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.

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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 (arsB1), and an arsenate reductase (arsC1) located immediately downstream from arsC1. Additional arsenic 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', arsB2, 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 layers, 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 aquaglyceroporins (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 arsenate 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 pF258 and pSX267 (24).

* Corresponding author. Mailing address: Área 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.
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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 (ars4 and arsB, respectively), together with an arsenate reductase (arsC). This operon was initially discovered in *E. coli* plasmids R773 and R46 (17) and then on plasmid pKWS301 from *Acidiphilum 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 *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 *ars1*, *ars2*, and *ars3* in *S. douglasi* (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 stress-formation 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 (MCM) (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 *in oue* method (28), and mobilization of plasmids from *E. coli* strain 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 on a shaking platform (250 rpm) at an optical density at 600 nm (OD600) of approximately 0.5. Ten microliters 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 

**Construction of recombinant plasmids.** To clone the whole *ars1* (arsR1-B1-C1’)-1 and *ars2* (arsR2-B2-C2) operons, 2.8- and 2.1-kb fragments were amplified by PCR from the total DNA of *C. glutamicum* strain ATCC 13032 using primer pairs ars1/ar5 and ars2/ar5 for *arsC1* (37), respectively (see Table S1 in the supplemental material). Primers were designed to include 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 Smal-digested bifunctional plasmid pECM2 (Table 1), affording plasmid pECAS1. The 2.1-kb PCR-amplified DNA fragment (ars2) was ligated to the Smal-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* strain 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 pK818m0 digested with BamHI and HindIII was ligated with the internal fragments of the arsenate 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 pMPA24 and pEGFP (Table 1) to afford plasmids pMP2/EPC/GFP (permease promoter) and pMP2/EPC/GFP/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 TSB medium supplemented with sodium arsenate [AsO₃³⁻, As(V)] or sodium arsenite [AsO₂⁻, (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...
| Strains | Relevant genotype or description | Source or reference |
|---------|--------------------------------|---------------------|
| 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’ (pRD-mE) | 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 ArsB2-B3 | ArsB3 derivative mutant, containing plasmid pOJA1 integrated in the arsB1 gene | This work |
| C. glutamicum ArsB3-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 | Relevant genotype or description | Source or reference |
|----------|--------------------------------|---------------------|
| 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 containing 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-corynebacterium 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.
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 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* DH5ax      | 5                     | 100                 |
| *Pseudomonas fluorescens*     | 6                     | 200                 |
| *Pseudomonas putida*          | 7                     | 200                 |
| *Corynebacterium glutamicum* 13032 | 12                  | >500                |
| *Corynebacterium glutamicum* 13869 | 12                  | >500                |
| *Rhodococcus fascians*        | 14                    | >500                |

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 pT258 (31) and pSX267 (48), *C. glutamicum* (NC_003450), *C. efficiens* (NC_004369), and *R. erythropolis* plasmid pBD2 (NC_005073). The five-gene operon (*arsRD4BC*), encoding an arsenite-inducible repressor (*arsR*), a negative regulatory protein (*arsD*), an oxyanion-promoting 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*. 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).
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 \textit{ars}1 and \textit{ars}2, located distant from each other on the chromosome were found and both of them showed similar genetic structures (\textit{arsRBC}). The only difference was the presence of an additional gene (\textit{arsC1’, Cgl1455 in the database}) at the end of the \textit{ars1} operon (Fig. 1A).

Genes homologous to \textit{arsC1} have also been found downstream from \textit{arsC1} in \textit{Corynebacterium efficiens} (accession number NC_004369) and in plasmid pBD2 from \textit{Rhodococcus erythropolis} (accession number NC_005073). The organization of the \textit{ars} genes in different bacteria is shown in Fig. 1B.

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

The second genes, \textit{arsB1} and \textit{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 \textit{B. subtilis} (52), \textit{Saccharomyces cerevisiae} Acr3p (61), and the arsenic protein carrier from a \textit{Synechocystis} sp. (36) (see Fig. S3 in the supplemental material).

The rest of the genes in both clusters, \textit{arsC1}, \textit{arsC1’}, and \textit{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, \textit{Staphylococcus} (62) and \textit{Bacillus} (4) (see Fig. S4 in the supplemental material).

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

\textbf{Arsenite permeases \textit{ArsB1} and \textit{ArsB2} are involved in arsenic resistance in \textit{C. glutamicum}.} To confirm the involvement of the above-described genes in arsenic resistance in \textit{C. glutamicum}, the integrative plasmids pKA1, pKA2, and pKA3 (Table 1) containing internal fragments of the hypothetical arsenite permease genes \textit{arsB1}, \textit{arsB2}, and \textit{arsB3} were introduced separately into \textit{E. coli} strain S17-1 and transferred to \textit{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 \textit{ars1} and \textit{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 \textit{arsC1}, \textit{arsC1’}, and \textit{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 \textit{C. glutamicum} \textit{ArsB1}, \textit{C. glutamicum} \textit{ArsB2}, or \textit{C. glutamicum} \textit{ArsB3} (Table 1); the resistance to arsenite of these insertion mutant strains is shown in Table 3. When the above mutants were grown in TSA in the presence of arsenate, \textit{C. glutamicum} \textit{ArsB2} and \textit{C. glutamicum} \textit{ArsB3} were able to grow in up to 500 mM arsenate like \textit{C. glutamicum} RES167 whereas \textit{C. glutamicum} \textit{ArsB1} was resistant to only 200 mM.

To construct the double arsenite permease mutant \textit{C. glutamicum} \textit{ArsB1-B2}, \textit{C. glutamicum} \textit{ArsB1} was used as recipient
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 hypersensitive strain AW3110 was complemented by both *ars* operons, and its resistance level to arsenite 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 insertion 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-
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
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 \textit{ars} B1 gene, a perfect inverted repeat (TGTCGATATT-N\textsubscript{12}-AATATCGA CA) was found. Similarly, 56 nucleotides upstream from the \textit{ars} B2 gene, another inverted repeat with a unique mismatch (ATGTCGCTCA-N\textsubscript{16}-TGACGcACAT) was also found. No similar sequences were found upstream from any other \textit{ars} genes by using the Palindrome program (http://bioweb.pasteur.fr/seqanal /interfaces/palindrome.html).

FIG. 5. The promoter of the \textit{ars} B1 gene is induced in the presence of arsenite or arsenate. \textit{C. glutamicum} [pGFP-EP] was grown in the presence of subinhibitory concentrations of arsenite (black bar) or arsenate (empty bar). \textit{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\textsubscript{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\textsubscript{600} was measured on a Biotek Synergy HT fluorimeter.

### DISCUSSION

The \textit{C. glutamicum} \textit{ars} system confers resistance to arsenic up to 12 mM As(III) and 500 mM As(V), and hence, \textit{C. glutamicum} is one of the most resistant microorganisms described to date. This could be due to the presence of two functional \textit{ars} operons (\textit{ars}1 and \textit{ars}2) and two accessory genes (\textit{ars}B3 and \textit{ars}C4) described and analyzed here. The existence of two chromosomal \textit{ars} operons involved in arsenic resistance and an additional arsenate reductase gene (\textit{ars}C3) scattered throughout the chromosome has also been described in \textit{P. putida} (Fig. 1B) (14), although the functionality of both operons was not studied.

An unusual characteristic of the \textit{ars}1 and \textit{ars}2 operons of \textit{C. glutamicum} is the orientation of \textit{ars}R; whereas in \textit{C. glutamicum}, \textit{ars}R (in both \textit{ars} operons) was located upstream and in the opposite orientation from \textit{ars}BC, in most of the analyzed bacteria, \textit{ars}R was located in the same orientation as \textit{ars}BC (Fig. 1B). Another exception is \textit{A. ferrooxidans} and \textit{Serratia marcescens}, where the chromosomal arsenic resistance operon contains two clusters (\textit{ars}CR-\textit{ars}BH for \textit{A. ferrooxidans} and \textit{ars}BC-\textit{ars}RH for \textit{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). \textit{ArsRs} from either \textit{C. glutamicum}, actinomycetes, or \textit{A. ferrooxidans} do not have a typical arsene binding motif (CXXXCC) present in the \textit{ArsR} proteins from the \textit{E. coli} plasmid R773.

Another structural difference between operons \textit{ars}1 and \textit{ars}2 is the presence of an extra arsenate reductase gene (\textit{arsC1'}) in operon \textit{ars}1. It has been claimed that a single \textit{arsC} was added to the original \textit{ars} operon (\textit{ars}BB) when molecular oxygen appeared in the atmosphere (46); subsequent horizontal gene transfer in the evolution of arsenate reductases and sequence divergence gave the distinct \textit{ars}C classes that exist today (29). However, a convergent evolution of the two major \textit{ArsC} branches cannot be discarded (40). Three families of arsenate reductases have been described previously (40), and those encoded by genes found in the \textit{C. glutamicum} \textit{ars}1 and \textit{ars}2 operons (and also in \textit{C. efficiens, R. erythropolis}, and \textit{Streptomyces coelicolor} A3 [2]) belong to the group of \textit{Bacillus} enzymes where arsenate reductases use thioredoxin and are similar to protein tyrosine phosphatases but form a separate subfamily (see Fig. 5 in the supplemental material). The arsenate reductase \textit{ArsC4} from \textit{C. glutamicum}, together with the \textit{ArsC4} from \textit{C. efficiens} and \textit{ArsC} from \textit{Corynebacterium diphtheriae}, seems to be more related to the R773 \textit{ArsC} purified from \textit{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 \textit{ars}C genes in the \textit{ars}1 operon from \textit{C. glutamicum} is also intriguing, although three \textit{ars}C genes are present in the unique \textit{ars} operon of \textit{C. efficiens} (accession number NC_004369) and \textit{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 \textit{C. glutamicum}, being the only case described up to date; permeases \textit{ArsB1} and \textit{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 arsenic 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: TGTCGATAAT-N12-AATATCAGACA for arsB1 and ATG TCCGTCAN8-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, aminonitrite (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.

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