cultures of exponentially growing cells in media lacking high levels of copper. Only two genes were upregulated in CuR1 relative to the wild type: Msed\_1525, a putative peroxiredoxin (cd3016 PFAM0578), and Msed\_0842, a hypothetical protein. Both were expressed at levels 2-fold above those in the wild-type strain. As orthologs of both genes are in the genomes of the copper-sensitive organisms *S. solfataricus* and *S. acidocaldarius*, they were not pursued further. Instead, other mechanisms were considered, such as a rapid regulatory response to metal challenge that resulted in high metal resistance. Transcriptomic analysis was conducted by challenging both strains with 2.0 mM copper, a relatively low dose of copper that did not inhibit the growth of either strain (Fig. 1B). The regulatory response to copper shock, measured after 15 min and 60 min, revealed distinct differences between the wild type and CuR1 *M. sedula* strains (Fig. 2). Two patterns were evident: a more rapid response to metal challenge in strain CuR1 and hyperinduction of several genes. The most responsive gene in the comparison between the wild-type and CuR1

strains 15 min after metal addition was Msed\_1076, a hypothetical protein that showed 47.3-fold upregulation in CuR1. This protein contains a lycopene cyclase region/domain that might have a role in carotenoid biosynthesis. After both 15 min and 60 min, the primary copper efflux protein, Msed\_0490, was 3-fold upregulated in CuR1. The expression of additional transporters that could promote metal efflux also was increased in CuR1, including Msed\_0805, Msed\_0960, and Msed\_1772. Their increased expression ranged from 5- to 9-fold. In addition, two differentially expressed transcription factors were identified. Msed\_1818 was upregulated 4.6-fold in CuR1 15 min after copper challenge. This

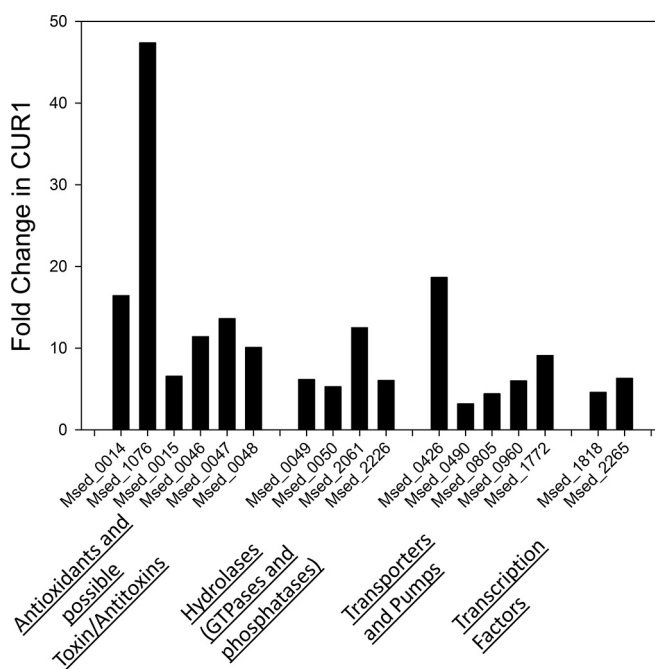


FIG 2 Transcriptomic profile of CuR1 in response to copper shock. Fold changes in CuR1 are relative to the level for the wild type and represent observed differences at 15 min postshock. In total, 40 genes showed increased expression (>2-fold), while only 1 showed decreased expression (>2-fold) relative to the wild type. Classes of affected genes are shown below the figure.



TABLE 1 Summary of sequence coverage and mutations

<i>M. sedula</i> strain	Depth of coverage by Illumina reads (fold)	No. of substitutions in ORFs	No. of substitutions in intergenic regions
Wild type	1,050	24	3
CuR1	821	17 <sup>a</sup>	3

<sup>a</sup> No overlap with mutations detected in wild-type *M. sedula*.

gene is annotated as a bacterium-opsin activator with an HTH domain and may be a metal response transcriptional regulator. Similarly, Msed\_2265 was upregulated 2-fold in CuR1 60 min after metal challenge (not shown). This gene also responds 15 min after metal challenge but with 6.3-fold upregulation (Fig. 2). Msed\_2265 is annotated as a zinc finger TFIIIB-type domain-containing protein and is a transcription initiation factor associated with RNA polymerase II. The expression of two other functional classes of proteins also were noted. These consisted of hydrolases (GTPases and phosphatases), including Msed\_0499, Msed\_0050, Msed\_2061, and Msed\_2226, whose increased expression ranged from 4- to more than 10-fold. Finally, a second group of proteins with probable antioxidant activity (in addition to Msed\_1076), along with class II toxin and antitoxin genes, were identified. These included Msed\_0014, Msed\_0015, Msed\_0046, Msed\_0047, and Msed\_0048. Increased expression of toxins could position the cell for rapid readjustment in transcript abundance as part of an overall response to metal challenge.

**Genome resequencing.** Differences in gene expression patterns between the wild-type and CuR1 strains could be caused by mutations arising during the isolation of CuR1. Therefore, genome resequencing was conducted to map possible sequence differences between these strains. At a genome size of 2.19 Mb (7), this resulted in depths of sequence coverage for the two strains of 1,050-fold and 821-fold, respectively (Table 1). Since the goal was to identify genetic changes causing the metal-resistant CuR1 phenotype, the only mutations that were examined were those that were distinct between the CuR1 strain, the resequenced wild-type genome, and the previously determined *M. sedula* genome deposited at NCBI (NC\_009440.1, GI:146302785). Of these, 20 were pursued further because they were most likely to alter gene function and were not merely synonymous changes in open reading frames or in intergenic regions. DNA spanning these 20 sequence differences then was PCR amplified from the CuR1 genome and resequenced to verify their occurrence. Seventeen of these putative mutations were confirmed. While 5 of these mutations mapped to unannotated open reading frames (ORFs), 12 mapped to 11 genes with annotated function. These included 7 nonsynonymous substitutions, 4 insertions, and 1 deletion. A tabulated summary is presented of the confirmed mutations along with gene annotation, gene/protein length, numerical position of the mutational change, and its proximity to identified domains and/or motifs (Table 2).

Mutations in the CuR1 genome were identified in two transcription factors, Msed\_1279 and Msed\_1338, both annotated as multiple antibiotic resistance regulator (MarR) family members. These changes could be responsible for the differences in transcriptional response to copper shock. The MarR family regulates the expression of proteins conferring resistance to oxidative stress agents, multiple antibiotics, organic solvents, and pathogenic factors (36, 37). While the mutation in Msed\_1338 resulted in the

loss of a charged residue (Glu133 to Gly133), it did not map to the putative MarR family domain. In contrast, mutation of Msed\_1279, another MarR family member belonging to the OhrR subfamily, was mutated from Ser115 to Leu115 within alpha helix 5 of the MarR domain. This change could impact DNA binding and alter transcription factor activity.

Two distinct genes were mutated in CuR1 encoding proteins with important metabolic function. Msed\_1213 encodes the alpha subunit of pyruvate dehydrogenase that catalyzes conversion of pyruvate into acetyl coenzyme A (CoA). Msed\_1213 underwent a frameshift mutation resulting from insertion of a G and corresponding premature chain termination such that the protein was reduced from 344 to 199 amino acids (aa). This event ablated the conserved domain. However, pyruvate-ferredoxin oxidoreductase may provide an alternative route for acetyl-CoA formation, as demonstrated in *S. solfataricus* (36, 37). Orthologs of this enzyme are found in *M. sedula* as Msed\_0305, Msed\_0306, Msed\_0307, Msed\_0308, and Msed\_0309. Msed\_0191 encodes ornithine cyclodeaminase, which is required for proline biosynthesis. In addition, Msed\_0191 had a nonsynonymous mutation replacing Glu230 with Lys (acidic to basic) within the region coding for the NADB-Rossman superfamily domain that overlaps both the ornithine cyclodeaminase and amino acid deaminase domains. This event may change proline abundance and resistance to metals (38).

Several genes were mutated in CuR1-encoding proteins involved in phosphorylation. Msed\_1343 contains the P-loop\_NTPase superfamily domain, and the adjacent Msed\_1342 has homology to chromosome partitioning proteins, suggesting these genes are involved in cell division processes. There is an 8-bp overlap between the Msed\_1343 and Msed\_1344 genes. The 1-nt deletion within Msed\_1343 extends the reading frame an additional 195 aa. Msed\_0545 is a small gene (68 amino acids) whose closest BLAST hit to a nonhypothetical protein was to an ROK (repressor, ORF, kinase) family protein. ROK is a RhoA-binding kinase that plays a role in actin and other facets of cytoskeletal reorganization (39). Expression studies have shown that upregulating ROK promotes formation of stress fibers and adhesion complexes, while an N-terminally truncated ROK instead induces disassembly of these.

The genes of the last functional group that were mutated in CuR1 were involved in transport. Msed\_0972 is annotated as a major facilitator transporter, but its substrate is not known. The mutation in this gene changed Ala28 to Val within the major facilitator superfamily (MFS) domain. The second mutated transporter was Msed\_0490, which encodes the CopA efflux protein required for copper resistance in *M. sedula* (27). A nonsynonymous mutation occurred within the leader sequence, changing Gly18 to Val. Since this region is likely to be removed upon membrane insertion, its impact on efflux activity should be nominal. The third transporter that underwent a mutation, Msed\_1516, had a change of Leu218 to Ser. Msed\_1516 shares homology with the Pho80 family of phosphate transporters.

The third example of a transporter that was altered in CuR1 involves Msed\_1517. In this case, however, the mutation appeared to result in a gain of function. In the genome sequence of *M. sedula*, Msed\_1517 is annotated as a pseudogene, and this was verified by genome resequencing of this strain. The pseudogene could produce a 75-aa protein. In strain CuR1, a 1-nucleotide (nt) insertion of a G at position 170 reverted a natural frameshift,

TABLE 2 Confirmed mutations in CuR1

Genome coordinate	Substitution and/or description	Msed ORF	Protein	Mutation location	Domain affected
155,137	C→T, nonsynonymous (Glu→Lys)	0191	Ornithine cyclodeanimase	nt 691/939; aa 231/312	NADB Rossman superfamily
448,008	C→A, nonsynonymous (Gly→Val)	0490	Heavy-metal translocating ATPase	nt 5/2235; aa 2/744	Leader sequence
500,688	C→T, nonsynonymous (Glu→Lys)	0545	Hypothetical	nt 187/207; aa 63/68	
898,334	C→T, nonsynonymous (Pro→Ser)	0972	Major facilitator transporter	nt 1009/1164; aa 337/387	MFS superfamily (in region with putative substrate translocation pores)
1,187,052	1-nt insertion of G; frameshift starting at aa 160 and premature stop after aa 199	1213	Pyruvate dehydrogenase	nt 478/1035; aa 160/344	TPP enzyme superfamily
1,255,967	C→T, nonsynonymous (Ser→Leu)	1279	Transcriptional regulator, MarR family	nt 344/453; aa 115/150	HTH superfamily
1,318,092	1-nt deletion; frameshift starting at aa73	1343	Hypothetical (close BLAST hit to nucleotide binding domain-containing protein)	nt 217/243; aa 73/81	Outside domains (close to C-terminal end of protein)
1,314,441	T→C, nonsynonymous (Leu→Pro)	1338	Hypothetical (close hit to bacterial regulatory protein, ArsR family)	nt 245/846; aa 82/281	Outside domains
1,377,170	C→T, nonsynonymous (Tyr→Asn)	1408	Hypothetical	nt 175/309; aa 59/103	
1,377,775	T→G, nonsynonymous (Thr→Pro)	1409	Hypothetical	nt 46/474, 16/157	
1,397,090	T→G	1427	Pseudogene	nt 597/1217	
1,479,994	T→C, nonsynonymous (Leu→Ser)	1516	Hypothetical (low-score BLAST hit to sodium-dependent phosphate transporter)	nt 806/975; aa 269/374	No conserved domains in protein
1,481,054	1-nt insertion of G; extends ORF by 627 nt	1517	Pseudogene	nt 170/854	Extends ORF to include PHO4 superfamily
1,783,624-1,783,625	2-nt insertion	1838	Pseudogene (upregulated in transcriptomic data but no coding sequence)	nt 631/2282	Pseudogene
1,783,847	1-nt insertion	1838	Pseudogene	nt 855/2282	Pseudogene
1,784,992	T→C	1838	Pseudogene	nt 1838/2282	Pseudogene
1,827,819	T→C, nonsynonymous (Leu→Ser)	1884	Glutamate synthase	nt 1512/2139; aa 504/712	Outside domains

thereby extending the coding sequence an additional 640 nucleotides. The deduced amino acid sequence encoded by the extended allele has high identity to the Pho4 superfamily of phosphate transporters along with strong identity to the PitA domain; over a coding length of 305, residues of this sequence matched the PitA (COG0306) phosphate/sulfate permeases (e value of 9.22e<sup>-29</sup>). The restored Msed\_1517 coding sequence identified in strain CuR1 also had high identity to homologs conserved in other *Metallorphaera* species. These alignments indicated the CuR1 Msed\_1517 allele remained truncated at the C-terminal end, as the reading frames of homologous sequences in related species were 36 amino acids longer (Fig. 3).

**Characterization of phosphate transport.** To determine whether Msed\_1517 was a phosphate transport protein, rates of radiolabeled phosphate uptake were determined for CuR1 versus wild-type *M. sedula* under physiologic conditions (pH 2.5, 75°C). *K<sub>m</sub>* values for phosphate uptake were 2 μM for wild-type *M. sedula* and 36 μM for CuR1. *V<sub>max</sub>* values (nmol/min) for the wild-type and CuR1 strains at the phosphate concentrations indicated in parentheses were 2.4 (0 μM), 11.8 (2 μM), 14.25 (20 μM), and

22.55 (200 μM). These values are similar to previously reported values for PitA in *E. coli* (33) and describe a low-affinity, high-velocity phosphate transport protein. A second hallmark of the secondary transporter PitA is its reliance on the proton motive force for energy (34). Gramicidin D treatment was used as described previously (32) to measure the impact on phosphate uptake through dissipation of the proton motive force. Treatment

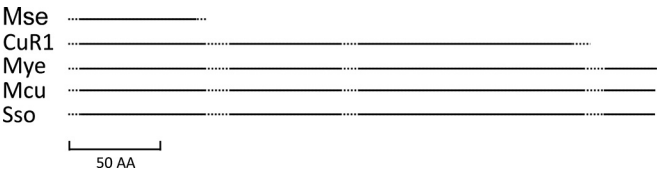


FIG 3 Amino acid alignment of Msed\_1517 variants from thermoacidophiles. The line length corresponds to the open reading frame. The solid portions indicate regions of conservation, and the dashed portions indicate regions of divergence. Msed, *Metallorphaera sedula* DSM5348T; CuR1, spontaneous copper resistant derivative of *M. sedula*; Mye, *Metallorphaera yellowstonensis* MK1; Mcu, *Metallorphaera cuprina* Ar-4; Sso, *Sulfolobus solfataricus* 98/2.

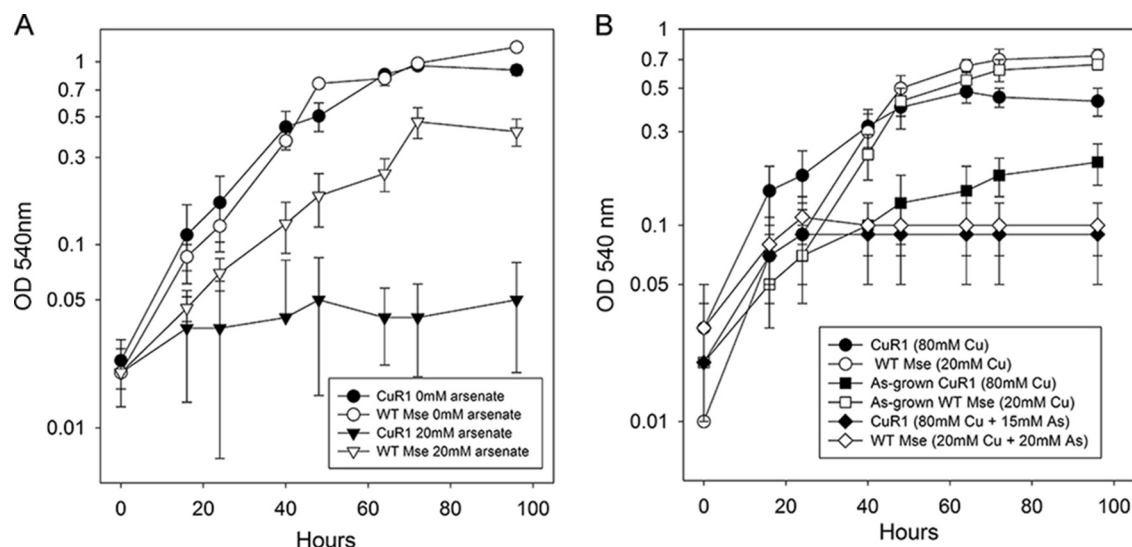


FIG 4 Response of *M. sedula* and CuR1 growth to arsenate challenge and its effect on copper resistance. (A) Effect of arsenate. (B) Effect of arsenate on growth at maximum tolerated levels of cupric ion.

for 5 min at 12.5  $\mu$ M concentration prior to addition of labeled phosphate reduced phosphate uptake to 16% of that of untreated cells for CuR1 and to 61% of that of untreated cells for the wild type, indicating a greater reliance of the PitA system on proton motive force than other unidentified phosphate transporters in this organism.

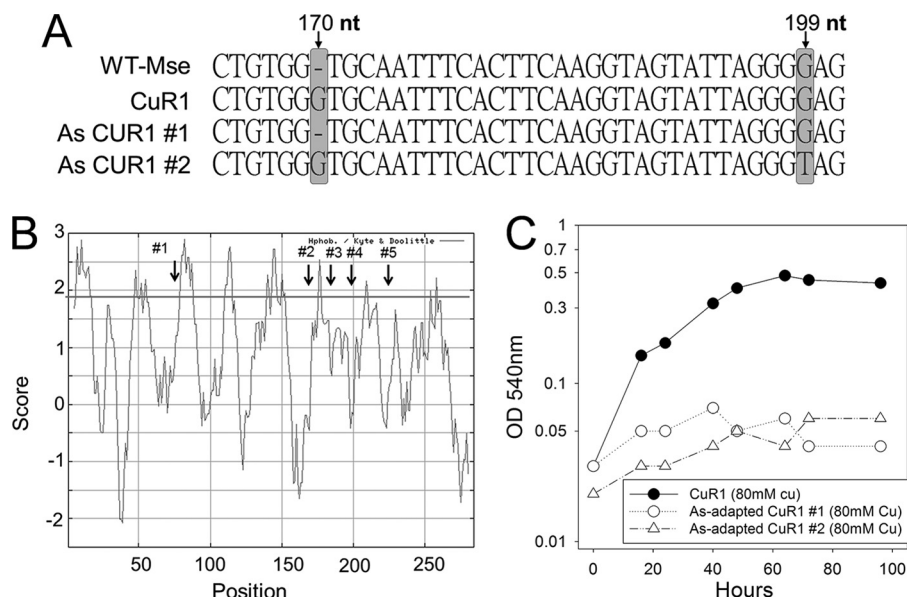
Studies of *E. coli* have shown that PitA transports arsenate in addition to phosphate and that PitA mutants are arsenate resistant (35). Rates of radiolabeled phosphate uptake also were measured under different arsenate concentrations to determine if there was competition for phosphate uptake by arsenate. The  $K_i$  values for arsenate were 16  $\mu$ M for the wild type and 32.4  $\mu$ M for CuR1. As expected, the  $K_i$  value is nearly 10-fold higher than the  $K_m$  value for the wild type, indicating that it has much higher affinity for phosphate than arsenate, while in CuR1 the affinity for phosphate and arsenate is very similar. CuR1 showed increased arsenate sensitivity relative to wild-type *M. sedula*, consistent with the allelic state of *pitA* (functional in CuR1, nonfunctional in the wild type) (Fig. 4A). CuR1 showed a decreased growth rate and lower cell yields at 15 mM arsenate, and growth was inhibited by 20 mM arsenate. In contrast, wild-type *M. sedula* showed only slight growth inhibition at 20 mM arsenate. This observation suggested a link between copper, arsenate, and Msd\_1517 and the possibility that arsenate, through its competition with phosphate, antagonizes copper resistance. This was tested by examining the effect of arsenate exposure on copper resistance (Fig. 4B). Arsenate-exposed CuR1 showed increased copper sensitivity, but arsenate-grown wild-type cells showed no difference in metal resistance between arsenate-exposed and unexposed cells. In contrast, growth in medium containing both copper and arsenate was inhibitory for both strains. Based on these observations, it was reasonable to propose that spontaneous arsenate-resistant derivatives of CuR1 arise by mutation of Msd\_1517. Seven spontaneous arsenate-resistant isolates were recovered, and DNA sequence analysis indicated each had undergone mutation in Msd\_1517. Mutant CuR1 As#1 underwent a single-nucleotide deletion recreating the wild-type Msd\_1517 truncated allele, while mutant

CuR1 As#2 underwent a G→T substitution at nt 199 (Fig. 5A). In both cases, these mutations produced highly truncated open reading frames. Three additional alleles were identified with nonsynonymous mutations that occurred much later in the Msd\_1517 coding sequence. Two of them comprised changes at two positions. These alleles consisted of the following changes at the indicated nucleotide positions: G→A, 77; T→G, 173; T→A, 180; T→A, 193; and G→C, 211 (Fig. 5B). Interestingly, all five of these mutations mapped to predicted membrane-spanning domains, as indicated by Kyte-Doolittle analysis (ExPasy) (Fig. 5B). The arsenate-resistant CuR1 derivatives also showed decreased copper resistance compared to the parental CuR1 strain (Fig. 5C) and a reduced rate of phosphate transport, resulting in 28% of the parental CuR1 strain transport rate. Taken together, these data identify Msd\_1517 as a PitA homolog and a critical component of the metal resistance mechanisms found in thermoacidophilic archaea. A model for copper resistance in *M. sedula* is presented (Fig. 6) that integrates these results with previous findings.

## DISCUSSION

Of the 17 mutations distinguishing *M. sedula* strain CuR1 from the wild type, the gain-of-function mutation in pseudogene Msd\_1517 was the most informative. This mutation restored nearly the entire reading length of a gene encoding the PitA/Pho4 domain that is conserved across the thermoacidophilic archaea. Therefore, wild-type *M. sedula* (DSM 5348T) would be a *pitA* mutant. Based on comparisons between cell lines, archaeal PitA appears to promote copper resistance and may do so by increasing phosphate-metal efflux. This type of resistance mechanism has been proposed as the basis for high-level metal resistance in *M. sedula* and related lithoautotrophic archaea (3). The ability to increase phosphate ion efflux in complex with copper ions would decrease intracellular metal concentrations. Phosphate ions would derive from polyphosphate granules shown previously to accumulate to high levels only in the highly metal-resistant group of thermoacidophilic archaea (21). In bacteria such as *E. coli*, inorganic phosphate transport involves two transport systems, in-





**FIG 5** Location and copper resistance of arsenate-resistant derivatives of CuR1. (A) DNA sequences of Msd\_1517 in wild-type *M. sedula*, strain CuR1, and two spontaneous arsenate-resistant mutants resulting in early sequence truncation. Bars indicate the location of sequence alterations. (B) Association of spontaneous arsenate-resistant mutations and predicted Msd\_1517 membrane-spanning regions. Membrane-spanning regions were determined using Kyte-Doolittle hydropathy analysis. (C) Growth in copper medium (80 mM).

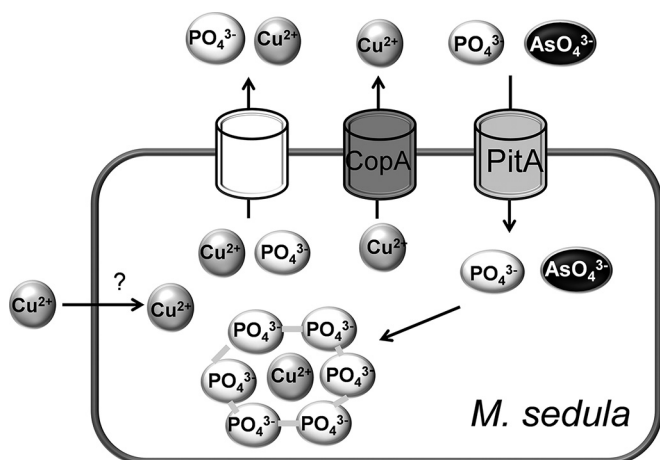
cluding a high-affinity, low-velocity Pst (phosphate-specific transport) system and a low-affinity, high-velocity Pit (Pi transport) system (35). These systems have been distinguished by their contribution to arsenate resistance; cells having only Pst (mutated for Pit) will grow in the presence of arsenate, while those having only Pit (mutated in Pst) cannot grow in the presence of an arsenate-to-phosphate ratio of 10 in the medium (35). If Msd\_1517 constitutes the archaeal Pit transport system, then restoration would support increased phosphate efflux and metal symport through the Pit-like system. This type of mechanism has been suggested to operate in *E. coli* (33, 40). In previous studies on metal resistance in thermoacidophilic archaea, the presence of

another transporter typified by the Pho84 domain was noted (21). Pho84, like Pit, belongs to the family of phosphate-proton symporters and the major facilitator superfamily. Perhaps acting together, Msd\_1517 and Pho84 comprise dual phosphate transporters that are inherent to these organisms and are critical for high-level metal resistance.

Genome resequencing of CuR1 also provided the first measure of mutation rate in lithoautotrophic archaea. After 24 generations over 6 passages, CuR1 accumulated a total of 20 mutations, of which 17 could be experimentally verified. This resulted in a mutation rate of  $3.44 \times 10^{-7}$  mutations per cell division and was comparable to mutation rates seen in heterotrophic thermoacidophilic archaea (41). This frequency will be of use to assess the mutation rates experienced by such organisms in natural settings.

Transcriptomic analysis of CuR1 also demonstrated that increased metal resistance reflected a more rapid and stronger response to metal challenge. The key genes involved in this response included two transcription factors, of which one, TFB, constitutes a generalized transcription factor. Changes in the abundance of both of these transcription factors are likely to change gene expression. In addition, several transporters exhibited increased expression and could assist in the export of metal ions to achieve metal homeostasis. Finally, the induction of toxin-antitoxin proteins could provide an additional mechanism to adjust the abundance of transcripts critical to meet the needs of a copper-challenged cell, much as has been described recently for heat shock in the related *S. solfataricus* (42).

The type species of *Metallosphaera sedula*, DSM 5348T, unexpectedly was found to be naturally mutated for Msd\_1517. The restoration of the function of this gene is a key component of the elevated copper resistance of the spontaneous copper-resistant derivative strain, CuR1. However, the gain-of-function mutation did not completely restore the full length of Msd\_1517 (*pitA*) relative to the alleles in other genome sequences of thermoacido-



**FIG 6** Copper resistance model in *M. sedula*. CopA catalyzes copper efflux. Archaeal PitA (Msd\_1517) catalyzes the uptake of both phosphate and arsenate. An unidentified transporter may catalyze the efflux of copper phosphate complexes. The location of intracellular copper is unknown but may be phosphate associated.

philic archaeal lineages. Like bacterial PitA, archaeal PitA was found to be a low-affinity, high-velocity secondary transporter dependent on the proton motive force. While a role for PitA has been described as a mediator of arsenate sensitivity and loss-of-function mutations result in arsenate resistance (43), archaeal PitA also is required for high-level copper resistance, and its inactivation resulted in a 3-fold reduction of adaptive levels of this trait (200 mM versus 76 mM) (27). To explain the combined roles of PitA as a transporter (of phosphate and arsenate) and a mediator of high-level copper resistance, it is necessary to suggest that intracellular phosphate is limiting in the absence of PitA. It has been suggested in extreme thermoacidophiles that intracellular polyphosphate provides a source of soluble phosphate that can be effluxed bound to copper ions (21, 44). Here, PitA could be shown to mediate both phosphate and arsenate uptake; therefore, it seems unlikely that PitA also mediates phosphate-copper complex efflux but rather that some unidentified transport protein is responsible for this process.

## ACKNOWLEDGMENTS

This research was supported by funding from the NIH (1R01GM090209), DTRA (HDTRA1-09-0030), and AFOSR (FA9550-13-1-0236) to R.K. and P.B. and the UNL Cell Development Facility to P.B.

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