Analysis of Genes Involved in Arsenic Resistance in *Corynebacterium glutamicum* ATCC 13032†

Efrén Ordóñez, Michal Letek, Noelia Valbuena, José A. Gil, and Luis M. Mateos*

Departamento de Ecología, Genética y Microbiología, Área de Microbiología, Facultad de Biología, Universidad de León, 24071 León, Spain

Corynebacterium glutamicum **is able to grow in media containing up to 12 mM arsenite and 500 mM arsenate and is one of the most arsenic-resistant microorganisms described to date. Two operons (*ars1* and *ars2*) involved in arsenate and arsenite resistance have been identified in the complete genome sequence of *Corynebacterium glutamicum*. The operons *ars1* and *ars2* are located some distance from each other in the bacterial chromosome, but they are both composed of genes encoding a regulatory protein (*arsR*), an arsenite permease (*arsB*), and an arsenate reductase (*arsC*); operon *ars1* contains an additional arsenate reductase gene (*arsC1′*) located immediately downstream from *arsC1*. Additional arsenite permease and arsenate reductase genes (*arsB3* and *arsC4*) scattered on the chromosome were also identified. The involvement of *ars* operons in arsenic resistance in *C. glutamicum* was confirmed by gene disruption experiments of the three arsenite permease genes present in its genome. Wild-type and *arsB3* insertional mutant *C. glutamicum* strains were able to grow with up to 12 mM arsenite, whereas *arsB1* and *arsB2* *C. glutamicum* insertional mutants were resistant to 4 mM and 9 mM arsenite, respectively. The double *arsB1-arsB2* insertional mutant was resistant to only 0.4 mM arsenite and 10 mM arsenate. Gene amplification assays of operons *ars1* and *ars2* in *C. glutamicum* revealed that the recombinant strains containing the *ars1* operon were resistant to up to 60 mM arsenite, this being one of the highest levels of bacterial resistance to arsenite so far described, whereas recombinant strains containing operon *ars2* were resistant to only 20 mM arsenite. Northern blot and reverse transcription-PCR analysis confirmed the presence of transcripts for all the *ars* genes, the expression of *arsB3* and *arsC4* being constitutive, and the expression of *arsR1*, *arsB1*, *arsC1*, *arsC1′*, *arsR2*, *arsB2*, and *arsC2* being inducible by arsenite.**

Corynebacterium glutamicum is a biotechnologically important microorganism that is widely used for the large-scale production of amino acids such as L-glutamate and L-lysine (25, 42). Together with other members of the genera *Rhodococcus*, *Gordonia*, *Nocardia*, and *Mycobacterium*, *Corynebacterium* belongs to the mycolata, a broad and diverse group of mycolic-acid-containing actinomycetes. Besides a thick peptidoglycan layer, the mycolata contain large amounts of mycolic acids and other lipids in their cell walls (43). Recently, the complete genome sequence of *C. glutamicum* strain ATCC 13032 was determined (44) and is predicted to contain 3,002 open reading frames (32).

Arsenic is one of the most prevalent toxic metals in the environment; it is mainly of geochemical origin (rocks and minerals) in an insoluble form but also derives from anthropogenic sources (41). In soluble forms, arsenic occurs as trivalent arsenite [As(III)] and pentavalent arsenate [As(V)]. Arsenate, a phosphate structural analogue, can enter the bacterial cell via the phosphate transport system. Its toxicity is due to its interference in normal phosphorylation processes by replacing cellular phosphate. It has recently been demonstrated that arsenite enters the cells, at neutral pH, by aqua-glyceroporins (glycerol transport proteins) in bacteria, yeasts, and mammals (41) and that its toxicity lies in its ability to bind sulfhydryl groups of cysteine residues in proteins, thereby inactivating them. Arsenite is considered to be more toxic than arsenate and can be oxidized to arsenate chemically or microbiologically (20). In some gram-negative bacteria, arsenite is converted to arsenate by an arsenite oxidase, a periplasmic membrane-bound enzyme member of the dimethyl sulfoxide reductase family of molybdoenzymes (51). The toxic properties of arsenic are well known and have been exploited in the production of antimicrobial agents, such as the first specific antimicrobial drug Salvarsan 606, in addition to the commonly used wood preservative chromated copper arsenate (27).

Bacteria have developed a variety of mechanisms to avoid the toxicity of arsenic: (i) minimizing the uptake of arsenate through the system for phosphate uptake (16), (ii) by peroxidation reactions with membrane lipids (1), and (iii) using the best characterized microbial arsenic detoxification pathway involving the *ars* operon (55).

Bacterial operons encoding analogous arsenic resistance determinants (*ars*) have been found on the chromosome as well as on transmissible plasmids from gram-positive and gram-negative microorganisms. These operons generally consist of either three (*arsRBC*) or five (*arsRDABC*) genes that have been organized into a single transcriptional unit (55). The three-gene system, encoding the arsenic transcriptional repressor (*arsR*), arsenite permease (*arsB*), and arsenate reductase (*arsC*), was present on the chromosome of *Escherichia coli*, *Pseudomonas aeruginosa* (13), and other enterobacteria (18). The operon of three genes is also present in the *Staphylococcus* plasmids pI258 and pSX267 (24).

* Corresponding author. Mailing address: Area de Microbiología, Departamento de Ecología, Genética y Microbiología, Universidad de León, 24071 León, Spain. Phone: 34-87-291126. Fax: 34-87-291409. E-mail: deglmd@unileon.es.

The five-gene operon (*arsRDABC*) encodes an arsenite-inducible repressor (*arsR*), a negative regulatory protein that provides the fine tuning of operon expression (*arsD*), an ATPase, and a membrane-located arsenite efflux pump (*arsA* and *arsB*, respectively), together with an arsenate reductase (*arsC*). This operon was initially discovered in the *E. coli* plasmids R773 and R46 (17) and then on plasmid pKW301 from *Acidiphilium multivorum* (57). In addition to the above-mentioned arsenic resistance operons, a broad diversity of four-gene operons have been described in different species, such as *Bacillus subtilis* (52), *Acidithiobacillus ferrooxidans* (11, 12), and a *Synechocystis* sp. (36). Two operons involved in arsenic resistance have recently been identified on the chromosome of the multiresistant *Pseudomonas putida*, although no molecular details were provided (14). In *Saccharomyces*, a cluster of three genes involved in arsenic resistance was identified; genes *acr1*, *acr2*, and *acr3* in *S. cerevisiae* (7) and genes *arr1*, *arr2*, and *arr3* in *S. douglasii* (37) seem to encode the regulator, arsenate reductase, and arsenite permease, respectively.

In view of the ubiquitous presence of arsenic in nature, we wished to determine whether the saprophytic soil bacterium *C. glutamicum* contained genes involved in resistance to arsenic and the possible use of *C. glutamicum* in the detoxification of episodic increases of arsenic in soil and water.

Here, we report the identification of genes involved in arsenic resistance in *C. glutamicum*, some of them forming two similar operons (called *ars1* and *ars2*), and two accessory genes (*arsB3* and *arsC4*) scattered throughout the chromosome. We also show that both operons (*ars1* and *ars2*) are functional and clearly involved in arsenic resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture, and transformation conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown in Luria-Bertani broth or Luria-Bertani agar (26) at 37°C. Corynebacterial strains were grown at 30°C in trypticase soy broth (TSB; complex medium), TSA (TSB supplemented with 2% agar), or minimal medium for corynebacteria (MMC) (33). When necessary, antibiotics were added at the following final concentrations: kanamycin, 50 μg/ml for *E. coli* and 25 μg/ml for corynebacteria; ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml for *E. coli* and 10 μg/ml for corynebacteria; apramycin, 50 μg/ml for *E. coli* and 25 μg/ml for corynebacteria. Transformation of *E. coli* strains was carried out by the Inoue method (28), and mobilization of plasmids from *E. coli* S17-1 (donor strain) to coryneform recipient strains in conjugation assays was essentially accomplished as described previously (39). Transconjugants were selected on TSA medium containing 50 μg/ml of nalidixic acid and an additional antibiotic (kanamycin, chloramphenicol, or apramycin), depending on the mobilizable plasmid used for the mating. For arsenic resistance tests, strains were grown on TSA-MMC plates and single colonies were transferred into TSB medium and grown at 30°C to an optical density at 600 nm (OD$_{600}$) of approximately 0.5. Ten microlitera of cell suspension was streaked on plates of TSA-MMC (supplemented with an appropriate volume of either arsenate or arsenite) and incubated for up to 2 days at 30°C. Resistance was determined by the appearance of growth and defined as the highest concentration of either arsenate or arsenite that permitted growth.

Molecular genetic methods. Restriction endonuclease digestion, agarose gel electrophoresis, Southern blot analysis, and molecular cloning techniques were performed by using standard procedures (34). Primers for PCR were generated by using the *C. glutamicum* strain ATCC 13032 genome sequence and are listed in Table S1 in the supplemental material.

Isolation of RNA from *C. glutamicum* strain RES167 grown in MMC [supplemented or not with 5 mM As(III)], electrophoresis, transfer to nylon membranes, and hybridization were performed as described previously (45). Probes corresponding to internal fragments of *arsB1*, *arsB2*, *arsC1*, *arsC2*, and *arsC1'* were obtained by PCR amplification using specific primers (see Table S1 in the supplemental material).

For reverse transcription (RT)-PCR analysis (two-step procedure), 1 μg of RNA was used as template to generate single-strand cDNA from the desired genes using the first-strand cDNA synthesis kit for RT-PCR (Roche) and following the manufacturer's recommendations. Primers used to generate cDNA from the corresponding transcripts were designed using the specific Applied Biosystems software (Primer express software, v2.0) and can be found in Table S1 in the supplemental material. cDNAs were used as template for the second PCR amplification reaction using specific primers (see Table S1 in the supplemental material); amplified DNAs were separated by electrophoresis through 1.6% agarose gels and stained with ethidium bromide. RNA samples were tested for contamination with genomic DNA by using each RNA sample as a template for PCR.

For quantitative-PCR (Q-PCR), 1/20 of each cDNA sample (from the first RT-PCR; see above) was used as template for amplification, adding a specific pair of primers for each gene (see Table S1 in the supplemental material), a suitable volume of the master reaction mix (Applied Biosystems), and water up to 25 μl. Reactions were performed in an ABI Prism 7000 sequence detection system (Applied Biosystems), and results were processed using specific software (ABI Prism 7000 SDS software). Results are indicated relative to the cycle threshold value. In all cases, the oligonucleotide primers used in Q-PCR were optimized to amplify fragments of the same length from each gene (around 50 nucleotides) and to ensure a similar melting temperature (59°C) according to the software instructions.

Computer analyses were performed with DNASTAR; database similarity searches were performed using BLAST and FASTA public servers (National Collection of Industrial Bacteria and European Bioinformatics Institute [EBI], Hixton Hall, United Kingdom), and multiple alignments of sequences were accomplished using ClustalW (EBI). Phylogenetic analyses were performed using MEGA2 (Molecular Evolutionary Genetics Analysis) software (35) by the neighbor-joining method with 21 *arsR* genes, 22 *arsB* genes, and 29 *arsC* genes; these analyses are shown in the supplemental material (Fig. S1, S3, and S4).

Construction of recombinant plasmids. To clone the whole *ars1* (*arsR1-B1-C1-C1'*) and *ars2* (*arsR2-B2-C2*) operons, 2.8- and 2.1-kb fragments were amplified by *Pfu* from the total DNA of *C. glutamicum* strain ATCC 13032 using primer pairs ars1/ars2 and ars37/ars38, respectively (see Table S1 in the supplemental material). Primers were designed to include at their 5′ ends sites for the restriction enzymes EcoRI and BamHI; the 2.8-kb PCR-amplified DNA band (*ars1*) was digested with EcoRI (Klenow fragment filled) and BamHI and ligated to the BglII- plus SmaI-digested bifunctional plasmid pECM2 (Table 1), affording plasmid pECAS1. The 2.1-kb PCR-amplified DNA fragment (*ars2*) was ligated to the SmaI-digested pECM2, obtaining the bifunctional plasmid pECAS2 (Table 1).

For the disruption of the *arsB1*, *arsB2*, and *arsB3* genes (encoding the three arsenite permeases present in the *C. glutamicum* genome), internal fragments of the genes were obtained by PCR amplification of total DNA from *C. glutamicum* ATCC 13032 by using primers ars3/ars4 for *arsB1* (310 bp), ars5/ars6 for *arsB2* (370 bp), and ars7/ars8 for *arsB3* (260 bp) (Fig. 1A; see Table S1 in the supplemental material). Primers were designed to include restriction sites for BamHI (upper primers) and HindIII (lower primers); plasmid pK18mob digested with BamHI and HindIII was ligated with the internal fragments of the arsenite permease genes to afford plasmids pKA1, pKA2, and pKA3 (Table 1). Similarly, the internal fragments from *arsB1* and *arsB2* were cloned into plasmid pOJ260 (Table 1) to obtain pOJA1 and pOJA2, respectively.

To identify promoters in the upstream region of *arsB1* and *arsC1*, DNA fragments were amplified by PCR with the primers ars9/ars10 (ParsB1, 158 bp) and ars11/ars12 (ParsC1, 198 bp) (see Table S1 in the supplemental material); restriction enzyme sites for EcoRI and NdeI were included in the up and down primers, respectively. Amplified fragments were cloned into the promoter probe plasmids pJMFA24 and pEGFP (Table 1) to afford plasmids pJMF-EP/pGFP-EP (permease promoter) and pJMF-ER/pGFP-ER (reductase promoter), respectively.

RESULTS

Coryneform bacteria are highly resistant to arsenic. Several gram-negative and gram-positive bacteria were assayed for their sensitivity/resistance to arsenic in TSA medium supplemented with sodium arsenate [AsO$_3^-$, As(V)] or sodium arsenite [AsO$_2^-$, (AsIII)]. Bacteria were classified as low resistant, moderately resistant, and highly resistant to arsenic (Table 2). As expected, the level of resistance to As(V) was

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description[a]	Source or reference[b]
Strains		
E. coli DH5α	r⁻ m⁻ used for general cloning	26
E. coli S17-1	Mobilizing donor strain, pro recA, which has an RP4 derivative integrated into the chromosome	53
E. coli W3110	K-12 F⁻ (rmD-rmE)	3
E. coli AW3110	E. coli W3110 strain lacking the chromosomal ars operon (Δars::cam)	15
C. glutamicum 13869	Wild-type strain	ATCC
C. glutamicum 13032	Wild-type strain	ATCC
C. glutamicum RES167	ATCC 13032 restriction-deficient derivative used as host of recombinant plasmids	59
C. glutamicum ArsB1	RES167 derivative containing plasmid pKA1 integrated in the arsB1 gene	This work
C. glutamicum ArsB2	RES167 derivative containing plasmid pKA2 integrated in the arsB2 gene	This work
C. glutamicum ArsB3	RES167 derivative containing plasmid pKA3 integrated in the arsB3 gene	This work
C. glutamicum ArsB1-B2	ArsB1 derivative mutant, containing plasmid pOJA2 integrated in the arsB2 gene	This work
C. glutamicum ArsB1-B3	ArsB3 derivative mutant, containing plasmid pOJA1 integrated in the arsB1 gene	This work
C. glutamicum ArsB2-B3	ArsB3 derivative mutant, containing plasmid pOJA2 integrated in the arsB2 gene	This work
Staphylococcus aureus 240	Wild-type strain	CECT
Rhodococcus fascians 3001	Wild-type strain	CECT
Nocardia corynebacterioides 14898	Wild-type strain	ATCC
Mycobacterium smegmatis mc² 155	Efficient plasmid transformation M. smegmatis strain	56
Bacillus subtilis 36	Wild-type strain	CECT
Streptomyces lividans 1326	Wild-type strain	JIC
Pseudomonas fluorescens 378	Wild-type strain	CECT
Pseudomonas putida KT2440	bdsMR	22
Plasmids		
pK18mob	E. coli mobilizable plasmid containing lacZ and kan	54
pKA1	pK18mob derivative carrying the 310-bp internal fragment of the arsB1 gene from C. glutamicum	This work
pKA2	pK18mob derivative carrying the 370-bp internal fragment of the arsB2 gene from C. glutamicum	This work
pKA3	pK18mob derivative carrying the 260-bp internal fragment of the arsB3 gene from C. glutamicum	This work
pOJ260	E. coli mobilizable plasmid containing lacZ and apm	6
pOJA1	pOJ260 derivative carrying the 310-bp internal fragment of the arsB1 gene from C. glutamicum	This work
pOJA2	pOJ260 derivative carrying the 370-bp internal fragment of the arsB2 gene from C. glutamicum	This work
pECM2	Mobilizable E. coli-Corynebacterium bifunctional plasmid containing kan and cat	30
pECAS1	pECM2 derivative carrying the whole ars1 operon (arsRBCC)	This work
pECAS2	pECM2 derivative carrying the whole ars2 operon (arsRBC)	This work
pJMFA24	E. coli promoter-probe vector containing bla and the promoterless kan gene from Tn5 as reporter gene	2
pJMF-EP	pJMFA24 derivative containing the putative promoter region of arsB1 (ParsB1)	This work
pJMF-ER	pJMFA24 derivative containing the putative promoter region of arsC1 (ParsC1)	This work
pEGFP	Bifunctional E. coli-corynebacteria promoter-probe vector containing kan as selective marker and egfp2 gene under the kan promoter (Pkan) as reporter	M. Letek and L. M. Mateos, unpublished data
pGFP-EP	pEGFP derivative containing the putative promoter region of arsB1 (ParsB1) instead of Pkan	This work
pGFP-ER	pEGFP derivative containing the putative promoter region of arsC1 (ParsC1) instead of Pkan	This work
pEGNC	pEGFP derivative vector containing the promoterless egfp2 gene	M. Letek and L. M. Mateos, unpublished data

[a] kan, apm, bla, cat, and egfp2 are respectively genes for kanamycin, apramycin, ampicillin, chloramphenicol, and green fluorescent protein.
[b] ATCC, American Type Culture Collection; CECT, Spanish Type Culture Collection; JIC, John Innes Centre, Norwich, United Kingdom.

FIG. 1. Schematic representation of genes involved in arsenic resistance in *C. glutamicum* ATCC 13032 (A) and in other bacteria (B). (A) Operons *ars1* and *ars2* are indicated. Arrows represent open reading frames. Dashed boxes indicate the probes used for the cloning of internal fragments of the desired genes obtained by PCR amplification. (B) The three-gene operon (*arsRBC*), encoding the transcriptional repressor (*arsR*), an arsenite permease (*arsB*), and an arsenate reductase (*arsC*), is present in the *E. coli* K-12 chromosome (accession number NC_000913), *Staphylococcus* plasmids pI258 (31) and pSX267 (48), *C. glutamicum* (NC_003450), *C. efficiens* (NC_004369), and *R. erythropolis* plasmid pBD2 (NC_005073). The five-gene operon (*arsRDABC*), encoding an arsenite-inducible repressor (*arsR*), a negative regulatory protein (*arsD*), an oxyanion-protruding ATPase, an arsenite efflux pump (*arsA* and *arsB*, respectively), and an arsenate reductase (*arsC*), is present in the *E. coli* plasmids R773 and R46 (10, 17) and in plasmid pKW301 from *A. multivorum* (57). The four-gene operon has been found in different species, such as the skin element of *B. subtilis* (52), *A. ferrooxidans* (11), plasmid R478 from *S. marcescens* (49), a *Synechocystis* sp. (36), and *Pseudomonas putida* (NC_002947). An isolated *arsC* gene is present in the genome of *Haemophilus influenzae* (NC_000907), and two operons involved in arsenic resistance have been identified on the chromosome of *P. putida* and *C. glutamicum*.

TABLE 2. Resistance levels of different microorganisms to arsenite (AsIII) and arsenate (AsV)

Microorganism	AsIII resistance (mM)	AsV resistance (mM)
Mycobacterium smegmatis	1	25
Nocardia corynebacteroides	2	100
Staphylococcus aureus	2	50
Bacillus subtilis	2.5	75
Streptomyces lividans	4	150
Escherichia coli DH5α	5	100
Pseudomonas fluorescens	6	200
Pseudomonas putida	7	200
Corynebacterium glutamicum 13032	12	>500
Corynebacterium glutamicum 13869	12	>500
Rhodococcus fascians	14	>500

10- to 20-fold (50 to 150 mM) higher than the resistance level to As(III), except for *C. glutamicum* and *Rhodococcus fascians*, whose resistance level reached 400 to 500 mM (close to the solubility level of sodium arsenate in the media).

From these experiments, it may be concluded that members of the coryneform group (*C. glutamicum* 13032, *C. glutamicum* 13869 [formerly *B. lactofermentum*], and *R. fascians* [formerly *Corynebacterium fascians*]) are much more resistant to arsenic than the rest of the bacteria analyzed. The genes responsible for the resistance to arsenic in *C. glutamicum* 13032 or *C. glutamicum* 13869 must be located within the bacterial chromosome because no plasmids bearing arsenic resistance genes have been described in these strains (38, 50, 58).

Several genes probably involved in arsenic resistance are present in *Corynebacterium glutamicum*. Once the complete nucleotide sequence of the *C. glutamicum* chromosome was available (44), and assuming that proteins of similar sequences

TABLE 3. Arsenite/arsenate resistance levels of different *E. coli* and *C. glutamicum* strains untransformed or transformed with plasmid pECAS1 (operon *ars1*) or pECAS2 (operon *ars2*)

Strain	As(III) resistance (mM)			As(V) resistance (mM)		
	Untransformed	pECAS1	pECAS2	Untransformed	pECAS1	pECAS2
E. coli						
DH5α	5	6	6	100	100	100
W3110	5	6	6	100	100	100
AW3110	0.2	6	6	2	20	20
C. glutamicum						
RES 167	12	60	20	500	500	500
ArsB1	4	60	15	200	500	500
ArsB2	9	60	20	500	500	500
ArsB3	12	60	20	500	500	500
ArsB1-B2	0.4	60	15	10	500	500

perform similar functions, the gene products possibly involved in arsenic resistance became available in the genome via homology-based analyses such as BLAST and FASTA (19). Thus, two operons, thereafter named *ars1* and *ars2*, located distant from each other on the chromosome were found and both of them showed similar genetic structures (*arsRBC*). The only difference was the presence of an additional gene (*arsC1'*, Cgl1455 in the database) at the end of the *ars1* operon (Fig. 1A). Genes homologous to *arsC1'* have also been found downstream from *arsC1* in *Corynebacterium efficiens* (accession number NC_004369) and in plasmid pBD2 from *Rhodococcus erythropolis* (accession number NC_005073). The organization of the *ars* genes in different bacteria is shown in Fig. 1B.

Based on BLAST analysis, the first genes of both clusters (*ars1* and *ars2*) are *arsR1* and *arsR2*, which encode putative arsenite regulatory proteins (*arsR1* was not described in the database, and *arsR2* was previously named Cgl 0275). ArsR1 and ArsR2 showed clear homologies to ArsR proteins from actinomycetes and *Acidithiobacillus ferrooxidans* (see Fig. S1 in the supplemental material). ArsR1 and ArsR2 from *C. glutamicum* (as in the ArsR from *A. ferrooxidans*) do not have the typical CXCXXC arsenite binding motif of R773 ArsR but have two joined cysteines close to the arsenite binding motif of R773 ArsR. Furthermore, ArsR1 has two additional cysteines (there is only one in ArsR2) at the N-terminal extension, like CadC (21) (see Fig. S2 in the supplemental material). Interestingly, the *arsR* in both operons is expressed divergently from the rest of the *ars* genes.

The second genes, *arsB1* and *arsB2*, described as encoding an arsenite efflux pump, might encode enzyme arsenite permeases (formerly named Cgl 1453 and Cgl 0258 respectively). These are homologous to the arsenic protein carriers from actinomycetes, the skin element from *B. subtilis* (52), *Saccharomyces cerevisiae* Acr3p (61), and the arsenic protein carrier from a *Synechocystis* sp. (36) (see Fig. S3 in the supplemental material).

The rest of the genes in both clusters, *arsC1*, *arsC1'*, and *arsC2* (previously named Cgl 1454, Cgl 1455, and Cgl 0259, respectively), encode predicted protein tyrosine phosphatases with sequence similarity to the thioredoxin-dependent arsenate reductases from actinomycetes and, to a lesser extent, *Staphylococcus* (62) and *Bacillus* (4) (see Fig. S4 in the supplemental material).

In addition to the above operons probably involved in arsenic resistance (operons *ars1* and *ars2*), another putative arsenite permease gene (*arsB3*, Cgl 1414) and a putative arsenate reductase gene (*arsC4*, Cgl 1049) were present in the *C. glutamicum* genome (Fig. 1A). ArsB3 did not show homologies with arsenite permeases from gram-negative bacteria and seems to be different from the ArsB clade from actinomycetes, the skin element from *B. subtilis*, *Saccharomyces cerevisiae* Acr3p, and the ArsB clade from a *Synechocystis* sp. ArsC4 seems to be unrelated to the rest of *C. glutamicum* ArsCs and showed homologies with ArsC from gram-negative bacteria (see Fig. S4 in the supplemental material).

Arsenite permeases ArsB1 and ArsB2 are involved in arsenic resistance in *C. glutamicum*. To confirm the involvement of the above-described genes in arsenic resistance in *C. glutamicum*, the integrative plasmids pKA1, pKA2, and pKA3 (Table 1) containing internal fragments of the hypothetical arsenite permease genes *arsB1*, *arsB2*, and *arsB3* were introduced separately into *E. coli* strain S17-1 and transferred to *C. glutamicum* strain RES167 by conjugation. The arsenite permease gene was chosen for gene disruption because of the possible polar effect of its disruption on both *ars1* and *ars2* operons (Fig. 1A). Kanamycin-resistant transconjugants were obtained in all cases, suggesting that the integrative plasmids would have been incorporated into the host chromosome and that therefore the homologous chromosomal genes (and downstream *arsC1*, *arsC1'*, and *arsC2* genes) might be disrupted. Plasmid integration was confirmed both by Southern hybridization and by PCR amplification (data not shown). Ten transconjugants from each conjugation experiment were analyzed for arsenite and arsenate resistance; because the behaviors of all the transconjugants obtained with a given suicide plasmid (pKA1, pKA2, or pKA3) were identical, only one transconjugant of each conjugation experiment was selected and was named *C. glutamicum* ArsB1, *C. glutamicum* ArsB2, or *C. glutamicum* ArsB3 (Table 1); the resistance to arsenite of these insertional mutant strains is shown in Table 3. When the above mutants were grown in TSA in the presence of arsenate, *C. glutamicum* ArsB2 and *C. glutamicum* ArsB3 were able to grow in up to 500 mM arsenate like *C. glutamicum* RES167 whereas *C. glutamicum* ArsB1 was resistant to only 200 mM.

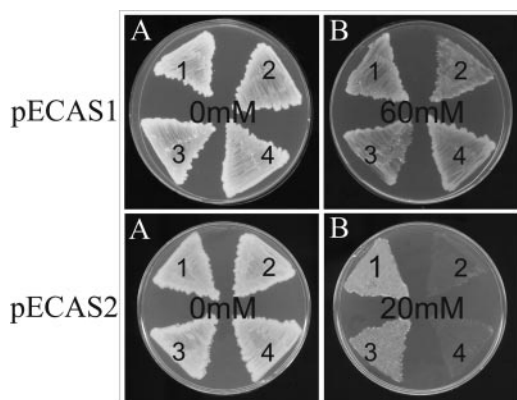To construct the double arsenite permease mutant *C. glutamicum* ArsB1-B2, *C. glutamicum* ArsB1 was used as recipient

FIG. 2. Complementation of *C. glutamicum arsB* mutants by the *ars1* (pECAS1) and *ars2* (pECAS2) operons. *C. glutamicum* RES167 (1), *C. glutamicum* ArsB1 (2), *C. glutamicum* ArsB2 (3), and *C. glutamicum* ArsB1-B2 (4) transformed with pECAS1 or pECAS2 were inoculated in TSA containing increasing amounts of arsenite (0 mM to 60 mM), incubated at 30°C for 36 h, and photographed. Please note that some strains containing plasmid pECAS2 were unable to grow in the presence of 20 mM arsenite and that none of them were able to grow in 60 mM arsenite.

in the conjugation assay, using the mobilizable plasmid pOJA2 (Table 1); mutants ArsB1-B3 and ArsB2-B3 were obtained after the transfer of plasmid pOJA1 (Table 1) and plasmid pOJA2, respectively, to the recipient strain *C. glutamicum* ArsB3. One transconjugant from each assay was selected and checked by Southern blot analysis. *C. glutamicum* mutants ArsB1-B3 and ArsB2-B3 behaved exactly like the single mutants *C. glutamicum* ArsB1 and *C. glutamicum* ArsB2, respectively. The behavior of the double mutant *C. glutamicum* ArsB1-B2 in relation to its resistance to arsenite is indicated in Table 3; furthermore, this mutant was also unable to grow in 10 mM arsenate. The resistance level of the double mutant ArsB1-B2 was reduced 30-fold (from 12 mM to 0.4 mM) for arsenite and 50-fold (from 500 mM to 10 mM) for arsenate.

These results suggest that resistance to arsenite [and perhaps also to arsenate through the conversion of As(V) into As(III)] in *C. glutamicum* would mainly be due to the activity of operons *ars1* and *ars2*, operon *ars1* being more crucial than *ars2*.

Gene dose effect of *ars1* and *ars2* on resistance to arsenic in *C. glutamicum* and *E. coli* strains. To confirm the involvement of the *ars1* and *ars2* operons in the resistance to arsenic, we attempted to analyze the effect of the *C. glutamicum ars1* and *ars2* operons in several *E. coli* strains (heterologous complementation) or in *C. glutamicum* ArsB mutants by using the multicopy bifunctional plasmids pECAS1 and pECAS2 (Table 1). Resistance levels to arsenite for transformed and untransformed *E. coli* and *C. glutamicum* strains are shown in Table 3.

As can be observed in Fig. 2, mutations in *arsB1* and *arsB2* were clearly complemented by plasmid pECAS1 because transconjugants of mutants *C. glutamicum* ArsB1, ArsB2, and ArsB1-B2 were able to grow in up to 60 mM arsenite (Table 3; Fig. 2) and in up to 500 mM arsenate (Table 3). When plasmid pECAS2 was introduced into the *C. glutamicum* strains, a different behavior was observed (Table 3; Fig. 2); the control strain RES167 and mutant ArsB2 were resistant up to 20 mM arsenite, but the strains affected in the *arsB1* gene (mutants

ArsB1 and ArsB1-B2) were resistant only up to 15 mM arsenite. In conclusion, the presence of several copies (ca. 30 copies/cell) of the *ars1* and *ars2* operons not only complements the respective *arsB1* and *arsB2* mutations but also confers on *C. glutamicum* hitherto undescribed levels of resistance to arsenite.

Transformation of *E. coli* with plasmids pECAS1 and pECAS2 indicated that the *ars* mutation present in *E. coli* AW3110 was clearly complemented by both the *ars1* and *ars2* operons (Table 3) whereas the resistance level of transformed *E. coli* DH5α and *E. coli* W3110 was only slightly increased (from 5 to 6 mM). Similar results were obtained when the transformed *E. coli* strains were analyzed for arsenate resistance; the supersensitive strain AW3110 was complemented by both *ars* operons, and its resistance level to arsenate increased from 2 mM to 20 mM.

Transcriptional analysis of the *ars* operons. The genetic organization of the *ars1* and *ars2* operons suggests that genes *arsB1-C1-C1'* and *arsB2-C2* might be transcribed together and divergently with the transcription of *arsR1* and *arsR2*, respectively. To analyze this possibility, *C. glutamicum* cells were grown in MMC to mid-exponential phase in the presence or absence of 5 mM arsenite and total RNA was isolated and used in Northern blot experiments.

As can be observed in Fig. 3, transcription of both operons was induced in the presence of arsenite and transcription of operon *ars1* was more efficient than that of operon *ars2*, corroborating our earlier results with the insertional mutants. The presence of transcripts of 2.4 kb and 1.1 kb when an internal fragment from *arsB1* was used as probe (Fig. 3A) suggests that *arsB1-C1-C1'* is transcribed mainly as a single 2.4-kb polycistronic unit and that the 1.1-kb transcript might correspond to a transcript of the gene coding for the ArsB1 permease. When an internal fragment from *arsC1* was used as probe (Fig. 3A), the same 2.4-kb transcript was observed, as well as an additional 1.2-kb transcript corresponding to the dicistronic *arsC1-C1'* (Fig. 3A). Similar results were obtained when the internal fragment from *arsC1'* was used as probe.

The transcriptional organization of the *ars2* operon was very similar, with a transcript of 1.5 kb (dicistronic *arsB2-C2*) when an internal *arsB2* fragment was used as probe (Fig. 3B) and transcripts of 1.5 kb (dicistronic *arsB2-C2*) and 0.4 kb (monocistronic *arsC2*) when an internal *arsC2* fragment was used (Fig. 3B).

To confirm the expression of both *ars* operons, RT-PCR was performed using mRNA from cultures growing in the absence or presence of 5 mM arsenite. As can be observed in Fig. 4, *arsB1*, *arsC1*, and *arsC1'* were induced in the presence of this compound. Similar results were obtained with the *ars2* operon (data not shown).

To quantify more precisely the level of expression of all the genes probably involved in resistance to arsenic, Q-PCR analysis was performed with all nine genes described in this work (*arsR1*, *arsB1*, *arsC1*, *arsC1'*, *arsR2*, *arsB2*, *arsC2*, *arsB3*, and *arsC4*) by using specific primers (see Table S1 in the supplemental material). As can be observed in Table 4, the estimated numbers of specific mRNA molecules for *arsB3* and *arsC4* were similar in the presence or absence of 5 mM arsenite whereas a number of molecules of specific mRNA for *arsR1*, *arsB1*, *arsC1*, *arsC1'*, *arsR2*, *arsB2*, and *arsC2* were clearly in-
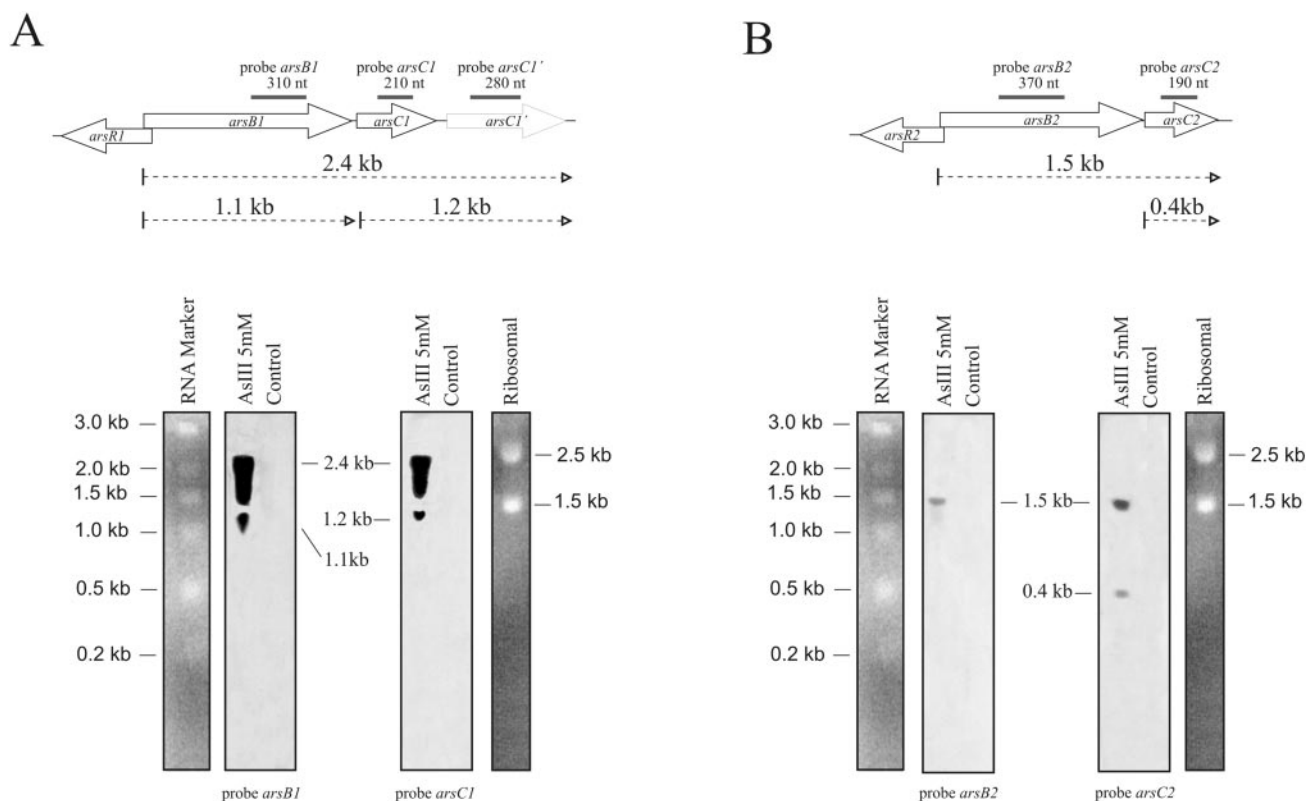
FIG. 3. Northern blot analysis of specific transcripts for *arsB1*, *arsC1*, *arsC1′* *arsB2*, and *arsC2* from *C. glutamicum*. Total RNA (15 μg) was isolated from *C. glutamicum* in the exponential phase (OD$_{600}$ = 1.5) in the absence (control) or presence of 5 mM arsenite, electrophoresed, transferred to nylon filters, and hybridized with internal fragments of *arsB1*, *arsC1*, *arsC1′*, *arsB2*, and *arsC2* obtained by PCR (dashed boxes) using the primers described in Table S1 in the supplemental material. Size controls corresponding to the migration of bands in the gel are indicated.
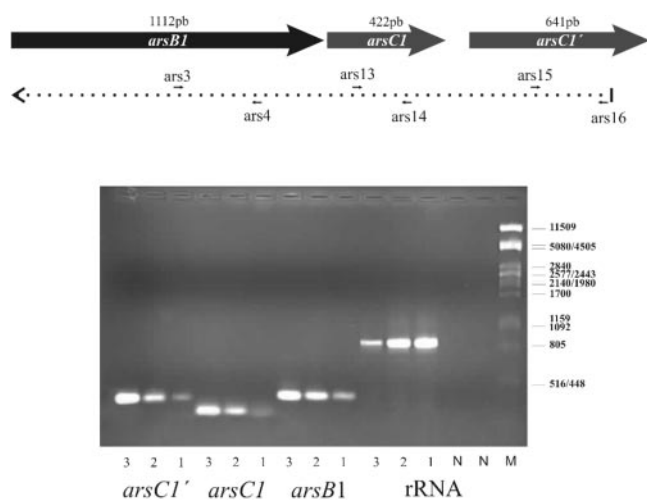
FIG. 4. Transcriptional analysis of *arsB1-C1-C1′* by RT-PCR. Total RNA isolated from *C. glutamicum* in the exponential phase (OD$_{600}$ = 1.5) in the absence (lanes 1) or presence (lanes 2) of 5 mM arsenite was used as the template in a reverse transcriptase reaction with primer ars16 to generate cDNA and then amplified with primers (ars3/4, ars13/14, and ars15/16) internal to each gene. A positive control using *C. glutamicum* chromosomal DNA instead of cDNA was included (lanes 3), as well as RT-PCR of the 16S rRNA (rRNA) using primer rrn2 for the synthesis of cDNA and primers rrn1 and rrn2 for PCR amplification (see Table S1 in the supplemental material). DNA contamination of the RNA samples was ruled out by performing PCR directly on the RNAs (N).

duced in the presence of 5 mM arsenite. From the results of this experiment, it is possible to conclude that the expression of *arsB3* and *arsC4* is constitutive whereas the expression of the rest of the genes is inducible by arsenite, the induction of both regulatory genes (*arsR1* and *arsR2*) being weaker than the rest of the *ars* genes.

Promoter region analysis. Taking into account the above results and the higher expression of the *ars1* operon (Fig. 3), we checked for the presence of DNA fragments with promoter activity upstream from *arsB1* or *arsC1* with a view to finding new regulated promoters for use in corynebacterial expression systems.

Plasmids pJMF-EP and pJMF-ER (containing the upstream regions of *arsB1* and *arsC1*, respectively) were transformed into *E. coli* DH5α, and transformants were able to grow in Luria-Bertani broth containing 200 and 100 μg/ml of kanamycin, respectively. This indicated that both DNA fragments (ParsB1 and ParsC1) have promoter activity in *E. coli*, the permease promoter being stronger than that of reductase. Both promoter regions also showed promoter activity in *C. glutamicum* when plasmids pGFP-EP and pGFP-ER (Table 1) were present. Promoter activity in *C. glutamicum* was quantified by the level of green fluorescent protein, and as in *E. coli*, ParsB1 was stronger than ParsC1; the expression of ParsB1 was clearly induced in the presence of 0.01 to 5 mM arsenite/arsenate (Fig. 5), but no induction was observed in the case of ParsC1. Because in many microorganisms, the *ars* operon is

TABLE 4. Quantitative PCR of genes involved in arsenic resistance

Gene	C_t^a		Ratio [+As(III)/−As(III)][b]
	− As(III)	+ As(III)	
arsR1	22.39	18.35	16
arsB1	26.70	21.35	121
arsC1	26.16	18.19	256
arsC1'	25.92	18.06	232
arsR2	23.12	19.07	17
arsB2	24.84	19.95	56
arsC2	23.12	16.33	111
arsB3	26.40	26.20	1.1
arsC4	24.28	23.68	1.5

[a] C_t is defined as the cycle at which fluorescence is determined to be statistically significant above background, being inversely proportional to the log of the initial copy number; this value was calculated automatically by the ABI Prism 7000 SDS software.

[b] +As(III)/−As(III) ratio indicates the relative number of specific mRNA molecules for each gene in the presence or absence of As(III). It was calculated assuming that at the threshold points, the number of amplified DNA molecules (N) in the presence or absence of arsenite would be the same as and proportional to the initial number of RNA molecules (n), as follows: $N = n \times 2^{C_t}$.

induced by antimonite and bismuth (5, 36, 47), we studied the possible effect of different elements or compounds such as bismuth, antimonite, phosphate, phosphite, nitrates, or nitrites on the induction of ParsB1 and no induction was observed in any case.

Interestingly, 26 nucleotides upstream from the *arsB1* gene, a perfect inverted repeat (TGTCGATATT-N$_{12}$-AATATCGA CA) was found. Similarly, 56 nucleotides upstream from the *arsB2* gene, another inverted repeat with a unique mismatch (ATGTCCGTCA-N$_8$-TGACGcACAT) was also found. No similar sequences were found upstream from any other *ars* genes by using the Palindrome program (http://bioweb.pasteur.fr/seqanal /interfaces/palindrome.html).
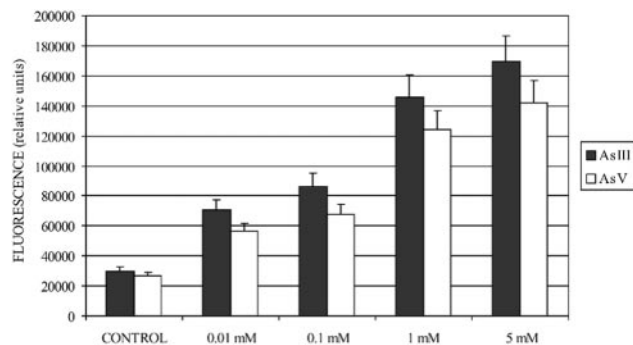

FIG. 5. The promoter of the *arsB1* gene is induced in the presence of arsenite or arsenate. *C. glutamicum* [pGFP-EP] was grown in the presence of subinhibitory concentrations of arsenite (black bar) or arsenate (empty bar). *C. glutamicum* [pEGNC] (Table 1) was used as a negative control, and its fluorescence level was subtracted from all the values obtained. In all cases, the fluorescence level of each sample was divided by the OD$_{600}$ of the sample. The values are the means of four determinations, and the standard deviation is indicated on the bar top. The fluorescence level ratio of green fluorescent protein/OD$_{600}$ was measured on a Biotek Sinergy HT fluorimeter.

DISCUSSION

The *C. glutamicum ars* system confers resistance to arsenic up to 12 mM As(III) and 500 mM As(V), and hence, *C. glutamicum* is one of the most resistant microorganisms described to date. This could be due to the presence of two functional *ars* operons (*ars1* and *ars2*) and two accessory genes (*arsB3* and *arsC4*) described and analyzed here. The existence of two chromosomal *ars* operons involved in arsenic resistance and an additional arsenate reductase gene (*arsC3*) scattered throughout the chromosome has also been described in *P. putida* (Fig. 1B) (14), although the functionality of both operons was not studied.

An unusual characteristic of the *ars1* and *ars2* operons of *C. glutamicum* is the orientation of *arsR*; whereas in *C. glutamicum*, *arsR* (in both *ars* operons) was located upstream and in the opposite orientation from *arsBC*, in most of the analyzed bacteria, *arsR* was located in the same orientation as *arsBC* (Fig. 1B). Another exception is *A. ferrooxidans* and *Serratia marcescens*, where the chromosomal arsenic resistance operon contains two clusters (*arsCR-arsBH* for *A. ferrooxidans* and *arsBC-arsRH* for *S. marcescens*) which are transcribed in the opposite direction (12). The possible significance of this particular gene organization remains unknown, but a divergent expression could mean an independent or differential regulation to optimize the adaptation of the microorganism to nutritional or environmental changes (9). ArsRs from either *C. glutamicum*, actinomycetes, or *A. ferrooxidans* do not have a typical arsenite binding motif (CXCXXC) present in the ArsR proteins from the *E. coli* plasmid R773.

Another structural difference between operons *ars1* and *ars2* is the presence of an extra arsenate reductase gene (*arsC1'*) in operon *ars1*. It has been claimed that a single *arsC* was added to the original *ars* operon (*arsRB*) when molecular oxygen appeared in the atmosphere (46); subsequent horizontal gene transfer in the evolution of arsenate reductases and sequence divergence gave the distinct *arsC* classes that exist today (29). However, a convergent evolution of the two mayor ArsC branches cannot be discarded (40). Three families of arsenate reductases have been described previously (40), and those encoded by genes found in the *C. glutamicum ars1* and *ars2* operons (and also in *C. efficiens*, *R. erythropolis*, and *Streptomyces coelicolor* A3 [2]) belong to the group of *Bacillus* enzymes where arsenate reductases use thioredoxin and are similar to protein tyrosine phosphatases but form a separate subfamily (see Fig. S4 in the supplemental material). The arsenate reductase ArsC4 from *C. glutamicum*, together with the ArsC4 from *C. efficiens* and ArsC from *Corynebacterium diphtheriae*, seems to be more related to the R773 ArsC purified from *E. coli* which requires reduced glutathione (GSH) and the small thiol transfer protein glutaredoxin (Grx) for arsenate reductase activity (23). The presence of two functional *arsC* genes in the *ars1* operon from *C. glutamicum* is also intriguing, although three *arsC* genes are present in the unique *ars* operon of *C. efficiens* (accession number NC_004369) and *R. erythropolis* (accession number NC_005073) (Fig. 1B), all of them belonging to the mycolata group and being the most arsenite-resistant bacteria described to date (Table 2).

Genes encoding three hypothetical arsenite permeases were located in the genome of *C. glutamicum*, being the only case described up to date; permeases ArsB1 and ArsB2 are related

to each other, whereas ArsB3 is phylogenetically more distant, but all the permeases belong to the same clade (see Fig. S3 in the supplemental material). The presence of multiple permeases could circumvent the absence of the oxyanion ATPase (ArsA) described in the five-gene system (Fig. 1B). The *arsA* gene was not found in the genome sequence of *C. glutamicum*, and hence, the mechanism of arsenic resistance could be driven by the ArsB permease, as has been described for several other microorganisms. In some cases, heterologous cloning of the *arsA* gene from *E. coli* resulted in increased resistance to arsenite in *Staphylococcus aureus*, as previously described (8).

Insertional mutation of genes encoding the three arsenite permeases found in the genome of *C. glutamicum* clearly indicates that *arsB1* and *arsB2* are involved in resistance to arsenite but that insertional mutant *C. glutamicum* ArsB3 behaves like the wild type. Nevertheless, Q-PCR analysis clearly indicates that *arsB3* is expressed constitutively and that it is considered an accessory gene, like *arsC4*. The distinct level of transcription of both *ars1* and *ars2* operons, observed in RNA/DNA hybridization experiments, indicates that operon *ars1* is transcriptionally more active than *ars2*. This was also confirmed when operons *ars1* and *ars2* were introduced into *C. glutamicum* (Table 3).

Operons *ars1* (pECAS1) and *ars2* (pECAS2) were also used to transform *E. coli* strains, and the resulting transformed strains were slightly more resistant to arsenite than the untransformed strains DH5α and W3110 (Table 3); operons *ars1* and *ars2* functionally replace the *ars* mutation of the supersensitive *E. coli* strain AW3110, which increases the resistance 30-fold (Table 3). These results confirmed the functionality of the *ars1* and *ars2* operons from *C. glutamicum* in *E. coli*.

The theoretical binding site for ArsR overlaps with the putative -10 and -35 promoter regions of the *arsB* genes, and both contain symmetric and nonidentical dyad sequences: TGTCGATATT-N_{12}-AATATCGACA for *arsB1* and ATG TCCGTCA-N_8-TGACGcACAT for *arsB2*. Dyad sequences have been found in multiple regulatory sites involved in the binding of ArsR in *E. coli* (60) and *Synechocystis* (36). Induction of ParsB1 in the presence of As(III)/As(V) was demonstrated when plasmid pGFP-EP was used in the reporter system; however, antimonite [Sb(III)] was not able to induce pGFP-EP, although all ArsR repressors so far described do respond to Sb(III).

In conclusion, operons *ars1* and *ars2* are involved in arsenic resistance in *C. glutamicum*. Operon *ars1* seems to be the main arsenic detoxification system according to different lines of experimental evidence: (i) *C. glutamicum* ArsB1 is less resistant to arsenite than mutant *C. glutamicum* ArsB2; (ii) RNA/DNA hybridization experiments reveal a higher expression of *ars1* than *ars2*; (iii) the arsenic-supersensitive mutant *C. glutamicum* ArsB1-B2 transformed with the whole *ars1* operon becomes resistant to 60 mM arsenite at five times the level of resistance of the wild-type strain; and (iv) *C. glutamicum* ArsB1-B2 transformed with operon *ars2* becomes resistant to 15 mM arsenite at 1.25 times the level of resistance of *C. glutamicum*.

ACKNOWLEDGMENTS

REFERENCES

1. **Abdrashitova, S. A., G. G. Abdullina, and A. N. Ilialetdinov.** 1986. Role of arsenites in lipid peroxidation in *Pseudomonas putida* cells oxidizing arsenite. Mikrobiologiya **55:**212–216.
2. **Adham, S. A., S. Rodriguez, A. Ramos, R. I. Santamaria, and J. A. Gil.** 2003. Improved vectors for transcriptional/translational signal screening in corynebacteria using the *melC* operon from *Streptomyces glaucescens* as reporter. Arch. Microbiol. **180:**53–59.
3. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36:**525–557.
4. **Bennett, M. S., Z. Guan, M. Laurberg, and X. D. Su.** 2001. *Bacillus subtilis* arsenate reductase is structurally and functionally similar to low molecular weight protein tyrosine phosphatases. Proc. Natl. Acad. Sci. USA **98:**13577–13582.
5. **Bhattacharjee, H., J. Li, M. Y. Ksenzenko, and B. P. Rosen.** 1995. Role of cysteinyl residues in metalloactivation of the oxyanion-translocating ArsA ATPase. J. Biol. Chem. **270:**11245–11250.
6. **Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene **116:**43–49.
7. **Bobrowicz, P., R. Wysocki, G. Owsianik, A. Goffeau, and S. Ulaszewski.** 1997. Isolation of three contiguous genes, ACR1, ACR2 and ACR3, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. Yeast **13:**819–828.
8. **Broer, S., G. Ji, A. Broer, and S. Silver.** 1993. Arsenic efflux governed by the arsenic resistance determinant of *Staphylococcus aureus* plasmid pI258. J. Bacteriol. **175:**3480–3485.
9. **Browning, D. F., C. M. Beatty, A. J. Wolfe, J. A. Cole, and S. J. Busby.** 2002. Independent regulation of the divergent *Escherichia coli* nrfA and acsP1 promoters by a nucleoprotein assembly at a shared regulatory region. Mol. Microbiol. **43:**687–701.
10. **Bruhn, D. F., J. Li, S. Silver, F. Roberto, and B. P. Rosen.** 1996. The arsenical resistance operon of IncN plasmid R46. FEMS Microbiol. Lett. **139:**149–153.
11. **Butcher, B. G., S. M. Deane, and D. E. Rawlings.** 2000. The chromosomal arsenic resistance genes of *Thiobacillus ferrooxidans* have an unusual arrangement and confer increased arsenic and antimony resistance to *Escherichia coli*. Appl. Environ. Microbiol. **66:**1826–1833.
12. **Butcher, B. G., and D. E. Rawlings.** 2002. The divergent chromosomal *ars* operon of *Acidithiobacillus ferrooxidans* is regulated by an atypical ArsR protein. Microbiology **148:**3983–3992.
13. **Cai, J., K. Salmon, and M. S. DuBow.** 1998. A chromosomal *ars* operon homologue of *Pseudomonas aeruginosa* confers increased resistance to arsenic and antimony in *Escherichia coli*. Microbiology **144:**2705–2713.
14. **Canovas, D., I. Cases, and V. de Lorenzo.** 2003. Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. Environ. Microbiol. **5:**1242–1256.
15. **Carlin, A., W. Shi, S. Dey, and B. P. Rosen.** 1995. The *ars* operon of *Escherichia coli* confers arsenical and antimonial resistance. J. Bacteriol. **177:**981–986.
16. **Cervantes, C., G. Ji, J. L. Ramirez, and S. Silver.** 1994. Resistance to arsenic compounds in microorganisms. FEMS Microbiol. Rev. **15:**355–367.
17. **Chen, C. M., T. K. Misra, S. Silver, and B. P. Rosen.** 1986. Nucleotide sequence of the structural genes for an anion pump. The plasmid-encoded arsenical resistance operon. J. Biol. Chem. **261:**15030–15038.
18. **Diorio, C., J. Cai, J. Marmor, R. Shinder, and M. S. DuBow.** 1995. An *Escherichia coli* chromosomal *ars* operon homolog is functional in arsenic detoxification and is conserved in gram-negative bacteria. J. Bacteriol. **177:**2050–2056.
19. **Durand, P., L. Canard, and J. P. Mornon.** 1997. Visual BLAST and visual FASTA: graphic workbenches for interactive analysis of full BLAST and FASTA outputs under MICROSOFT WINDOWS 95/NT. Comput. Appl. Biosci. **13:**407–413.
20. **Ehrlich, H. L.** 2002. Bacterial oxidation of As(III) compounds, p. 313–329. *In* W. T. Frankenberger, Jr. (ed.), Environmental chemistry of arsenic. Marcel Dekker Inc., New York, N.Y.
21. **Endo, G., and S. Silver.** 1995. CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. J. Bacteriol. **177:**4437–4441.
22. **Franklin, F. C., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. Proc. Natl. Acad. Sci. USA **78:**7458–7462.
23. **Gladysheva, T. B., K. L. Oden, and B. P. Rosen.** 1994. Properties of the arsenate reductase of plasmid R773. Biochemistry **33:**7288–7293.

24. **Gotz, F., J. Zabielski, L. Philipson, and M. Lindberg.** 1983. DNA homology between the arsenate resistance plasmid pSX267 from *Staphylococcus xylosus* and the penicillinase plasmid pI258 from *Staphylococcus aureus*. Plasmid **9:**126–137.

25. **Gourdon, P., and N. D. Lindley.** 1999. Metabolic analysis of glutamate production by *Corynebacterium glutamicum*. Metab. Eng. **1:**224–231.

26. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.

27. **Hingston, J. A., C. D. Collins, R. J. Murphy, and J. N. Lester.** 2001. Leaching of chromated copper arsenate wood preservatives: a review. Environ. Pollut. **111:**53–66.

28. **Inoue, H., H. Nojima, and H. Okayama.** 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene **96:**23–28.

29. **Jackson, C. R., and S. L. Dugas.** 2003. Phylogenetic analysis of bacterial and archaeal *arsC* gene sequences suggests an ancient, common origin for arsenate reductase. BMC. Evol. Biol. **3:**18.

30. **Jager, W., A. Schafer, A. Puhler, G. Labes, and W. Wohlleben.** 1992. Expression of the *Bacillus subtilis sacB* gene leads to sucrose sensitivity in the gram-positive bacterium *Corynebacterium glutamicum* but not in *Streptomyces lividans*. J. Bacteriol. **174:**5462–5465.

31. **Ji, G., and S. Silver.** 1992. Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid pI258. J. Bacteriol. **174:** 3684–3694.

32. **Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff, M. Bott, A. Burkovski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Kramer, B. Linke, A. C. McHardy, F. Meyer, B. Mockel, W. Pfefferle, A. Puhler, D. A. Rey, C. Ruckert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegrabe, and A. Tauch.** 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J. Biotechnol. **104:**5–25.

33. **Kaneko, H., and K. Sakaguchi.** 1979. Fusion of protoplasts and genetic recombination of *Brevibacterium flavum*. Agric. Biol. Chem. **43:**1007–1013.

34. **Kieser, T., M. J. Bibb, M. J. Buttner, B. F. Chen, and D. A. Hopwood.** 2000. Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, Conn.

35. **Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei.** 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics **17:**1244–1245.

36. **Lopez-Maury, L., F. J. Florencio, and J. C. Reyes.** 2003. Arsenic sensing and resistance system in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J. Bacteriol. **185:**5363–5371.

37. **Maciaszczyk, E., R. Wysocki, P. Golik, J. Lazowska, and S. Ulaszewski.** 2004. Arsenical resistance genes in *Saccharomyces douglasii* and other yeast species undergo rapid evolution involving genomic rearrangements and duplications. FEMS Yeast Res. **4:**821–832.

38. **Martin, J. F., R. I. Santamaria, H. Sandoval, G. del Real, L. M. Mateos, J. A. Gil, and A. Aguilar.** 1987. Cloning systems in amino acid-producing corynebacteria. Bio/Technology **5:**137–146.

39. **Mateos, L. M., A. Schafer, J. Kalinowski, J. F. Martin, and A. Puhler.** 1996. Integration of narrow-host-range vectors from *Escherichia coli* into the genomes of amino acid-producing corynebacteria after intergeneric conjugation. J. Bacteriol. **178:**5768–5775.

40. **Mukhopadhyay, R., and B. P. Rosen.** 2002. Arsenate reductases in prokaryotes and eukaryotes. Environ. Health Perspect. **110**(Suppl. 5)**:**745–748.

41. **Mukhopadhyay, R., B. P. Rosen, l. T. Phung, and S. Silver.** 2002. Microbial arsenic: from geocycles to genes and enzymes. FEMS Microbiol. Rev. **26:** 311–325.

42. **Nakayama, K., K. Araki, and H. Kase.** 1978. Microbial production of essential amino acid with *Corynebacterium glutamicum* mutants. Adv. Exp. Med. Biol. **105:**649–661.

43. **Ochi, K.** 1995. Phylogenetic analysis of mycolic acid-containing wall-chemotype IV actinomycetes and allied taxa by partial sequencing of ribosomal protein AT-L30. Int. J. Syst. Bacteriol. **45:**653–660.

44. **Ohnishi, J., S. Mitsuhashi, M. Hayashi, S. Ando, H. Yokoi, K. Ochiai, and M. Ikeda.** 2002. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Appl. Microbiol. Biotechnol. **58:**217–223.

45. **Ramos, A., M. P. Honrubia, D. Vega, J. A. Ayala, A. Bouhss, D. Mengin-Lecreulx, and J. A. Gil.** 2004. Characterization and chromosomal organization of the *murD-murC-ftsQ* region of *Corynebacterium glutamicum* ATCC 13869. Res. Microbiol. **155:**174–184.

46. **Rosen, B. P.** 2002. Biochemistry of arsenic detoxification. FEBS Lett. **529:** 86–92.

47. **Rosen, B. P., H. Bhattacharjee, and W. Shi.** 1995. Mechanisms of metal-loregulation of an anion-translocating ATPase. J. Bioenerg. Biomembr. **27:**85–91.

48. **Rosenstein, R., K. Nikoleit, and F. Gotz.** 1994. Binding of ArsR, the repressor of the *Staphylococcus xylosus* (pSX267) arsenic resistance operon to a sequence with dyad symmetry within the *ars* promoter. Mol. Gen. Genet. **242:**566–572.

49. **Ryan, D., and E. Colleran.** 2002. Arsenical resistance in the IncHI2 plasmids. Plasmid **47:**234–240.

50. **Santamaria, R. I., J. A. Gil, J. M. Mesas, and J. F. Martin.** 1984. Characterization of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*. J. Gen. Microbiol. **130:**2237–2246.

51. **Santini, J. M., and R. N. vanden Hoven.** 2004. Molybdenum-containing arsenite oxidase of the chemolithoautotrophic arsenite oxidizer NT-26. J. Bacteriol. **186:**1614–1619.

52. **Sato, T., and Y. Kobayashi.** 1998. The *ars* operon in the skin element of *Bacillus subtilis* confers resistance to arsenate and arsenite. J. Bacteriol. **180:**1655–1661.

53. **Schafer, A., J. Kalinowski, R. Simon, A. H. Seep-Feldhaus, and A. Puhler.** 1990. High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. J. Bacteriol. **172:** 1663–1666.

54. **Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.

55. **Silver, S., and L. T. Phung.** 1996. Bacterial heavy metal resistance: new surprises. Annu. Rev. Microbiol. **50:**753–789.

56. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.

57. **Suzuki, K., N. Wakao, T. Kimura, K. Sakka, and K. Ohmiya.** 1998. Expression and regulation of the arsenic resistance operon of *Acidiphilium multivorum* AIU 301 plasmid pKW301 in *Escherichia coli*. Appl. Environ. Microbiol. **64:**411–418.

58. **Tauch, A., A. Puhler, J. Kalinowski, and G. Thierbach.** 2003. Plasmids in *Corynebacterium glutamicum* and their molecular classification by comparative genomics. J. Biotechnol. **104:**27–40.

59. **Wehmeier, L., O. Brockmann-Gretza, A. Pisabarro, A. Tauch, A. Puhler, J. F. Martin, and J. Kalinowski.** 2001. A *Corynebacterium glutamicum* mutant with a defined deletion within the *rplK* gene is impaired in (p)ppGpp accumulation upon amino acid starvation. Microbiology **147:**691–700.

60. **Wu, J., and B. P. Rosen.** 1993. Metalloregulated expression of the *ars* operon. J. Biol. Chem. **268:**52–58.

61. **Wysocki, R., P. Bobrowicz, and S. Ulaszewski.** 1997. The *Saccharomyces cerevisiae* ACR3 gene encodes a putative membrane protein involved in arsenite transport. J. Biol. Chem. **272:**30061–30066.

62. **Zegers, I., J. C. Martins, R. Willem, L. Wyns, and J. Messens.** 2001. Arsenate reductase from *S. aureus* plasmid pI258 is a phosphatase drafted for redox duty. Nat. Struct. Biol. **8:**843–847.