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Organoarsenicals inhibit bacterial peptidoglycan biosynthesis by targeting the essential enzyme MurA

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Abstract

Trivalent organoarsenicals such as methylarsenite (MAs(III)) are considerably more toxic than inorganic arsenate (As(V)) or arsenite (As(III)). In microbial communities MAs(III) exhibits significant antimicrobial activity. Although MAs(III) and other organoarsenicals contribute to the global arsenic biogeocycle, how they exert antibiotic-like properties is largely unknown. To identify possible targets of MAs(III), a genomic library of the gram-negative bacterium, *Shewanella putrefaciens* 200, was expressed in *Escherichia coli* with selection for MAs(III) resistance. One clone contained the *S. putrefaciens murA* gene (*SpmurA*), which catalyzes the first committed step in peptidoglycan biosynthesis. Overexpression of *SpmurA* conferred MAs(III) resistance to *E. coli.* Purified SpMurA was inhibited by MAs(III), phenylarsenite (PhAs(III)) or the phosphonate antibiotic fosfomycin but not by inorganic As(III). Fosfomycin inhibits MurA by binding to a conserved residue that corresponds to Cys117 in SpMurA. A C117D mutant was resistant to fosfomycin but remained sensitive to MAs(III), indicating that the two compounds have different mechanisms of action. New inhibitors of peptidoglycan biosynthesis are highly sought after as antimicrobial drugs, and organoarsenicals represent a new area for the development of novel compounds for combating the threat of antibiotic resistance.

Keywords: Organoarsenicals, peptidoglycan, methylarsenite, fosfomycin, bacterial resistance

1. Introduction

Arsenic is among the most prevalent toxic elements in the environment. Life first arose in environments with substantial concentrations of inorganic arsenic. Although evolution of genes for arsenic tolerance was a first priority for primordial life, the ubiquity of arsenic provided an opportunity for microbes to adapt those pathways to produce organoarsenical antimicrobial compounds that could give them a competitive advantage over other members of microbial communities (Zhu et al., 2014). Microbial arsenic biotransformations include redox cycles, methylation, thiolation and incorporation into complex organic molecules such as arsenolipids and arsenosugars, creating a global arsenic biogeochemical cycle (Zhu et al., 2017). As a result, a variety of organisms produce a variety of arsenic-containing natural products that differ in toxicity (Sup. Table 1). In general, pentavalent methylarsenicals are less toxic than inorganic As(III), with the exception of thiolated species (Fan et al., 2018) and the recently-discovered pentavalent organoarsenical antibiotic arsinothricin (Nadar et al., 2019). In contrast, trivalent organoarsenical natural products such as MAs(III) and dimethylarsenite (DMAs(III)) are much more toxic than As(III) (Petrick et al., 2000; Naranmandura et al., 2011; Yoshinaga and Rosen, 2014). These organoarsenicals are products of the enzyme As(III) *S*-adenosylmethionine (SAM) methyltransferase (ArsM in microbes and AS3MT in animals). ArsM is postulated to have co-evolved with the MAs(III) efflux permease ArsP in an anoxic environment prior to the Great Oxygenation Event (GOE) (Chen et al., 2017). We proposed that the antibiotic-like properties of its products provide a competitive advantage for producers, over other members of anaerobic microbial communities (Li et al., 2016; Chen et al., 2018). Following the GOE, trivalent arsenicals were generally oxidized to their less toxic pentavalent forms such as methylarsenate (MAs(V)). In response, members of aerobic microbial communities adapted ways to reduce MAs(V), giving them a competitive advantage over MAs(III)-sensitive microbes (Yoshinaga et al., 2011). Yet other members of aerobic microbial communities developed oxygen-dependent

resistance to MAs(III) such as ArsH, which oxidizes MAs(III) to MAs(V) (Chen et al., 2015a) and the C-As bond cleavage dioxygenase ArsI, which demethylates MAs(III) to less toxic As(III) (Yoshinaga and Rosen, 2014), suggesting that this competition between microbial communities is a driving force behind the arsenic biogeocycle. Indeed, the MAs(III) efflux permease ArsP is another pathway for resistance (Chen et al., 2015b).

The goal of this study was to identify possible targets of MAs(III) inhibition that can explain its strong antibiotic properties. We chose to search for new genes in the environmentally ubiquitous gram-negative facultative anaerobe *S. putrefaciens* 200 because it has a number of *ars* operons with many known and putative genes for arsenic resistance and a wide capacity for organoarsenic biotransformation and transport, suggesting evolution in an environment containing MAs(III) (Chen et al., 2015b; Chen and Rosen, 2016). We constructed a genomic expression library from *S. putrefaciens* 200 and selected for MAs(III) resistance clones in *E. coli.* Overexpression of target enzymes have been shown to confer resistance to antibiotics such as triclosan, trimethoprim, penicillin and other β-lactams (Henze and Berger-Bächi, 1996; Palmer and Kishony, 2014). One MAs(III)-resistant clone with an insert of 2.8 kb was sequenced and shown to express the *S. putrefaciens murA* gene. MurA catalyzes transfer of enolpyruvate from phosphoenolpyruvate (PEP) to uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), the first committed step in peptidoglycan biosynthesis (Barreteau et al., 2008). Thus, we considered the possibility that MurA is a target of MAs(III), which could inhibit peptidoglycan biosynthesis, and increasing MurA expression could overcome the antimicrobial activity of MAs(III). From the use of arsenic-containing minerals in ancient China to Paul Erlich's synthesis of the aromatic arsenical salvarsan, the first chemotherapeutic agent for syphilis, arsenic-containing drugs have been used extensively throughout history (Lloyd et al., 2005; Liu et al., 2008). In modern medicine, arsenic trioxide (ATO) is an FDA-approved, effective anti-cancer agent for promyelocytic leukemia (APL) (Ralph, 2008). With the increasing threats posed by antibiotic resistance, the bacterial

peptidoglycan biosynthetic pathway has been a recent focus for development of new inhibitors (Brackman et al., 2016; Chang et al., 2017). The identification of cell wall biosynthesis as a potential target of MAs(III) affords the prospect for development of new arsenic-containing antimicrobial agents.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from MilliporeSigma (Burlington, MA) unless otherwise mentioned. For *in vivo* assays, MAs(V) was reduced to MAs(III) by a slight modification of a previous procedure (Johnson, 1971). Briefly, 0.2 mM MAs(V) was mixed with 27 mM $Na_2S_2O_3$, 66 mM $Na_2S_2O_5$, and 82 mM H_2SO_4, followed by pH adjustment to 6 using NaOH. For *in vitro* assays, because the reagents used to reduce MAs(V) ($Na_2S_2O_3$, $Na_2S_2O_5$ and H_2SO_4) ablated MurA activity, the methylarsonous acid iodide derivative ($MAs(III)I_2$) was synthesized as previously described (Stice et al., 2016).

2.2. Strain, plasmids, media, and growth conditions

S. *putrefaciens* 200 used for total DNA extraction was provided to us as a gift from Flynn Picardal, Indiana University. Expression vector pET26b containing *S. aureus* SH1000 (WP_000358012.1) MurA was kindly provided by Dr. Alex O'Neill (University of Leeds, Leeds, UK). *E. coli* One Shot™ TOP10 (Thermo Fisher Scientific, Waltham, MA) (F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) was used for library construction and *in vivo* arsenical resistance assays. *E. coli* strain BL21 (fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5) was used for expression and purification of *S. putrefaciens* 200 wild-type and C117D MurA, as well as *in vivo* fosfomycin resistance assays. *E. coli* strains were grown aerobically at either 30 °C or 37

°C in either lysogeny broth (LB) or 2x ST medium (20-fold concentrated ST 10^{-1} media, 10 g L^{-1} Difco Bacto Peptone and 1 g L^{-1} yeast extract) supplemented with 0.2% glucose (Maki et al., 2006).

2.3. Isolation of S. putrefaciens 200 *DNA fragment conferring MAs(III) resistance*

To construct a genomic library of *S. putrefaciens* 200, total DNA was extracted using DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) and digested with *Hind*III (New England Biolabs, Ipswich, MA). Following digestion and ethanol precipitation, the pelleted DNA was suspended in water and layered on a discontinuous sucrose gradient (10%, 20%, 30%, 40%) buffer containing 50 mM Tris (pH8.0 adjusted by HCl), 0.1 M EDTA, and 0.1 M NaCl. The gradients were then centrifuged at 210,000 × *g* for 5 h at 17 °C to separate DNA fragments by size. After the centrifugation, the solution was carefully removed from the top of the layer and separated into fractions. Fractions were run on 1% agarose gels to confirm their size, and fractions containing DNA fragments larger than 2 kbp were combined and concentrated via ethanol precipitation. The resulting DNA fragment mixture was ligated overnight at 16 °C into *Hind*III-digested cloning vector pUC118 using LONG ligase (TaKaRa, Kusatsu, Japan). Ligation mixture was transformed into *E. coli* TOP10 using electroporation, and transformants were spread on LB media agar plates containing 100 µg/mL ampicillin. Ligation and transformation steps were repeated twice more to attain a total of 3 plates of approximately 2,000 colonies each. A glycerol stock of the library was made from LB plates and aliquoted, followed by flash freezing and storage at -80 °C. To ensure complete coverage of the *S. putrefaciens* 200 genome during MAs(III) resistance selection, average fragment sizes were calculated by purifying plasmid DNA from several transformants using E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA), and digesting plasmids with *Hind*III, followed by gel electrophoresis using 1% agarose gels. Average insert size was found to be 6.7 kbp, hence approximately 2,000 colonies were spread to cover the 5 Mbp of the *S. putrefaciens* 200 genome.

Clones were selected for resistance to MAs(III). After obtaining approximately 2,000 colonies on LB plates, colonies were replicated onto 1, 2, and 3 μM MAs(III) ST 10^{-1} media (0.5 g L^{-1} Difco Bacto Peptone and 0.05 g L^{-1} yeast extract) agar plates supplemented with 50 μg/mL ampicillin and 0.2% glucose, and incubated at 30 °C for 48 h. To exclude MAs(III) clones that had acquired the gene for the ArsP MAs(III)-selective efflux pump (of which *S. putrefaciens* 200 has two copies), colonies growing on 2 and 3 μM MAs(III) plates were picked and analyzed for the presence of the *arsP* gene by colony PCR using forward primers GATGATGATCCATGGGGATGAATCCTGAAACCCTAGCC (*arsP1*) and GATGATGATCCATGGGGATGCTGCAAATATTTTCAGATTTAGCGAGTTGG (*arsP2*) and reverse primers GATGATGATGTCGACGCTAAATACATAGCTATAAAGAAACCCAGAGCCG (*arsP1*) and GATGATGATGTCGACCAAAATGGGGCTGATCCCGTT (*arsP2*). Colonies lacking *arsP* genes were assayed for their arsenic biotransformation capacity using high performance liquid chromatography (HPLC) (Series 2000; Perkin-Elmer, Waltham, MA) coupled with inductively coupled plasma mass spectrometry (ICP-MS) (ELAN DRC-e; Perkin-Elmer, Waltham, MA) (vide infra). A single clone isolated lacking both *arsP* genes and arsenic biotransformation activity was streaked to confirm homogeneity, plasmid DNA was purified from two colonies, and partial sequence information of the insert fragment was determined. The whole sequence of the insert fragment was obtained through nucleotide BLAST search against the *S. putrefaciens* 200 genome (GCA_000169215.2), and the same fragment was obtained from plasmids of both colonies.

To compare the level of MAs(III) resistance conferred by the DNA fragments to known MAs(III) resistance mechanisms, pTrcHis2A vector carrying *S. putrefaciens* 200 *arsP* (pTrcHis2A-ArsP) (Chen et al., 2015b) and pUC19 vector carrying *Bacillus sp.* MD1 *arsI* (pUC19-ArsI) (Yoshinaga and Rosen, 2014) were transformed into *E. coli* TOP10 and grown overnight in LB media supplemented with 100 μg/mL ampicillin. Overnight cultures were washed by 2x ST medium, and

cell density was adjusted to a starting absorbance (A_{600nm}) of 0.05 in 2x ST media containing indicated concentrations of MAs(III) and supplemented with 50 µg/mL ampicillin and 0.2% glucose. Cells were incubated at 30 °C shaking for 5 h, after which growth was estimated from the absorbance at 600 nm.

2.4. Arsenic transformation by HPLC-ICP-MS

To determine whether the DNA fragment isolated from *S. putrefaciens* 200 conferred the ability to transform MAs(III) into less toxic species, cells with that construct were compared with cells expressing the ArsI C-As lyase. *E. coli* TOP10 cells carrying pUC19-ArsI (Yoshinaga and Rosen, 2014), and two colonies of *E. coli* pUC118 cells carrying the *S. putrefaciens* 200 DNA fragment, and the parent plasmid (pUC118) were cultured overnight in 3 mL LB media supplemented with 100 µg/mL ampicillin. Overnight cultures were centrifuged and suspended in 2 mL ST 10^{-1} medium supplemented with 0.2% glucose containing 2 µM MAs(III). Cells were incubated for 4 h at 30 °C. After incubation, culture media was filtered by centrifugation at 20,000 × *g* for 10 min at 4 °C using Amicon Ultra Centrifugal Filters with a 3 kDa membrane (MilliporeSigma, Burlington, MA). Arsenic from filtrates was speciated by HPLC-ICP-MS using a reverse-phase C18 column (250 mm × 4.6 mm; Thermo-Fisher, Waltham, MA), as described previously (Qin et al., 2006).

2.5. Identifying the gene responsible for MAs(III) inhibition

Tyrosine-to-stop codon mutations were introduced individually into *bolA* and *murA* genes in the purified plasmid DNA via site-directed mutagenesis using forward primers 5'-TCGGATGGTAGCCATTAGAAAGTCATCGCCGTAG-3' (*bolA*) and 5'-CGTTATTGGCACGCTAGGGTACCGCTGACG-3' (*murA*) and reverse primers 5'-CTACGGCGATGACTTTCTAATGGCTACCATCCGA-3' (*bolA*) and 5'-CGTCAGCGGTACCCTAGCGTGCCAATAACG-3' (*murA*). Primers were designed using the QuikChange® primer design tool (https://www.agilent.com/store/primerDesignProgram.jsp). For

mutagenesis, a PCR mixture containing 180 ng of template DNA, 0.5 µM of forward and reverse primers, 2.5 mM of dNTP mix, 5% DMSO, and 100U of Pfu Turbo (with the appropriate buffer) was used. An initial denaturing step was performed at 94 °C for 5 min, followed by 18 cycles of denaturing (50 s at 94 °C), annealing (50 s at 60.4 °C), and elongation (4 min at 72 °C). A final elongation step was performed at 72 °C for 10 min. Following mutagenesis, plasmids were digested with *Dpn*I at 37 °C overnight. Digestion mixtures were concentrated and transformed into *E. coli* TOP10 using heat-shock. Transformants were spread on LB plates supplemented with 100 µg/mL ampicillin and incubated overnight at 37 °C. Plasmid DNA was purified from several colonies and sequenced using forward primers 5'-GATGATGATGAGCTCATGGAATGCAGCTTAATCGAACAG-3' (*bolA*) and 5'-GATGATGATGAGCTCATGGCGGGAGTATTGGCAGAGACC-3' (*murA*) and reverse primers 5'-GATGATGATCGGCCGTCACTAGCTAGGCATGTTGAA-3' (*bolA*) and 5'-GATGATGATCGGCCGTTATTGTACGCGCTCTACATGCGC-3' (*murA*). After obtaining the appropriate mutants, MAs(III) resistance assays were assayed in 2x ST media, as described previously. DNA sequencing was performed by Sequetech (Mountain View, CA).

2.6. S. putrefaciens 200 *MurA expression and purification*

Codon-optimized *S. putrefaciens* 200 wild-type *murA* was commercially synthesized and cloned into expression vector pET28a (GenScript, Piscataway, NJ). This construct was transformed into *E. coli* BL21 using heat-shock. Transformants were cultured in 10 mL of LB media supplemented with 25 µg/mL kanamycin and incubated at 37 °C overnight. An overnight culture was used to inoculate 1 L of LB media supplemented with 25 µg/mL kanamycin, and MurA expression was induced using 0.3 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1.5 h at 37 °C at an $A_{600\ nm}$ = 0.6. Cells were collected and suspended in 5 mL per gram of wet cells in buffer A (50 mM MOPS, 20% glycerol (vol/vol), 0.5 M NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol). Cells were broken by a single passage through a French press cell at 20,000 psi. Cell lysate was

ultra-centrifuged at 160,000 × *g* for 1 h at 4 °C and the supernatant was loaded onto a nickel-NTA resin column (Thermo-Fisher Scientific, Waltham, MA) at a flow rate of 1.0 mL/min. The column was washed with buffer A and eluted with buffer A containing 100 mM imidazole into fractions. MurA purity was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), and fractions containing MurA were combined and concentrated using an Amicon Ultra-30 centrifugal filter (MilliporeSigma, Burlington, MA) by centrifugation at 5,000 x *g*. Imidazole was removed from MurA fractions by diluted to sub-micromolar concentrations and concentration twice with buffer A without imidazole. Protein concentration was estimated using the Bradford assay using bovine serum albumin as standard (Bradford, 1976). MurA was aliquoted, flash frozen, and stored at -80 °C.

A Cys117 to aspartate mutation (C117D) was introduced into the *murA* gene via site-directed mutagenesis. Primers used were 5'-ATTGCCGGGCGGCGATGCCATTGGCGCG-3' (forward) and 5'-CGCGCCAATGGCATCGCCGCCCGGCAAT-3' (reverse). Plasmid digestion with *Dpn*I, purification, and sequencing was performed as described previously, as were expression and purification of C117D MurA.

2.7. In vitro MurA activity and inhibition

MurA activity was determined from the release of inorganic phosphate (P_i) with purine-nucleoside phosphorylase (PNP), as previously described with minor modifications (Blake et al., 2009). Briefly, 760 µl of reaction mixture containing 50 mM HEPES, 100 µM dithiothreitol (DTT), 90 nM MurA, 300 µM UDP-*N*-acetylglucosamine (UDP-GlcNAc), 250 µM 7-methyl-6-thioguanosine (MESG), 0.1U/mL PNP and inhibitors (Fos, MAs(III), PhAs(III)) was added to wells on a 96-well plates (190 µL), and the reaction was started by adding 10 µl of 2 mM phospho*enol*pyruvate (PEP) at a final concentration of 100 µM. The increase in absorbance at 360 nm was monitored at room temperature in real time using a Synergy™ H4 (BioTek, Winooski, VT) plate reader. For co-incubation of MAs(III) (50 µM) and *N*-ethylmaleimide (NEM) (450 µM), DTT was removed from

247 the reaction mixture to allow for NEM binding to cysteine residues, and mixtures were incubated
248 with NEM for 15 min at room temperature prior to adding MAs(III).
249

250 ## 3. Results

251 ### *3.1. Selection of an S. putrefaciens 200 gene that confers MAs(III) resistance*

252 Most bacterial genes involved in arsenic detoxification and transformation are found in *ars*
253 operons; however, we expected that the targets of antimicrobial compounds such as MAs(III)
254 would likely be unrelated to arsenic biochemistry. Thus, to isolate potential targets of MAs(III)
255 inhibition, a genomic library of *S. putrefaciens* 200 was constructed and expressed in *E. coli*.
256 Selection for resistance to 3 μM MAs(III) yielded a colony (Clone 1) that was confirmed to confer
257 MAs(III) resistance after purification by streaking onto LB agar plates (Fig. 1).

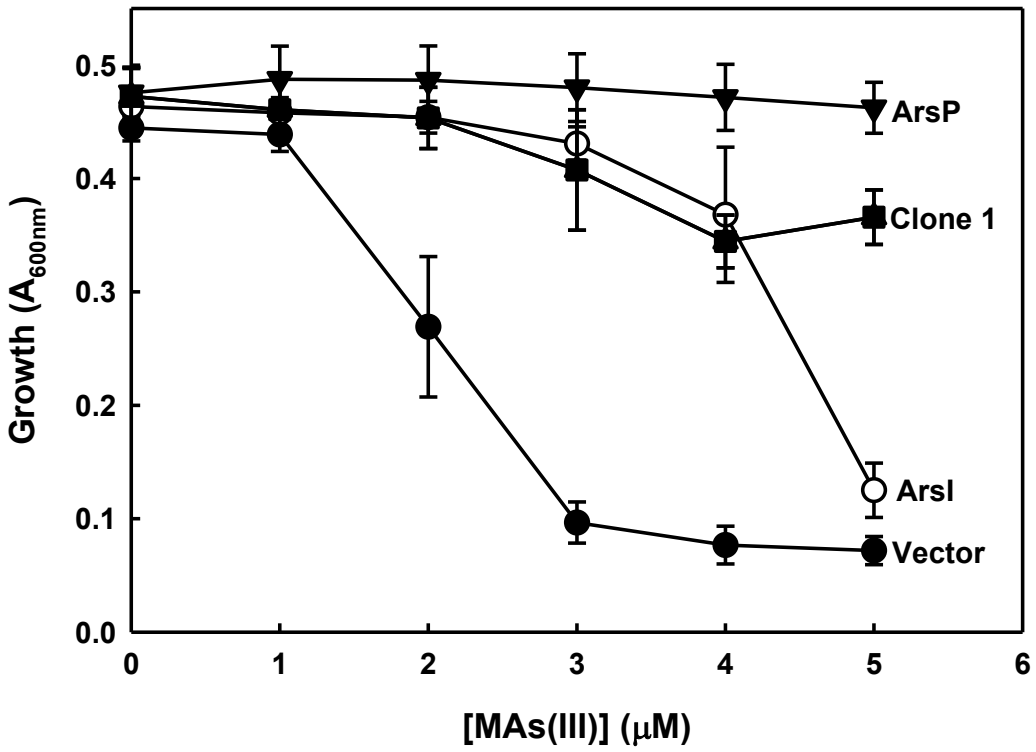


Fig. 1. DNA isolated from an *S. putrefaciens* 200 genomic library confers MAs(III) resistance. Overnight cultures of *E. coli* TOP10 carrying either, pUC118-Clone 1 (■), pUC19-ArsI (○), pTrcHis2A-ArsP (▼), or plasmid pUC118 (●) were adjusted to a starting absorbance at 600nm of 0.05 in 2x ST media supplemented with 0.2% glucose containing the indicated concentrations of MAs(III). Growth was estimated from A_{600nm} after 5 h of incubation at 30 °C. Data are the mean ± SE (*n* = 3).

Clone 1 did not biotransform MAs(III) (Fig. S1). Plasmid DNA was isolated from Clone 1, and the DNA insert was sequenced and aligned to the *S. putrefaciens* 200 genome (accession number: GCA_000169215.2). A 2.8 kbp fragment was identified, which contained two full-length open

reading frames that were annotated as *bolA,* encoding a transcriptional regulator, and *murA*, annotated to encode a UDP-*N*-acetylglucosamine enolpyruvyl transferase. Neither gene was in an *ars* operon of *S. putrefaciens* 200, and neither has been previously reported to be involved in arsenic resistance. To determine which of these genes confers the MAs(III)-resistant phenotype, stop codons were introduced via site-directed mutagenesis into the sequences of each gene. Cells carrying the mutant *bolA* plasmid remained resistant to MAs(III), whereas cells carrying the mutant *murA* plasmid lost resistance (Fig. 2), demonstrating that *murA* is responsible for the MAs(III)-resistance phenotype.

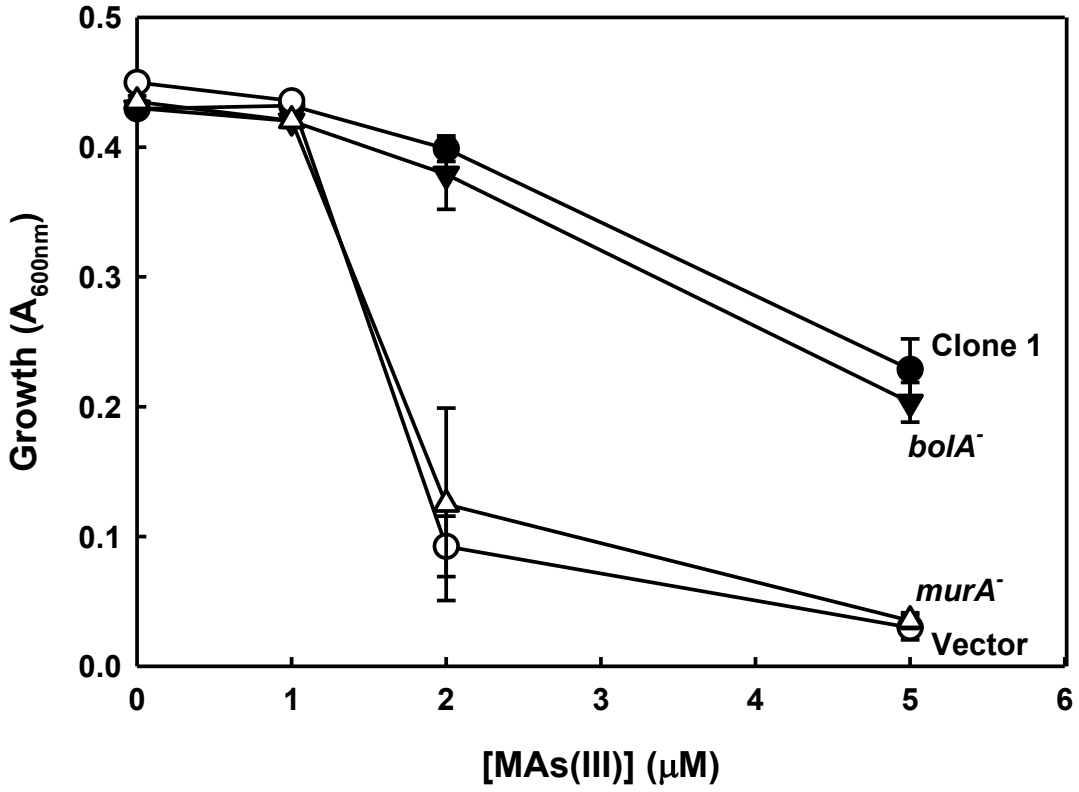


Fig. 2. Expression of *murA* confers resistance to MAs(III) *in vivo*. Overnight cultures of *E. coli* TOP10 carrying either pUC118-Clone 1, which contains wild-type *S. putrefaciens* 200 *murA* and *bolA* (●), vector carrying a *murA* tyrosine-to-stop codon mutant (Δ), vector carrying a *bolA* tyrosine-to-stop codon mutation (▼), or parental plasmid pUC118 (○) were adjusted to a starting

283 $A_{600\ nm}$ of 0.05 in 2x ST media supplemented with 0.2% glucose containing the indicated
284 concentrations of MAs(III). Growth was estimated from the A_{600nm} after 5 h incubation at 30 °C.
285 Data are the mean ± SE (*n* = 3).

286

287 *3.2. MurA is selectively inhibited by organoarsenicals*

288 MurA catalyzes the first committed step of bacterial peptidoglycan biosynthesis and is essential
289 for both gram-negative and gram-positive bacteria (Barreteau et al., 2008). The enzyme catalyzes
290 transfer of the enolpyruvate moiety of phosphoenolpyruvate (PEP) to the 3' hydroxyl group of
291 UDP-*N*-acetylglucosamine (UDP-GlcNAc), generating UDP-*N*-acetylglucosamine-enolpyruvate
292 and inorganic phosphate (P_i) (Eschenburg et al., 2003). SpMurA was purified from cells of *E. coli*
293 and assayed for Pi release using a coupled assay (Webb, 1992; Blake et al., 2009). MurA activity
294 was inhibited by MAs(III) but not inorganic As(III) (Fig. 3). The aromatic organoarsenical PhAs(III)
295 inhibited MurA more strongly than MAs(III), consistent with the observation that aromatic
296 arsenicals have higher affinity for arsenic-binding enzymes than MAs(III) (Pawitwar et al., 2017).

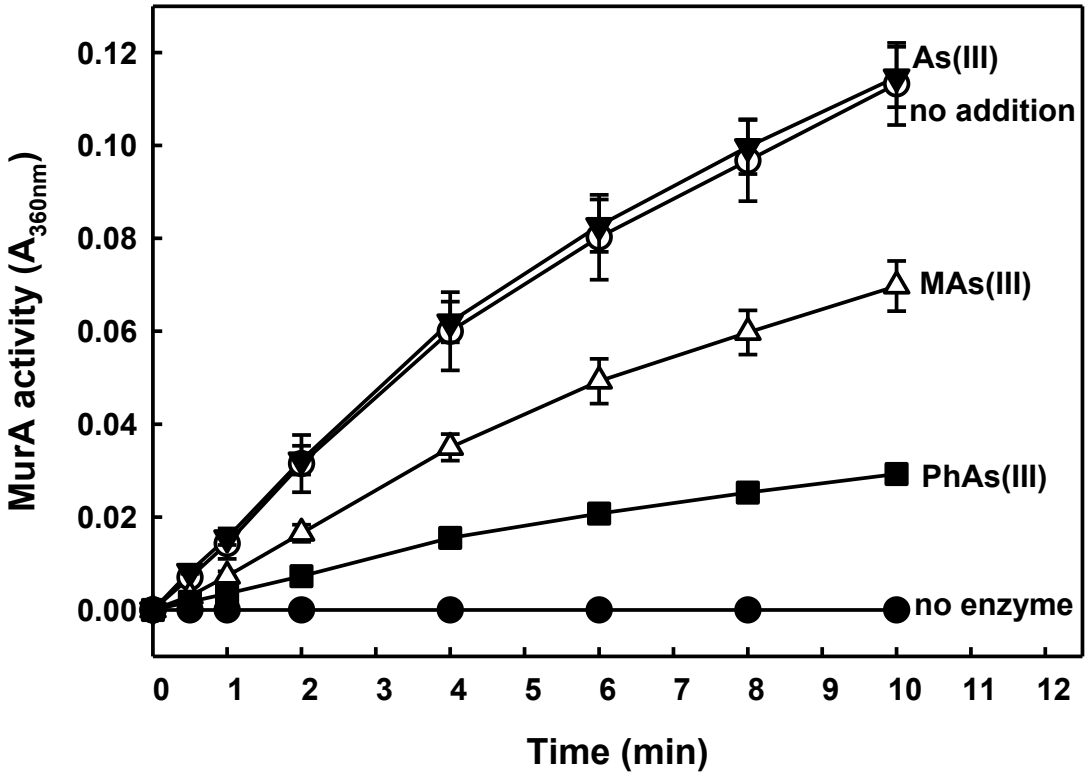
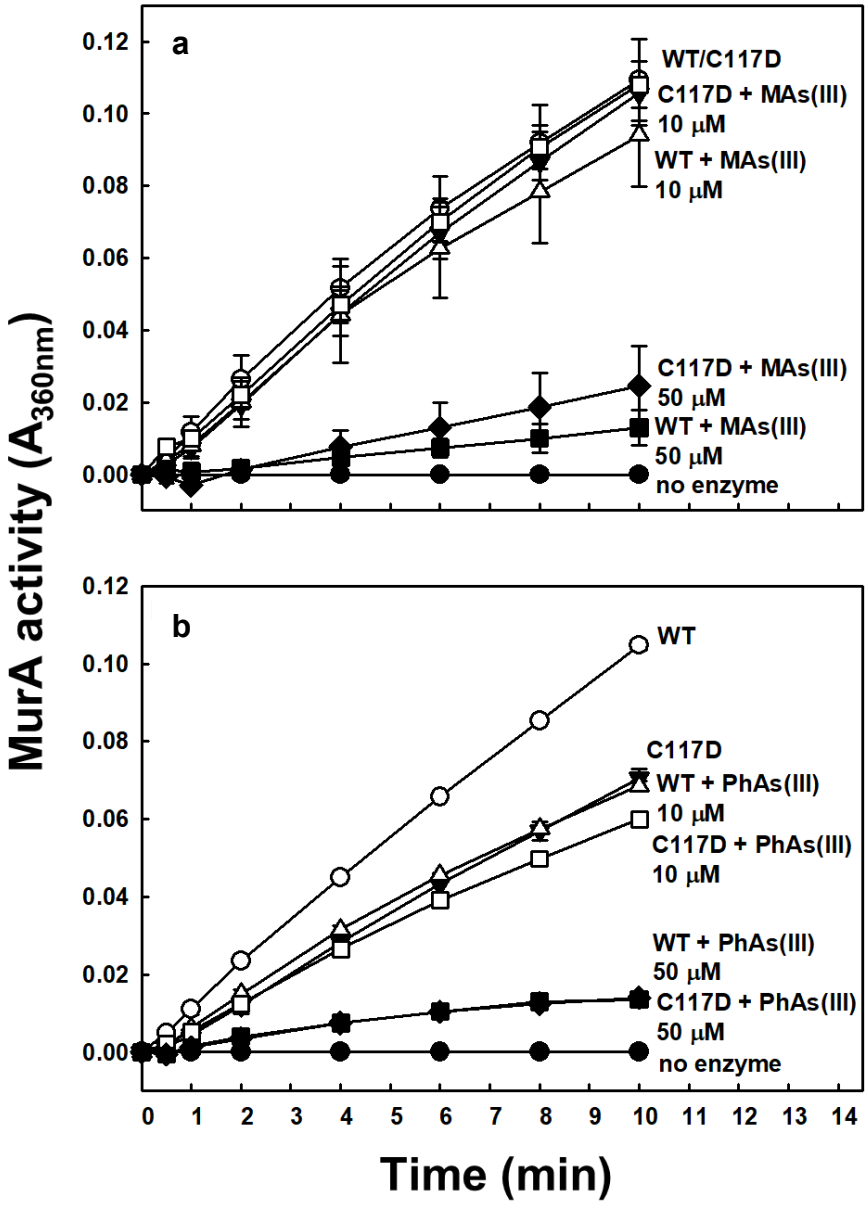


Fig. 3. SpMurA is selectively inhibited by organoarsenicals. Purified wild-type *S. putrefaciens* 200 SpMurA and UDP-*N*-acetylglucosamine (UDP-GlcNAc) were incubated with purine-nucleoside phosphorylase (PNP) and its substrate, methylthioguanosine (MESG), whose purine base product absorbs at λ = 360 nm. The reaction was initiated by addition of PEP at a final concentration of 0.1 mM, and the release of P_i in the absence (○) or presence of 20 μM As(III) (▼), 20 μM MAs(III) (Δ), or 20 μM PhAs(III) (■) was monitored by the increase in $A_{360\ nm}$. Data were normalized to the activity of the control reaction lacking MurA (●). Data are the mean ± SE (*n* = 3).

3.3. Cysteine 117 is not required for organoarsenical inhibition of SpMurA activity

MurA is inhibited by the broad-spectrum antibiotic fosfomycin (Fos) (Kahan et al., 1974), which is a competitive inhibitor of PEP that covalently binds to a cysteine residue conserved in most but not all bacterial species (Fig. S2). Overexpression of MurA confer resistances to Fos (Couce et al., 2012). Growth of cells of *E. coli* expressing a wild-type *SpmurA* gene was inhibited by Fos (Fig. S3A). Similarly, the activity of purified wild-type MurA was inhibited by Fos (Fig. S3B). The *murA* gene of *Mycobacterium tuberculosis* has an aspartic acid residue in the equivalent position of this cysteine, and this bacterium is resistant to Fos (De Smet et al., 1999). *E. coli* cells, which have a chromosomally-encoded wild-type EcMurA with Cys115, are sensitive to Fos, and a C115D mutation has been shown to confer Fos resistance (Kim et al., 1996). To examine whether Cys117 in SpMurA is involved in organoarsenical inhibition of *S. putrefaciens* 200 MurA, Cys117 was changed by mutagenesis to an aspartic acid residue. Cells expressing the C117D mutant are resistant to Fos (Fig. S3A), and purified $SpMurA_{C117D}$ was not inhibited by Fos (Fig. S3B). In contrast, both wild-type SpMurA and the C117D derivative are equally inhibited by either MAs(III) or PhAs(III) (Fig. 4). These results indicate that Cys117 is not involved in inhibition by trivalent organoarsenicals, suggesting that MAs(III) and fosfomycin have different mechanisms of action.



325

326 **Fig. 4.** Cys117 is not required for organoarsenical inhibition. The C117D SpMurA was purified,
327 and inhibition by MAs(III) compared with wild-type SpMurA. As described in the legend to Fig. 3.,
328 activity of SpMurA was monitored in the absence ((o), wild-type; (▼), C117D) or presence of
329 either MAs(III) **(a)** or PhAs(III) **(b)** at either 10 μM (Δ), (wild type; (□), C117D) or 50 μM ((■), wild
330 type; (♦), C117D). Data were normalized to the activity of the control reaction lacking MurA (●).
331 Data are the mean ± SE (*n* = 3).

3.4. *Organoarsenical inhibition of SpMurA involves cysteine residues*

While Cys117 is apparently not involved in MAs(III) inhibition, *S. putrefaciens* 200 MurA and various homologs have several other non-conserved cysteine residues (Fig. S2). SpMurA has eight other cysteine residues in addition to Cys117. To examine whether any cysteine residues are involved in organoarsenical inhibition, purified SpMurA activity was assayed in the presence of the cysteine modifying reagent N-ethylmaleimide (NEM), which completely inhibited enzymatic activity (Fig. 5).

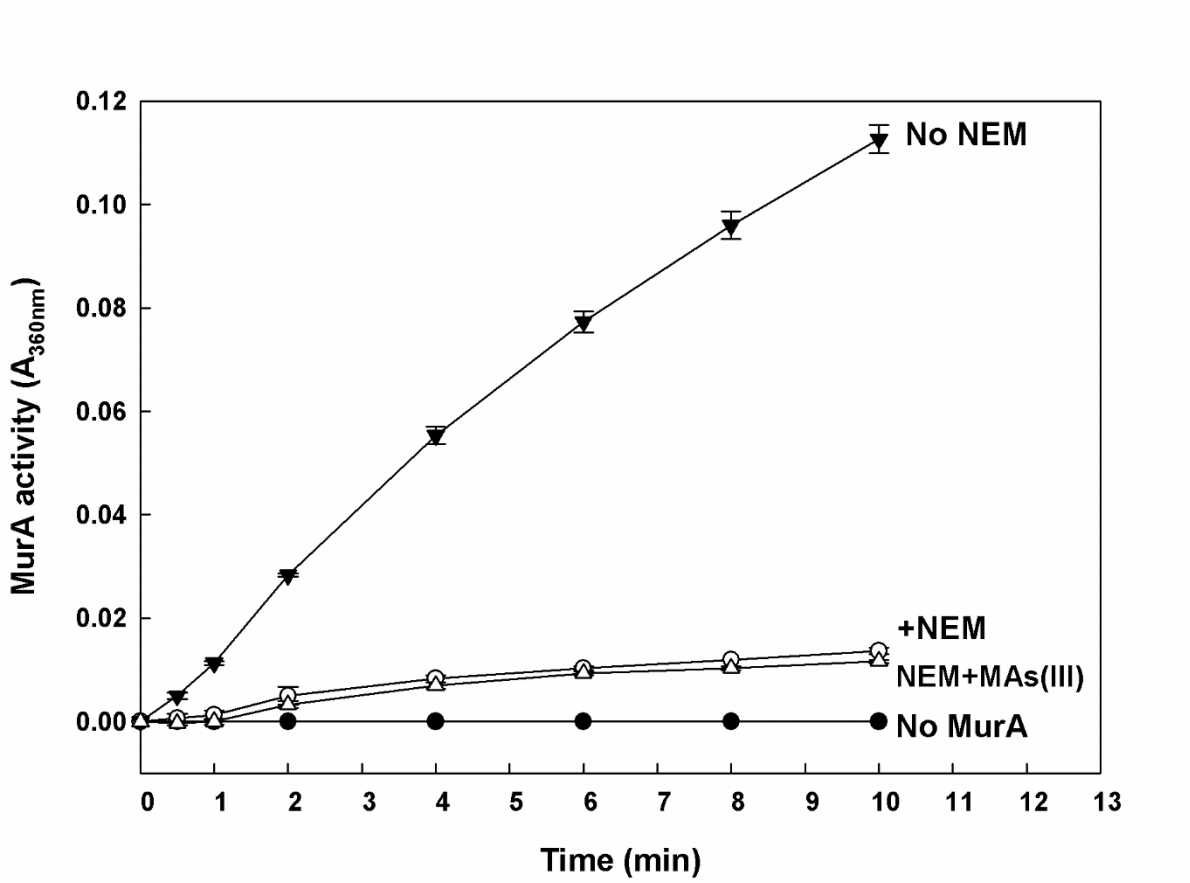
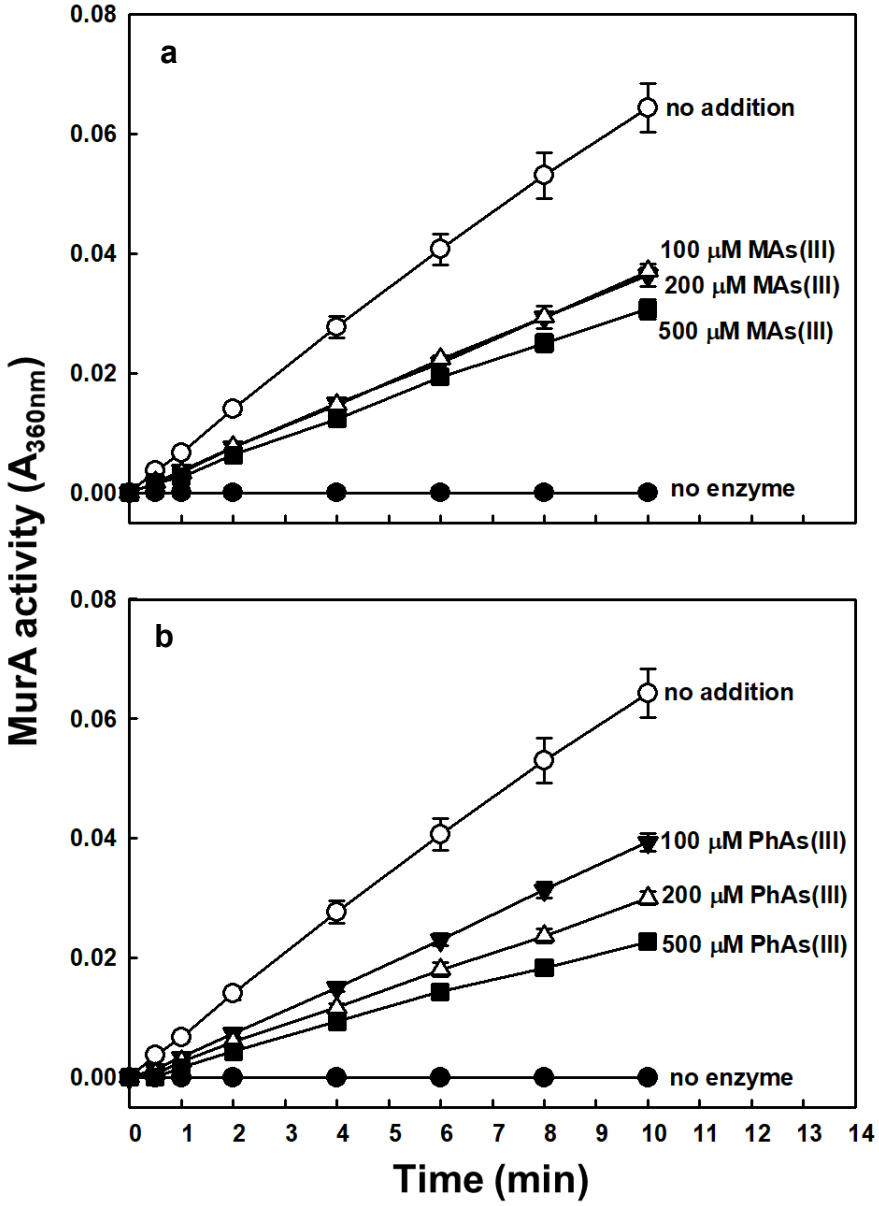


Fig. 5. NEM modification of non-conserved cysteines in SpMurA results in loss of activity. Purified wild type SpMurA was assayed, as described in the legend to Fig. 3., in the presence (○) or

absence (▼) of NEM or in the presence of both 50 MAs(III) and NEM (Δ). Data were normalized to the activity of the control reaction lacking MurA (●). Data are the mean ± SE (*n* = 3).

In contrast, MurA from *Staphylococcus aureus* (SaMurA) contains only the single conserved cysteine. The *SamurA* gene was expressed in *E. coli,* and SaMurA purified. SaMurA was sensitive to Fos (Fig. S4) but relatively resistant to trivalent organoarsenicals compared with SpMurA (Fig. 6). These results are consistent with our idea that the conserved cysteine residue is not involved in MAs(III) inhibition, in contrast to its role in Fos inhibition.



352

353 **Fig. 6.** A MurA ortholog with a single conserved cysteine confers trivalent organoarsenical
354 resistance. Purified *S. aureus* SaMurA that contains only the single conserved cysteine required
355 for fosfomycin inhibition was assayed for organoarsenical inhibition in the absence (○) or
356 presence of either MAs(III) **(a)** or PhAs(III) **(b)** at 100 μM (▼), 200 μM (Δ), or 500 μM (■). Data
357 were normalized to the activity of the control reaction lacking MurA (●). Data are the mean ± SE
358 (*n* = 3).

These results indicate that organoarsenicals, while not dependent on the canonical conserved cysteine used for PEP binding, still inhibit MurA in a cysteine-dependent manner, and that one or more of the other cysteine residues in MurA sequences may play a structural role or be otherwise indirectly involved in catalysis.

4. Discussion

MAs(III) has antibiotic-like properties and is produced by a number of bacterial species in microbial communities as a way to gain a competitive advantage (Chen et al., 2018). Trivalent arsenicals, including As(III) and MAs(III), have rather nonspecific toxic effects by depletion of cellular glutathione and small thiol proteins such as thioredoxin and glutaredoxin (Lin et al., 2001), and inhibition of thiol-containing enzymes such as lipoamide dehydrogenase (Bergquist et al., 2009). These shut down energy metabolism and increase reactive oxygen species. However, targets specific for bacteria, which could be used for the development of new antibiotics or antimicrobial drugs, have not been identified. In this report we identify the inhibition of MurA UDP-*N*-acetylglucosamine enolpyruvyl transferase by the trivalent natural product MAs(III) and the synthetic organoarsenical PhAs(III), and propose their antibiotic properties are at least partially a result of this inhibition.

As the first committed step in that pathway, MurA is an essential enzyme for many bacteria, which makes it an excellent drug target. Several classes of inhibitors such as sesquiterpene lactones, 2-aminotetralones and quinazolinones have been developed against MurA (Bachelier et al., 2006; Dunsmore et al., 2008; Hrast et al., 2017). MurA is also inhibited by fosfomycin, a broad-spectrum antibiotic used to combat Staphylococci, Pseudomonads and other enteric gram-negative pathogens (Raz, 2012). A genome-wide screen using the complete *E. coli* open reading frame library identified *murA* as the only chromosomal gene that confers clinical-level of fosfomycin

384 resistance by overexpression, and resistance is achieved at a low fitness cost (Couce et al.,
385 2012). Nonetheless, only a few reports have described enhanced *murA* expression associated
386 with fosfomycin resistance in clinical isolates, suggesting that overexpression of *murA* is rare in
387 clinical settings (Castañeda-García et al., 2013). However, the mechanism of action of those
388 MurA inhibitors requires a cysteine residue that is conserved in many but not all bacteria. Species
389 that lack the conserved cysteine residue such as *M. tuberculosis* are resistant to these antibiotics
390 (De Smet et al., 1999). Furthermore, in limited cases, even MurA orthologs that possess the
391 conserved cysteine exhibit fosfomycin resistance via other amino acid substitutions (Kumar et al.,
392 2009; Takahata et al., 2010). Thus, it is important to develop new antibiotics that act on MurA
393 independent of the consensus cysteine residue. It is significant that trivalent organoarsenicals do
394 not require the consensus cysteine residue to inhibit the *S. putrefaciens* 200 MurA enzyme. We
395 propose that inhibition of peptidoglycan biosynthesis is the primary target of the biologically-
396 generated antibiotic MAs(III). Future goals are to determine how MAs(III) binds to and inhibits
397 SpMurA, and to examine whether MurA inhibition by trivalent organoarsenicals is widespread
398 among pathogenic bacteria. These studies will advance our understanding of arsenical targets in
399 bacteria and provide valuable information to design new MurA inhibitors.

400

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406

407 ## 6. Conflicts of interest

408 The authors declare that they have no conflict of interest.

7. Credit author statements

L.D.G. conducted the experiments. L.D.G., B.P.R. and M.Y. designed the experiments and wrote the manuscript.

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