

# 1       **Functional and structural characterization of AntR, an** 2                   **Sb(III) responsive transcriptional repressor**

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22 **Key words:** Antimony, AntR, ArsR family, transcriptional repressor, gene  
23 regulation. *Comamonas testosterone*

24 **Abbreviations:** Arsenite, As(III); antimonite, Sb(III); methylarsenite, MAs(III);  
25 methylarsenate, MAs(V) or MSMA; dimethylarsenate, DMAs(V); Tris 2-  
26 carboxyethyl)phosphine, TCEP; sodium dodecyl sulfate poly acrylamide gel  
27 electrophoresis, SDS PAGE; isothermal titration calorimetry, ITC; HPLC, high  
28 performance liquid chromatography; ICP-MS, inductively coupled mass  
29 spectroscopy; electrophoresis mobility shift assay, EMSA.

30 **RUNNING TITLE:** AntR, an Sb(III) responsive transcriptional repressor

31 **Summary**

32 The *ant* operon of the antimony-mining bacterium *Comamonas testosteroni* JL40  
33 confers resistance to Sb(III). The operon is transcriptionally regulated by the product  
34 of the first gene in the operon, *antR*. AntR is a member of ArsR/SmtB family of  
35 metal/metalloid-responsive repressors resistance. We purified and characterized *C.*  
36 *testosteroni* AntR and demonstrated that it responds to metalloids in the order  
37 Sb(III)>methylarsenite (MAs(III))>>As(III). The protein was crystallized, and the  
38 structure solved at 2.1 Å resolution. The homodimeric structure of AntR adopts a  
39 classical ArsR/SmtB topology architecture. The protein has five cysteine residues, of  
40 which Cys103<sub>a</sub> from one monomer and Cys113<sub>b</sub> from the other monomer, are  
41 proposed to form one Sb(III) binding site, and Cys113<sub>a</sub> and Cys103<sub>b</sub> forming a  
42 second binding site. This is the first report of the structure and binding properties of  
43 a transcriptional repressor with high selectivity for environmental antimony.

## 44 **Introduction**

45 Antimony is a toxic element that is considered a priority environmental pollutant by  
46 the United States Environmental Protection Agency (EPA) and the European Union  
47 (EU, 1976, USEPA, 1979). Exposure to antimony leads to a number of human  
48 disorders, including cancer, liver, cardiovascular and respiratory diseases. Antimony  
49 is found in two oxidation states, highly toxic antimonite (Sb(III)) and much less toxic  
50 antimonate (Sb(V)). Antimony is located below arsenic on the periodic table, and  
51 both metalloids have similar chemical and toxicological properties (Li *et al.*, 2016).

52

53 Bacterial resistance to arsenic is conferred by *ars* operons. These genes also usually  
54 confer antimony as well. Recently an antimony-specific *antRCA* operon was  
55 identified in *Comamonas testosterone*, which was isolated from an antimony mine  
56 (An *et al.*, 2021). The *antA* gene encodes an Sb(III)-translocating P<sub>1B</sub>-type ATPase  
57 that extrudes the toxic metalloid from the cells, giving resistance. AntC is a small  
58 protein that is proposed to be an Sb(III) chaperone for the AntA efflux pump.

59

60 AntR is a negative repressor that regulates the *ant* operon. It responds to Sb(III) and  
61 MAs(III) but only weakly to As(III) and not at all to Sb(V) or As(V). AntR (124 amino  
62 acid residues, 13.5 kDa, accession number WP034375793.1) is a member of the  
63 ArsR/SmtB family of metal(oid)-responsive transcriptional repressors (Busenlehner  
64 *et al.*, 2003, Chen & Rosen, 2014). These transcriptional repressors respond to a  
65 variety of toxic metal(oid) ions, including four different types of ArsR repressors  
66 (As(III), Sb(III), Bi(III)) (Chen *et al.*, 2017, Ordóñez *et al.*, 2008, Qin *et al.*, 2007, San  
67 Francisco *et al.*, 1990), SmtB (Zn(II)) (Huckle *et al.*, 1993), CadC (Cd(II)) (Ye *et al.*,  
68 2005, Ji & Silver, 1992), and CmtR (Cd(II)) (Cavet *et al.*, 2003). In the absence of

69 metal ions, the homodimeric repressor binds to the promoter, preventing  
70 transcription. When substrate binds, the repressor dissociates from the promoter,  
71 allowing gene expression (Busenlehner *et al.*, 2003, Chen & Rosen, 2014).  
72 Members of the ArsR/SmtB family share common features such as structural  
73 topology, dimerization and functional activity. AntR homologs form a separate clade  
74 from the four known types of ArsR repressors (An *et al.*, 2021). Members of the four  
75 ArsR groups have evolved four different types of As(III)/Sb(III) binding sites. Each  
76 type of binding site is composed of two or three cysteine residues, but the location  
77 of those residues differs among the groups, reflecting convergent evolution of As(III)  
78 binding sites. In some ArsRs the binding site residues are within the same monomer  
79 such as the plasmid R773 ArsR (Shi *et al.*, 1994) and *Corynebacterium glutamicum*  
80 CgArsR (Ordóñez *et al.*, 2008). In others both chains contribute cysteine residues to  
81 the binding site that are in close spatial proximity such as *Acidithiobacillus*  
82 *ferrooxidans* AfArsR (Qin *et al.*, 2007).

83

84 Here we describe the structural and functional properties of the *C. testosterone*  
85 AntR. The results of binding assays performed with isothermal titration calorimetry  
86 (ITC) and the fluorescence quenching of a single tryptophan derivative demonstrate  
87 that AntR is highly selective for Sb(III) and MAs(III) over As(III). AntR has five  
88 cysteine residues. From the results of mutagenesis, Cys103 and Cys113 appear to  
89 be involved in Sb(III)/MAs(III) binding. AntR was crystallized, and the apo structure  
90 solved at 2.1 Å resolution. It is a homodimer with a ArsR/SmtB topology  
91 architecture. The structure shows that Cys103 from one monomer is opposed to  
92 Cys113 from the other monomer. We proposed that these two cysteine residues  
93 form a two-coordinate binding site for Sb(III)/MAs(III), with two binding sites in the

94 homodimer. This report of the structure and binding properties of a transcriptional  
95 repressor with high selectivity for environmental antimony sheds light on how life  
96 has adapted to the presence of this toxic metalloid.

97

## 98 **Results**

### 99 ***AntR is a new member of the ArsR/SmtB family of metal(loid)-responsive*** 100 ***transcription factors***

101 Recently an antimony resistance operon was identified in the chromosome of *C.*  
102 *testosterone* JL40 from an antimony mine in Lengshuijiang, Hunan Province, China  
103 (Li *et al.*, 2013). The *antRCA* operon encodes an Sb(III) efflux system that confers  
104 resistance by pumping the metalloid from the cells. The first gene in the operon  
105 encodes the AntR repressor that controls expression of itself and the *antCA* genes  
106 by sensing environmental Sb(III) (An *et al.*, 2021). From phylogenetic analysis, AntR  
107 belongs to the ArsR/SmtB family of negative repressors (Xu & Rosen, 1999,  
108 Busenlehner *et al.*, 2003). Members of this family are homodimers that bind  
109 transition metals, heavy metals or metalloids, including As(III), Sb(III), Cd(II), Pb(II),  
110 Zn(II), Co(II), and Ni(II). Binding dissociates the repressor from the promoter DNA,  
111 resulting in transcription of the resistance genes. The regulatory sites are often  
112 three- or four-coordinate metal binding sites composed of cysteine thiolates and  
113 histidine imidazole nitrogens. Reflecting shared evolution, they have a common  
114 backbone structure. Unexpectedly, however, the binding sites are found in quite  
115 different locations in various structures. We have hypothesized that those sites are  
116 the result of parallel evolution and arose in response to different environmental  
117 pressures. For example, the three-coordinate As(III) binding site in the *E. coli* and  
118 plasmid R773 ArsRs is more closely related to the four coordinate Cd(II)/Pb(II)/Zn(II)

119 binding site of the CadC repressor of the *S. aureus* pI258 plasmid *cadCA* operon (Ye  
120 *et al.*, 2005) than to other ArsR repressors such as CgArsR or AfArsR (Ordóñez *et al.*,  
121 2008, Qin *et al.*, 2007, Prabakaran *et al.*, 2019). AntR appears to more closely  
122 related to the SmtB Zn(II)-responsive repressor than to ArsRs (Fig. 1), so the  
123 location of the Sb(III) binding site could not be predicted from the structure of ArsR  
124 repressors.

### 125 ***AntR is a Sb(III)/MAs(III)-selective repressor***

126 To determine the inducer specificity of AntR, a two-plasmid biosensor was  
127 constructed that utilizes the gene for *antR* under control of the arabinose promoter  
128 in one plasmid (pBAD-*CtantR*), and the *ant* promoter controlling *gfp* expression in  
129 the other (pACYC184-*ParsO-gfp*) (Fig. S1) using the system described previously  
130 (Chen *et al.*, 2012). In cells of *E. coli* AW3110 $\Delta$ *ars* bearing both plasmids, addition of  
131 arabinose drives expression of AntR, which binds to the *antO* promoter and inhibits  
132 the expression of *gfp* from pACYC184-*ParsO-gfp* (Fig. 2). In the absence of  
133 arabinose, *gfp* is constitutively expressed. Induction by addition of 0.2% arabinose,  
134 represses *gfp* expression, and the cells are not fluorescent. The biosensor with wild  
135 type AntR exhibited little *gfp* expression upon addition of 5  $\mu$ M As(III). Addition of 5  
136  $\mu$ M Sb(III) or MAs(III) induced *gfp* expression. Pentavalent arsenicals (5  $\mu$ M of As(V),  
137 MAs(V) or DMAs(V)) were not inducers (data not shown).

138

### 139 ***Role of cysteine residues in in vivo response to metalloids***

140 ArsR repressors typically have two- or three-coordinate binding sites per monomer  
141 (Moinier *et al.*, 2014). AntR has five cysteine residues, Cys12, Cys58, Cys91, Cys103  
142 and Cys113. Only Cys103 and Cys113 are conserved in the 10 closest AntR  
143 homologs, so we focused on these two cysteine residues (Fig. S2). To examine their

144 involvement in Sb(III) sensing, two mutant repressors were constructed, C103S and  
145 C113S. Each mutant gene was put into the biosensor plasmids, and their response  
146 to metalloids examined. The response of biosensor with wild AntR to metalloids was  
147 proportional to the concentration of Sb(III) and MAs(III) with little or no response to  
148 MAs(V) or As(III) or As(V) (Fig. 3A). Both mutants had reduced responses to Sb(III)  
149 and MAs(III) compared with the wild type (Fig. 3C and D). These results are  
150 consistent with a role of both Cys103 and Cys113 in binding and sensing of Sb(III)  
151 and MAs(III).

152

### 153 ***Binding of metalloids and role of cysteine residues in in vitro response to*** 154 ***metalloids***

155 AntR was purified by NiNTA affinity chromatography, and binding of metalloids was  
156 assayed by two assays that apply different physical principles. First, the intrinsic  
157 tryptophan fluorescence of the AntR Y96W derivative was quenched by addition of  
158 metalloids. This method has been used to examine binding specificity in proteins  
159 that bind As(III) and Sb(III) such as the ArsA ATPase (Zhou & Rosen, 1997, Ruan *et*  
160 *al.*, 2006), the ArsD As(III)/Sb(III) metallochaperone (Yang *et al.*, 2010) and the ArsM  
161 As(III) S-adenosylmethionine methyltransferase (Marapakala *et al.*, 2012). From  
162 titration of the fluorescence quenching with metalloid, the apparent  $K_d$  values for  
163 both Sb(III) and MAs(III) were approximately 5  $\mu$ M (Fig. 4). The estimated  $K_d$  for  
164 As(III) of approximately 160  $\mu$ M is at the limit of the method.

165

166 Second, the binding parameters of purified AntR for Sb(III), MAs(III) and As(III)  
167 determined by ITC (Fig. 5). The apparent  $K_d$  values for Sb(III) and MAs(III) were  
168 observed to be 57  $\mu$ M and 77  $\mu$ M, respectively. The  $K_d$  for As(III) was calculated as



169 440  $\mu\text{M}$ , nearly an order of magnitude lower affinity. It should be pointed out that  
170 the 10-fold differences between apparent values with the two assays are probably  
171 due to the 10-fold different protein concentrations used in each, so these apparent  
172 binding constants should not be considered actual  $K_d$  values but are useful for  
173 comparative analysis of different ligands. However, the results from both assays  
174 clearly demonstrate that AntR is an Sb(III)/MAs(III)-selective repressor with low  
175 affinity for As(III).

176

### 177 ***Binding of AntR to the ant promoter***

178 AntR binding to the operator/promoter site and the response to inducers were  
179 examined by EMSA. The 193 bp upstream sequence of the *antR* gene containing the  
180 operator/promoter sequence was amplified by PCR using a 6-FAM-labeled primer  
181 and purified AntR was used for the EMSA assays. The mobility of the probe was  
182 gradually retarded when increasing amounts of AntR were added, reflecting binding  
183 of the repressor to its cognate promoter (Fig. 6A). The effect of metal(loids) on  
184 AntR binding was assayed (Fig. 6B-F). The mobility shift decreased with increasing  
185 concentration of Sb(III). Neither pentavalent metalloids nor other metals evoked a  
186 response. These results are consistent with the results of binding experiments  
187 demonstrating that ArsR is selective for Sb(III).

188

### 189 ***Structural architecture of AntR***

190 Members of the ArsR/SmtB family are homodimers that adopt a winged helix  
191 structural topology. The AntR homodimer crystallized in the  $P12_11$  space group with  
192 cell dimensions of  $a=51.7 \text{ \AA}$ ,  $b=58.5 \text{ \AA}$  and  $c=53.5 \text{ \AA}$ . The structure refined with  
193 acceptable stereochemistry and a final R-factor of 0.22 ( $R_{\text{free}}=0.26$ ) at  $2.1 \text{ \AA}$

194 resolution (PDB ID 6UVU). The details of data collection and refinement are given in  
195 Table 1. The structure consists of chain A from residues 11 to 124 and chain B from  
196 residues 11-121 (Fig. 7). N terminal residues (1-10) are not visible in either chain.  
197 The overall structure is composed of five  $\alpha$ -helices and two anti-parallel  $\beta$ -strands  
198 connected through coils ( $\alpha$ 1- $\alpha$ 2- $\alpha$ 3- $\alpha$ 4- $\beta$ 1- $\beta$ 2- $\alpha$ 5). All five cysteine residues are  
199 visible in both chains. The two conserved cysteines, Cys103 and Cys113, are visible  
200 in helix  $\alpha$ 5 at the dimer interface. The sulfur atom of Cys103<sub>a</sub> is 10.9 Å from that of  
201 Cys113<sub>b</sub>, and Cys113<sub>a</sub> sulfur atom is 11.2 Å from the sulfur atom of Cys103<sub>b</sub>.  
202 Combining the mutagenesis results described above with these structural data are  
203 consistent with Cys103 and Cys113 from each monomer forming two 2-coordinate  
204 Sb(III)/MAs(III) binding sites between the two dimers.

205

## 206 **Discussion**

207 In this report we characterized the AntR transcriptional repressor that controls  
208 expression of the *antRCA* operon of *Comamonas testosterone* JL40, which was  
209 isolated from an antimony mine (Li *et al.*, 2013). The results of biosensor and  
210 binding assays clearly demonstrate that the repressor is highly selective for Sb(III),  
211 as well as MAs(III), which has been described as a primordial antibiotic (Chen *et al.*,  
212 2019). AntR is a member of the ArsR/SmtB family of metal(loid)-responsive  
213 transcriptional regulatory proteins. Most ArsR repressors also bind and respond to  
214 Sb(III). In contrast, AntR has little response to As(III). What governs the differential  
215 selectivity of the AntR and ArsR repressors? AntR is more closely related to SmtB  
216 than to any characterized ArsR (Fig. 1B). Since SmtB is a Zn(II)-responsive  
217 repressor, there is no reason to expect that AntR would bind As(III) (Fig. 6C) any  
218 better than the non-inducer Zn(II) (Fig. 6F). Typical ArsR repressors bind As(III) with

219 a trigonal pyramidal molecular geometry (Prabaharan *et al.*, 2019). Two recent  
220 crystal structures of ArsR repressors from *A. ferrooxidans* (AfArsR) and *C.*  
221 *glutamicum* (CgArsR), respectively, show two different three-cysteine As(III) sites. In  
222 AfArsR As(III) is bound to Cys95, Cys96 and Cys102 from the same monomer of the  
223 homodimer, while, in the CgArsR structure, As(III) is bound to Cys15 and Cys16 from  
224 one monomer and Cys55 from the other monomer (Prabaharan *et al.*, 2019).

225

226 Those ArsR repressors can also bind Sb(III) and MAs(III), but is a three-coordinate  
227 site required to bind those more thiophilic metalloids? An atypical ArsR is the  
228 MAs(III)-responsive SpArsR from *Shewanella putrefaciens* 200 that regulates  
229 expression of the MAs(III) resistance genes *arsP* and *arsH* (Chen *et al.*, 2017).  
230 SpArsR is induced by MAs(III), with little response to As(III). SpArsR is closely related  
231 to AfArsR from *A. ferrooxidans*, in which As(III) is bound to a three-coordinate site  
232 composed of residues Cys95, Cys96 and Cys102, which are located at the C-  
233 terminus of the repressor (Qin *et al.*, 2007). In SpArsR the residue equivalent to  
234 Cys102 is not a cysteine, and MAs(III) is bound to a two-coordinate site composed of  
235 Cys101 and Cys102. This illustrates how replacing a single cysteine residue can  
236 alter an  $S_3$  site into an  $S_2$  site that changes selectivity from As(III) to MAs(III). SpArsR  
237 and AntR both respond to MAs(III) but are not closely related, indicating that this is  
238 probably convergent evolution of two different two-coordinate binding sites.

239

240 In AntR there are only two cysteine residues that could reasonably form a binding  
241 site, Cys103 and Cys113. This idea is supported by 1) the fact that only these two  
242 cysteine residues of the five in AntR are conserved; 2) mutagenesis of either of  
243 those residues reduces *in vivo* biosensing; 3) the proximity of those two residues in

244 the crystal structure. It should be pointed out, however, that the distance between  
245 the Cys103 sulfur atom in one monomer and the Cys113 sulfur atom in the other  
246 monomer of the apo-AntR structure is approximately 10-11 Å. In CgArsR and AfArsR  
247 As(III) is bound with a trigonal pyramidal molecular geometry with As-S distances of  
248 approximately 2.25 Å. Typical Sb-S bond lengths fall in the range of 2.4-2.5 Å. To  
249 form a similar trigonal pyramidal binding site in AntR in which Sb(III) or MAs(III) is  
250 bound to two sulfurs and a hydroxyl replacing a third, the cysteine residues would  
251 have to move closer to each other. Even though distances are too long in the apo  
252 structure, it is reasonable to propose that binding of Sb(III)/MAs(III) would bring the  
253 a6 helices close enough to each other to form the binding site. In fact, this might  
254 propagate a conformational change in the repressor that would result in dissociation  
255 from the promoter DNA and hence derepression. Since the *ant* operon is found in an  
256 antimony-mining organism, it seems reasonable to consider that the physiological  
257 inducer for AntR is Sb(III) even though it responds to MAs(III). In summary, this is  
258 the first report of the structure and function of a transcriptional regulatory element  
259 that controls detoxification of antimony and illustrates how microbes adapted to the  
260 presence this highly toxic environmental metalloid.

261

## 262 **Experimental procedures**

### 263 ***Strains, plasmids, medium and reagents***

264 *E. coli* Stellar™ (Clontech Laboratories, Mountain View, CA) (*F2, endA1, supE44, thi-*  
265 *1, recA1, relA1, gyrA96 phoA, lacZΔ M15, Δ(lacZYA-argF)U169,*  
266 *Δ(mrrhsdRMSmcrBC), ΔmcrA, k<sup>-</sup>*) was used for plasmid DNA construction and  
267 replication. *E. coli* AW3110(DE3) (*Δars::cam F2IN(rrn-rrnE)*) (Carlin *et al.*, 1995) in  
268 bearing two plasmids, pACYC184-*PantO-gfp* and pBAD-*CtantR* was constructed for

269 use as an Sb(III)/MAs(III) biosensor (Chen *et al.*, 2012). For most experiments  
270 cultures of *E. coli* bearing the indicated plasmids were grown aerobically in lysogeny  
271 broth (LB) medium or M9 medium at 37°C supplemented with 100 µg/mL ampicillin  
272 or 34 µg/mL chloramphenicol, as required (Sambrook *et al.*, 1989). Bacterial growth  
273 was monitored by measuring the absorbance at 600 nm ( $A_{600nm}$ ).

274

275 Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich. MAs(V)  
276 was obtained from Thermo Fisher Acros Organics Division (Waltham, MA). MAs(III)  
277 was reduced as described (Reay & Asher, 1977). Briefly, 0.2 mM of pentavalent  
278 arsenicals were mixed with 27 mM  $Na_2S_2O_3$ , 66 mM  $Na_2S_2O_5$ , and 82 mM  $H_2SO_4$ ,  
279 following which the pH was adjusted to 6.0 with NaOH. The reduced products were  
280 not thiolated, as determined by simultaneous As and S analysis by high pressure  
281 liquid chromatography (HPLC) inductively coupled mass spectroscopy (ICP-MS)  
282 using an ELAN DRC-e (PerkinElmer, Waltham, MA). The dynamic reaction cell  
283 detects arsenic as  $^{91}AsO^+$  and sulfur as  $^{48}SO^+$  with high sensitivity.

284

### 285 **Plasmid construction**

286 The *antR* gene from *C. testosterone* JL40 was chemically synthesized with 5'-*NcoI*  
287 and 3'-*Sall* sites and codon optimization for expression in *E. coli* GenScript (NJ, USA).  
288 The gene was cloned into pBAD/myc-HisA, generating plasmid pBAD-*CtantR* (Fig.  
289 S1). The *antR* (WP\_034375793) promoter corresponding to the sequence of the  
290 genomic DNA of *C. testosteroni* JL40 was chemically synthesized together with a *gfp*  
291 gene originated from vector plasmid pGreen (Hellens *et al.*, 2000). The *gfp* reporter  
292 was under control of the AntR promoter in vector plasmid pACYC184, generating  
293 plasmid pACYC184-*PantO-gfp*. To purify AntR for biochemical characterization and

294 crystallization, *antR* was excised from pBAD-*CtantR* with *NcoI* and *XhoI* restriction  
295 enzymes and subcloned into plasmid pET-29a(+) using the same restriction sites in  
296 frame with a C-terminal six-histidine residue tag.

297

### 298 **Biosensor assays**

299 Plasmids pBAD-*CtantR* and pACYC184-*PantO-gfp* were co-expressed in *E. coli*  
300 AW3110 $\Delta$ *ars* (Carlin *et al.*, 1995), generating a biosensor that could be used to  
301 determine the binding affinity of AntR for antimonials and arsenicals, as previous  
302 reported (Chen *et al.*, 2012). Transcriptional activity of the biosensor was estimated  
303 from metalloid-responsive expression of *gfp*. Cultures of the biosensor were grown  
304 to mid-exponential phase in M9 medium at 37 °C with 100  $\mu$ g ml<sup>-1</sup> ampicillin and 34  
305  $\mu$ g ml<sup>-1</sup> chloramphenicol with shaking. Glycerol (0.5%) was added as carbon source  
306 and for constitutive expression of *gfp*. The *CtantR* gene was induced by addition of  
307 0.2% arabinose for 5 h. Derepression was produced by simultaneous addition of  
308 arabinose and metalloid for 5 h. Cell densities were normalized by dilution or  
309 suspension to the same  $A_{600nm}$ , and expression of *gfp* was assayed from the  
310 fluorescence of cells using a Photon Technology International spectrofluorometer  
311 with an excitation wavelength of 470 nm and emission wavelength of 510 nm.

312

### 313 **Mutagenesis of the *antR* gene**

314 The codons for residues Cys58, Cys103 and Cys113 were changed to serine codons,  
315 generating single cysteine AntR mutants. The mutations were generated by site-  
316 directed mutagenesis using a Quick-Change mutagenesis kit (Stratagene, La Jolla,  
317 CA). The forward and reverse mutagenic oligonucleotides used for both strands and  
318 the respective changes introduced (underlined) are as follows: C103S forward, 5'-

319 GCATCGTGGACCCGAGCGTGCTGAGAATG-3' and reverse 5'-  
320 CATTCTCAGCACGCTCGGGTCCACGATGC-3'; C113 forward, 5'-  
321 GAATGCTCGAACTTGGGCTAAGCTTATCGAGGAG-3' and reverse 5'-CTCCTCG  
322 ATAAGGCTTAGCCCAAGTTCGAGCATTC-3'. Each mutation was confirmed by DNA  
323 sequencing (Sequetech, Mountain View, CA).

324

### 325 ***Protein purification***

326 Cells of *E. coli* BL21(DE3) bearing *CtantR* in plasmid pET-29a(+) were grown in LB  
327 medium containing 100 µg/ml kanamycin with shaking at 37 °C. At an  $A_{600nm}$  of 0.6,  
328 0.3 mM isopropyl β- d-1-thiogalactopyranosidewas added as an inducer, and the  
329 culture was grown for an additional 4 h at 37 °C. The cells were harvested and  
330 suspended in 5 ml/g of wet cells in buffer A (50 mM 4-morpholinepropanesulfonic  
331 acid, 20 mM imidazole, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 20% glycerol  
332 (vol/vol), pH 7.5 and 2 mM Tris 2-carboxyethyl)phosphine (TCEP)). The cells were  
333 broken by a single passage through a French pressure cell at 20,000 psi, and  
334 immediately treated with the protease inhibitor diisopropyl fluorophosphate (2.5 µl/  
335 g wet cell). Membranes and unbroken cells were removed by centrifugation at  
336 150,000 g for 1 h, and the supernatant solution was loaded onto a Ni<sup>2+</sup>-  
337 nitrilotriacetic acid column (Qiagen,Valencia, CA) at a flow rate of 0.5 ml/min. The  
338 column was washed with more than 25 column volumes of buffer A. CtAntR was  
339 eluted with a liner gradient of 0-0.2 M imidazole in buffer A, and purity was analyzed  
340 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on a 15%  
341 gel. Protein concentrations were estimated from  $A_{280nm}$  ( $\epsilon=1880 M^{-1} cm^{-1}$ ). CtAntR-  
342 containing fractions were divided into small portions, rapidly frozen and stored at -

343 80°C until use. On gel filtration purified AntR eluted at the position of a dimer,  
344 consistent with the dimeric structure of ArsR repressors (Prabaharan *et al.*, 2019)

345

### 346 ***Phylogenetic analysis***

347 Multiple alignment of AntR with selected members of the ArsR/SmtB family was  
348 performed using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). AntR,  
349 SmtB, CadC and ArsR sequences with conserved cysteines were selected for  
350 phylogenetic analysis. Acquisition of sequences was performed by searching a list of  
351 reference organisms. Phylogenetic analysis was performed to infer the evolutionary  
352 relationship among the arsenic repressor of various organisms. The phylogenetic  
353 tree was constructed using the Neighbor-Joining method with MEGA 6.0.1 (Saitou &  
354 Nei, 1987). The statistical significance of the branch pattern was estimated by  
355 conducting a 1000 bootstrap.

356

### 357 ***Binding affinity for metalloids***

358 The binding affinity of AntR with metalloids was assayed by two methods. The first  
359 measured the quenching of intrinsic protein fluorescence of AntR upon binding of  
360 metalloids. AntR has no tryptophan residues and low intrinsic fluorescence. A single  
361 tryptophan mutant was constructed by changing Tyr96 to a tryptophan residue, and  
362 the AntR Y96W derivative was used for metalloid quenching assays. A temperature-  
363 controlled Quanta-Master UV–vis QM-4 steady state spectrofluorometer (Photon  
364 Technology International, Birmingham, NJ) was used for fluorescence  
365 measurements. The fluorescence of 5  $\mu$ M AntR Y96F and or derivatives was assayed  
366 in a buffer consisting of 50 mM MOPS, pH7.8, 0.5 M NaCl and 2 mM TCEP at  
367 excitation and emission wavelengths of 295 and 331 nm, respectively. Sb(III) (25,



368 50, 100, 150, 200  $\mu\text{M}$ ) or MAs(III) (2.5, 5, 7.5 10, 15  $\mu\text{M}$ ) were added, and quenching  
369 of fluorescence was measured. For all assays, the temperature was 23  $^{\circ}\text{C}$ , and the  
370 spectrum of the buffer solution alone was subtracted to correct for background  
371 fluorescence and Raman scattering. For determination of relative affinities of Sb(III),  
372 MAs(III) and As(III), fluorescence spectra were acquired at various concentrations of  
373 metalloids, and the affinity for each was calculated according to the method of  
374 Rosenthal (Rosenthal, 1967) by plotting  $(\Delta F/\Delta F_{\text{max}})/[L]$  as a function of  $\Delta F/\Delta F_{\text{max}}$ ,  
375 where  $\Delta F/\Delta F_{\text{max}}$  is the fractional change in fluorescence at 345 nm,  $\Delta F$  is the  
376 quenching at a particular concentration of arsenical ligand,  $[L]$ , and  $\Delta F_{\text{max}}$  is the  
377 quenching at the highest (saturating) arsenical concentration.

378

379 In the second assay, quantitative binding parameters were determined by  
380 isothermal titration calorimetry (ITC). Assays were performed at 30  $^{\circ}\text{C}$  with a stirring  
381 speed of 350 rpm using a MicroCal iTC<sub>200</sub> instrument (GE Healthcare Bio Sciences) in  
382 a freshly prepared buffer solution consisting of 50 mM MOPS, 20% glycerol, 0.5 M  
383 NaCl, 20 mM imidazole and 1 mM TCEP. Each assay contained 0.3 ml of 70  $\mu\text{M}$  AntR,  
384 with sequential addition of 45  $\mu\text{l}$  aliquots of 0.5 mM Sb(III), MAs(III) or As(III) in the  
385 same buffer that had been filtered through 0.2  $\mu\text{M}$  filters, as indicated. MAs(III) was  
386 freshly prepared by chemical reduction of MAs(V) before use.

387

### 388 ***Electrophoretic mobility shift assays***

389 AntR binding to the operator/promoter site and the response to inducers were  
390 examined by electrophoretic mobility shift assays (EMSA). A DNA fragment was  
391 amplified by PCR using primers AntA-EMSA-F and AntA-EMSA-R consisting of the 193  
392 nt upstream of the start of the *antR* gene containing the *ant* operator/promoter

393 sequence. The forward primer was synthesized with 5'-labeling with the fluorophore  
394 6-fluorescein phosphoramidite (6-FAM) (Integrated DNA Technologies). AntR purified  
395 as described above was used for EMSA assays. All reaction mixtures with or without  
396 metalloids were incubated at 28 °C for 30 min in a buffer consisting of 20 mM Tris-  
397 HCl, pH 7.0, 50 mM NaCl, 1 mM dithiothreitol, 10 mM MgCl<sub>2</sub> and 0.1 mg/ml bovine  
398 serum albumin. The binding solution was loaded onto a 6 % native PAGE gel. After 3  
399 h at 80 V in Tris-glycine-EDTA (TGE) buffer (12 mM Tris, 0.95 M glycine and 5 mM  
400 EDTA, the gels were analyzed using a Fujifilm FLA-5100 fluorescence imaging  
401 system.

402

#### 403 ***Crystallization, data collection and structure determination***

404 Purified AntR (15 mg/ml) was used for initial crystallization screens using sitting  
405 drop vapor diffusion and hanging drop methods using crystallization screens from  
406 Hampton Research, Molecular Dimensions and Emerald BioSystems, Inc. Small  
407 plate-like crystals were grown in crystal screen kit condition 41 (0.1 mM HEPES  
408 sodium, pH 7.5, 10% v/v 2-propanol, 20% w/v polyethylene glycol 4,000) in sitting  
409 drops. Addition of a 1:1 ratio of protein:reservoir solution led to a slight precipitate  
410 that cleared by addition of 1 µl of 0.1 M TCEP. Diffraction-quality crystals were  
411 obtained with Hampton additive screen condition 47 within a week. High quality  
412 crystals were flash cooled in liquid nitrogen for data collection. X-ray diffraction data  
413 were collected at the Advanced Light Source (ALS), Lawrence Berkeley National  
414 Laboratory, Berkeley, California and the Southeast Regional Collaborative Access  
415 Team (SER-CAT) facility at the Advanced Photon Source (Sapsford *et al.*, 2006),  
416 Argonne National Laboratory. The structures were determined by molecular  
417 replacement (McCoy, 2007). Structural refinement of each data set was performed

418 with REFMAC5 (Vagin *et al.*, 2004) implemented with the CCP4 suite (Winn *et al.*,  
419 2011). The model and electron density map were visualized using COOT software  
420 (Emsley & Cowtan, 2004).

421

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441

442 **Data Availability Statement:** Data available on request from the authors

443

444

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542

543

544 ***Supporting information***

545 Additional supporting information may be found in the online version of this article  
546 at the publisher's web-site.



547 **Table 1. Crystal data and refinement statistics.** The numbers in parentheses  
 548 are the data for the highest resolution shell.

<b>Data Collection</b>	
Diffraction source	ALS BEAMLINE 8.2.2
Wavelength (Å)	0.999
Resolution range (Å)	47.11- 2.1 (2.175- 2.1)
Space group	P 1 21 1
a, b, c (Å)	51.7, 58.5, 53.5
$\alpha$ , $\beta$ , $\gamma$ (°)	90 118.28 90
Total reflections	32773 (3283)
Unique reflections	16526 (1653)
Multiplicity	2.0 (2.0)
Completeness (%)	98.79 (99.94)
Mean I/sigma(I)	13.87 (4.62)
Wilson B-factor	27.99
R-merge	0.0437 (0.155)
R-meas	0.0618 (0.219)
R-pim	0.0437 (0.155)
CC1/2	0.986 (0.95)
CC*	0.997 (0.99)
<b>Refinement</b>	
Reflections used in refinement	16348 (1652)
Reflections used for R-free	872 (56)
R-work (%)	0.22
R-free (%)	0.26
Number of non-hydrogen atoms	1812
Macromolecules	1701
Water	111
Protein residues	225
RMS(bonds)	0.008
RMS(angles)	1.22
Ramachandran favored (%)	98.64
Ramachandran allowed (%)	1.36

Ramachandran outliers (%)	0.00
Rotamer outliers (%)	7.18
Clash score	4.30
Average B-factor (Å <sup>2</sup> )	31.66
Macromolecules	31.42
Water	35.27
PDB ID	6UVU

549

550

551 **Figure legends**

552

553 **Figure 1: Multiple sequence alignment of AntR with selected members of**  
554 **the ArsR//SmtB family. A. Multiple alignment (accession numbers in**  
555 **parentheses).** *C. testosteroni* JL40 AntR (WP034375793.1); *Synechocystis* sp. PCC  
556 7942 SmtB (CAA45872); *C. glutamicum* CgArsR1 (CAF21518); *A. ferrooxidans*  
557 (ACK80311), *S. putrefaciens* SpArsR (ADV53698); plasmid R773 ArsR (CAA34168);  
558 and plasmid pl258 CadC (P20047). The multiple alignment was calculated with  
559 CLUSTAL W. Cysteine residues are highlighted in yellow. Identical (black highlight)  
560 and conservative replacements (grey highlight) are identified. **B. Evolutionary**  
561 **relationships of AntR with other metal (metalloid) repressors from**  
562 **members of other bacterial species.** A neighbor joining phylogenetic tree shows  
563 that AntR is more close to SmtB, not arsenic responsive repressor ArsR.

564

565 **Figure 2. The bacterial biosensor with the *antR* gene responds only to**  
566 **Sb(III) and MAs(III).** Conditions for constitutive, repressed or derepressed *gfp*  
567 expression. In cells of *E. coli* AW3110 with both plasmids, *antR* is not expressed in  
568 the absence of arabinose, and *gfp* expression is constitutive, producing cellular  
569 fluorescence. In the presence of arabinose, *antR* is expressed, and *gfp* is repressed,  
570 so the cells are not fluorescent. In the presence of both arabinose and arsenical  
571 inducer, *gfp* expression is derepressed, and the cells are fluorescent. Expression of  
572 the *gfp* reporter gene was assayed as described under Experimental Procedures.  
573 Cells of *E. coli* strain AW3110(DE3) bearing wild type *antR* were grown without  
574 arabinose, 0.2% arabinose, or 0.2% arabinose and metalloids at the indicated

575 concentrations. Fluorescence intensities were quantified by spectrofluorometry. The  
576 data are the mean  $\pm$  SE (n = 3).

577

578 **Figure 3. Role of cysteine residues in binding of Sb(III) and MAs(III) to**  
579 **AntR.** Expression of the *gfp* reporter gene was assayed as described under  
580 Experimental Procedures. Cells were grown in low phosphate medium for 14 h with  
581 0.5% glycerol as carbon source. Cells of *E. coli* strain AW3110(DE3) bearing  
582 plasmids with wild type AntR, C103S or the C113S mutant *in trans* with reporter  
583 plasmid pACYC184-ParsP-gfp were grown with 0.2% arabinose and the indicated  
584 concentrations of metalloids. **(A)**, Comparison of the response of the bacterial  
585 biosensor to arsenicals and antimonite. Response mutant biosensors **(B)** C58S; **(C)**  
586 C103S; and **(D)** C113S to the indicated inducers indicated. Fluorescence intensities  
587 of cell suspensions were quantified using a Photon Technology International  
588 spectrofluorometer with an excitation wavelength of 470 nm and emission  
589 wavelength of 510 nm. The data are the mean  $\pm$  SE (n=3).

590

591 **Figure 4. Metalloid binding affinities by AntR determined by the quenching**  
592 **of the single tryptophan AntR Y96W derivative.** The relative affinities for  
593 Sb(III), MAs(III) and As(III) were estimated from the quenching of tryptophan  
594 fluorescence at the indicated concentrations of metalloids, as described under  
595 Materials and Methods.

596

597 **Figure 5: Protein-ligand interactions determined by isothermal titration**  
598 **calorimetry.** ITC was performed with purified AntR as described under materials  
599 and methods with **A)** Sb(III); **B)** MAs(III) and **C)** As(III).

600

601 **Figure 6. AntR negatively regulates expression of the *ant* promoter.** (A) The  
602 FAM-labeled *antR* promoter probe interacts with purified AntR, retarding the  
603 electrophoretic mobility. The amounts of DNA probes and AntR are shown above  
604 each panel. Binding of substrate releases AntR from the DNA, increasing the  
605 electrophoretic mobility. Metal(loids) were (B) Sb(III); (C) As(III); (D), As(V); (E)  
606 Cu(II); or (F), Zn(II) at the indicated concentrations. The amount of DNA probe was  
607 100 ng, the amount of AntR was 1.0  $\mu$ M.

608

609 **Figure 7: The structural architecture of AntR.** The structure of the AntR  
610 homodimer is shown in ribbon representation. Chain A is shown in green, and Chain  
611 B is shown in cyan. Structural elements are labeled. Cysteine residues are shown in  
612 ball-and-stick. Dashed lines indicate the distances between the sulfur atoms of  
613 Cys103 and Cys113.