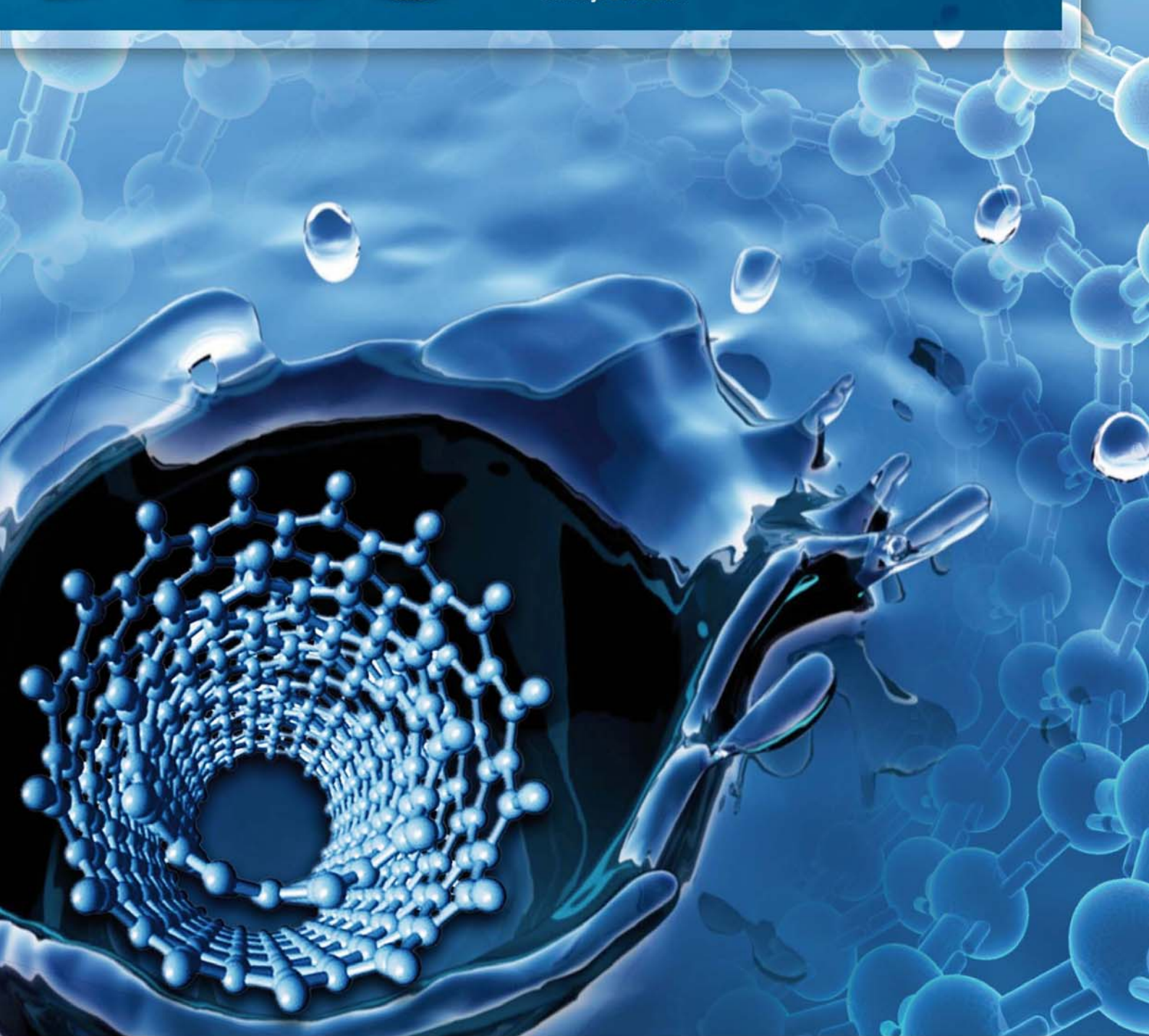


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## Function of *arsA**Torf7orf8* of *Bacillus* sp. CDB3 in arsenic resistance

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### Abstract

*Bacillus* sp. CDB3 isolated from an arsenic contaminated cattle dip site possesses an uncommon arsenic resistance (*ars*) operon bearing eight genes in the order of *arsRYCDATorf7orf8*. We investigated the functions of *arsA*, *arsT*, *orf7* and *orf8* in arsenic resistance using a plasmid-based gene knockout approach in the *ars* gene deficient *Escherichia coli* strain AW3110. The CDB3 *arsA* gene was shown to play a significant role in resistance, suggesting that the encoded ArsA may couple with the arsenite transporter, forming an ArsAY complex that can enhance arsenite extrusion efficiency. The disruption of either *arsT* or *orf7* was not observed to affect arsenic resistance in the heterologous *E. coli* host, but their involvement in arsenic resistance can not be excluded. The *orf8* gene is predicted to encode a putative dual-specificity protein phosphatase which also shares certain homology to arsenate reductases. The function loss of *orf8* resulted in a remarkable decrease in resistance to arsenate, though not arsenite. To examine if this effect was due to the reduction of arsenate by *orf8*, the *arsC* gene within the 8-gene operon was disrupted. The resulting abolishment of arsenate resistance suggests that the involvement of *orf8* in arsenic resistance is not via reductase activity.

**Key words:** *ars* genes; arsenic resistance assay; gene knockout

**DOI:** 10.1016/S1001-0742(12)60154-5

### Introduction

Arsenic, a toxic metalloid element existing in the form of either organic or inorganic salts, is widely spread in the environment (Ye et al., 2012). Arsenite and arsenate are two primary oxidation states, which are toxic to living organisms. The pressure of survival in arsenic-containing environments has prompted the evolution of detoxification systems in almost all microorganisms (Achour-Rokbani et al., 2010). As a result, many such organisms have been found to possess arsenic resistance (*ars*) genes (Huang et al., 2012; Kim and Rensing, 2012; Srivastava et al., 2012).

The most common *ars* genes involved in the mechanism of bacterial arsenic resistance are *arsR*, *arsB*, *arsC*, *arsD* and *arsA*. The *arsR* gene codes for a trans-acting repressor regulating the expression of *ars* genes (Shi et al., 1994). ArsD was first shown to be a second repressor (Chen and Rosen, 1997) and was later demonstrated to also function as a metallochaperone (Lin et al., 2006). The *arsC* gene encodes an arsenate reductase, reducing arsenate to arsenite prior to extrusion (Ding et al., 2005; Rosen, 2002). Arsenite is then extruded out of the cells via chemiosmotic arsenite specific transmembrane transporters. Two major

families of membrane arsenite transporters have been identified in bacteria. The first group is composed of the *Escherichia coli* pR773 ArsB protein and its homologs, which utilize the membrane potential of the cell to extrude arsenite and antimonite from the cytosol (Kuroda et al., 1997; Rosen, 2002, 1999). The second group, the YqcL (ArsY) family first discovered in *Bacillus subtilis*, has been shown to be specific to arsenite, lacking the activity for antimonite (Rosen, 1999; Sato and Kobayashi, 1998). ArsA, an anion translocating ATPase initially identified in *E. coli* pR773 has been found to be able to couple with ArsB and the complex improving the efficiency of arsenite extrusion (Dey et al., 1994a, 1994b; Dey and Rosen, 1995).

Apart from these well characterized *ars* genes, a number of less common genes involved in arsenic resistance have also been reported. *arsH* was first identified in the *Yersinia enterocolitica* virulence plasmid pYV (Neyt et al., 1997), and has since been found in many other bacteria including *Thiobacillus ferrooxidans* (Butcher et al., 2000), *Acidithiobacillus caldus* (Dopson et al., 2001), *Serratia marcescens* (Ryan and Colleran, 2002), *Cyanobacterium Synechocystis* (Kaneko et al., 1996; López-Maury et al., 2003) and *Staphylococcus* sp. strain NBRIEAG-8 (Srivastava et al., 2012). The function of *arsH* however has not yet

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been demonstrated consistently. A recent investigation into ArsH function in *Synechocystis* has suggested its role in dealing with the damage caused by arsenite by means of reducing semiquinone radicals or oxidized quinones (Hervás et al., 2012). *arsM*, a putative arsenite-methyltransferase gene located on the large extrachromosomal replicon pNRC100 in *Halobacterium* sp. strain NRC-1 was demonstrated to be involved in arsenic resistance via gene knockout assay (Wang et al., 2004). Further investigation has shown that purified ArsM is capable of catalysing the transfer of methyl groups from *S*-adenosylmethionine to arsenite (Qin et al., 2006). Bacteria that express *arsM* have been demonstrated capable of removing arsenic via volatilization of methylated form(s) from the contaminated soil (Liu et al., 2011). A recent study has also revealed that the expression of *arsM* of cyanobacteria is not regulated by the *ars* repressor but likely to be constitutive (Ye et al., 2012). The flavin-binding monooxygenase encoded by *arsO* is probably involved in the mechanism of arsenic resistance in *Streptomyces* sp. FR-008 but is yet to be functionally characterized (Butcher et al., 2000; Wang et al., 2006). The *arsK* gene identified in *B. subtilis* has been evidenced to contribute to resistance against low concentrations of arsenite and high concentrations of arsenate by an in-frame deletion and plasmid-insertion disruption (Sato and Kobayashi, 1998), but the mechanisms by which *arsK* is involved in arsenic resistance is not yet clear. *arsT*, encoding a putative thioredoxin reductase, has been identified in some *Streptomyces* strains and ArsT is assumed to provide reductive power for the thioredoxin-coupled arsenate reductases (Wang et al., 2006).

A *Bacillus* strain designated CDB3 isolated from an arsenic contaminated cattle dip site (Chopra et al., 2007), has been found to possess a novel *ars* cluster with eight genes in the order of *arsRYCDATorf7orf8*, one of the longest *ars* operons identified to date (Bhat et al., 2011). This *ars* operon has been shown capable of significantly enhancing the resistance to arsenic of transformed arsenic-

sensitive *E. coli* strain AW3110, but exhibited little effect on the resistance to antimonite (Bhat et al., 2011). Apart from the first five common genes *arsRYCDA*, the cluster harbours a thioredoxin reductase gene (*arsT*), and two other novel genes, *orf7* and *orf8*, which have not been reported in any previously characterised *ars* clusters. The theoretical translation of *orf7* suggests a HesB-like domain whilst *orf8* is predicted to be a dual-specificity protein phosphatase (Bhat et al., 2011). In this study, we examined the functions of *arsT*, *orf7* and *orf8* in arsenic resistance via plasmid-based gene mutant assays in the *ars* gene deficient *E. coli* AW3110. In addition, the function of ArsA was also investigated, confirming its role in coupling with ArsY to enhance arsenite extrusion.

## 1 Materials and methods

### 1.1 Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. *E. coli* strains with plasmids transformed were grown in Luria-Bertani (LB) broth medium (Sambrook et al., 1989) containing ampicillin (Sigma, USA; 100 µg/mL) and varying concentrations of sodium arsenite or sodium arsenate (Sigma, USA).

### 1.2 General DNA manipulation

Plasmid DNA isolation, restriction endonuclease digestion, agarose gel electrophoresis, ligation and *E. coli* transformation were performed using standard methods (Sambrook et al., 1989). All enzymes were obtained from Promega (USA). DNA sequencing reactions were carried out using the Big Dye Terminator v3.1 cycle sequencing kit (Perkin-Elmer, USA) following the manufacturer's instructions and the extended DNA fragments analysed on an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, USA).

**Table 1** Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<i>E. coli</i> AW3110	K-12 F-IN ( <i>rrnD-rrnE</i> ) <i>ars::cam</i> (Cm <sup>r</sup> , the chromosomal <i>arsRBC</i> deleted)	Carlin et al., 1995; gift of Barry P. Rosen
<i>E. coli</i> JM109	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> (rk <sup>-</sup> , mk <sup>+</sup> ), <i>supE44</i> , <i>relA1</i> , Δ ( <i>lac-proAB</i> ), [F <sup>+</sup> , <i>traD36</i> , <i>proAB</i> , <i>lacIqZΔM15</i> ]	Promega, USA
<i>Bacillus</i> sp. CDB3	Isolated from cattle dip sites	Chopra et al., 2007
pGEM7Zf(+)	Cloning vector (Ap <sup>r</sup> )	Promega, USA
pRYCDATorf7,8	A 7600 bp <i>Sau3AI</i> fragment containing the CDB3 <i>ars</i> cluster 1 cloned into pGEM7Zf(+) vector at <i>Bam</i> HI site	Bhat et al., 2011
pRYC <sup>Δ</sup> DATorf7,8	<i>arsC</i> mutant of pRYCDATorf7,8	This study
pRYCDA <sup>Δ</sup> Torf7,8	<i>arsA</i> mutant of pRYCDATorf7,8	This study
pRYCDA <sup>Δ</sup> orf7,8	<i>arsT</i> mutant of pRYCDATorf7,8	This study
pRYCDATorf7 <sup>Δ</sup> ,8	<i>orf7</i> mutant of pRYCDATorf7,8	This study
pRYCDATorf7,8 <sup>Δ</sup>	<i>orf8</i> mutant of pRYCDATorf7,8	This study
pRorf8	<i>arsYCDATorf7</i> deleted from pRYCDATorf7,8	This study
pRorf8 <sup>Δ</sup>	<i>arsYCDATorf7</i> deleted from pRYCDATorf7,8 <sup>Δ</sup>	This study

### 1.3 Mutagenesis of *ars* genes

Although chromosomal gene knockout is still a common approach in investigating gene function for bacteria, plasmid-based analysis in heterologous hosts has also been used by many for its simplicity and proved effective. For instance, the functional analysis of the *amarsA1*, *amarsA2* and *acr3* genes of *Alkaliphilus metalliredigens* conducted in *E. coli* has demonstrated their involvements and interactions in arsenic resistance (Fu et al., 2010). With previous attempts to knockout the *ars* genes in *Bacillus* sp. CDB3 unsuccessful (unpublished) and with the finding that the transcriptional pattern of CDB3 *arsI* in *E. coli* was similar to that in *Bacillus* sp. CDB3 (not shown), the plasmid-based heterologous host method was adapted in this study.

The open-reading frames of target CDB3 *ars* genes were disrupted to generate respective gene function knockout mutants in pRYCDATorf7,8. pRYCDATorf7,8 and pGEM7Zf(+) were positive and negative controls respectively throughout this study. For creating the *arsC*, *A* and *T* mutants (*arsC*<sup>Δ</sup>, *A*<sup>Δ</sup> and *T*<sup>Δ</sup>), the target sequences between two appropriate restriction enzyme sites were replaced with synthetic mutant sequences (GeneArt, Germany). Thus the *arsC* gene was disrupted from the fifth codon (Lys) with a mutant *KpnI/CspI* DNA fragment carrying a stop codon at the fifth position; *arsA* was deleted from codon one to 552 with a *CspI/NcoI* fragment; and *arsT* was disrupted from the third codon (Lys) by replacement with a mutant *NcoI/PstI* DNA fragment. For *orf7* and *orf8* mutants (*orf7*<sup>Δ</sup> and *orf8*<sup>Δ</sup>), the respective internal *BamHI* and *BstXI* restriction sites were cut and blunted with T4 DNA polymerase (Promega, USA) before re-ligation to generate frame shifts from the 61st and 90th codons respectively, resulting in loss-of-function mutants. To create pRorf8 and pRorf8<sup>Δ</sup>, pRYCDATorf7,8 and pRYCDATorf7,8<sup>Δ</sup> were opened with *EcoRV* and *BamHI*, with the *BamHI* overhangs filled by Klenow activity followed by re-ligation resulting in deletions in both plasmids from codon 15 of *arsY* to codon 41 of *orf7*.

### 1.4 Arsenic resistance assays

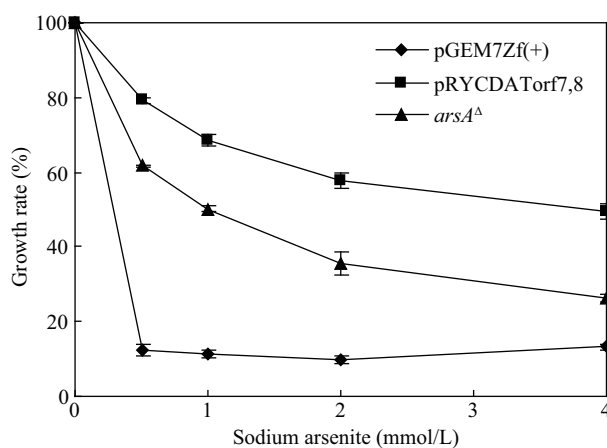
Overnight liquid cultures of *E. coli* JM109 or AW3110 strains carrying different plasmids were adjusted to equal optical densities and diluted 100 fold in LB broth with 100 μg/mL ampicillin. Aliquots of 500 μL were then transferred to 1.5 mL microcentrifuge tubes, with varying concentrations of sodium arsenate (Na<sub>2</sub>HAsO<sub>4</sub>) or sodium arsenite (NaAsO<sub>2</sub>). These tubes were agitated at 37°C, for 2–3 hr with the endpoint of incubation for each determined by the 0 mmol/L arsenic sample reaching an OD<sub>600 nm</sub> of 0.5. Cell density for each sample set was then determined at OD<sub>600 nm</sub>. Absorption readings were recorded using a SpectraMax Plus 384 Microplate Spectrophotometer (Biostrategy, Australia). Growth inhibition as a measurement for sensitivity to arsenic was expressed as a percentage of the OD reading observed for the 0 mmol/L arsenic-treated

samples. The assays were conducted in triplicates with the data subjected to analysis of variance (ANOVA).

## 2 Results and discussion

### 2.1 Function of *arsA* and *arsT* in arsenic resistance

*E. coli* AW3110 strains bearing the wild type (WT) operon plasmid (pRYCDATorf7,8) and the *arsA* loss-of-function mutant (*arsA*<sup>Δ</sup>) were assayed for resistance to arsenite. The resistance curve of AW3110 harbouring *arsA*<sup>Δ</sup> was observed to be much lower than that of AW3110 containing pRYCDATorf7,8 but significantly higher than that of the negative control, AW3110 harbouring pGEM7Zf(+) (Fig. 1), indicating that ArsA plays a significant role in arsenite resistance. Sequence alignment demonstrates significant homology between the CDB3 ArsA and *E. coli* pR773 ArsA, with the metalloid- and nucleotide-binding domains and signal transduction domains conserved (data not shown). It has previously been shown that the *E. coli* ArsA can combine with ArsB in the cell to form an ArsAB complex pumping arsenite out of the cytosol more efficiently than ArsB alone (Dey et al., 1994a, 1994b; Dey and Rosen, 1995). In contrast, the CDB3 *ars* operon bears a gene encoding a transmembrane protein designated ArsY (a homolog of Acr3) rather than ArsB. In comparison to ArsB which possesses 12 membrane-spanning regions, ArsY only contains 10 (Sato and Kobayashi, 1998; Wu et al., 1992; Wysocki et al., 1997). Our result suggests that the CDB3 ArsA is capable of coupling with ArsY to form an ArsAY complex which, like ArsAB, also acts to enhance extrusion efficiency. This is in agreement with a recent study demonstrating the interaction between ArsA and Acr3 in *Alkaliphilus metalliredigens* QYMF (Fu et al., 2010); interestingly, the functional ArsA of *A. metalliredigens* QYMF is assembled by two polypeptides (halves, AmArsA1 and AmArsA2) which are encoded separately.



**Fig. 1** Growth inhibition by sodium arsenite of *E. coli* AW3110 harbouring pGEM7Zf(+), pRYCDATorf7,8 and *arsA*<sup>Δ</sup> (pRYCDA<sup>Δ</sup>Torf7,8), respectively. Growth inhibition was expressed as a percentage of the OD reading observed for the 0 mmol/L arsenite-treated samples. Vertical bars represent standard deviation.

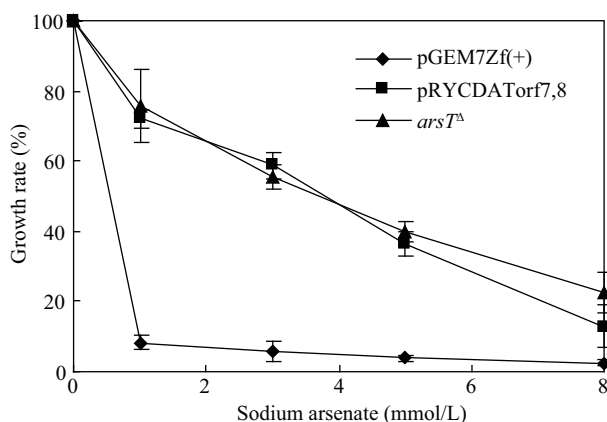
ArsT has previously been suggested to serve as a thioredoxin reductase, functioning through the provision of reductive power for thioredoxin-coupled arsenate reductases, like CDB3 ArsC (Bhat et al., 2011; Li et al., 2007; Messens et al., 2002; Wang et al., 2006). However, no loss of resistance against arsenate was observed for the AW3110 cells containing *arsT<sup>Δ</sup>* compared to the WT (Fig. 2). Since the CDB3 ArsT shares significant homology to thioredoxin reductases of many species, including those of *E. coli*, possessing all domains and the CXXX active site responsible for reductive activity (Fig. 3), it is speculated that the thioredoxin reductases encoded by the *E. coli* AW3110 chromosome may have compensated for the loss of ArsT function, acting in regenerating the CDB3 ArsC. This mirrors the result of Wang et al. (2006) who reported that mutation of *arsT* in the *ars* operon of pJTU91 of *Streptomyces griseus* IMRU3570 resulted in no

significant decrease in resistance to arsenate.

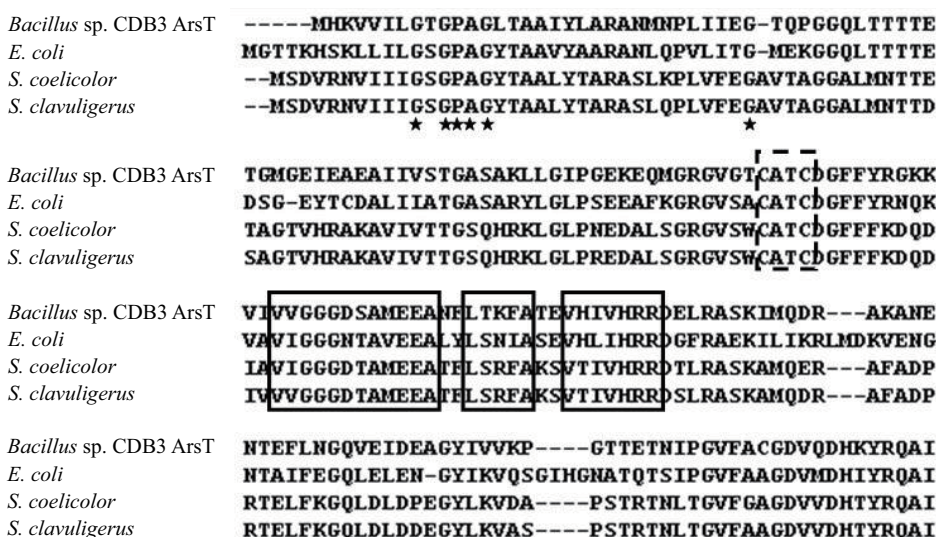
## 2.2 Involvement of *orf8* in arsenic resistance

The CDB3 *ars* cluster additionally contains two novel *orfs* downstream of *arsT*: *orf7* and *orf8*. *Orf7* has a HesB-like domain, demonstrating sequence similarities with proteins involved in metallo-sulphur cluster assembly, while *orf8* is predicted to encode a dual specificity protein phosphatase (Bhat et al., 2011). To date, neither protein family has been implicated in arsenic resistance. As illustrated in Fig. 4, the bacterial cells harbouring *orf7<sup>Δ</sup>* exhibited an almost identical resistance growth curve to that of the WT under both arsenite and arsenate treatments. The cells harbouring *orf8<sup>Δ</sup>*, however, exhibited a remarkable decrease in resistance to arsenate, although not arsenite, compared to the WT, indicating a significant contribution by this gene to the overall arsenate resistance of *arsRYCDATorf7,8*.

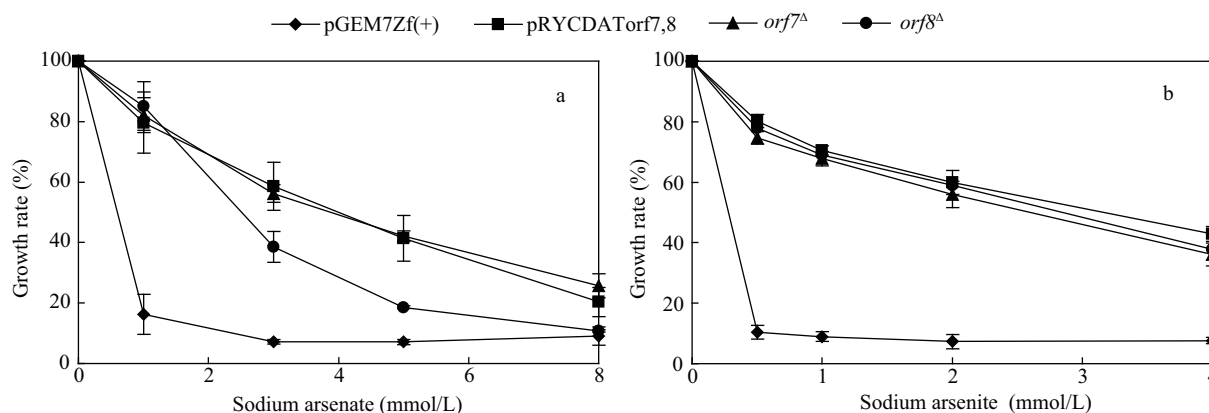
Taking into account the relationship between protein tyrosine phosphatases and arsenate reductases, it was possible that *orf8* was functioning as an arsenate reductase (Bennett et al., 2001; Zhou et al., 2006). In an attempt to ascertain whether *orf8* possessed this activity, an *arsC<sup>Δ</sup>* construct (pRYC<sup>Δ</sup>DATorf7,8) was generated. The growth of *E. coli* AW3110 harbouring *arsC<sup>Δ</sup>* was totally inhibited in media supplemented with >1 mmol/L arsenate (Fig. 5a), suggesting *orf8* could not compensate for the loss of the reductase function of ArsC while both the WT and *arsC<sup>Δ</sup>* constructs demonstrated similar resistance levels to arsenite (Fig. 5b). These results suggest that ArsC is the only protein encoded by the CDB3 *ars* cluster possessive of arsenate reductase activity and this is in line with our original hypothesis that *orf8* may function as a phosphatase (Bhat et al., 2011).



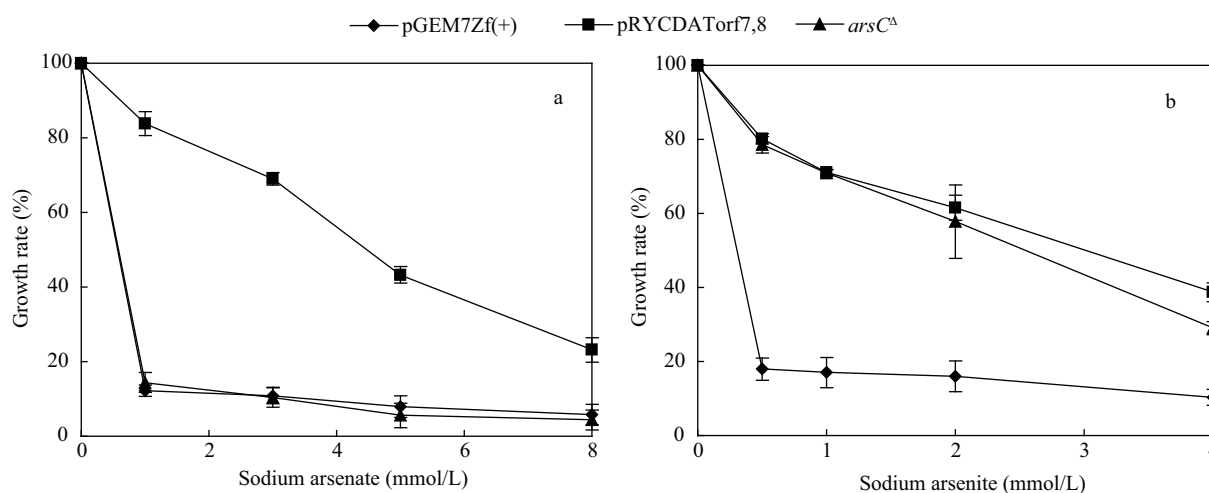
**Fig. 2** Growth inhibition by sodium arsenate of *E. coli* AW3110 harbouring pGEM7Zf(+), pRYCDATorf7,8 and *arsT<sup>Δ</sup>* (pRYCDAT<sup>Δ</sup>orf7,8), respectively. Vertical bars represent standard deviation.



**Fig. 3** Partial sequence alignment of CDB3 ArsT with some homologous proteins. The alignment was performed using ClustalW (Thompson et al., 1994). FAD binding site 1, FAD binding site 2, reactive site and NADP(H) binding site are indicated in stars, line, broken box and intact box, respectively. The accession numbers for CDB3 ArsT, thioredoxin reductase of *Streptomyces coelicolor* A3(2), *Streptomyces clavuligerus* and *E. coli* are AF178758, CAA63076, CAA79940 and ADR26238, respectively.



**Fig. 4** Growth inhibition by sodium arsenate (a) and sodium arsenite (b) of *E. coli* AW3110 harbouring pGEM7Zf(+), pRYCDATorf7,8, *orf7* $\Delta$  (pRYCDATorf7 $\Delta$ ,8) and *orf8* $\Delta$  (pRYCDATorf7,8 $\Delta$ ) respectively. Vertical bars represent standard deviation.



**Fig. 5** Growth inhibition by sodium arsenate (a) and sodium arsenite (b) of *E. coli* AW3110 harbouring pGEM7Zf(+), pRYCDATorf7,8 and *arsC* $\Delta$  (pRYC $\Delta$ DATorf7,8), respectively. Vertical bars represent standard deviation.

### 2.3 Contribution of *orf8* to arsenate resistance in transformed *E. coli* JM109

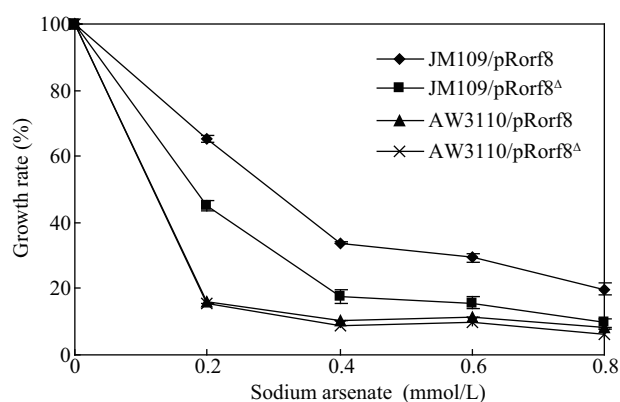
Having established evidence for the contribution of Orf8 to arsenate resistance, its role was further investigated by testing whether the gene alone could confer elevated levels of resistance to transformed *E. coli*. pRorf8 and pRorf8 $\Delta$  were thus constructed and transformed into both AW3110 and JM109 strains because of the different backgrounds they provide. As mentioned previously, AW3110 lacks any *ars* resistance elements (Carlin et al., 1995). In contrast, JM109 possesses a chromosomal *arsRBC* operon; thus any contribution of *orf8* to resistance could be identified as either independent or as requiring the basic *arsRBC* components. An initial assay used high concentrations of arsenate (1–8 mmol/L) as applied in other resistance assays of this study, but the bacterial strains were all found almost completely inhibited (data not shown). The subsequent assays with lower concentrations of arsenate (0.2–0.8 mmol/L) revealed a better bacterial growth and the effect of *orf8* expression. The AW3110 cells harbouring either pRorf8 or pRorf8 $\Delta$  could barely grow in

arsenate supplemented media (Fig. 6), indicating that the expression of *orf8* had no effect on arsenate resistance in the *arsRBC* lacking mutant host. In contrast, the JM109 harbouring pRorf8 grew remarkably better than the control cells carrying pRorf8 $\Delta$  (Fig. 6). This again suggested the role of Orf8 in combating the toxic metalloids. Whether this effect is due to a direct influence of *orf8* on the arsenic resistance machinery or a flow-on effect of its function in regard to other cellular stress responses to arsenic warrants further investigation.

### 3 Conclusions

This study has confirmed the involvements of *arsA* and *orf8* genes of the newly identified 8-gene CDB3 cluster in arsenic resistance. It is proposed that the CDB3 ArsA is capable of coupling with ArsY to enhance the extrusion of arsenite. Although ArsT has not been evidenced as required for arsenate resistance in an *E. coli* system, its involvement in arsenate reduction in *Bacillus* sp. CDB3 may still be assumed since it appears in the presence





**Fig. 6** Growth inhibition by sodium arsenate of *E. coli* JM109 and AW3110 harbouring pRorf8 or pRorf8<sup>Δ</sup>. Vertical bars represent standard deviation.

of a thioredoxin-coupled reductase. *orf8* was shown to contribute significantly to resistance to arsenate but not arsenite. It did not complement the function loss of *arsC* suggesting that its role is not via arsenate reductase activity but another unknown mechanism, possibly the predicted phosphatase activity. Further work is in progress to investigate the biochemical function of Orf7 and *orf8*.

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