

arsRBOCT Arsenic Resistance System Encoded by Linear Plasmid pHZ227 in *Streptomyces* sp. Strain FR-008

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In the arsenic resistance gene cluster from the large linear plasmid pHZ227, two novel genes, *arsO* (for a putative flavin-binding monooxygenase) and *arsT* (for a putative thioredoxin reductase), were coactivated and cotranscribed with *arsRI-arsB* and *arsC*, respectively. Deletion of the *ars* gene cluster on pHZ227 in *Streptomyces* sp. strain FR-008 resulted in sensitivity to arsenic, and heterologous expression of the *ars* gene cluster in the arsenic-sensitive *Streptomyces* strains conferred resistance on the new hosts. The pHZ227 ArsB protein showed homology to the yeast arsenite transporter Acr3p. The pHZ227 ArsC appears to be a bacterial thioredoxin-dependent ArsC-type arsenate reductase with four conserved cysteine thioredoxin-requiring motifs.

Arsenic is a toxic metalloid and a cause of cancer that exists in natural and polluted industrial environments. Arsenic resistance genes have been identified in circular plasmids and chromosomes in bacteria (10). Arsenic resistance gene clusters in the eukaryotic model organisms *Saccharomyces cerevisiae* and *Saccharomyces douglasii* consist of three genes, *ACR1*, *ACR2*, and *ACR3* (2, 5, 21), whose functions are similar to those of *arsR*, *arsC*, and *arsB*, respectively, of bacteria (10). However, none of the three gene products shows significant sequence similarity to the bacterial *ars* gene products.

New arsenic resistance gene clusters other than the initial *arsRDABC* type continue to emerge, and varied mechanisms seem to occur in diverse biological systems. The *ars* operon in the *skin* (for *sigK*-intervening) element of *Bacillus subtilis* contains four genes in the order *arsR*, *ORF2*, *arsB*, and *arsC*. Although *ORF2* homologs have not been found (17), it is known to be essential for arsenic resistance. The large extrachromosomal replicon pNRC100 in *Halobacterium* sp. strain NRC-1 harbors an *arsADRC-R2M*-type arsenic resistance gene cluster involving a putative arsenite(III)-methyltransferase (20). The circular plasmid pWCFS103 in *Lactobacillus plantarum* carries a uniquely organized *arsRDDDB*-type arsenic resistance gene cluster (13) that lacks the arsenate reductase gene *arsC* and contains two copies of the *arsD* regulatory gene.

Linear plasmids have been reported to encode diverse biological functions, yet arsenic resistance systems in linear plasmids have not been studied in detail. Here, we report the analysis of an arsenic resistance (*ars*) gene cluster from a large linear plasmid, pHZ227, in *Streptomyces* sp. strain FR-008. This determinant is compared with the putative *ars* gene clusters from *Streptomyces coelicolor* A3(2) (1) and *Streptomyces* sp. strain F2 (GenBank accession number AY943951, submitted by another group).

General methods and techniques. The general methods used for handling *Escherichia coli* and *Streptomyces* species have been described previously (11, 16). Sequencing was performed at the Chinese National Human Genome Center, Shanghai, China. The sequence data were analyzed with FramePlot 3.0 beta. DNA and deduced protein sequence homology searches were performed by using the BLAST and FASTA programs (<http://www.ncbi.nlm.nih.gov>).

Cosmids covering pHZ227 and pHZ228 from the *Streptomyces* sp. strain FR-008 genomic library. The linear plasmid pHZ227 band was cut and isolated from a pulsed-field gel electrophoresis gel and labeled with α -³²P as a probe to hybridize against a *Streptomyces* sp. strain FR-008 genomic library in *Escherichia coli* comprising 1,920 colonies. Hybridizing cosmids (46) were obtained, and eight overlapping cosmids (pHZ1239 to pHZ1246) were aligned and mapped to form a contig. Three positive clones were also obtained for the smaller linear plasmid pHZ228 of *Streptomyces* sp. strain FR-008, which has not been mapped in detail.

Generation of a deletion in the *ars* gene cluster by targeted gene replacement. The *ars* gene cluster was removed from a 7,568-bp PstI fragment (GenBank accession number DQ231520) by digestion with NruI and PmaCI and replaced with the apramycin resistance gene [*acc(3)IV*] as a blunt-ended SmaI-EcoRV fragment from pHGF9827 (L. Bai, personal communication). This construct was cloned into pHZ1358 (19) to yield pJTU1908 for gene replacement. pJTU1908 was transferred by conjugation from *E. coli* ET12567(pUZ8002) into *Streptomyces* sp. strain FR-008. Thiostrepton-sensitive and apramycin-resistant colonies were selected from the initial thiostrepton-resistant exconjugants after two rounds of apramycin-selective growth. The selected thiostrepton-sensitive and apramycin-resistant strain (WL1) was shown to have the expected gene replacement by PCR amplification using oligonucleotide primers WL1-1 (5'-GGTCCTTCGCCTCCAA CA-3') and WL1-2 (5'-ATCACCGCCAGGTCCAAG-3').

RT-PCR analysis. Fresh spores of *Streptomyces* sp. strain FR-008 were separately inoculated into 10 ml TSBY growth medium (3% Difco tryptic soy broth powder, 34% sucrose, 1% Difco yeast extract, pH 7.2) in three batches with 250 μ M

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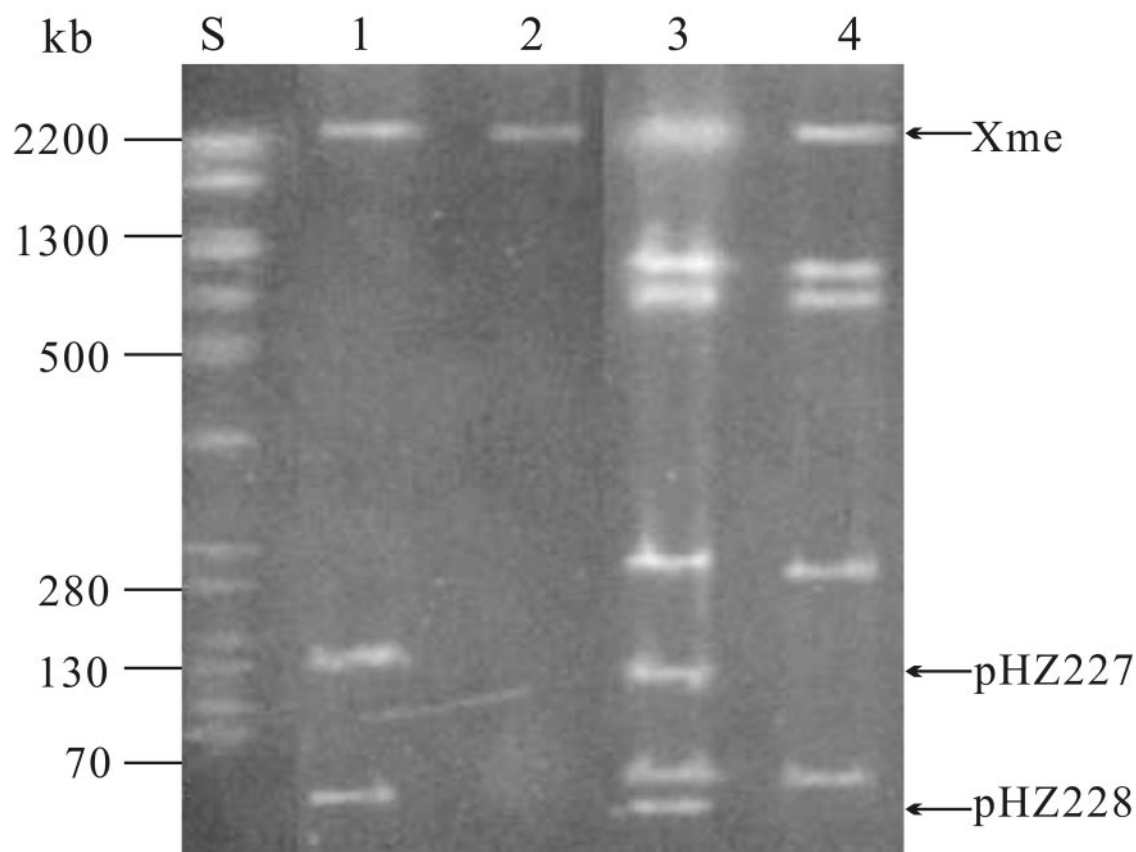


FIG. 1. Identification of linear plasmids in *Streptomyces* sp. strain FR-008 by PFGE. Undigested chromosomes and plasmids (pHZ227 and pHZ228) of *Streptomyces* sp. strain FR-008 (lane 1) and *S. griseus* IMRU3570 (lane 2) and intact samples (lanes 1 and 2) digested with endonuclease *VspI* (lanes 3 and 4) were run in parallel with size standards (S). Xme, undigested chromosomes.

As(III), with 500 μ M As(V), and without arsenic as a control, respectively. The cultures were incubated with agitation at 30°C for 30 h. Total RNA was isolated using the RNeasy minikit (QIAGEN) and treated with DNase I for 7 h. Reverse transcription (RT)-PCR was performed in a Thermo Hybaid PCR system (0.2 S) using the OneStep RT-PCR kit and Q-Solution (QIAGEN).

Genomic and phenotypic comparisons of the two *Streptomyces* strains producing identical candicidin complexes. *Streptomyces* sp. strain FR-008 and *Streptomyces griseus* IMRU3570 (a gift from José Gil, Universidad de León, Spain) are independent isolates, one from China and the other from Spain, known to synthesize the antibiotic complex of the candicidin compounds (4). A comparison of the strains by pulsed-field gel electrophoresis analysis revealed two additional bands (ca. 130 and 30 kb) that were present in *Streptomyces* sp. strain FR-008 but absent in *S. griseus* IMRU3570 (Fig. 1), apart from chromosomal bands. Two-dimensional pulsed-field gel electrophoresis revealed no further separation of these bands after UV irradiation. These bands were also found to be sensitive to ExoIII exonuclease, but not to λ exonuclease, consistent with the properties of other reported linear plasmids and chromosomes in *Streptomyces* (reference 12 and data not shown). The two linear plasmids of *Streptomyces* sp. strain FR-008 were named pHZ227 and pHZ228 (Fig. 1).

When *Streptomyces* sp. strain FR-008 and *S. griseus* IMRU3570 were compared for sensitivity to heavy metals, including mer-

curic chloride, sodium arsenite, and sodium arsenate, neither grew on SFM (2% soy flour, 2% mannitol, 2% agar) plates containing 50 μ M mercuric chloride. However, *Streptomyces* sp. strain FR-008 grew on the plates supplemented with arsenite and arsenate at concentrations of 5 mM and 100 mM (data not shown).

Linear plasmid pHZ227 is involved in arsenic resistance. Four representative overlapping cosmids (pHZ1239, pHZ1240, pHZ1244, and pHZ1246) covering almost the entire region of pHZ227 and three cosmid derivatives carrying fragments from pHZ228 were introduced into *S. griseus* IMRU3570 (6) by conjugation. Interestingly, cosmids pHZ1239 and pHZ1240 enabled *S. griseus* IMRU3570 to grow on SFM plates supplemented with 5 mM arsenite and 100 mM arsenate (data not shown).

Identification and sequence analysis of the *ars* gene cluster. The fact that arsenic resistance genes were likely located on the overlapping region between pHZ1239 and pHZ1240 prompted us to sequence cosmid pHZ1239. Six putative genes likely to be involved in *Streptomyces* arsenic resistance (designated *arsR2*, *arsO*, *arsB*, *arsR1*, *arsC*, and *arsT*) were located on an internal 7,568-bp *PstI* fragment (Fig. 2). The DNA and deduced protein sequences had been deposited in GenBank under the accession number DQ231520. The overall G+C content of the six *ars* genes is 69.9%. They are arranged in an unusual manner, with *arsR1*, *arsB*, and *arsO* constituting one operon and *arsC* and *arsT* as another divergently transcribed

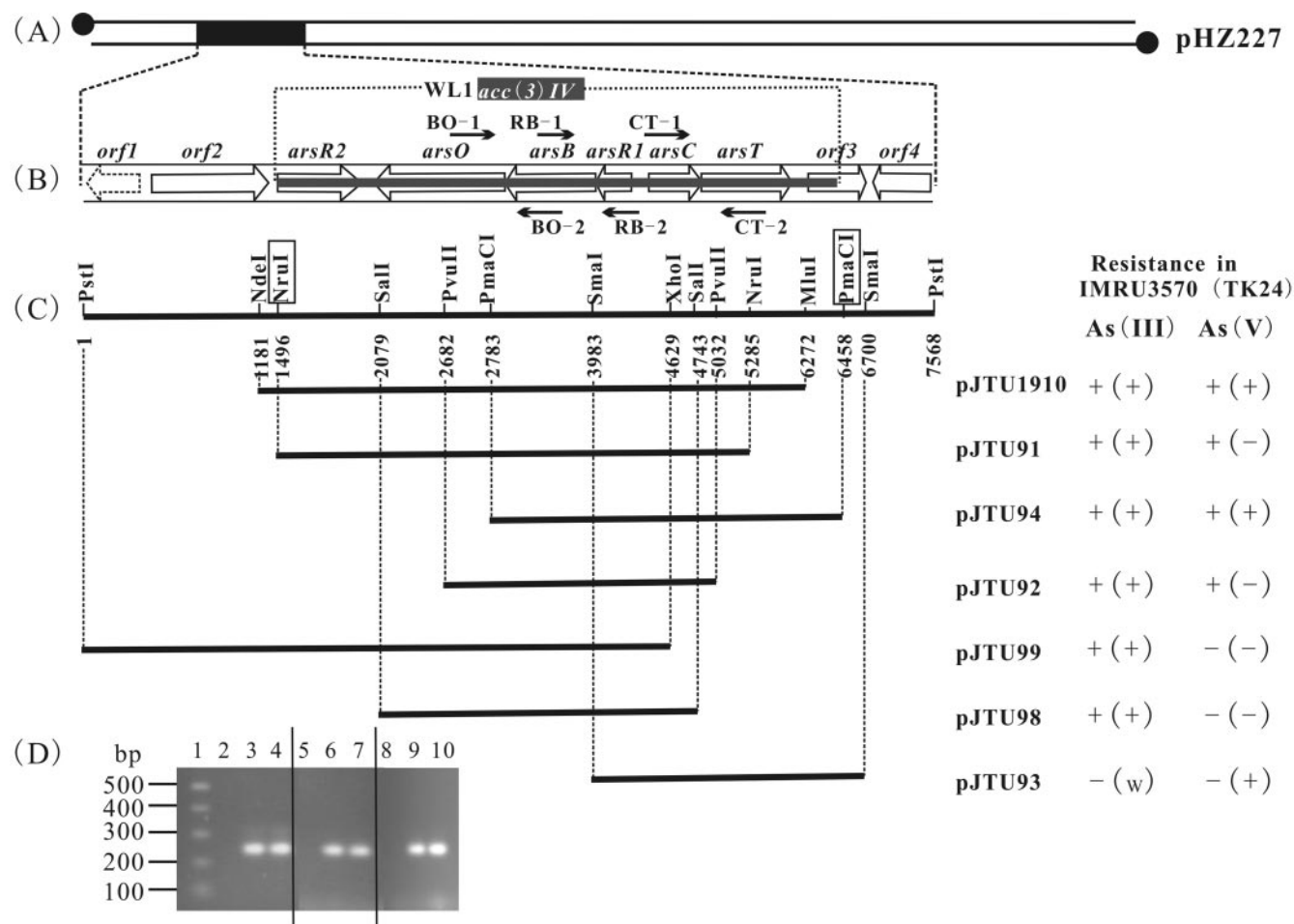


FIG. 2. (Top) Schematic presentation of the linear plasmid pHZ227 with putative terminal proteins. (A) The arsenic resistance gene cluster (thick shaded bar) is located in the overlapping region as a PstI fragment between pHZ1239 and pHZ1240. (B) Genetic organization of the *ars* gene cluster, with the region indicated by open arrows replaced by the 1.4-kb *acc(3)IV* gene to obtain mutant WL1. (C) A series of deletion clones for the characterization of arsenite [As(III)] and arsenate [As(V)] resistance in *S. griseus* IMRU3570 and in *S. lividans* TK24 (in parentheses). +, able to confer arsenic resistance; -, unable to confer arsenic resistance; w, grew weakly. (D) RT-PCR analysis of cotranscriptions of *arsRIBO* and *arsCT*, using primers schematically located above or below the putative *ars* gene cluster in panel B. PCR products were obtained using template RNA extracted from *Streptomyces* sp. strain FR-008 under uninduced (lanes 2, 5, and 8), arsenite-induced (lanes 3, 6, and 9) or arsenate-induced (lanes 4, 7, and 10) conditions. Primers RB-1 (5'-GATCGAGTCGTCCCCACCC-3') and RB-2 (5'-CGGAGCCAAGCAG CCAACC-3') (lanes 2 to 4), BO-1 (5'-TCGGCCGGGAGAACAGGTG-3') and BO-2 (5'-CGGTCCTGATCGGCCTGGTC-3') (lanes 5 to 7), and CT-1 (5'-CGTCTTCCCCGGCAAGCGCTA-3') and CT-2 (5'-CGAACAGCACGGGCCTCAGC-3') (lanes 8 to 10) were used as primer pairs. Results obtained using different primer pairs are separated by vertical lines.

operon. The 3' end of *arsB* and the 5' end of *arsO* overlapped by 4 bp (GTGA). The same 4-bp overlap (GTGA) was also observed at the 3' end of *arsC* and the 5' end of *arsT*. An extensive NCBI Conserved Domain search was performed for these putative proteins. ArsB belongs to the yeast arsenite efflux pump Acr3p family. It showed remarkable homology to Acr3p from *Saccharomyces cerevisiae* (33% amino acid identity) and *Saccharomyces douglasii* (31% identity), but not to bacterial *arsB* gene products, such as those encoded by *E. coli* plasmid R773 and *Staphylococcus aureus* plasmid pI258.

In contrast, ArsC was a homolog of bacterial arsenate reductases, such as ArsC from *S. aureus* plasmid pI258 (9) (36% amino acid identity) and *Staphylococcus xylosus* plasmid pSX267 (15) (35% amino acid identity), but shared no homology with the Acr2p yeast arsenate reductases from *S. cerevisiae* and *S. douglasii*. ArsO was homologous to flavin-binding monooxygenases, as it was 57% identical to a putative monooxy-

genase (ZP_00420634) from *Burkholderia vietnamiensis* G4 and 48% identical to a putative monooxygenase (NP_744078) from *Pseudomonas putida* KT2440. ArsT showed extensive homology to numerous thioredoxin reductases, such as thioredoxin reductases from *Streptomyces coelicolor* A3(2) (71% amino acid identity to CAA63076) and *Streptomyces clavuligerus* (73% amino acid identity to CAA79940). The other two putative proteins (ArsR1 and ArsR2) showed significant homology to ArsR proteins from many arsenic systems, suggesting their roles as repressors for the arsenic resistance genes. Several genes flanking both sides of the six *ars* genes could not be assigned to a role in arsenic resistance, including the complete *orf2* and incomplete *orf1* (whose putative products showed homology to monooxygenases involved in K⁺ transport) and the complete *orf3* and *orf4* (whose putative products showed no significant homology to known proteins in the protein database) (Fig. 2).

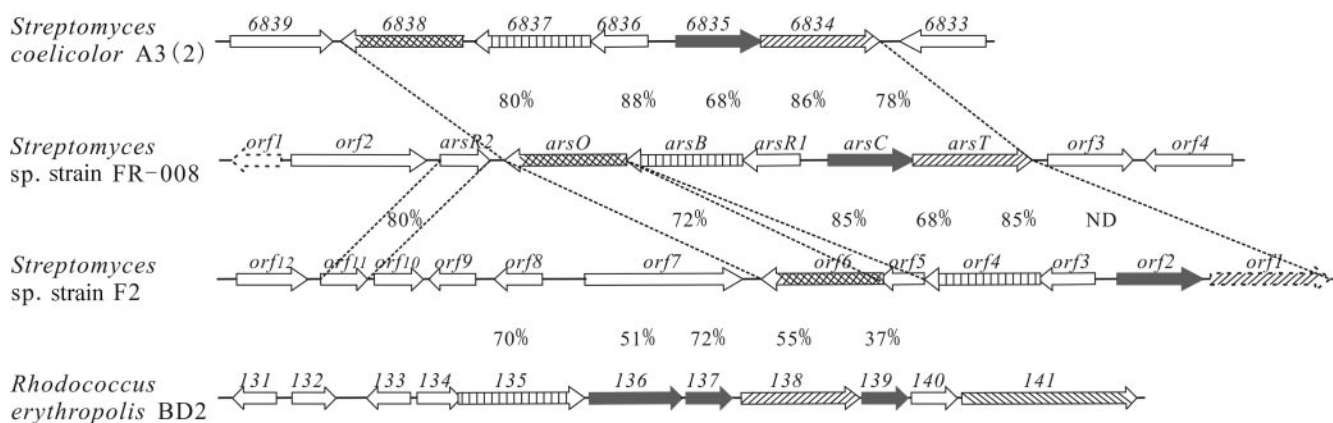


FIG. 3. Genetic organizations of genes involved in arsenic resistance in *Streptomyces* sp. strain FR-008 (GenBank accession number DQ231520) with *S. coelicolor* A3(2) (GenBank accession number AL939129), *Streptomyces* sp. strain F2 (GenBank accession number AY943951), and *Rhodococcus erythropolis* BD2 (GenBank accession number AY223810). The gene numbers correspond to the numbers in the genome database. Only one of the two arsenic resistance gene clusters (SCO6834 to SCO6838, harboring full-length *arsT* but lacking *arsR2* homology) from *S. coelicolor* A3(2) is shown. Homologous genes are aligned with crosshatching (*arsO*), vertical lines (*arsB*), hatching (*arsT*), and gray (*arsC*) for comparison. The numbers between the dotted lines and above *R. erythropolis* BD2 are the percentages of amino acid sequence identity between the related *ars* proteins of *Streptomyces* sp. strain FR-008.

Localization and functional analyses of the *ars* gene cluster.

The 5,091-bp NdeI-MluI fragment, containing only six putative *ars* genes, was cloned into a phage ΦC31-derived integrative vector, pSET152 (11), for the construction of pJTU1910. When pJTU1910 was introduced into *S. griseus* strain IMRU3570, the derivative strain gained resistance to both 5 mM arsenite and 100 mM arsenate, implying that this NdeI-MluI fragment contained the complete arsenic resistance gene cluster.

Evidence that the above-mentioned six *ars* genes were directly responsible for arsenic resistance in their native host, *Streptomyces* sp. strain FR-008, came from deletion of the putative *ars* gene cluster from pHZ227 by targeted gene replacement. The resulting mutant, WL1, displayed sensitivity to 5 mM arsenite and 100 mM arsenate comparable to that of *S. griseus* IMRU3570. Arsenic resistance was restored when pHZ1239 and pHZ1240 were introduced into WL1, while the control cosmids (pHZ1244 and pHZ1246) had no such effect. Thus, the *arsRBOCT* gene cluster lying on linear plasmid pHZ227 was demonstrated to be responsible for arsenic resistance in *Streptomyces* sp. strain FR-008.

To determine the functions of individual genes in the pHZ227 arsenic resistance gene cluster, a series of pSET152 derivative clones carrying different sets of the six putative *ars* genes were assayed for arsenic resistance in *S. griseus* IMRU3570 (Fig. 2). Vector pSET152 was used as a negative control throughout. Plasmids pJTU91 (lacking *arsT*), pJTU94 (lacking *arsR2* and *arsO*), and pJTU92 (lacking *arsR2*, *arsO*, and *arsT*) conferred resistance to both arsenite and arsenate, suggesting that a PvuII fragment carrying an *arsB-arsR1-arsC* gene cassette (pJTU92) was sufficient for conferring resistance to As(III), as well as As(V), on *S. griseus* IMRU3570. Additionally, further partial removal of *arsC* (pJTU98 and pJTU99) abolished resistance to As(V) but not to As(III) (data not shown), while a clone (pJTU93) removing *arsB* but maintaining *arsC* and *arsR1* lost resistance to As(III) and to As(V) completely in *S. griseus* IMRU3570 (data not shown). The most striking difference in the heterologous expression of these clones in *Streptomyces lividans*

TK24 was that the absence of *arsT* (as in pJTU91 and pJTU92 [Fig. 2C]) abolished resistance to As(V).

ArsB appears to be an arsenite efflux pump protein required for resistance to both arsenite and arsenate, while ArsC is only necessary for resistance to arsenate. This is in accord with the known function of ArsC as an arsenate reductase for the reduction of As(V) to As(III), which is then effluxed by ArsB. Thioredoxin is required for arsenate reduction by ArsC of *S. aureus* plasmid pI258 (8), and an independent functional *trxA* (thioredoxin) gene in the *E. coli* chromosome is required for *T. ferrooxidans* ArsC-mediated arsenate resistance in *E. coli* (3). An equivalent function is likely to be mediated by *arsT*, a putative thioredoxin reductase gene in the pHZ227 *ars* gene cluster. This agrees well with the proposal that the thioredoxin reductase gene participates in the reduction of arsenate to arsenite with the reducing power from NADPH → thioredoxin reductase → thioredoxin → ArsC → arsenate (8).

***arsR1BO* and *arsCT* constitute two cotranscribed operons.** Total RNA was isolated from arsenite-induced, arsenate-induced, and uninduced mycelia of *Streptomyces* sp. strain FR-008. RT-PCR results using different sets of primers are shown in Fig. 2D. As expected, *arsR1*, *arsB*, and *arsO* were cotranscribed, as RT-PCR with two sets of primers (RB-1 and RB-2 and BO-1 and BO-2) resulted in two expected 249-bp fragments. Likewise, the expected 250-bp fragment was obtained using CT-1 and CT-2, specific for the putative *arsC-arsT*. No transcription was observed for these genes under uninduced conditions. We therefore concluded that *arsR1BO* and *arsCT* constitute two independently cotranscribed operons.

With the rapidly increasing information from genome-sequencing projects, a number of potential *ars* gene clusters have been annotated in chromosomes. An extensive BLAST search using the pHZ227 *ars* gene cluster against NCBI databases identified related putative *ars* gene clusters (Fig. 3). Among these, pHZ227 *ars* genes showed the highest similarities to putative *ars* genes from the *S. coelicolor* A3(2) genome open reading frames (ORFs). One of the two sets of putative arsenic

resistance genes in *S. coelicolor* A3(2) (SCO6834 to SCO6838 of GenBank accession no. AL939129) (Fig. 3) is located in a tRNA region, suggesting acquisition from an exogenous genomic island (1). The other *ars* gene cluster (SCO3696 to SCO3701 of GenBank accession no. AL939117) contains an *arsR2* homolog (ORF SCO3696) and a partial *arsT* (ORF SCO3701). The *ars* genes from pHZ227 also showed significant homology with the genes of the putative arsenic resistance gene cluster (G+C content 69.4%) from *Streptomyces* sp. strain F2, recently deposited in GenBank by a different Shanghai research group (Fig. 3), although the starting *orf1* encoding a putative thioredoxin reductase is an incomplete gene sequence. An extra gene (*orf5*), whose role is unknown, was found between *arsB* and *arsO* homologs. Though the arsenic resistance genes in *S. coelicolor* A3(2) and *Streptomyces* sp. strain F2 have not been functionally analyzed, wild-type *S. coelicolor* A3(2) has been shown to be resistant to arsenic (7).

Additionally, pBD2, a linear plasmid from *Rhodococcus erythropolis* BD2, which displayed an arsenic resistance phenotype, carries several arsenic resistance gene homologs (18) whose genetic organizations differ dramatically from that of pHZ227 (Fig. 3). First, linear plasmid pBD2 carries an *arsA* homolog (ORF 141 in Fig. 3) encoding an ATPase typical of arsenic resistance gene clusters in gram-negative bacteria that is absent in pHZ227. In contrast to three independent ORFs (ORFs 136, 137, and 139 in Fig. 3), encoding putative ArsC in pBD2, there is only one *arsC* gene in pHZ227. In addition, pBD2 contains a putative thioredoxin reductase gene (an *arsT* homolog) but apparently does not have a homolog for a flavin-binding monooxygenase gene (*arsO*).

The genetic arrangement of *arsO* and *arsT* in pHZ227 implies that they are coupled with *arsR1-arsB* and *arsC*, respectively. Nonetheless, plasmids pJTU91 lacking *arsT* and pJTU94 lacking *arsO* both conferred resistance to arsenic on *S. griseus* IMRU3570, although the *arsT* function was absolutely required for arsenate resistance in *S. lividans* TK24 (Fig. 2C). Since the putative thioredoxin reductase activity of ArsT is known to be widely distributed in bacteria [e.g., ArsT is highly homologous to at least five putative thioredoxin reductases, ORFs CAB71847, CAB42713, CAA63076, CAA07451, and CAA63075 in GenBank accession number AL645882, in *S. coelicolor* A3(2)], it is possible that such activity is recruited from endogenous chromosomal ArsT homologs. This may explain why most known arsenic resistance gene clusters harboring a thioredoxin-dependent *arsC* do not contain an *arsT* (thioredoxin reductase) gene or an *arsO* (flavin-binding monooxygenase) homolog.

A flavin-binding monooxygenase has not previously been characterized in known arsenic resistance systems. Although no obvious similarity was found, the role of ArsO in Acr3p-type arsenite efflux systems may be similar to that of ArsH, a protein showing weak homology to oxidoreductases (3, 14), in the arsenic resistance systems of some gram-negative bacteria.

All of the *ars* gene clusters in *Streptomyces* have high G+C contents (ca. 70%), a general feature of *Streptomyces* genomes which nevertheless contrasts with the generally lower (< 50%) G+C contents of arsenic gene clusters found in other organisms. Moreover, the *arsT* gene (encoding a possible thioredoxin reductase) was found only in a relatively high-G+C-content arsenic resistance gene cluster (65%) from the linear plasmid pBD2 of gram-positive *Rhodococcus erythropolis* BD2. Also unusual for

pHZ227 *ars* clusters are the hypothetical flavin-binding monooxygenase ArsO, the similarity of the arsenite transporter (ArsB) to that of the yeast Acr3p family, and the similarity of the arsenate reductase (ArsC) to bacterial ArsC. Taken together, these features may constitute an unusual entity specific to *Streptomyces* and other gram-positive organisms with high G+C contents.

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