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Published in:
Frontiers in Bioscience

DOI:
10.3389/fmolb.2015.00070

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Liu, H., Zhuang, W., Zhang, S., Rensing, C. G. T., Huang, J., Li, J., & Wang, G. (2015). Global regulator IscR positively contributes to antimonite resistance and oxidation in Comamonas testosteroni S44. DOI: 10.3389/fmolb.2015.00070
Global Regulator IscR Positively Contributes to Antimonite Resistance and Oxidation in Comamonas testosteroni S44

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Antimonial compounds can be found as a toxic contaminant in the environment. Knowledge on mechanisms of microbial Sb oxidation and its role in microbial tolerance are limited. Previously, we found that Comamonas testosteroni S44 was resistant to multiple heavy metals and was able to oxidize the toxic antimonite [Sb(III)] to the much less toxic antimonate [Sb(V)]. In this study, transposon mutagenesis was performed in C. testosteroni S44 to isolate genes responsible for Sb(III) resistance and oxidation. An insertion mutation into iscR, which regulates genes involved in the biosynthesis of Fe-S clusters, generated a strain called iscR-280. This mutant strain was complemented with a plasmid carrying iscR to generate strain iscR-280C. Compared to the wild type S44 and iscR-280C, strain iscR-280 showed lower resistance to Sb(III) and a lower Sb(III) oxidation rate. Strain iscR-280 also showed lower resistance to As(III), Cd(II), Cu(II), and H2O2. In addition, intracellular γ-glutamylcysteine ligase (γ-GCL) activity and glutathione (GSH) content were decreased in the mutated strain iscR-280. Real-time RT-PCR and lacZ fusion expression assay indicated that transcription of iscR and iscS was induced by Sb(III). Results of electrophoretic mobility shift assay (EMSA) and bacterial one-hybrid (B1H) system demonstrated a positive interaction between IscR and its promoter region. The diverse defective phenotypes and various expression patterns suggest a role for IscR in contributing to multi-metalloid resistance and Sb(III) oxidation via Fe-S cluster biogenesis and oxidative stress protection. Bacterial Sb(III) oxidation is a detoxification reaction.

Keywords: IscR, Comamonas testosteroni, multi-metal resistance, antimonite oxidation, oxidative stress, iron-sulfur cluster

INTRODUCTION

Antimony (Sb) belongs to subgroup 15 of the periodic table along with nitrogen (N), phosphorus (P), arsenic (As), and bismuth (Bi). The most common oxidation states found in nature are Sb(III) and Sb(V) (Li et al., 2013). Sb and its compounds are considered as pollutants by the Environmental Protection Agency of the United States (USEPA) and the European Union (Herbst et al., 1985;
Filella et al., 2002). Nowadays, Sb is dramatically increased in bogs and arctic polar ice cores showing the serious degree of Sb pollution due to anthropogenic activities (Shotyk et al., 2005). Molecular mechanisms of Sb resistance were mostly studied in the protozoan parasite Leishmania. In vitro studies suggested that genes encoding AQP1, PGPA, TDR1, and ACR2 in parasites are required for Sb(III) resistance (Decuyper et al., 2012). The best-known mechanism of resistance to Sb involves the detoxification of Sb(III) via conjugation to trypanothione [T(SH)2], which is a thiol compound in the protozoan parasite (Legare et al., 2001).

Sb shares similar chemical and toxicological properties with arsenic. However, unlike bacteria-arsenic interactions, bacteria-Sb interactions have not been elucidated in great detail. The glycerol transporter GlpF in Escherichia coli is reported to be responsible for Sb(III) uptake (Meng et al., 2004), while the ArsB protein, Acr3p family, and ABC transporter superfamily are involved in Sb(III) efflux (Filella et al., 2007). To our knowledge, only a few studies have reported biological Sb(III) oxidation, either as a cellular detoxification mechanism in bacteria and algae (Torma and Gabra, 1977; Lehr et al., 2007) or as a possible chemo-autotrophic process for the bacterium Stibio bacter senarmontii (Lilakova, 1974). Recently, the arsenite oxidase AioAB in Agrobacterium tumefaciens was found to be able to oxidize Sb(III) (Wang et al., 2015). The oxidoreductase AnoA was reported to be responsible for bacterial Sb(III) oxidation (Li et al., 2015). However, the disruption of both of these genes only led a partial loss of Sb(III) oxidation, thereby indicating the existence of unknown mechanisms related to bacterial Sb(III) oxidation.

Three regulatory systems have been reported to affect Fe-S cluster assembly, Isc (iron-sulfur cluster), Suf (sulfur formation), and Nif (nitrogen fixation) (cluster assembly, Isc (iron-sulfur cluster), Suf (sulfur formation), oxidoreductase AioAB in Agrobacterium tumefaciens was found to be able to oxidize Sb(III) (Wang et al., 2015). The oxidoreductase AnoA was reported to be responsible for bacterial Sb(III) oxidation (Li et al., 2015). However, the disruption of both of these genes only led a partial loss of Sb(III) oxidation, thereby indicating the existence of unknown mechanisms related to bacterial Sb(III) oxidation.

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TABLE 1 | Bacteria and plasmids used in this study.

| Strains and plasmids | Relevant property or derivation | Source or reference |
|----------------------|--------------------------------|--------------------|
| Comamonas testosteroni S44 | Wild type, Sb(III) oxidizing | This study |
| iscR-280 | iscR mutant with TrnG insertion | This study |
| iscR-280C | iscR complementary strain | This study |
| E. coli DH5α (pNPR) | F′ Φ80lacZ ΔM15 Δ(argF-lacZYA) U169 relA1 hisdR17 deoR thi-1 supE44 gyrA96 recA1/lpr | Miller and Mekalanos, 1988 |
| S17-1 (pNPR) | Tn5 recA thi pro hisdR2− hisdM− R4, 2Tc, Km, T7, tpir | Simon et al., 1983 |
| BL21 (DE3) | F− ompT hsdS (rB mB) gal dcm (DE3) | Laboratory collection |
| DH5α (pLSP-k2lacZ) | DH5α with empty LacZ-fusion vector pLSP, Km | This study |
| DH5α (piscR + lacZ) | DH5α containing pLSP with piscR and piscR promoter region, Km | This study |
| DH5α (piscR− + lacZ-S44) | DH5α containing pLSP with iscR and iscR promoter region, Km | This study |
| DH5α (piscR− + lacZ-280) | DH5α containing pLSP with mutant iscR and iscR promoter region, Km | This study |
| XL1-Blue | B1H system reporter strain, Km | Guo et al., 2009 |
| C-+ | Positive control of B1H system composed of co-transformants with pBX-M2031p/pTRG-Fv3133c, Km, Cm, Tef, Str | Guo et al., 2009 |
| C-− | Negative controls of B1H system composed of co-transformants with pBXcmT/pTRG, pBX-iscRp/pTRG and pBXcmT/pTRG-iscR, Km, Cm, Tef | This study |
| pTRG-iscR/pBxcmT-iscRp | XL1-Blue containing pTRG-iscR and pBxcmT with iscR promote region, Km, Cm, Tef, Str | This study |

PLASMIDS

- pRL27-Cm: Transposon vector, oriR6K, Cm
- pCPP30: Broad host complementary vector, tetA
- pCPP30:iscR: pCPP30 with 680bp long DNA sequence cloned from strain S44, starting from +81 bp relative to iscR start codon, Tef
- pLSP-k2lacZ: Km, ori, LacZ-fusion vector
- pTRG: B1H system vector, tetA
- pBxcmT: B1H system vector, Cm
- pET-28a+: Expression vector for iscR
- pET-28a+:iscR: pET-28a+ with iscR coding region
- Cm: chloramphenicol resistant; Tef: tetracycline resistant; Km: kanamycin resistant; Tp: trimethoprim resistant; Str: streptomycin resistant; Cm: chloramphenicol sensitive; Tef: tetracycline sensitive.

Described previously (Lehr et al., 2007). For growing cells, overnight cultures of strains S44, iscR-280, and iscR-280C (OD600 ~1.0) were inoculated into CDM-A liquid medium supplemented with 50 μM Sb(III) and incubated at 37°C for 7 d. For culture supernatant and cell-free extract, overnight cultures (100 mL) were harvested (OD600 ~1.0) by centrifugation and two equal parts of culture supernatant and cell-free extract were supplemented with 1 μM Sb(III) and incubated at 37°C for 5 d. Culture supernatant and cell-free extract were prepared as described previously (Liu et al., 2015). Minimum inhibitory concentration (MIC) was performed in LB medium supplemented without Sb(III) and with different concentration of NaAsO2 [As(III)], CuSO4 [Cu(II)], and CdCl2 [Cd(II)] at 37°C for 48 h. Spotting assays using serially diluted bacterial suspensions were performed to determine the cellular resistance to H2O2 (0.3 mM).

Co-transcription of iscRSUA and IscR-DNA Interaction Analysis

Total RNA was isolated using Trizol reagent (Invitrogen, Grand Island, NY, USA) and purified by RNA clean-up kit (Omega Bio-Tek, Georgia, USA) according to the manufacturer’s instructions. Confirmation of no genomic DNA contaminating the RNA was also performed. The ranges of coding sequences used for in vitro co-transcription of iscRSUA were designed as described in Figure 1.

The iscR promoter region was predicted using Berkeley Drosophila Genome Project (BDGP, http://www.fruitfly.org/seq_tools/promoter.html, Reese, 2001). The potential binding motifs of IscR in different species were further analyzed by comparing with a 25bp consensus sequence extracted from the RegPrecise database (http://regprecise.lbl.gov/RegPrecise/sites.jsp?regulog_id=1935, Novichkov et al., 2010). Multiple alignments of IscRs from different bacteria were performed using Clustal_X algorithm (Thompson et al., 1997).

A B1H system was constructed to detect in vivo IscR-DNA interactions. A 200 bp PCR product of predicted iscR promoter region was subcloned into pBxcmT (Guo et al., 2009) to yield pBx-iscRp. A 600 bp PCR product of iscR coding region was subcloned into pTRG (Guo et al., 2009) to yield pTRG-IscR. Then, pBX-iscR and pTRG-IscR were co-transformed into the E. coli XL1-Blue MRF' Kan reporter strain (XL1-Blue, Stratagene). Rv3133c has been previously shown to bind to motif sequences upstream of the acr (Rv2031 = M12031) coding region.
and its C-terminal HTH DNA-binding domain is essential for the binding. The sequence-specific interaction between Rv3133c and the promoter of M12031 was commonly used as a positive control to test the new B1H system (Guo et al., 2009; Gao et al., 2012). Co-transformants containing pBXcmT/pTRG-IscR, pBX-iscRp/pTRG and pBXcmT/pTRG were used as negative controls. The transformants were incubated on His-selective medium supplemented with 0.4% glucose, 200 µM adenine-HCl, 0.1% His Do sup [His Dropout Supplement (BD/Clontech, Cat. #630415)], 100 µM CaCl₂, 400 µM IPTG, 1 mM MgSO₄, 1 mM Thiamine HCl, 10 µM ZnSO₄, 50 µg/ml Km, 50 µg/ml Cm, 12.5 µg/ml tetracycline (Tet), 5 mM 3-amino-1,2,4-triazole (3-AT) and 16 µg/ml streptomycin (Str). LB medium supplemented with 50 µg/ml Km, 50 µg/ml Cm, and 12.5 µg/ml Tet was used as a control. His-selective plate and LB agar plate were aerobically incubated at 28°C for 7 d.

EMSA was also performed to confirm the in vitro interaction between IscR and its promoter. The intact coding region of iscR was subcloned into a His-tag expression vector, pET-28a(+) (Novagen, Madison, WI), to yield pET-28a(+)–IscR (Table 1). The His-tagged IscR was then expressed in E. coli BL21 (DE3), and purified by affinity chromatography (Ni-Sepharose 6 Fast Flow, GE Healthcare, UK). Each DNA (0.2 µg) was incubated with the purified IscR (0.8–6.4 µg) for 30 min at 30°C in a 20 µL reaction mixture containing binding buffer [100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM DTT, Tween 20, 1% (w/v), 150 mM KCl]. The binding reaction was stopped by the addition of 4 × loading buffer [0.25 × TBE buffer, 60%; glycerol, 40%, bromphenol blue, 0.2% (w/v)]. Predicted iscR promoter

FIGURE 1 | Gene organization of the isc operon and schematic representation of iscRSUA co-transcription. (A) The isc operon of strain S44 was composed of the iscRSUA–iscB–fdx genes located in contig61 with accession number, ADVO1000059; protein_id, EF608623–EF608629. (B) DNA electrophoresis of iscRSUA for co-transcription assay of strains S44 (WT) and iscR-280 (mutant). The white arrows marked for cDNA of strains S44 and iscR-280. The hollow arrows represent predicted coding regions of the Isc cluster genes. No. 1–7 represent coding regions of iscR (242 bp), and cross-linked regions of arrow, and M represent putative type I IscR-binding motif, transcription initiation and DNA marker, respectively.
**RESULTS**

**Isolation and Identification of Sb(III) Sensitive Mutants**

Transposon mutagenesis was performed using a Tn5-tagging method as described previously (Larsen et al., 2002). Approximately 5000 transformants were isolated and tested for Sb(III) resistance on CDM-A agar plates. Six mutants with different Tn5 insertion position within *iscRS* were found. Four mutants have an insertion within *iscR* and two mutant have an insertion between *iscR* and *iscS*. All of the six mutants showed a lower resistance to Sb(III) (data not shown). A mutant strain *iscR-280* showed the lowest Sb(III) resistance and was chosen for this study.

Analysis of the *C. testosteroni* S44 genome (ADVQ00000000, Xiong et al., 2011) revealed only one Isc system containing *iscRSUA-hscBA-fdx* (protein_id: EFI60623–EFI60629, protein_id: EFI60623–EFI60629, protein_id: EFI60623–EFI60629, protein_id: EFI60623–EFI60629).
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FIGURE 3 | B1H system and EMSA analysis for IscR interaction with DNA. (A) B1H system for iscR. Co-transformants containing pBX-Mt2031p/pTRG-Rv3133c were employed as positive controls (CK+), while co-transformants containing pBXcmT/pTRG-IscR, pBX-iscR/pTRG, and pBXcmT/pTRG were used as negative controls (CK-). Cells of CK+, pBX-iscR/pTRG-IscR, and CK- were grown to OD$_{600}$ of $\sim$1.0 and 4 $\mu$L of each was spotted onto His-selective medium (+3AT, +Str$^r$) and LB plate (-3AT, -Str$^r$). (B) SDS-PAGE gel of purified IscR. Bands 1–3 show un-induced protein, IPTG-induced protein and the purified IscR, respectively. The protein size marker (kDa, Thermo Scientific) is shown on the left. (C) EMSA analysis of IscR interaction with DNA. Band 1–4 represent negative controls, 1, DNA probe containing no IscR binding motif (non-specific DNA probe) added with IscR; 2, non-specific DNA probe only; 3, DNA probe containing putative IscR binding motif (specific DNA probe) added with heat-inactivated IscR; 4, specific DNA probe only; Band 5–8 represent the 0.8, 1.6, 3.2, and 6.4 $\mu$g of IscR added with specific DNA probe, respectively.

ADVQ01000059, Figure 1A) putatively involved in Fe-S cluster biogenesis. In vitro transcription assays indicated that iscR was co-transcribed with iscSUA (Figure 1B). No genomic DNA contaminated the total RNA (Figure S1). Multiple alignments of bacterial IscRs (Figure S2) exhibited an analogous organization among different species, such as E. coli, V. vulnificus, A. vinelandii, E. chrysanthemi, and P. aeruginosa (Rincon-Enriquez et al., 2008; Lim and Choi, 2014; Romsang et al., 2014). The residues (C92, C98, C104, and H107) involved in [Fe-S] ligation (Fleischhacker et al., 2012) were all highly conserved.

Defective Phenotypes were Restored by iscR-Complementation

An iscR complemented strain iscR-280C was generated as described above. Growth, multi-metal(loid)s resistance and Sb(III) oxidation rates were examined in strains S44, iscR-280, and iscR-280C. Without addition of Sb(III), strain iscR-280 showed the same growth rate in LB medium compared with strains S44 and iscR-280C (Figure 2A). However, growth of strain iscR-280 was strongly inhibited when Sb(III) was added (Figure 2B). Assays of bacterial Sb(III) oxidation rates were performed using liquid CDM-A which by itself exhibited no Sb(III) oxidation. The Sb(III) oxidation rate of strain iscR-280 in CDM-A medium was much lower than strains S44 and iscR-280C (Figures 2C–E), which might at least in part be attributed to diminished growth of strain iscR-280. Furthermore, Sb(III) oxidation occurred in the culture supernatant with the oxidation rates being highest in S44 > iscR-280C > iscR-280 (Figure S3A). The cell-free extract as a control showed no significant Sb(III) oxidation (Figure S3B), which suggested Sb(III) oxidation mainly occurred outside of cells. Strain iscR-280 displayed lower MICs compared to strain S44 for Sb(III) (300–100 $\mu$M), As(III) (14–10 mM), Cd(II) (2–0.9 mM), and Cu(II) (4–3 mM). The MICs for Sb(III), As(III), and Cu(II) of the strain iscR-280C could be restored almost completely (Table S2). As for bacterial H$_2$O$_2$ resistance, iscR-280 was less resistant than strains S44 and iscR-280C (Figure S4).
IscR Interacted with iscRSUA Promoter Region

The predicted promoter region sufficient for IscR binding was located within −181 to −117 nt upstream from the predicted iscR start codon. The B1H system assay revealed the interaction between IscR and its promoter region (Figure 3A). Each co-transformant with pBX-Mt2031p/pTRG-Rv3133c and pBX-iscR/pTRG-IscR grew well in the screening medium. By contrast, no growth was observed for the negative controls. All of the co-transformants showed a normal growth trend on LB plates.

EMSA coupled with a purified recombinant IscR (Figure 3B) and a predicted regulatory sequence confirmed that IscR was able to bind the promoter region of the isc operon (Figure 3, Band 5–8). But negative controls did not show any lagging bands (Figure 3, Band 1–4). Comparative analysis using the RegPrecise database revealed two putative type I IscR-binding motifs in C. testosteroni S44 (Table S2, Figure 4A), denoted as site A (TTACCCGACAAAATTGATGGGGAAT, −182 to −158 bp relative to iscR start codon) and site B (ATACTCGCCTCAAACACTCAACAAC, −152 to −128 bp relative to iscR start codon), which was consistent with the predicted region in BDGP. Sequence blast of site A and site B against C. testosteroni S44 genome revealed some sequence hits (12–13 bp long) distributed before or within multiple genes (Table S3). Table S4 showed that the nucleotide sequences of IscR-binding motifs varied among different species (data extracted from the RegPrecise database, Novichkov et al., 2010).

In order to determine the precise binding sequence of IscR on its target genes, two 30-bp-sequences (Site A and Site B) of IscR-binding motifs (Figure 4B), were synthesized to proceed with EMSA. As expected, both of them could bind IscR (Figure 4C). Site-directed mutations were constructed to determine their effect on DNA-IscR interaction. Site B is representative of the Type I IscR binding site. No binding activity was detected after the highly conserved bases of ATA within the site B were substituted with GCG (Figure 4B). Site A is not as well-conserved and does not resemble other known IscR binding sites. However, mutations to the base pairs located at positions −169 (T), −170 (A), −165 (T), and −166 (A) within site A, eliminated IscR binding (Figure 4C).

Sb(III) Induced Transcription of iscRS

Real-time RT-PCR analyses revealed that the transcription of iscR and iscS in strain S44 was significantly promoted by Sb(III) (Figures 5A,B), respectively. As expected, iscR transcription in iscr-280C was also induced by Sb(III). Under non-inducing conditions, the transcription level of iscS in iscr-280 was 3.8-fold higher than in strain S44 (Figure 5B). Sb(III) slightly enhanced the iscS expression in strain iscr-280 (Figure 5B). The multicopy-based complementation of iscR in strain iscr-280C showed strong repression of iscS transcription (Figure 5B).

IscR Negatively Regulated iscRSUA Expression

The β-galactosidase activities were measured in planktonic cultures of E. coli DH5α containing the different lacZ fusions (Figures 5C,D). Figure 5D showed background β-galactosidase
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FIGURE 5 | Analyses of real-time RT-PCR and LacZ reporter fusions for iscRS and γ-GCL activity/GSH content assay. (A,B) represent real-time RT-PCR results for iscR and iscS, respectively. The relative mRNA expression levels of iscR and iscS induced by Sb(III) (black bars) and without induction (white bars) were determined as described in Materials and Methods. (C) The location of the DNA segments used to generate lacZ fusions. (D) β-galactosidase activity was measured from E. coli DH5α containing different lacZ fusions. LacZ reporter fusions were constructed by ligating pLSP-kt2LacZ with iscR promoter (PiscR′-lacZ); with iscR promoter and iscR coding region of strain S44 (PiscR′-lacZ-S44); and with iscR promoter and iscR coding region of strain iscR-280 (PiscR′-lacZ-280). The empty vector pLSP-kt2LacZ was used as a control. (E,F) represent the determination of γ-GCL activity and GSH content, respectively. γ-GCL activity and GSH concentrations were calculated against a standard curve of GSH and expressed as the GSH concentration (nmol GSH/mg protein) obtained from cell free extract of the exponentially growing cells (mid-log phase at 10 h and stationary phase at 12 h). Data are expressed as mean ± SD, N = 3. Error bars represent standard deviations of triplicate tests. **Indicates a significant difference from the control (p < 0.01, Student’s t-test).

activity in pLSP-kt2lacZ (empty vector control) and PiscR′-lacZ-S44 (vector harboring intact iscR and iscR promoter), indicating that IscR negatively regulated transcription of iscRSUA. The lacZ fusions of both PiscR′-lacZ (vector harboring only the iscR promoter) and PiscR′-lacZ-280 (vector harboring Tn5-insertional mutant of iscR and the iscR promoter) exhibited high β-galactosidase activity (Figure 5D).

γ-GCL Activity and GSH Content were Both Decreased in the iscR Mutant
In order to determine whether thiol metabolism is related to the stress caused by Sb(III), we determined both the γ-GCL activity and GSH content simultaneously. The results demonstrated that the γ-GCL activity of iscR-280 was much lower than that of S44 and iscR-280C (Figure 5E), and the GSH content was positively correlated with γ-GCL activity (Figure 5F). The γ-GCL activities of iscR-280 at the two time points (10 h and 12 h) were nearly the same (Figures 5E,F), while the GSH content in iscR-280 was obviously reduced after being incubated for two more hours which might partially correspond to a point where there is an increased demand for GSH. The results indicated the iscR mutation led to a decreasing γ-GCL activity and less GSH content. Thiol metabolism might be influenced by the Isc system.

DISCUSSION
C. testosteroni S44 displayed resistance to multiple metal(loid)s, such as Sb(III), As(III), Cd(II), and Cu(II) (Xiong et al., 2011) and was also able to oxidize Sb(III). Whole genome sequencing of strain S44 revealed three As(III)/Sb(III) resistance genes including two putative arsB and one glpF. Although As and Sb
share similar physicochemical properties, strain S44 displayed no As(III) oxidation. This is different as in the case of *A. tumefaciens* 5A, in which both As(III) and Sb(III) oxidation were found to occur in the same strain (Lehr et al., 2007). Moreover, the whole genome of strain S44 did not contain genes encoding the putative As(III) oxidase gene *aioBA* (Wang et al., 2015), which indicated that there were different mechanisms for bacterial oxidation of Sb(III) or As(III).

We observed that the disruption of *iscR* resulted in a lower level of resistance to Sb(III), As(III), Cd(II), Cu(II), and H$_2$O$_2$ and a reduced Sb(III) oxidation rate. This indicated that IscR is an important component of resistance to multiple metal(loids) and also Sb(III) oxidation. Our finding also suggested *C. testosteroni* IscR plays an important role in the H$_2$O$_2$-induced oxidative stress response, similar as reported in *P. aeruginosa* and *V. vulnificus* (Lim and Choi, 2014; Romsang et al., 2014). Heavy-metals such as Cd(II) and Cu(II) could cause considerable oxidative stress by damaging Fe-S cluster assembly thereby generating free Fe(II) (Chillappagari et al., 2010; Xu and Imlay, 2012; Zheng et al., 2014). Sb(III) and As(III) might also lead to the destruction of Fe-S clusters. This again would increase unincorporated iron leading to substantially accelerated hydroxyl radical (OH-) formation by the Fenton reaction (Kohanski et al., 2007).

Our results showed that IscR could positively contribute to $\gamma$-GCL activity and GSH formation, possibly through regulating IscS-mediated cysteine desulfurization (Giel et al., 2006). Recent studies discovered that GSH alone can coordinate and stabilize Fe-S cluster formation under physiological conditions, and maturation of cytosolic Fe-S proteins required GSH (Qi et al., 2012; Wang et al., 2012). The Fe-S assembly protein IscU catalyzed formation of [2Fe-2S](GS)$_4$ from Fe and S ions in the presence of GSH (Qi et al., 2012). Thus, in strain iscR-280, the inactivation of IscR resulted in up-regulation of the isc operon consuming more GSH during a specific period and eliminating more S from cysteine. Moreover, the GSH synthesis was partially blocked because of cysteine shortage. Thiol metabolisms protect microbe from oxidative damage caused by due oxidants such as heavy metals (Rouhier et al., 2008). Glutathione is one of the most abundant thiolates in proteobacteria and cyanobacteria serving as a protecting agent (Fahey and Sundquist, 1991). Antimonites [Sb(III) compounds], cadmic compound [Cd(II) compounds], and cupric compounds [Cu(II) compounds] were able to inactivate [Fe-S] enzymes via a oxidative-stress-dependent disabling of their [Fe–S] catalytic clusters (Calderon et al., 2009; Chillappagari et al., 2010; Xu and Imlay, 2012). A reducing environment and the presence of GSH

![FIGURE 6](image) A hypothetical model of IscR role in Sb(III) resistance and oxidation. (i) Transport, Sb(III) is taken up by GlpF/ABC-like transporter and pumped out by ArsB (Meng et al., 2004); (ii) Toxicity, Sb(III)-caused toxicity disturbs the cellular redox homeostasis and damages the Fe-S cluster biogenesis. Meanwhile, impairment of IscR and elevated expression of IscS will result in a decrease of $\gamma$-GCL activity and GSH content; (iii) Restoration, intact IscR positively regulates the $\gamma$-GCL activity and GSH biosynthesis, and in turn the GSH promotes Fe-S cluster assembly and reduces the toxicity of Sb(III). Expelling Sb(III) is oxidized to less toxic Sb(V) by Fe-S containing enzymes.
within the cells was assumed to be a vital component of the bacterial oxidative stress response, indicating that IscR played global roles in resistances to multi-metalloids. In addition, the decrease of Sb(III) oxidation rate and in vitro Sb(III) oxidation suggested that Sb(III) was potentially oxidized to less toxic Sb(V) as a detoxification mechanism by soluble extracellular enzyme.

IscR was reported to suppress the transcription initiation of the isc operon, including iscR (Schwartz et al., 2001). In this study, B1H system and EMSA analysis implied the auto-regulation of iscR expression indicating that IscR auto-regulated the isc pathway for biosynthesis of its cofactor [Fe–S], which developed a connection between IscR activity and Fe–S clusters demand. Besides, we further confirmed that two putative IscR-binding motifs are essential for the recognition of IscR. Real-time RT-PCR analysis indicated the fact IscR may function as both transcriptional repressor and activator depending on cellular redox state. Under non-inducing conditions, IscR may function as a transcriptional repressor to maintain Fe–S cluster homeostasis. Under Sb(III) stress, iscRS expression in strain S44 was significantly promoted as IscR may function as a transcriptional activator. It has been reported that ROS can induce iscR expression (Imlay, 2006). The Fe–S cluster assembly system of isc (iscRUA-hscBA-fdx) was also up-regulated in the bacterial transcriptional response to oxidative stress (Wang et al., 2009). These indicated that intracellular oxidative stress originating from cellular exposure to Sb(III) might target Fe–S clusters and disable the [Fe–S] assembly. Meanwhile, under oxidative stress, most IscRs existed as apo-IscRs and functioned as an activator (Yeo et al., 2006), which in turn led to an increased expression of the isc system to meet the demands for Fe–S biogenesis and for the acclimation to Sb(III)-induced toxicity.

In summary, based on the literatures and our data, we show a hypothetical model of IscR role in Sb(III) resistance and oxidation (Figure 6): (i) Sb(III) is taken by GlpF-like transporter, and secreted by ArsB in bacteria (Meng et al., 2004); (ii) Sb(III) induced toxicity disturb the cellular redox homeostasis and damage the Fe–S cluster biogenesis. Destruction of Fe–S clusters increases free iron enough to accelerate Fenton reaction to produce more toxic OH· (Dwyer et al., 2009). Therefore, a number of Fe–S proteins or enzymes, vital for cell growth and metabolism, are impaired or inactivated. Meanwhile, impairment of IscR and elevated expression of IscS result in a decrease of γ-GCL activity and GSH content. (iii) The complemented IscR positively regulates the γ-GCL activity and GSH biosynthesis, and in turn GSH promotes the Fe–S cluster assembly and stability. Sb(III) is finally oxidized to less toxic Sb(V) potentially by extracellularly secreted proteins employing Fe–S cluster as cofactors. Consequently, Fe–S cluster synthesis, holo-IscR assembling and Fe–S protein function are progressively restored, which leads to the recovery of cellular redox homeostasis.

AUTHOR CONTRIBUTIONS

HL and WZ designed and performed the experiments and wrote the manuscript. SZ, JH, and JL participated in the experiments. CR helped to design the study and revise the manuscript. GW design the study and revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31170106) for GW, the National Natural Science Foundation of China (31500085) for HL, and the Open Project of State Key Laboratory of Agricultural Microbiology (AMLKF201503) in Huazhong Agricultural University for HL.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmolb.2015.00070

REFERENCES

Calderon, I. L., Elias, A. O., Fuentes, E. L., Pradenas, G. A., Castro, M. E., Arenas, F. A., et al. (2009). Tellurite-mediated disabling of [4Fe-4S] clusters of Escherichia coli dehydrogenases. Microbiology 155, 1840–1846. doi: 10.1099/mic.0.026260-0

Chillappagari, S. A., Trip, H., Kuipers, O. P., Marahiel, M. A., and Miethke, M. (2010). Copper stress affects iron homeostasis by destabilizing iron-sulfur cluster formation in Bacillus subtilis. J. Bacteriol. 192, 2512–2524. doi: 10.1128/JB.00585-10

Decuyper, S., Vanaerschot, M., Brunker, K., Imamura, H., Muler, S., Khaliad, B., et al. (2012). Molecular mechanisms of drug resistance in natural Leishmania populations vary with genetic background. PLoS Negl. Trop. Dis. 6:e1514. doi: 10.1371/journal.pntd.0001514

Dwyer, D. J., Kohanski, M. A., and Collins, J. J. (2009). Role of reactive oxygen species in antibiotic action and resistance. Curr. Opin. Microbiol. 12, 482–489. doi: 10.1016/j.mib.2009.06.018

Fahey, R. C., and Sundquist, A. R. (1991). “Evolution of glutathione metabolism,” in Advances in Enzymology and Related Areas of Molecular Biology, Vol. 64, ed A. Meister (Hoboken, NJ: John Wiley & Sons, Inc.), 1–53. doi: 10.1002/978047123102.2ch1

Filella, M., Belzile, N., and Chen, Y. W. (2002). Antimony in the environment: a review focused on natural waters I. Occurrence. Earth Sci. Rev. 57, 125–176. doi: 10.1016/S0012-8252(01)00070-8

Filella, M., Belzile, N., and Lett, M. C. (2007). Antimony in the environment, A review focused on natural waters. III. Microbiota relevant interactions. Earth Sci. Rev. 80, 195–217. doi: 10.1016/j.earscirev.2006.09.003

Fleischhacker, A. S., Stubna, A., Hsueh, K. L., Guo, Y., Teter, S. J., Rose, J. C., et al. (2012). Characterization of the [2Fe–2S] cluster of Escherichia coli transcription factor IscR. Biochemistry 51, 445–4462. doi: 10.1021/bi3003204

Gao, C., Yang, M., and He, G. (2012). Characterization of a novel ArsR-like regulator encoded by Rv2034 in Mycobacterium tuberculosis. PLoS ONE 7:e36255. doi: 10.1371/journal.pone.0036255

Giel, J. L., Nesbit, A. D., Mettert, E. L., Fleischhacker, A. S., Wanta, B. T., and Killey, P. J. (2013). Regulation of iron-sulphur cluster homeostasis through transcriptional control of the isc pathway by [2Fe–2S]-IscR in Escherichia coli. Mol. Microbiol. 87, 478–492. doi: 10.1111/mmi.12052

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Xu, F. F., and Imlay, J. A. (2012). Silver(I), mercury(II), cadmium(II), and zinc(II) target exposed enzymic iron-sulfur clusters when they toxify *Escherichia coli*. *Appl. Environ. Microbiol.* 78, 3614–3621. doi: 10.1128/AEM.07368-11

Yeo, W. S., Lee, J. H., Lee, K. C., and Roe, J. H. (2006). IscR acts as an activator in response to oxidative stress for the *suf* operon encoding Fe-S assembly proteins. *Mol. Microbiol.* 61, 206–218. doi: 10.1111/j.1365-2958.2006.05220.x

Zheng, S., Su, J., Wang, L., Yao, R., Wang, D., Deng, Y., et al. (2014). Selenite reduction by the obligate aerobic bacterium *Comamonas testosteroni* S44 isolated from a metal-contaminated soil. *BMC Microbiol.* 14:204. doi: 10.1186/s12866-014-0204-8

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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