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Characterization of the *ars* Gene Cluster from Extremely Arsenic-Resistant *Microbacterium* sp. Strain A33▽†

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The arsenic resistance gene cluster of *Microbacterium* sp. A33 contains a novel pair of genes (*arsTX*) encoding a thioredoxin system that are cotranscribed with an unusual *arsRC2* fusion gene, *ACR3*, and *arsC1* in an operon divergent from *arsC3*. The whole *ars* gene cluster is required to complement an *Escherichia coli ars* mutant. ArsRC2 negatively regulates the expression of the pentacistronic operon. ArsC1 and ArsC3 are related to thioredoxin-dependent arsenate reductases; however, ArsC3 lacks the two distal catalytic cysteine residues of this class of enzymes.

Arsenic is widely dispersed in the environment and occurs primarily in two oxidation states, arsenate [As(V)] and arsenite [As(III)], and both are toxic to the majority of living organisms. The frequent abundance of arsenic in all environmental compartments has guided the evolution of detoxification systems in almost all microorganisms. Of these, the arsenic resistance system (*ars*) appears to be widely distributed among prokaryotes. It involves an arsenate reductase (ArsC), an arsenite efflux pump (ArsB or ACR3), and a transcriptional repressor (ArsR) (32), encoded by a set of genes that display large variations in their number and genomic organization. The early identified *ars* system of *Escherichia coli* plasmid R773 (41) has two additional components, ArsA, which acts as the catalytic subunit of the ArsAB arsenite extrusion pump (33), and ArsD, a metallochaperone protein that transfers As(III) to ArsA (18). In addition to these well-studied *ars* components, a variety of *ars* clusters contain additional genes whose functions in arsenic resistance have not been clearly established in many cases (31).

Members of the *Microbacterium* lineage of actinobacteria that can tolerate various metals, including nickel, chromium, and uranium (1, 16, 25), have been isolated from metal-rich environments. New examples of arsenic-resistant isolates of *Microbacterium* are continuously being reported (1, 2, 8, 10, 12, 21). In each case, however, the tolerance mechanism was not investigated, probably due to the lack of efficient genetic systems in this genus. Among actinobacteria, only *Streptomyces* sp. FR-008 (40) and *Corynebacterium glutamicum* ATCC 13032 (28) have been subjected to molecular characterization of determinants of defense against arsenic. In the former, the linear plasmid pHZ227 carries an arsenic resistance gene cluster with two novel genes, the *arsO* and *arsT* genes, which encode a putative flavin-binding monooxygenase and a putative thioredoxin reductase, respectively (40). The latter strain was recently shown to possess two members of a new class of arsenate reductases (Cg_ArsC1 and Cg_ArsC2) (30) and a transcriptional repressor (Cg_ArsR1) with a metalloid binding site unrelated to other previously characterized members of the ArsR/SmtB metalloregulatory proteins (29).

The present study focuses on *Microbacterium* sp. strain A33, a soil isolate previously shown to tolerate high concentrations of arsenite and arsenate (2). Here, we report on the isolation and functional characterization of an original set of arsenic resistance genes from this strain. Distinctive attributes of the *ars* cluster include the placement of two genes encoding a thioredoxin (Trx) system within the cluster, one gene encoding a putative Trx-coupled arsenate reductase lacking two catalytic cysteines, and a fusion gene whose product is a multidomain protein consisting of a transcription factor and an arsenate reductase.

Resistance of *Microbacterium* sp. strain A33 to arsenic. *Microbacterium* sp. strain A33 was assayed for tolerance toward arsenic oxyanions in TRIS low-phosphate (LP) medium (27). Overnight cultures were diluted 1:100 in fresh LP medium supplemented with increasing concentrations of arsenate [As(V)] or arsenite [As(III)] and incubated at 28°C for 24 h before measurement of absorbance at 600 nm (A_{600}). The concentrations of As(V) and As(III) that caused 50% growth inhibition (IC_{50}s) were 400 mM and 28 mM, respectively. Cells were still able to grow (5% of maximum growth; A_{600} = 0.15) at 800 mM As(V) and 50 mM As(III), even without preexposure to lower concentrations of the oxyanions (Fig. 1). Similar and higher levels of tolerance to arsenite have been reported only for a few microorganisms, including *Ochrobactrum tritici* (6), *Ferroplasma acidarmanus* (5), an arsenite oxidizing bacterium identified as *Microbacterium lacticum* (24), and, recently, several *Microbacterium* isolates retrieved from deep-sea sediments (10). To our knowledge, however, the arsenate resistance level exhibited by *Microbacterium* sp. A33 appears to be the highest described so far.

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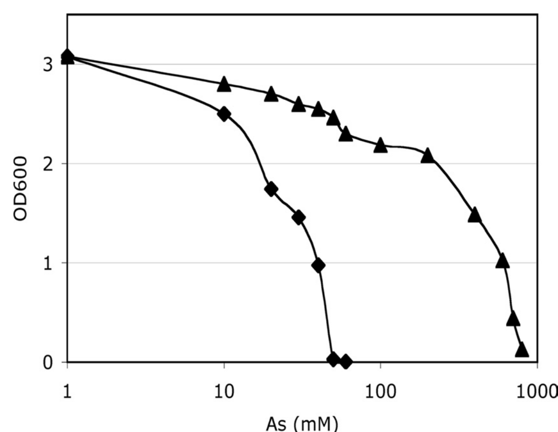


FIG. 1. Arsenic tolerance of *Microbacterium* sp. strain A33. Resistance to arsenate (▲) and arsenite (◆) was assayed by monitoring growth (OD_{600}) in low-phosphate medium after 24 h of incubation. Data are representative of two independent experiments.

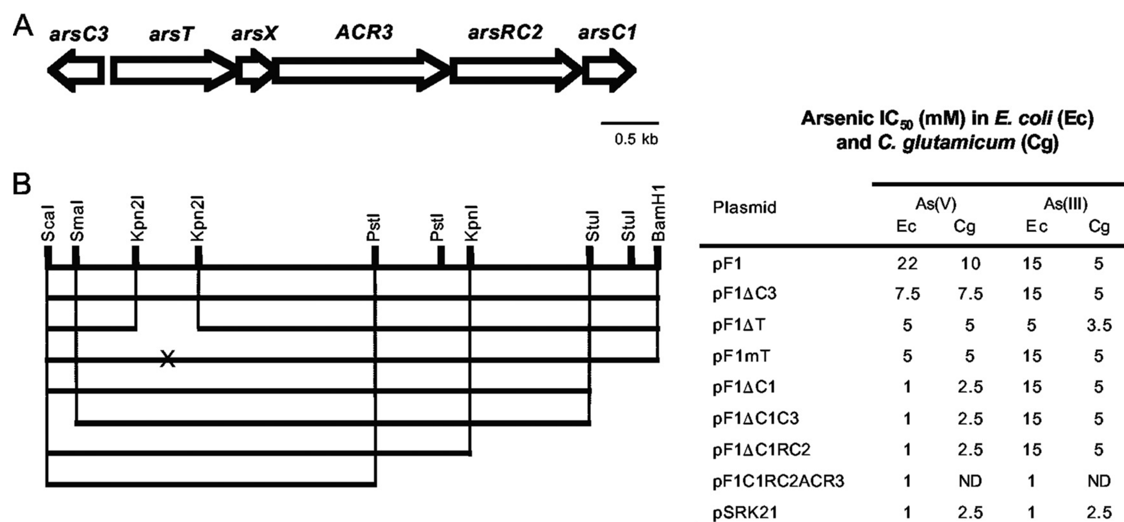
Cloning arsenic resistance genes by heterologous complementation. To investigate the nature of the genetic determinants responsible for the extreme arsenic tolerance of *Microbacterium* sp. A33, a genomic library was constructed by ligation of partially digested Sau3A fragments (7 to 10 kb) in the BamHI site of plasmid pSRK21 (39) and was subsequently introduced into the arsenic-sensitive strains *E. coli* MA1 (Table 1) and *Corynebacterium glutamicum* ArsB1-B2 (28) by electroporation (11, 38). *E. coli* transformants were selected on LB plates supplemented with 4 mM As(V) or 2 mM As(III), while a concentration of 12 mM As(III) was used for *C. glutamicum*, which is intrinsically less sensitive to arsenic than *E. coli* (28). A total of 126 arsenic-resistant transformants (91 with *E. coli* and 35 with *C. glutamicum*) were obtained and further analyzed for their plasmid content. Restriction mapping revealed that they all had common regions. Furthermore, PCR amplification using the dacr1F and dacr1R primers (2) produced a single amplicon of 0.75 kb in each case, indicating that all clones possessed the targeted *ACR3* gene. Low-stringency Southern hybridization experiments with *Microbacterium* sp. A33 total DNA using this PCR fragment as a probe confirmed that the strain contains only one copy of the *ACR3* gene (data not shown).

Sequence analysis of the *ars* cluster. The DNA region encompassing the *ACR3* gene was sequenced from a subset of clones of the gene library conferring arsenate and arsenite resistance to both *E. coli* and *C. glutamicum*. Six putative genes were identified, five of which were designated *arsC3*, *arsT*, *ACR3*, *arsRC2*, and *arsC1* on the basis of their homology to known *ars* genes, and the remaining gene was called *arsX*. The organization of this six-gene cluster is depicted in Fig. 2A. *arsC3* is transcribed divergently from the five other genes which appear to form an operon (see the transcription analyses below). An extensive BLAST search against the NCBI nr database was performed for the six putative encoded proteins.

ArsC1 and ArsC3 were found to share sequence similarity to Trx-dependent arsenate reductases from high-G+C-content, Gram-positive bacterial groups, such as *Streptomyces* sp. FR-008 (40) (70% and 40% amino acid identity, respectively), and, to a lesser extent, to the archetypical ArsC protein from *Staphylococcus aureus* plasmid pI258 (37% and 28% amino acid identity, respectively). Both proteins possess the consensus N-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or genotype	Reference or source
Strains		
Microbacterium sp. A33	Soil isolate, high arsenic resistance	2
C. glutamicum ArsB1-B2	Double arsenite permease mutant derivative from *C. glutamicum* RES167	28
E. coli W3110	K-12 F^- IN(*rrnD-rrnE*)	4
E. coli AW3110	W3110 Δ*ars*::*cam*	9
E. coli DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*araA leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
E. coli LF20012	*arsB*::*luxAB* chromosomal fusion; *ars* Tet^r	7
E. coli MA1	*ars* derivative of DH10B prepared by P1*vir* transduction from LF20012; Tet^r	This study
E. coli JW3470	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) LAM^- Δ*arsC759*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	3
Plasmids		
pSRK21	*E. coli*/*C. glutamicum* shuttle vector; Km^r	39
pBAD18	*E. coli* cloning and expression vector; Amp^r	15
pPROBE-NT	Broad-host range promoter-probe vector; Km^r	23
pF1	pSRK21 carrying the whole *ars* gene cluster	This study
pF1ΔC3	pF1 with a 0.2-kb SmaI-XbaI fragment (containing *arsC3*) deletion	This study
pF1ΔT	pF1 with a 0.49-kb Kpn2I fragment (containing *arsT*) deletion	This study
pF1ΔC1	pF1 with a 0.24-kb Eco147I fragment (containing *arsC1*) deletion	This study
pF1ΔC1RC2	pF1 with a 1.26-kb KpnI fragment (containing *arsC1-RC2*) deletion	This study
pF1ΔC1RC2ACR3	pF1 with a 1.9-kb PstI fragment (containing *arsC1-RC2-ACR3*) deletion	This study
pF1mT	pF1 with *arsT* Cys137 and Cys140 codons replaced by serine codons	This study
pT-GFP	PCR amplified *arsT-arsC3* intergenic region obtained with ProT/ProC3 primers inserted into pPROBE-NT; *arsT-gfp* fusion	This study
pC3-GFP	PCR-amplified *arsT-arsC3* intergenic region obtained with ProT/ProC3 primers inserted into pPROBE-NT; *arsC3-gfp* fusion	This study
pBAD-arsRC2	PCR-amplified *arsRC2* gene obtained with ExpRC2F/ExpRC2R primers cloned in pBAD18	This study



Arsenic IC_{50} (mM) in *E. coli* (Ec) and *C. glutamicum* (Cg)

Plasmid	As(V)		As(III)	
	Ec	Cg	Ec	Cg
pF1	22	10	15	5
pF1ΔC3	7.5	7.5	15	5
pF1ΔT	5	5	5	3.5
pF1mT	5	5	15	5
pF1ΔC1	1	2.5	15	5
pF1ΔC1C3	1	2.5	15	5
pF1ΔC1RC2	1	2.5	15	5
pF1C1RC2ACR3	1	ND	1	ND
pSRK21	1	2.5	1	2.5

FIG. 2. Organization and functional analysis of the *ars* gene cluster of *Microbacterium* sp. strain A33. (A) Open arrows show gene orientations. (B) The restriction map of the BamHI-ScaI DNA fragment cloned into the vector pSRK21 to yield pF1 is indicated. For functional mapping, deletion mutants of pF1 were constructed and tested for their ability to confer arsenate [As(V)] and arsenite [As(III)] resistance to *E. coli* AW3110 (Ec) and *C. glutamicum* ArsB1-B2 (Cg). Values are presented as IC_{50}s. The X symbol in construct pF1mT indicates mutations of two cysteine codons introduced in *arsT* by site directed mutagenesis. ND, not determined.

terminal presumed CX_5R oxyanion-binding motif of Trx-coupled arsenate reductases (22). However, only the ArsC1 protein has the two additional cysteines (Cys88 and Cys91) required for the catalytic activity of this family of arsenate reductases. In addition to *arsC1* and *arsC3*, a third *arsC*-like sequence fused to an *arsR*-like sequence (*arsRC2*) that could encode a single polypeptide of 331 amino acids was identified. The C-terminal portion (approximately 140 amino acids) of the predicted ArsRC2 protein is related to Trx-linked arsenate reductases from actinomycetes, like the above-mentioned ArsC1 protein. The three putative ArsC proteins share rather low sequence similarity, the best level of identity (52%) being between ArsC1 and the C-terminal domain of ArsRC2 and the least (39%) between ArsC1 and ArsC3. The 120-amino-acid N-terminal portion of ArsRC2 shows clear homology to transcriptional regulators of the ArsR family and shares 53% sequence identity with its closest homologue, ArsR from *Thermobifida fusca* XY (YP_289615.1). This domain contains both a putative arsenite binding motif (ESCVCDL) nearly identical to that of *E. coli* R773 ArsR and an adjacent DNA binding helix-turn-helix motif (35). *ACR3* is located immediately upstream of *arsRC2*. It encodes a putative arsenite transporter of the Acr3(1)p subfamily (2) showing significant homology with previously characterized ArsB1 from *C. glutamicum* (58.2% identity) and Acr3p from *Streptomyces* sp. FR-008 (66.3% identity) (40).

The remaining genes (*arsT* and *arsX*) are located proximally in the *arsT-arsX-ACR3-arsRC2-arsC1* cluster. They were found to overlap by a 4-bp sequence involving fusion of the termination codon of *arsT* with initiation codon of *arsX* (ATGA), suggesting that they are translationally linked. An identical 4-bp overlap was also observed between *arsX* and *ACR3*. ArsT is homologous to thioredoxin reductases and mostly relates to those from actinobacterial groups such as *Brevibacterium linens* BL2 (65.7% identity to ZP_00377913) or the recently identified ArsT protein from *Streptomyces* sp. FR-008 (58.9% identity). ArsX is a member of the thioredoxin family. As with the ArsT protein, the closest homolog of ArsX is from *B. linens* BL2 (72% identity to ZP_00377914). However, BLAST searches revealed that these two putative thioredoxins are more distantly related to other actinobacterial thioredoxins than those from alphaproteobacteria, which showed the best hits (data not shown). To our knowledge, this is the first description of a bacterial *ars* cluster harboring genes for a putative thioredoxin system.

Functional characterization of the *ars* cluster. A BamHI-ScaI DNA fragment carrying the whole *ars* cluster was cloned in the pSRK21 vector to yield plasmid pF1. Deletion mutagenesis was used to elucidate the function of the *ars* genes that contributed to arsenic tolerance. For this purpose, plasmid pF1 was digested with restriction endonucleases as indicated in Fig. 2B and Table 1. The constructs, as well as pF1 and empty pSRK21 control plasmids, were inserted into *E. coli* AW3110, a strain lacking the chromosomal *arsRBC* operon (9), and the transformants were assayed for arsenic resistance in LP medium. Introduction of pF1 in cells of AW3110 led to a restoration of both arsenate and arsenite resistances (Fig. 2B). Compared to what was observed for pF1, deletion of *arsC1* to yield plasmid pF1ΔC1 completely abolished resistance to As(V) but not to As(III). Removal of *arsC3* (pF1ΔC3) resulted in a similar phenotype; however, only partial loss of As(V) resistance was observed. These results support the hypothesis that ArsC1 and ArsC3 function as arsenate reductases, which are known to be required for As(V) detoxification. ArsC1 appears to be the major contributor of arsenate resistance in *E. coli.* Cells carrying plasmid pF1ΔC1RC2 (lacking *arsC1* and *arsRC2*) exhibited the same levels of resistance to As(V) and As(III) as those containing pF1ΔC1; therefore, no clear function could be inferred from that construct for ArsRC2. Extending the deletion to include *ACR3* (pF1ΔC1RC2ACR3)

resulted in a total loss of tolerance toward As(III) and As(V). This is reminiscent of what is observed in most bacterial *ars* systems when arsenite transporter genes are inactivated, i.e., hypersensitivity to arsenite and arsenate due to the inability of cells to extrude As(III) subsequent to As(V) reduction by ArsC. Cells transformed with pF1ΔT (lacking *arsT*) partly lost resistance to arsenate, in agreement with the predicted function of ArsT as a thioredoxin reductase and its possible involvement in the electron transfer to arsenate reductase. However, the deletion also led to a decreased level of arsenite resistance, which may be attributed to a polar effect on downstream genes. To test this possibility, Cys137 and Cys140 residues within the highly conserved CXXC motif that characterizes the active site of thioredoxin-related proteins were replaced with serine residues by using a megaprimer-based mutagenesis method (37). The first PCR product obtained with the proC3/C137-140Santi primer pair (see the supplemental material) was used as a megaprimer, together with primer TR, in a second round of PCR. The resulting product was digested with Kpn2I to give a mutated fragment, which was inserted into the Kpn2I site of pF1ΔT. *E. coli* harboring this *arsT*-mutated plasmid (pF1mT) displayed the same reduction in arsenate resistance level as with pF1ΔT, while tolerance to arsenite remained unaffected (Fig. 2B). This result provides substantial evidence that ArsT contributes to arsenate tolerance via a thioredoxin reductase activity that depends on the presence of the CXXC redox motif. Heterologous expression of the various *ars* constructs in the arsenic-sensitive strain *C. glutamicum* ArsB1-B2 produced the same phenotypes as those observed in AW3110, albeit with a less pronounced effect (Fig. 2B).

The ability of ArsRC2 to play a role in arsenate resistance in *E. coli* was tested with *E. coli* JW3470, a strain lacking *arsC* (3). The complete *arsRC2* coding region was amplified from plasmid pF1 by using the ExpRC2F/ExpRC2R primer pair, digested with HindIII and XbaI, and ligated into plasmid pBAD18 (15) under the control of the P_{BAD} promoter to create pBAD-arsRC2. Cells of JW3470 harboring pBAD-arsRC2 or pBAD18 exhibited the same low level of arsenate resistance whether or not they were cultured under inducing conditions, i.e., with added 0.2% arabinose. Considering that the fused gene product is actually produced in the pBAD-arsRC2 construct (see below), the inability to complement the *arsC* mutant suggests that ArsRC2 has no significant arsenate reductase activity in *E. coli*.

Transcription analysis. Reporter gene studies using *arsT-gfp* and *arsC3-gfp* fusions were performed to study the expression of the divergent *ars* gene cluster in *E. coli*. A fragment encompassing the *arsT-arsC3* intergenic region and 66 and 102 bp downstream of the initiation codons of *arsT* and *arsC3*, respectively, was PCR amplified using primers ProT and ProC3. The PCR product was digested with EcoRI and ligated in both orientations into the promoter-probe vector pPROBE-NT (23) to generate plasmids pT-GFP and pC3-GFP (Table 1). The orientation of the insert in recombinant clones was established by PCR with primers ProT and ProC3 used in concert with vector-specific primer PGFP. These constructs were electroporated into *E. coli* DH10B, together with the compatible plasmid pBAD-arsRC2, which provided the putative regulator in *trans*. For induction assays, cultures of transformants were exposed or not exposed to arsenate and/or 0.2% arabinose

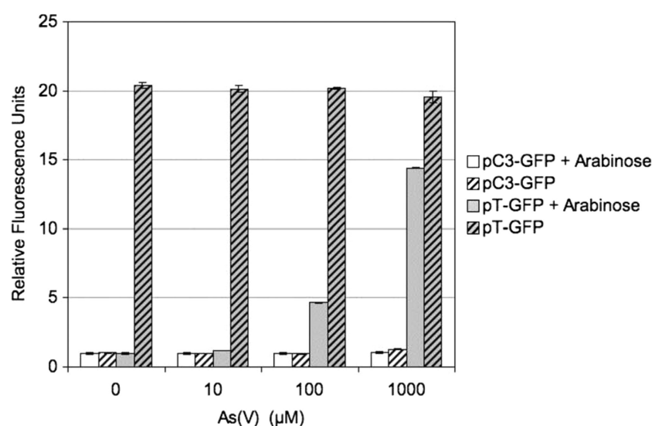


FIG. 3. Role of *arsRC2* in the transcription regulation of divergent *arsC3* and *arsT*. Cultures of *E. coli* DH10B harboring pBAD-ArsRC2 and plasmid pT-GFP (*arsT-gfp* fusion) or pC3-GFP (*arsC3-gfp* fusion) were incubated for 4 h in the presence or absence of 0.2% arabinose and various concentrations of arsenate before fluorescence determination. Data are presented as the mean fluorescence intensity normalized to the OD_{600} (relative fluorescence units) for duplicate assays ± the average deviation from the mean.

before the normalized fluorescence level (relative level of green fluorescent protein [GFP]/optical density at 600 nm [OD_{600}]) was measured with a FLX Xenius spectrofluorimeter (SAFAS, Monaco). In the absence of arabinose, the *arsT-gfp* fusion (pT-GFP) produced a high level of fluorescence independent of the concentration of added arsenate (Fig. 3). Expression was nearly abolished in the presence of arabinose alone and further increased in a dose-dependent manner upon arsenate addition. Arsenite and antimonite were found to elicit equivalent responses (data not shown). In contrast, low levels of fluorescence were measured for the *arsC3-gfp* fusion regardless of the presence or absence of arabinose or arsenate (Fig. 3). From these findings, we concluded that ArsRC2 serves as a transcriptional repressor of *arsT* but not *arsC3* and that As(V), As(III), and Sb(III) are inducers. A 17-bp imperfect inverted repeat (TTGTATCGATAAGTGTC-N_6-GACACATGTCGA TTCAA) that may constitute an ArsRC2 binding site was found in front of *arsT*, overlapping the ATG initiation codon. Another inverted repeat sequence (TCCGGCGGGC-N_5-GCC CGCCGGA) was present 15 nucleotides upstream of the putative GTG initiation codon of *arsC3*, suggesting the existence of a specific transcription regulator for this gene in *Microbacterium* sp. A33. Neither of these two dyad sequences resembles the ArsR binding sites identified so far in *E. coli*, *Synechocystis*, and *C. glutamicum* (20, 29, 35).

As mentioned above, the organization of the *ars* gene region suggested that *arsT-arsX-ACR3-arsRC2-arsC1* might form an operon divergently transcribed from *arsC3*. Reverse transcription-PCR (RT-PCR) experiments were performed to test this possibility. Total RNA was isolated from 5-ml samples of *Microbacterium* sp. A33 cultures at the mid-exponential phase, grown for 4 h in LP medium containing 5 mM As(V) or no arsenic. RNA was extracted using Nucleospin RNAII (Macherey-Nagel Duren, Germany) with DNase treatment according to the kit's manual. First-strand cDNA was synthesized from 2 μg RNA with Moloney murine leukemia virus (MMLV) re-

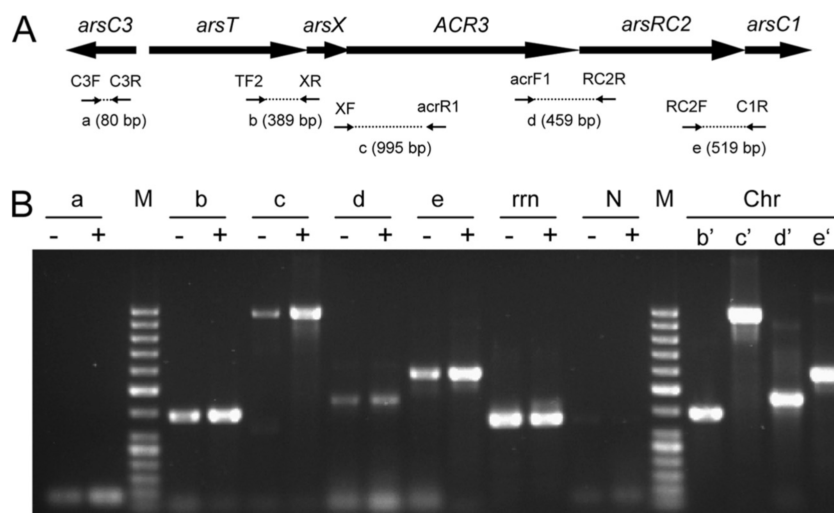


FIG. 4. RT-PCR analysis of *ars* genes of *Microbacterium* sp. strain A33. (A) Map positions of *ars* genes and the primers for RT-PCR analysis. Predicted RT-PCR products are represented by dotted lines under the genes and are labeled with lowercase letters, with predicted product sizes shown in parentheses. (B) Cotranscription determined by RT-PCR. Total RNA isolated from *Microbacterium* sp. A33 grown in the absence (−) or presence (+) of 5 mM arsenate was reverse transcribed and then amplified with the primer pairs shown in panel A. Letters "a" to "e" above the gel lanes correspond to predicted RT-PCR products. Lane rrn, RT-PCR of 16S rRNA with primers rrnF1 and rrnR; lane N, control reaction without reverse transcriptase for 16S rRNA amplified as in lane rrn; lanes b' to e', control PCR using chromosomal (Chr) DNA as a template; M, 50-bp DNA ladder marker.

verse transcriptase (Invitrogen) and random hexamers in accordance with the recommendations of the supplier. Synthesized cDNA was used as the template in subsequent PCR amplification reactions with primers designed to span the *ars* gene region or targeting the 16S rRNA gene. RT-PCR products of the expected sizes were obtained for primer pairs covering the region from *arsT* to *arsC1*, showing that these five genes form a single transcriptional unit (Fig. 4). An internal 80-bp *arsC3* product was also obtained, indicating that this gene is indeed expressed in *Microbacterium* sp. A33.

To provide an individual assessment of expression of the six *ars* genes, we performed real-time RT-PCR experiments with the same cDNA as that described above. Transcriptional induction by As(III) and Sb(III) was not tested, since these two metalloids were shown (see above) to act as inducers of the operon comprising *arsT* to *arsC1*. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm) to yield PCR products ~100 bp in length. Amplifications were carried out with a Bio-Rad iCycler iQ with 50-μl reaction mixtures containing 2 μl cDNA, 500 nM forward and reverse primers, and iQ SYBR green Supermix (Bio-Rad) according to the manufacturer's instructions and conducted under the following conditions: 4 min at 95°C, followed by 40 cycles of 15 s at 95°C and 45 s at 58°C. Each reaction was run in duplicate, and amplification efficiencies for each primer pair were determined from standard curves generated with serial dilutions of cDNA. Because the PCR efficiencies for all genes were close to each other (95 to 115%), relative mRNA expression levels were normalized for input RNA against the level of 16S rRNA gene transcripts and quantified by use of the comparative cycle threshold ($2^{\Delta\Delta Ct}$) method (19). The exposure of *Microbacterium* sp. A33 cells to arsenate (5 mM) resulted in a 14-fold increase in *arsC3* expression compared to the level for the control. The expression levels of both *arsT* and *arsX* were highly enhanced (98- and 60-fold, respectively) by arsenate treatment, while the induction levels of the downstream *ACR3*, *arsRC2*, and *arsC1* genes were less pronounced (7.7-, 7.3-, and 5.3-fold, respectively). Similar polar expression effects have been reported for the *ars* operons from *Synechocystis* sp. PCC 6803 (20) and *Desulfovibrio desulfuricans* G20 (17) but not for the two *ars* operons from *C. glutamicum* (28).

The facts that *arsT* and *arsX* occupy the two first positions in the *ars* operon and that they show the most-dramatic transcriptional induction by arsenic are indicative of their importance in the response of *Microbacterium* A33 to arsenic stress. The presence of a putative thioredoxin reductase in an arsenic resistance gene cluster has been reported only for the linear plasmids pHZ227 and pREL1 in *Streptomyces* sp. FR-008 and *Rhodococcus erythropolis* PR4, respectively (34, 40). We examined the occurrence of similar gene association in annotated bacterial genome sequences available in the nonredundant databases in the Integrated Microbial Genomes (IMG) system (Joint Genome Institute, Walnut Grove, CA [http://img.jgi.doe.gov/cgi-bin/pub/main.cgi]). In addition to the two above-mentioned cases, *arsT* homologs were found in the vicinity of *ars* genes in *Streptomyces coelicolor* A3(2) (NP_630905), plasmid pBD2 from *Rhodococcus erythropolis* BD2 (NP_898753), *Arthrobacter* sp. FB24 (YP_829715), *Arthrobacter aurescens* TC1 (YP_949317) and its plasmid TC2 (YP_950223), and *Brevibacterium linens* BL2 (ZP_00377913) as well as in the two gammaproteobacterial strains *Stenotrophomonas maltophilia* R551-3 (ZP_01642200) and *Acinetobacter baumannii* AYE (YP_001715387). For the latter strain, there is some evidence that the *ars* genes are located in a genomic resistance island presumably acquired by horizontal gene transfer (13). The only case of collocation of thioredoxin carrying *arsX* homolog and *ars* genes was found in the genome of *B. linens* BL2 (ZP_00377914). Interestingly, all of these clusters were found to contain at least one *arsC*-like gene encoding a

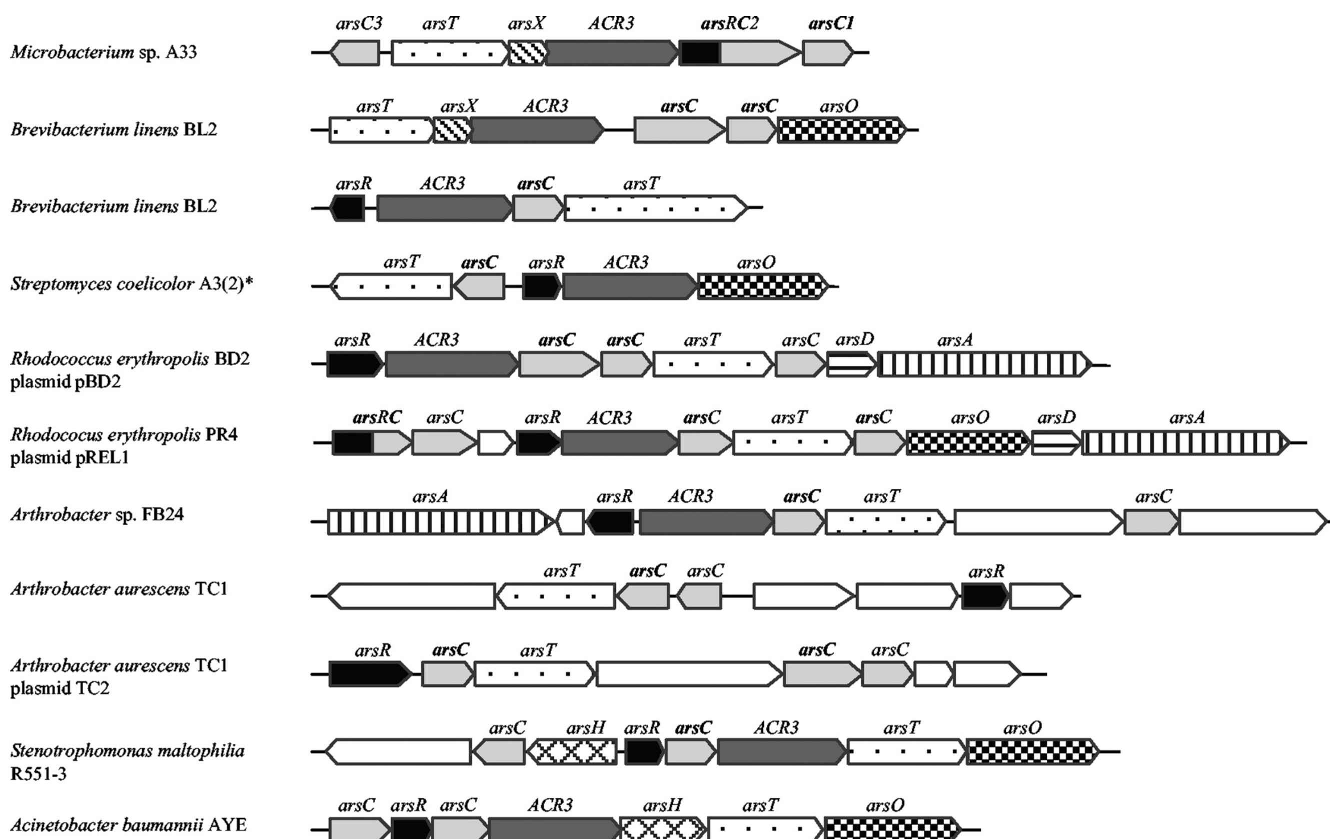


FIG. 5. Genetic arrangement of putative arsenic resistance genes in the neighborhood of *arsT* and *arsX* genes (numbers in parentheses are GenBank accession numbers) in *Microbacterium* sp. A33 (AM283030), *Brevibacterium linens* BL2 (NZ_AAGP01000058 and NZ_AAGP01000042), *Streptomyces coelicolor* A3(2) (NC_003888), *Rhodococcus erythropolis* BD2 plasmid pBD2 (NC_005073), *R. erythropolis* PR4 plasmid pREL1 (NC_007491), *Arthrobacter* sp. FB24 (NC_008541), *A. aurescens* TC1 (NC_008711), *A. aurescens* TC1 plasmid TC2 (NC_008713), *Stenotrophomonas maltophilia* R551-3 (NC_011071), and *Acinetobacter baumannii* AYE (NC_010410). Open boxes indicate open reading frames with no sequence similarity to known *ars* genes. The asterisk indicates that *S. coelicolor* A3(2) and *Streptomyces* sp. strain FR-008 *ars* gene clusters share the same genetic organization (40). *arsC* genes encoding putative thioredoxin-coupled arsenate reductases are marked in bold letters.

thioredoxin-linked arsenate reductase in the same orientation as *arsT* or *arsX* homologs (Fig. 5). Assuming that operons are composed of genes that cooperate in the same biological process, we hypothesize that coexpression of a thioredoxin system and arsenate reductase would enhance the efficiency of arsenate reduction and/or contribute to a better protection against arsenic-induced oxidative stress. Nevertheless, the evolutionary significance of the predominant occurrence of such an unusual genetic arrangement in actinobacteria awaits further clarification.

Another unique feature of the *Microbacterium* sp. A33 *ars* genes lies in *arsC3*. We showed that this gene, together with *arsC1*, could rescue the arsenate sensitivity phenotype of *E. coli* AW3110 (Fig. 2B) and that its expression is inducible by arsenate in *Microbacterium*, suggesting that it encodes a functional arsenate reductase. However, ArsC3 lacks two of the three catalytic cysteine residues of Trx-dependent arsenate reductases and does not exhibit recognizable sequence similarity to ArsC from *E. coli* plasmid R773, the representative of the second major class of glutathione-linked arsenate reductases (22). It seems unlikely that ArsC3 belongs to this class, since glutathione is generally absent from the actinomycetes, which instead produce mycothiol (26). Whether ArsC3 is a member of the recently identified third class of mycothiol-dependent arsenate reductases (30) is also improbable. Indeed, ArsC3 exhibits only 32% amino acid identity with the two representative enzymes of this novel class, Cg_ArsC1 and Cg_ArsC2 from *C. glutamicum*, and seems to be functional in *E. coli*, which rules out its dependence on mycothiol. This raises the question of the precise function of the ArsC3 protein and the way it contributes to arsenate resistance.

The *arsRC2* gene also constitutes an unusual feature of the *ars* cluster. Fusion of the ArsR and ArsC domains has been described only for *Leptospirillum ferriphilum* (36) and could therefore be considered a rare event. However, a CDART (14) search identified 73 proteins with the same domain architecture, essentially from alphaproteobacteria (37), high-G+C-content Gram-positive bacteria (23), and green nonsulfur bacteria (7) and 13 proteins with an inverse ArsCR architecture, exclusively from gammaproteobacteria. Such a wide distribution suggests that these fusions are nonrandom events and presumably confer a selective advantage to their host (42). Although most *ars* operons are inducible by arsenate and arsenite, the ArsR repressor has been shown to control the expression in response to As(III), so that As(V) has to be reduced *in vivo* to induce the *ars* system. We therefore speculate that the physical coupling of functional ArsC and ArsR

domains in a fusion protein might alleviate the problem of diffusion of As(III) to the inducer binding site and increase the efficiency of transcription in response to As(V). In this situation, the ArsC domain would primarily serve to provide As(III) to the repressor rather than to the ACR3 efflux protein, which would not necessarily require a high level of arsenate reductase activity. This may explain both the inability of *arsRC2* to complement an *arsC* mutation in *E. coli* JW3470 and the presence of the additional *arsC1* gene, which appears to play the major role in the detoxication of arsenate.

In summary, the *ars* system of *Microbacterium* sp. A33 exhibits remarkable features, including (i) the presence of genes required to provide electrons for the reduction of arsenate in a single operon rather than being dispersed in the genome, (ii) an ArsC3 arsenate reductase which contributes to arsenate resistance while lacking typical catalytic cysteine residues of related Trx-dependent enzymes, and (iii) an unusual ArsRC2 fusion protein which acts as an arsenic-dependent transcriptional repressor. Because the *ars* cluster has been found to confer arsenic tolerance to heterologous hosts, it is most probably functional in its natural host. However, its actual contribution to the exceptional arsenic tolerance of *Microbacterium* sp. A33 still remains to be determined. The development of effective genetic systems for *Microbacterium* would greatly facilitate addressing these questions.

Nucleotide sequence accession number. The sequence of the *ars* gene cluster from *Microbacterium* sp. A33 has been deposited in the EMBL database under accession number AM283030.

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