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Bacillus sp. CDB3 isolated from cattle dip-sites possesses two *ars* gene clusters

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Abstract

Contamination of soil and water by arsenic is a global problem. In Australia, the dipping of cattle in arsenic-containing solution to control cattle ticks in last centenary has left many sites heavily contaminated with arsenic and other toxicants. We had previously isolated five soil bacterial strains (CDB1-5) highly resistant to arsenic. To understand the resistance mechanism, molecular studies have been carried out. Two chromosome-encoded arsenic resistance (*ars*) gene clusters have been cloned from CDB3 (*Bacillus* sp.). They both function in *Escherichia coli* and cluster 1 exerts a much higher resistance to the toxic metalloid. Cluster 2 is smaller possessing four open reading frames (ORFs) *arsRorf2BC*, similar to that identified in *Bacillus subtilis* Skin element. Among the eight ORFs in cluster 1 five are analogs of common *ars* genes found in other bacteria, however, organized in a unique order *arsRBCDA* instead of *arsRDABC*. Three other putative genes are located directly downstream and designated as *arsTIP* based on the homologies of their theoretical translation sequences respectively to thioredoxin reductases, iron-sulphur cluster proteins and protein phosphatases. The latter two are novel of any known *ars* operons. The *arsD* gene from *Bacillus* species was cloned for the first time and the predict protein differs from the well studied *E. coli* ArsD by lacking two pairs of C-terminal cysteine residues. Its functional involvement in arsenic resistance has been confirmed by a deletion experiment. There exists also an inverted repeat in the intergenic region between *arsC* and *arsD* implying some unknown transcription regulation.

Key words: arsenic toxicity; bacterial resistance; ars operons; gene cloning

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Introduction

Arsenic is a toxic metalloid and the resistance to this toxicant has been evolved widely in living organisms. Bacteria arsenic resistance (*ars*) genes commonly exist in the form of *ars* operons (Jackson et al., 2003; Mukhopadhyay et al., 2002; Rosen, 1999; Silver and Phung, 2005). To date, a number of *ars* operons present on both plasmids and chromosomes of bacteria have been characterized at molecular level. Among the five common and mostly studied *ars* genes *R*, *B*, *C*, *D* and *A*, *arsR* and *arsD* encode trans-acting repressors, *arsC* encodes a small cytoplasmic reductase for arsenate and the rest of two, *arsA* and *arsB*, encode respectively the ATPase and membrane transporter of arsenite pump (Rosen, 1999). A recent investigation has assigned a second role, arsenic metallochaperon, to ArsD (Lin et al., 2006). YqcL (referred as "ArsY" from now on) is a functional homolog of ArsB found firstly in *Bacillus subtilis* (Sato and Kobayashi, 1998) and later also in some other bacteria (Achour et al., 2007; Cai et al., 2009). In yeast *Saccharomyces cerevisiae* a similar arsenite pump Acr3 more homologous to YqcL has been identified (Bobrowicz et al., 1997). The Y type arsenite transporters are structurally quite diverged from ArsB and no associated ArsA subunits have been identified (Rosen, 1999).

There are some other *ars* genes reported which are less common or yet to be characterized in terms of function of the gene products. *arsM* represents a best characterized sample. The methylation of arsenic has been assumed to be an important pathway in cellular detoxification process but the responsible gene has only recently been identified in *Halobacterium* sp. strain NRC-1 (Wang et al., 2004) and *Rhodopseudomonas palutris* (Qin et al., 2006). A gene called *arsH* has been found in several species including *Yersinia enterocolitica* (Neyt et al., 1997), *Acidithiobacillus ferrooxidans* (Butcher et al., 2000), *Serratia marcesens* (Ryan and Colleran, 2002), *Synechocystis* sp. (Lopez-Maury et al., 2003), *Sinorhizobium meliloti* (Yang et al., 2005), *Pseudomonas aeruginosa* (Parvatiyar et al., 2005)

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and *Ochrobactrum tritici* (Branco et al., 2008). Some studies, have demonstrated its role in arsenic resistance, probably by regulating other *ars* genes (Neyt et al., 1997; Ryan and Colleran, 2002; Yang et al., 2005; Branco et al., 2008). *aqpS*, reported from the study of *Sinorhizobium meliloti* by Yang et al. (2005) codes for an aquaglyceroporin. Its involvement in conferring arsenate resistance was demonstrated when coupled with reduction by *arsC*, although the function of aquaglyceroporin channels is thought to facilitate arsenite uptake which would increase the cell's arsenic-sensitivity. Wang et al. (2006) identified two other novel genes from *Streptomyces* sp. strain FR-008. *arsO* presumably codes for a putative flavin-binding monooxygenase and *arsT*, for a putative thioredoxin reductase. Only *arsT* was demonstrated as having a definitive role in resistance with an assumed function in coupling with the arsenate reductase which requires thioredoxin for regenerating activity. Another gene with no designated name yet was the one specifying the second open reading frame (*orf2*) identified in the ars operon on the SKIN element of *Bacillus subtilis* (Sato and Kobayashi, 1998) and its role in arsenic resistance has not yet been demonstrated. It appears that the bacterial genes involved in arsenic resistance are quite diverge and large in number.

The most common source of elevated arsenic concentrations in the Australian environment is attributable to anthropogenic activities and the dipping of cattle in arsenic-containing solution to control cattle ticks in last centenary represents such an activity which has left many sites heavily contaminated with arsenic along with other toxicants used (Smith et al., 2003). The contamination of these sites with soil content of arsenic as high as over 2000 mg/kg has become a major concern to public health but no efficient remediation method has been available. As part of a survey on the site's biota, we isolated five soil bacterial strains from two old cattle dip sites in Northern New South Wales and they were identified as *Ochrobactrum* sp., *Arthrobacter* sp., *Serratia* sp., and two *Bacillus* spp., respectively (Chopra et al., 2007). To understand the mechanism by which these bacterial strains can survive under such harsh arsenic stress, molecular studies have been carried out. This article reports the character of two ars clusters we have identified from *Bacillus* sp. CDB3.

1 Materials and methods

1.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth medium (Sambrook et al., 1989) with ampicillin (Sigma, USA; 100 μg/mL) or kanamycin (Sigma, USA; 50 μg/mL) added as required.

1.2 DNA manipulations

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) unless otherwise indicated. Total DNA isolated from *Bacillus* sp. CDB3 (Chopra et al., 2007) was partially digested with *Sau*3AI and fragments between 2 and 8 kb were isolated after separation on a low-melting point agarose gel (0.8%) and cloned into pJKKmf(-) (Kirschman and Cramer, 1988) or pGEM7Zf(+) (Promega, USA) which had been digested with *Bam*HI and subjected to dephosphorylation by calf intestine alkaline phosphatase. Arsenic resistant transformants of *E. coli* strain AW3110 (Carlin et al., 1995) were selected on LB agar containing kanamycin or ampicillin and 4 mol/L arsenate. DNA sequencing reactions were carried out using the Big Dye Terminator v3.1 cycle Sequencing kit (Perkin-Elmer, USA) following the manufacture's instructions and the extended DNA fragments analyzed on an ABI PRISM^{TM} 377 DNA sequencer (Applied Biosystems, USA). A primer-walking strategy was taken and all the primers were synthesized by Sigma (USA) according to progressively obtained sequence data. The DNA sequences reported in this article have been submitted to GenBank under the accession numbers AF178758 and GQ866968.

1.3 Large-plasmid separation by gel electrophoresis and DNA hybridization

One milliliter of CDB3 cultured overnight was lysed and subjected to the agarose gel eletrophoresis-based detection for large plasmids as described by Kado and Liu (1981). For genomic DNA hybridization, the isolated DNA was digested with an appropriate restriction enzyme before gel electrophoresis. After gel electrophoresis, DNA was

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
E. coli AW3110	K-12 F-IN (*rrn*D-*rrn*E) *ars*::*cam* (Cm^r, the chromosomal *arsRBC* deleted)	Carlin et al., 1995
E. coli JM109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (rk$^-$, mk$^+$), *sup*E44, *rel*A1, Δ(lac-proAB), [F', *tra*D36, proAB, *lac*IqZΔM15]	Promega, USA
Bacillus sp. CDB3	Isolated from cattle dip-sites	Chopra et al., 2007
pJKKmf(-)	Cloning vector (Km^r)	Kirschman and Cramer, 1988
pGEM7Zf(+)	Cloning vector (Ap^r)	Promega, USA
pRYCDATORF7,8	A 7600 bp *Sau*3AI fragment containing the CDB3 *ars* cluster 1 cloned into pGEM7Zf(+) vector at *Bam*HI site	This study
pR2ORF2Y2C2	A 4557 bp *Sau*3AI fragment containing the CDB3 cluster 2 cloned into pGEM7Zf(+) vector at *Bam*HI site	This study
pRYCD$^{\Delta}$ATORF7,8	A 105 bp sequence of the *arsD* deleted from pRYCDATIP	This study

transferred from gel to a nylon membrane (HybondTM-N^+, Amersham, UK) in 0.4 mol/L NaOH. DNA probes were prepared using a PCR DIG-labeling kit (Roche, Switzerland) with specifically PCR-amplified DNA fragments as templates. Southern hybridization was carried out in DIG easy hybridization solution (Roche, Switzerland)) and washed according to the manufacture's instruction.

1.4 Arsenic and antimony resistance assays

Overnight cultures of *E. coli* AW3110 strains harboring different plasmid constructs were diluted 100 fold in fresh LB medium containing appropriate antibiotics and varying amounts of sodium arsenate, sodium arsenite or potassium antimonite (Sigma, USA). Cells incubated at 37°C for 5–7 hr were measured for growth rate under metalloid stress as described previously (Chopra et al., 2007).

1.5 Sequence similarity search and comparison

Searching for homologous sequences in data bank was carried out using Blast programs (Altschul et al., 1997). Homologous sequences to the cloned CDB3 *ars* genes from different organisms were retrieved from Genbank and multiple alignments were conducted using ClustalW (Thompson et al., 1994).

1.6 Generation of gene deletion constructs

To generate loss-of-function *arsD* mutants, pRYC-DATIP was digested with *Rsr*II which cleaves *arsD* uniquely at 53 bp downstream of the start codon ATG. An Erase-A-Base deletion kit (Promega, USA) utilizing *Exo*III/*S*1 nucleases was employed to delete nucleotides to both directions from the cut point and then plasmid was self-ligated after a Klenow fill-in reaction.

2 Results and discussion

2.1 Two *ars* clusters cloned from *Bacillus* sp. CDB3

A large number of *E. coli* AW3110 transformants exhibited elevated arsenic resistance on plates after the shotgun cloning. Restriction enzyme digestion mapping of 61 such resistant clones revealed two distinct classes; 33 of them were mapped to one and 28 to the other. Sequencing data obtained from a few overlapping clones of each compiled 7071 and 3512 bp, respectively, revealing two different gene clusters. Cluster 1 consists of eight open reading frames and cluster 2 consists of four. Southern blot and PCR analyses confirmed that the gene arrangements in the two clusters cloned are the same as in the CDB3 bacterial genome (result not shown).

2.2 Sequence homology and characters

Out of the twelve ORFs recognized in the two clusters, ten (six in cluster 1 and all four in 2) were found to specify proteins showing significant homologies to proteins encoded by known *ars* genes (Table 2). In cluster 1, *orf 1, 2, 3, 4* and *5* encode protein homologs of characterized ArsR, YqcL (ArsY), ArsC, ArsD and ArsA, respectively. The predicted ArsD matched well in sequence with the functionally well characterized *E. coli* R773 ArsD except lacking two pairs of cysteines near the C-termini ($Cys^{112,113}$ and $Cys^{119,120}$).

Orf6 of cluster 1 is predicted to specify a protein highly homologous to thioredoxin reductase (TrxB) with all the domains conserved (not shown). Bearing in mind that the ArsCsa family of arsenate reductases to which CDB3 ArsC belongs (Table 2) require thioredoxin hence thioredoxin reductase for regeneration in reaction (Messens et al., 2002) it is not a surprise to find a gene in the cluster coding for a *TrxB* (called *arsT*). Recently such a gene has also been identified in *ars* operons of *Streptomyces* spp. (Wang et al., 2006). It is interesting, however, to note that CDB3 is the only *Bacillus* strain so far known to contain *trxB* in an *ars* cluster although several *Bacillus* strains belonging to different species possess otherwise identical clusters.

The two putative proteins (ORF7 and ORF8) did not match any known *ars* gene products. ORF7 was found to contain a HesB particularly sharing sequence similarities with IscA-like proteins (Fig. 1a). The sequence homology search of ORF8 points to the protein tyrosine phosphatase super family, especially the dual-specificity protein phosphatase (DSP) group, with best hits to some characterized mammalian mitochondrial protein tyrosine phosphatases PTPMT1 (Pagliarini and Dixon, 2006) (Fig. 1b). IscA, as an iron-chaperon, is involved in the biosynthesis of iron-sulfur clusters which are required by many proteins for their cellular function (Ayala-Castro et al., 2008). Oxidative damage resulted from arsenic stress to bacteria can cause instability of Fe-S clusters (Parvatiyar et al., 2005) and this may justify the inclusion in an *ars* cluster

Table 2 Sequence homologies (% of similarity) of theoretical translation products of CDB3 *ars* clusters 1 and 2 to known Ars proteins[a]

Source of homologous sequence	ArsR	ORF2	ArsB	ArsC	ArsD	ArsA	ArsT
CDB3 *ars* cluster 1							
CDB3 *ars* cluster 2	52.8	–[b]	76.9, YqcL	67.7, ArsCsa	–	–	–
Halobacterium sp. pNRC100	29.2	–	–	22.9	34.6	34.2	–
Acidophillum multivorum pKW301	25.2	–	21.0	28.1	37.6	49.7	–
Escherichia coli pR773	33.0	–	22.3	20.5	35.0	50.4	–
Bacillus subtilis Skin element	42.3	–	85.6, YqcL	75.2, ArsCsa	–	–	–
Staphylococcus aureus pI258	40.6	–	19.3	70.1, ArsCsa	–	–	–
Streptomyces coelicolor A3(2)	50.0	–	69.0, YqcL	49.0, ArsCsa	–	–	63.4
CDB3 *ars* cluster 2							
Escherichia coli pR773	44.7	–	41.5	42.0	–	–	–
Bacillus subtilis Skin element	89.0	77.0	85.0, YqcL	72.0, ArsCsa	–	–	–

[a] The YqcL type of arsenite transporters and ArsCsa type of arsenate reductases are indicated. [b] "–": protein not found.

a

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E. coli IscA          MSITLSDSAAARVNTFLANRGKGFGLRLGVRTSGCSGMAYVLEFVDEPTPEDIVFEDKGV
A. ferooxidans IscA   MALTLSESAARQVRKSIAKRGKGLGIRIGVKTSGCSGLSYVMEFVDVPNPEDLVFPHDDV
B. sp. CDB3 ORF7      --MNITDKAKEFIETAMKENGVST-LRFTFDGAGCCGPSYGINLGEAQE-NDVTETVNGI
                               *           *     *      ** *  *           *

E. coli IscA          KVVVDGKSLQFLDGTQLDFVKEGLNEGFKFTNPNVKDECGCGESFHV 107
A. ferooxidans IscA   NLFVDPKSLIYLDGTELDFTREGLNEGFRFNNPNVKDACGCGESFTT 107
B. sp. CDB3 ORF7      QVAMDPKVAEIVNTLTLDYVEDQQGAGLVISG---GSNC-C------  93
                          * *         **        *           * *
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b

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M. musculus PTPMT1    MAASAWLEAGLARVLFYPTLLYTVFRGRVRGPAHRDWYHRIDHTVLLGALPLKNMTRRLV
H. sapiens PTPMT1     MAATALLEAGLARVLFYPTLLYTLFRGKVPGRAHRDWYHRIDPTVLLGALPLRSLTRQLV
B. sp. CDB3 ORF8      -----------------MTNYHELVKGKV----------------YIGGV----------
                                      *       *                    *

M. musculus PTPMT1    LDENVRGVITMNEEYETRFLCNTSKEWKKAGVEQLRLSTVDMTGVPTLANLHKGVQFALK
H.sapiens PTPMT1      QDENVRGVITMNEEYETRFLCNSSQEWKRLGVEQLRLSTVDMTGIPTLDNLQKGVQFALK
B. sp. CDB3 ORF8      --DAIQDAVKKHGVTEVFDLRSGGQEPEGFPVEAKRHAYPIVEGVEGEDESVKNAIGAVK
                                 *   *     *     **  *       *             * *

M. musculus PTPMT1    YQ-ALGQCVYVHCKAGRSRSATMVAAYLIQV-HNWSPEEAIEAIAKIRSHISIRPSQLEV
H. sapiens PTPMT1     YQ-SLGQCVYVHCKAGRSRSATMVAAYLIQV-HKWSPEEAVRAIAKIRSYIHIRPGQLDV
B. sp. CDB3 ORF8      EAVERGEKVFFHCSGGRNRTGTVATGLLVELGHASNVEEAEQKVKEIRSIINIKPEMKQV
                            *  *  **  ** *  *     *    *    ***      *** * * *    *

M. musculus PTPMT1    LKEFHKEITARAAKN         193
H. sapiens PTPMT1     LKEFHKQITARATKDGTFVISKT 201
B. sp. CDB3 ORF8      LKRLYV                  141
                      **
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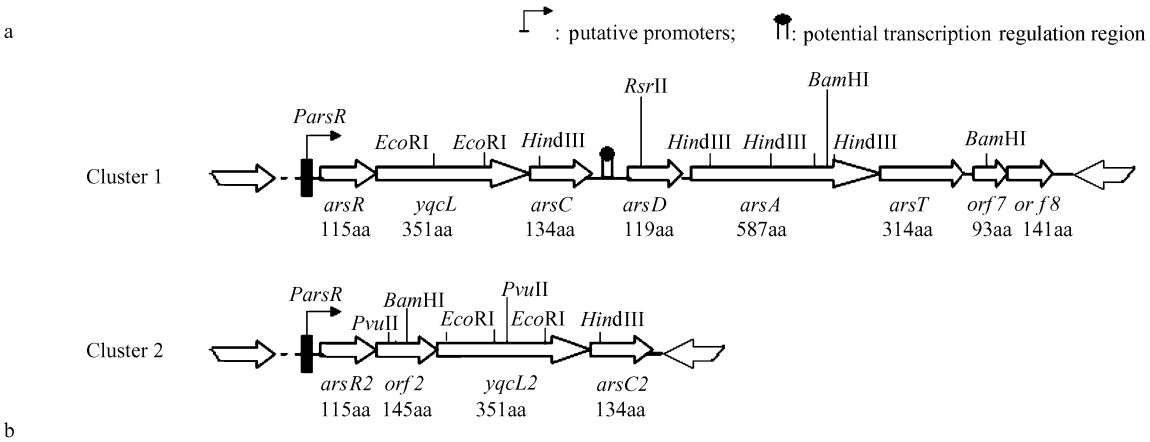
Fig. 1 Sequence alignments (Clustal W; Thompson et al., 1994) of the predicted CDB3 cluster 1 ORF7 (a) and ORF8 (b) proteins, respectively, with functionally known homologous proteins. An asterisk indicates all proteins have an identical residue, a blank space indicates a sequence gap. The number at the end of each sequence indicates the length of the protein. The conserved active site signature motif HCXXGXXR of protein tyrosine phosphatases is highlighted. The Accession numbers for the aligned proteins are: IscA of *E. coli* W3110, BAA16422; IscA of *Acidithiobacillus ferrooxidans* ATCC 23270 ACK78248; protein-tyrosine phosphatase (mitochondrial 1; PTPMT1) of mouse, Q66GT5; and protein-tyrosine phosphatase (mitochondrial 1, isoform 1) of human, Q8WUK0.

of a gene which can contribute to the production of these clusters. The need of thioredoxin system for iron binding to IscA (Ding et al., 2005) may also link the function of TrxB encoded by the upstream *arsT* gene. However, while bacterial Fe-S cluster assembly genes (*isc* or *suf* systems) have been found clustered together in operons, no other *isc* genes were recognized near the putative *arsI* gene (unpublished). Its function hence remains to be elucidated. Interestingly, *orf8* has been found to specify a polypeptide of 141 amino acids very homologous to protein tyrosine phosphatases, especially the subgroup of dual specificity protein phosphatases (Fig. 1b). There was no evidence that protein phosphorylation is involved in arsenic resistance. Evolutionarily, PTPs are thought to be ancestors of arsenic reductases. Through protein structural studies, many arsenic reductases have been found to belong to the protein tyrosine phosphatase super family and some even still possess the phosphatase activity (Mukhopadhyay et al., 2003). However, in *Bacillus* sp. CDB3, an *arsC* gene is already present in the gene cluster. It will be interesting to reveal weather the *orf8* gene product has the enzymatic activity of phosphatase and more importantly weather it actually functions in cell under the metalloid stress.

The four predicted proteins encoded by cluster 2 are highly homologous to the products of *arsR*, *orf2*, *yqcL* and *arsC* identified on the *B. subtilis* SKIN element (Sato and Kobayashi, 1998) (Table 2). The amino acid sequence identities between the two sets of predicted ArsR, YqcL and ArsC specified by the CDB3 operons 1 and 2 are 52.8%, 76.9% and 67.7%, respectively (Table 2). Both of the putative CDB3 arsenate reductases showed much higher identities to the ArsCsa family than to ArsCec family, indicating they may require therodoxin rather than glutaredoxin for reduction catalysis (Messens et al., 2002).

As shown in Fig. 2, at the beginning of each cluster, there is a promoter region similar to most known *ars* operons. However, an invert repeat of 9-bases also exists between the *arsC* and *D* coding sequences of cluster 1 (Fig. 2c). There are also some interesting organizational characters in CDB3 *ars* cluster 1. *arsD* and *arsA* are located downstream of *arsC*, rather than between *arsR* and *arsB* (*ycqL*) as other *arsD* and *arsA* containing clusters, for example, the *E. coli* pR773 *ars* operon. This gene organization seems conserved among *Bacillus* species (search result not shown). The intergenic region between *arsC* and *arsD* possesses a 9-bp inverted repeat that may function as a transcription regulatory region, raising the possibility that this group of arsenic resistance genes may not be expressed as a single operon simply like cluster 2 and most other bacterial *ars* clusters, but involve some complicated



b

Cluster 1: 5'-TACGTN$_{17}$TATAAGN$_{36}$ATG

-35 -10

Cluster 2: 5'-ATTAAGN$_{19}$TATAAAN$_{35}$GTG

-35 -10

c

5'-$_{19}$AGCCGAGAAN$_5$TTCTCGGCTN$_{29}$

Fig. 2 Organizations and restriction maps of two *ars* gene clusters present in *Bacillus* sp. CDB3. The protein length (amino acid residue numbers) of each predicted *ars* gene product is presented. Some restriction enzyme sites and putative transcription regulatory elements are labeled. (b) Putative promoter regions identified in both cluster 1 and 2. (c) Sequence of an invert repeat present in the intergenic region between *arsC* and *arsD* of cluster 1.

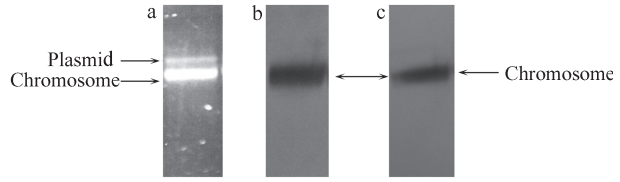


Fig. 3 Southern blot analysis to localize CDB3 *ars* clusters 1 and 2. (a) Ethidium bromide stained 0.7% TBE agarose gel image showing the separation of plasmid(s) from the chromosomal DNA of CDB3; (b) X-ray film showing specific band corresponding to the chromosomal DNA after hybridization with probe 1 (cluster 1 specific); (c) X-ray film showing specific band corresponding to the chromosomal DNA after hybridization with probe 2 (cluster 2 specific).

mechanism.

2.3 Chromosomal localization of CDB3 ars clusters 1 and 2

After gel separation, a clear band was observed above the CDB3 chromosomal DNA band (Fig. 3a) suggesting some large plasmid(s) may be present in the bacterium. DNA blot analysis was carried out to localize the two *ars* clusters. Both DIG labeled cluster-specific probes 1 and 2 hybridized to the lower chromosomal band (Fig. 3b, c), indicating that both CDB3 *ars* clusters 1 and 2 are located on the bacterial chromosome.

2.4 Metalloid resistance of *E. coli* strains transformed with CDB3 ars clusters

Both *Bacillus* CDB3 *ars* clusters functioned in *E. coli*, as they were identified in arsenic-resistance elevated transformants. By comparison, transformants of *E. coli* AW3110 harboring plasmid pRYCDATIP (cluster 1) showed a much higher resistance to arsenate than those harboring pR2ORF2Y2C2 (cluster 2) (Fig. 4a). Only moderate resistance to antimonite at concentrations 0.2–0.4 mol/L was detected with AW3110/pRYCDATIP (Fig. 4b). This result is in accordance with the test results of *S. cerevisiae* which employs ACR3 to extrude arsenite (Bobrowicz et al., 1997) and *B. subtilis* employing YqcL (Sato and Kobayashi, 1998). While the ArsB type of arsenite transporters (represented by the *E. coli* ArsB pump) can extrude both arsenite and antimonite out of bacterial cells, the YqcL type of arsenite transporters is assumed with no function on antimonite transport in general. Worth noting is also the presence of *arsA* gene in cluster 1, first case for its link to a functional *yqcL*. Assumbly, its product will couple with the YqcL transporter to pump out arsenite, same as the functional coupling of ArsA and ArsB in *E. coli* (Rosen et al., 1999), although still exists the possibility that there may be other *arsB* gene(s) present in CDB3 which can interact with *arsA* gene.

2.5 Function of CDB3 *arsD*

Sequence alignment of the known ArsD molecules (Lin et al., 2007) indicated the universal conservation of Cys^{12}, Cys^{13} and Cys^{18}, and only partial conservation of other cysteine residues. Since CDB3 ArsD lacks the two C-terminal vicinal pairs of cysteine ($Cys^{112,113}$ and $Cys^{119,120}$) and at least one pair have been demonstrated to be required for the repressor function of *E. coli* ArsD (Li et al., 2001), we generated *E. coli* AW3110/pRYCD$^{\Delta}$ATIP and examined its degree of resistance to arsenic in comparison with *E. coli* AW3110/pRYCDATIP. The decline in resistance of the ArsD mutant strain confirms that

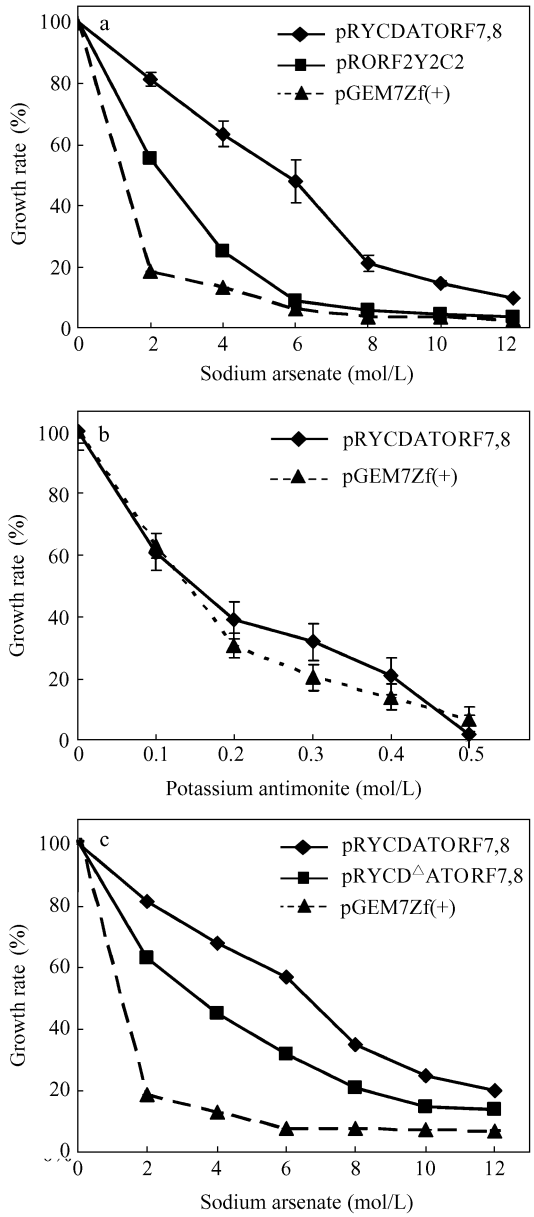


Fig. 4 Growth inhibition by arsenic and antimony of *E. coli* AW3110 harboring different *ars* gene-containing plasmids. (a) Comparison of growth between AW3110/pRYCDATORF7,8 (CDB3 *ars* cluster 1), /pR2ORF2Y2C2 (CDB3 *ars* cluster 2) and /pGEM7Zf(+) (vector control) in sodium arsenate-containing LB media; (b) comparison of growth between AW3100/pRYCDATORF7,8 and /pGEM7Zf(+) in potassium antimonite-containing media; (c) growth inhibition by sodium arsenate of *E. coli* AW3110 harboring plasmids pRYCD$^{\triangle}$ATORF7,8 (D knock-out), pRYCDATORF7,8 and pGEM7Zf(+).Vertical bars representing the standard deviation (n = 3) are mostly small enough to be hidden behind the data points.

CDB3 ArsD is functional (Fig. 4c). The *E. coli* ArsD has recently been found to act as a metallochaperone and those C-terminal cycteine residues are not essential for this chaperone activity (Lin et al., 2006; Lin et al., 2007). With this regard, it is reasonable to assume that CDB3 ArsD is also a metallochaprone for arsenite. However, its role as the second transcriptional repressor, lacking the two C-terminal vicinal pairs of cysteine, warrants further investigation.

3 Conclusions

Bacillus sp. CDB3 represents a rare bacterium which harbors two functional *ars* gene clusters. The eight-gene cluster 1 appears to be the largest characterized so far in bacteria and features by possessing both novel genes and a possible subtle regulatory mechanism. This study has revealed another novel *ars* gene cluster and again demonstrated the diversity of *ars* operons in controlling the arsenic resistance of bacterial cells. Further work is in progress to study the function of these novel genes and possible subtle regulatory mechanism.

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