Appl Microbiol Biotechnol (2014) 98:10143–10152
DOI 10.1007/s00253-014-6055-2

Engineered coryneform bacteria as a bio-tool for arsenic remediation

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Received: 24 July 2014 / Revised: 21 August 2014 / Accepted: 27 August 2014 / Published online: 11 September 2014

Abstract Despite current remediation efforts, arsenic contamination in water sources is still a major health problem, highlighting the need for new approaches. In this work, strains of the nonpathogenic and highly arsenic-resistant bacterium *Corynebacterium glutamicum* were used as inexpensive tools to accumulate inorganic arsenic, either as arsenate (As^{V}) or arsenite (As^{III}) species. The assays made use of "resting cells" from these strains, which were assessed under well-established conditions and compared with *C. glutamicum* background controls. The two mutant As^{V}-accumulating strains were those used in a previously published study: (i) ArsC1/C2, in which the gene/s encoding the mycothiol-dependent arsenate reductases is/are disrupted, and (ii) MshA/C mutants unable to produce mycothiol, the low molecular weight thiol essential for arsenate reduction. The As^{III}-accumulating strains were either those lacking the arsenite permease activities (Acr3-1 and Acr3-2) needed in As^{III} release or recombinant strains overexpressing the aquaglyceroporin genes (*glpF*) from *Corynebacterium diphtheriae* or *Streptomyces coelicolor*, to improve As^{III} uptake. Both genetically modified strains accumulated 30-fold more As^{V} and 15-fold more As^{III} than the controls. The arsenic resistance of the modified strains was inversely proportional to their metal accumulation ability. Our results provide the basis for investigations into the use of these modified *C. glutamicum* strains as a new bio-tool in arsenic remediation efforts.

Keywords *Corynebacterium* · Arsenate · Arsenite · Mycothiol · Aquaglyceroporins · ICP-MS

Introduction

The severe toxicity of arsenic (As) accounts for its ranking at the top of the Substance Priority List of the Superfund Amendments and Reauthorization Act (http://www.atsdr.cdc.gov/SPL/index.html). Frequent exposure to arsenic species is associated with various pathologies, including diabetes and cancer. These typically develop after chronic exposure, such as the daily consumption of water with arsenic levels higher than the established limit of 10 ppb (EPA-2006, USA). In Bangladesh, for example, consumption of arsenic-contaminated well water accounts for a mortality rate of around 20 % (Argos et al. 2010). Two inorganic arsenic species are generally found in soils and in surface waters: arsenite (As^{III}) and arsenate (As^{V}). Under oxic conditions, As^{V} is the most prevalent form. Its toxicity can be explained by the resemblance of its tetrahedral oxyanion structure to phosphate, such that it uncouples conversions in intermediary metabolism, e.g., oxidative phosphorylation (Kruger et al. 2013). As^{III} is more toxic than As^{V}, disturbing redox homeostasis because of its ability to react with free thiols; in addition, it promotes the generation of reactive oxygen species, which can damage proteins and DNA (Liu et al. 2001; Tsai et al. 2009).

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-6055-2) contains supplementary material, which is available to authorized users.

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Although chemical and physical arsenic remediation processes are widely available, they are expensive and in undeveloped countries difficult to apply. Moreover, they are not completely effective, leaving behind trace quantities of the metal (Chwirka et al. 2000). Therefore, over the years, several alternative arsenic bioremediation strategies have been developed. The most common biological processes make use of eukaryotic microorganisms, such as yeast (Shah et al. 2010; Singh et al. 2008a; Tsai et al. 2012), but others have used aquatic or terrestrial plants (Wojas et al. 2010) to achieve arsenic dispersion from contaminated water or soil by phytoremediation (Tsai et al. 2009).

The specific advantages of microbial remediation depend on the microorganism used as sorbent, but they include the small size of the microorganisms, their metabolic capacity, the large number of available genome sequences that can be exploited, and the simplicity and low cost of the process (Tsai et al. 2009). Some microbial species serve as unspecific chelators, binding arsenic species to the cell surface; however, there are also species that while they take up As^{V}, they extrude the more toxic As^{III} (Tsai et al. 2009). Nevertheless, there are only a few studies describing the use of bacteria, mostly *Escherichia coli*, for arsenic immobilization-accumulation and/or speciation analysis. Three strategies have so far been developed, and they can be combined to obtain a higher accumulation yield: the expression of arsenic-chelating proteins that retain arsenic inside the cell, the expression of membrane channels or porins that increase arsenic uptake, and the deletion of genes involved in As^{III} efflux. Chelating proteins include those that are arsenic-specific, such as the arsenic repressor protein (ArsR), but also those that chelate several metals (Kostal et al. 2004; Singh et al. 2008a, b). Both can result in significant arsenic accumulation. For example, recombinant *E. coli* strains overexpressing metallothioneins (metal-chelating proteins) from the marine alga *Fucus vesiculosus* accumulated 30- and 26-fold more As^{III} and As^{V}, respectively, than the original strain (Singh et al. 2008b). The same authors described a three-fold increase in accumulated arsenic by the overexpression in *E. coli* of the *EcglpF* gene, encoding the aquaglyceroporin GlpF (involved in As^{III} uptake). The heterologous coexpression in *E. coli* of the *Schizosaccharomyces pombe* phytochelatin synthase (*SpPCS*) and the γ-glutamylcysteine synthase (*Spgsh*) genes increased arsenic accumulation 36-fold (Singh et al. 2010). Removal of genes involved in As^{III} efflux was also shown to enhance accumulation by 80-fold in an *E. coli* strain coexpressing *Spgsh* and *EcglpF* genes (Singh et al. 2010). However, *E. coli* is not a generally recognized as safe (GRAS) microorganism according to the US FDA, thus hindering its release into the environment for remediation purposes.

Corynebacterium glutamicum is a gram-positive GRAS bacterium (Meiswinkel et al. 2013) of the actinobacteria-corynebacteria group. Its two functional and inducible *ars* operons (*ars1* and *ars2*) are organized as a larger *arsR-acr3-arsC* operon (with slight modifications) that encodes the repressor proteins [ArsR1 (NCgl1452) and ArsR2 (NCgl0257)], the arsenite permease efflux pumps [Acr3-1 (NCgl1453) and Acr3-2 (NCgl0258], and the arsenate reductases [ArsC1 (NCgl1454) and ArsC2 (NCgl0259)] (Villadangos et al. 2011). ArsC1 and ArsC2 reduce As^{V} using the mycothiol/mycoredoxin-1 redox system (Ordoñez et al. 2009). In addition, the *C. glutamicum ars1* operon constitutively expresses the gene for ArsC1' (NCgl1455) at very low levels. ArsC1' reduces As^{V} to As^{III} using electrons from the thioredoxin/thioredoxin reductase system (Villadangos et al. 2011). Cellular exposure to As^{V} is followed by its entry into the cell via phosphate uptake systems (Pst/Pit), its reduction to As^{III} by the arsenate reductases ArsC1/ArsC2 (principally), and the extrusion of this reduced form by the arsenite permeases Acr3-1/Acr3-2, with energy supplied by the membrane potential (Villadangos et al. 2012).

In previous reports, we described the kinetic uptake of As^{V} and As^{III} in *C. glutamicum* wild-type and mutant strains (Feo et al. 2007; Villadangos et al. 2010) and determined their intracellular equilibrium parameters. Most of the mutant strains used in those studies were further exploited in the present work (see Table 1), in particular *C. glutamicum* 2Δars (lacking the two *ars* operons; Ordóñez et al. 2008), *C. glutamicum* ArsC1-C2 (double mutant of arsenate reductases; Ordoñez et al. 2009), *C. glutamicum* MshA/C (mutants unable to synthesize mycothiol; Ordoñez et al. 2009), and *C. glutamicum* 2Acr3 (double mutant of arsenite permeases; Villadangos et al. 2012). Based on the knowledge obtained with those strains, we re-engineered *C. glutamicum* to obtain As^{V}- and As^{III}-accumulating cells and then examined the potential of these newly created strains as effective biocontainers to decontaminate arsenic-polluted water sources.

Materials and methods

Bacterial strains

The bacterial strains and plasmids used in this work are described in Table 1. All the strains (wild type and mutants) described in the present work are deposited in the public strain collection located at the Microbiology Area of the University of León (Spain).

Cell cultures, chemicals, and general methods for nucleic acid, proteins, and staining

E. coli was grown in Luria-Bertani (LB) complex medium and incubated at 37 °C with shaking (220 rpm). *C. glutamicum*

Table 1 Bacterial strains and plasmids

Strain	Genotype or description	Source or reference
E. coli Top10	Strain used for general cloning	Invitrogen, California, USA
E. coli S17-1	Mobilizing donor strain for conjugation assays	(Ordóñez et al. 2005)
S. coelicolor A3(2)	Wild-type strain	Prof. Hopwood, J.I.I. , Norwich, UK
C. glutamicum 13032	Wild-type strain	ATCC
C. glutamicum RES167	13032 restriction-deficient derivative used as recipient in conjugations experiments	(Ordóñez et al. 2005)
C. glutamicum ArsC1	RES167 derivative with a disrupted *CgarsC1* gene; Kan^R	(Ordoñez et al. 2009)
C. glutamicum ArsC2	RES167 derivative with a disrupted *CgarsC2* gene; Apr^R	(Ordoñez et al. 2009)
C. glutamicum ArsC1-C2	RES167 derivative with disrupted *CgarsC1* and *CgarsC2* genes; Kan^R, Apr^R	(Ordoñez et al. 2009)
C. glutamicum 2Acr3	RES167 derivative with disrupted *Cgacr3-1* and *Cgacr3-2* genes (previously named ArsB1-B2); Kan^R, Apr^R	(Villadangos et al. 2012)
C. glutamicum 2Δars	RES167 derivative with deleted *ars1* and *ars2* operons	(Ordoñez et al. 2009)
C. glutamicum MshA	RES167 derivative with a deleted *CgmshA* gene	(Ordoñez et al. 2009)
C. glutamicum MshB	RES167 derivative with a deleted *CgmshB* gene	(Feng et al. 2006)
C. glutamicum MshC	RES167 derivative with a deleted *CgmshC* gene	(Feng et al. 2006)
C. glutamicum MshD	RES167 derivative with a deleted *CgmshD* gene	(Feng et al. 2006)
Plasmid	Description	Reference
pXHisNpro	Plasmid derived from pIJ2925, containing the *ΔxysA-His*$_6$-P_{kan} and flanked by transcriptional terminators T1 and T2; *amp* gene	(Ordoñez et al. 2009)
pECM2	Mobilizable and bifunctional plasmid (*E. coli* and *Corynebacterium*); *kan* and *cat* genes	(Ordoñez et al. 2009)
pEppk2A	pECM2 derivative containing the *Cgppk2A* gene under the control of P_{kan}	This work
pEppk2B	pECM2 derivative containing the *Cgppk2B* gene under the control of P_{kan}	This work
pEglpCd	pECM2 derivative containing the *CdglpF* gene from *C. diphtheriae* under the control of P_{kan}	This work
pEglpSc	pECM2 derivative containing the *ScglpF* gene from *S. coelicolor* under the control of P_{kan}	This work
pEarsR1	pECM2 derivative containing the *CgarsR1* gene under the control of P_{kan}	This work
pEarsR2	pECM2 derivative containing the *CgarsR2* gene under the control of P_{kan}	This work
pET28a-Ars D109	pET28a derivative containing the first 109 amino acids of the *EcarsD* gene (functional fragment)	(Yang et al. 2011); gift from B.P. Rosen
pEarsD	pECM2 derivative containing the first 109 amino acids of the *EcarsD* gene (functional fragment) under the control of P_{kan}	This work

was grown in complex medium TSB (tryptone soy broth) at 30 °C or with 2 % agar (TSA; Oxoid, Hampshire, UK) added as needed (Ordóñez et al. 2005). The cells were cultured in minimal medium for corynebacteria low in phosphate (MMCLP) when As^V resistance was assayed (Villadangos et al. 2010). As required, the medium was supplemented with 50 μg kanamycin/ml, 100 μg ampicillin or apramycin/ml for *E. coli*, and 8 μg chloramphenicol/ml, 12.5 μg kanamycin or apramycin/ml for *C. glutamicum*. Cell growth was evaluated by monitoring the absorbance of the cultures at 600 nm (A_{600nm}) on a UV-visible spectrophotometer (Hitachi V-200, Tokio, Japan).

All chemicals were of analytical reagent grade and obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich (Gillingham, UK). Arsenic stock solutions were freshly prepared by dissolving sodium arsenite ($NaAsO_2$; As^{III}) or disodium hydrogen arsenate ($HNa_2AsO_4{\cdot}7H_2O$; As^V) in water. Freshly prepared transport assay buffer (TAB), containing 75 mM HEPES-KOH (pH 7.3), 150 mM K_2SO_4, and 1 mM $MgSO_4$, was used in the arsenic retention analyses (Villadangos et al. 2010).

The Neisser staining technique was carried out as previously described (Alcántara et al. 2014).

DNA purification and manipulation, plasmid constructions, *E. coli* transformation, and plasmid transfer from *E. coli* to *C. glutamicum* strains by conjugation were carried out as previously described (Mateos et al. 1994; Ordóñez et al. 2005).

For protein analysis, appropriate *C. glutamicum* was grown in TSB until A_{600nm}=1. The harvested cells were broken using Fast Prep FP120 (Thermo BIO101, MA). The amount of protein present in the samples was measured in a Bradford assay. The protein pattern was verified by SDS-PAGE followed by Coomassie blue staining (Villadangos et al. 2012).

Construction of recombinant plasmids

The gene structure of the *ars1* and *ars2* operons from *C. glutamicum* is depicted inside the Fig. 4. The following genes were PCR-amplified using the corresponding primer pairs described in Table 2 and DNA from *C. glutamicum* ATCC 13032, *Corynebacterium diphtheriae* NCTC 13129, *Streptomyces coelicolor* A3(2), or plasmid pET28a-ArsD109 as templates: (i) *Cgppk2A* (NCgl0880), *Cgppk2B* (NCgl2620), *CgarsR1*, and *CgarsR2* from *C. glutamicum*; (ii) *glpF* from *C. diphtheriae* (*CdglpF*; DIP2236) and *S. coelicolor* (*ScglpF*; SCO1659); and (iii) the *E. coli arsD109* gene (a truncated functional fragment) from the *E. coli* R773-*ars* operon (*EcarsD*; GenBank: U13073.1). The PCR-amplified fragments (993-bp *Cgppk2A*; 951-bp *Cgppk2B*; 368-bp *CgarsR1*; 469-bp *CgarsR2*; 768-bp *CdglpF*; 822-bp *ScglpF*; 327-bp *EcarsD*) were *Nde*I-*Xho*I digested and subcloned into the *E. coli* plasmid pXHisNpro. The recombinant derivatives of this plasmid were *Bgl*II-digested, yielding expression cassettes that were cloned into the bifunctional vector pECM2 (*BamH*I-digested). The resulting plasmids, pEppk2A, pEppk2B, pEarsR1, pEarsR2 pEglpCd, pEglpSc, and pEarsD (Table 1), were used to transform *E. coli* S17-1 and then individually transferred to *C. glutamicum* background strains (Ordóñez et al. 2005).

Arsenic accumulation by "resting cells"

C. glutamicum was grown overnight in TSB medium containing the appropriate antibiotics. The preculture was used to inoculate TSB (As^{III} assays) or MMCLP (As^{V} assays) medium; these cultures were grown until A_{600nm}=2. The cells were harvested, washed, and resuspended to a density corresponding to A_{600nm}=60; under these conditions, the cells are in a nongrowing state and hence considered as resting cells.

Arsenic accumulation was assayed by adding 100 μl of "resting cells" to 900 μl of TAB containing the desired As^{III} or As^{V} concentration, for a total reaction mix volume of 1 ml. At the indicated times, 100-μl aliquots of the reaction mix were withdrawn and filtered through 0.45-μm nitrocellulose filters (Millipore, Darmstadt, Germany). Cells retained on the filters were washed twice with 5 ml of TAB. Filtering and washing were carried out using a "ten place filter manifold" (Hoefer-FH 225V, San Francisco, CA, USA). The filters, which contained the retained cells, were treated with 70 % nitric acid at 70 °C until total digestion. The digested material was diluted with HPLC-quality water to bring each sample to a final volume of 10 ml. The amount of arsenic accumulated by the resting cells was directly quantified by ICP-MS (Varian, San Francisco, CA, USA). All experiments were carried out in triplicate.

Arsenic resistance analysis

C. glutamicum controls and mutants were grown overnight in TSB medium with the appropriate antibiotics. This preculture was then used to inoculate TSB or MMCLP medium containing the indicated concentrations of As^{III} or As^{V}, respectively. After about 16 h of incubation, cell growth was evaluated at

Table 2 Primers used in the present work

Primer	Target gene	Nucleotide sequence (5′→3′)[a]	Restriction sites
ppk2Aup	*Cgppk2A*	G**GAATTC CATATG**GTGGGTAAACTTCCC	*Eco*RI/*Nde*I
ppk2Adown	*Cgppk2A*	CCG**CTCGAG**ACCTTCTACTGAAAG	*Xho*I
ppk2Bup	*Cgppk2B*	GGAATTC**CATATG**CGAAAGAAAAAAGACG	*Nde*I
ppk2Bdown	*Cgppk2B*	CCG**CTCGAG**CTATTTCTTGGACTTC	*Xho*I
CdglpFup	*CdglpF*	TAT**TTTAAA CATATG**ACCGCCCTACAAGCATTC	*Dra*I/*Nde*I
CdglpFdown	*CdglpF*	TAT**TTTAAA CTCGAG**CTACAGCACTACAGTG	*Dra*I/*Xho*I
ScglpFup	*ScglpF*	TAT**TTTAAA CATATG**GTGTCCAGCTCCGAC	*Dra*I/*Nde*I
ScglpFdown	*ScglpF*	TTA**TTTAAA CTCGAG**TCAGGCGAACGCG	*Dra*I/*Xho*I
arsR1up	*CgarsR1*	G**GAATTC CATATG**ACCACTCTCCACAC	*Eco*RI/*Nde*I
arsR1down	*CgarsR1*	TAA**TTTAAACTCGAG**TAATTCCATTGAGCCGATC	*Dra*I/*Xho*I
arsR2up	*CgarsR2*	G**GAATTC CATATG**ACCGCACCCATCC	*Eco*RI/*Nde*I
arsR2down	*CgarsR2*	CCG**CTCGAG**TTATCCGATTTGCAGGATTG	*Xho*I
EcarsDup	*EcarsD*	TAT**TTTAAACATATG**AAAACGTTAATGGTATTTGACCC	*Dra*I/*Nde*I
EcarsDdown	*EcarsD*	TAT**TTTAAA CTCGAG**TTAAGGCGCTAATCCC	*Dra*I/*Xho*I

[a] Restriction sites are in bold.

by measuring the A_{600nm}. The arsenic resistance value for a strain cultured in broth was defined as the arsenic concentration that accounted for a 20 % of the A_{600nm} compared to the value obtained for the same strain grown in the same medium without arsenic (100 %; Villadangos et al. 2012). All experiments were carried out in triplicate.

Cellular dry weight calculation

Different volumes of resting cells were added to preweighed nitrocellulose filters (0.45-μm pore diameter; Millipore). The filters were dried at 65 °C and weighed again (Mettler AE240, Illinois-USA; sensitivity of ±0.01 mg). The reported results are the average of ten independent assays. A relationship between the cell volume and dry weight of the resting cells was determined with respect to a standardized curve; thus, the dry cell weight (DCW) of a 100-μl resting cell suspension (A_{600nm}=6) was 0.24 mg.

Results

Engineered ***C. glutamicum*** ArsC1-C2 mutants specifically accumulate arsenate

The natural As^V resistance of *C. glutamicum* is mediated by its efficient arsenate reductases, ArsC1 and ArsC2 (each from one *ars* operon), two major cellular mycothiol-based single-cysteine reductases (Ordoñez et al. 2009). By contrast, the three-cysteine homodimer ArsC1' (encoded by *CgarsC1'* of the *ars1* operon) is constitutively expressed but at low basal levels (Villadangos et al. 2011). To determine whether arsenate reductase mutant strains accumulate As^V, resting cell suspensions were incubated with 0.1 mM As^V for 2 h. The three tested *C. glutamicum* mutant strains carried single or double mutations obtained by independent or double disruptions of *CgarsC1* and *CgarsC2* (respectively, *C. glutamicum* ArsC1, ArsC2, and ArsC1-C2; Table 1) (Ordoñez et al. 2009). As^V retention by the *C. glutamicum* ArsC1-C2 mutant (28.50 nmol As/mg DCW) was about 28-fold higher than that of the control strain RES167 (Fig. 1a and Supplemental Table S1). Differences between *C. glutamicum* RES167 and either of the single mutants (*C. glutamicum* ArsC1 or ArsC2) were not significant (Fig. 1a). The mutant *C. glutamicum* 2Δars, lacking both the *ars1* and *ars2* operons (Table 1), accumulated the same amount of As^V (28.80 nmol/mg) as the ArsC1-C2 mutant. The increase in As^V accumulation by the ArsC1-C2 and 2Δars mutants was concomitant with their hypersensitivity to this arsenic species (0.10 mM; Fig. 1a). Moreover, their ability to accumulate relatively large amounts of As^V suggested that the constitutively expressed arsenate reductase ArsC1' is hardly involved in the reduction of As^V to As^{III}, as noted elsewhere (Villadangos et al. 2011).

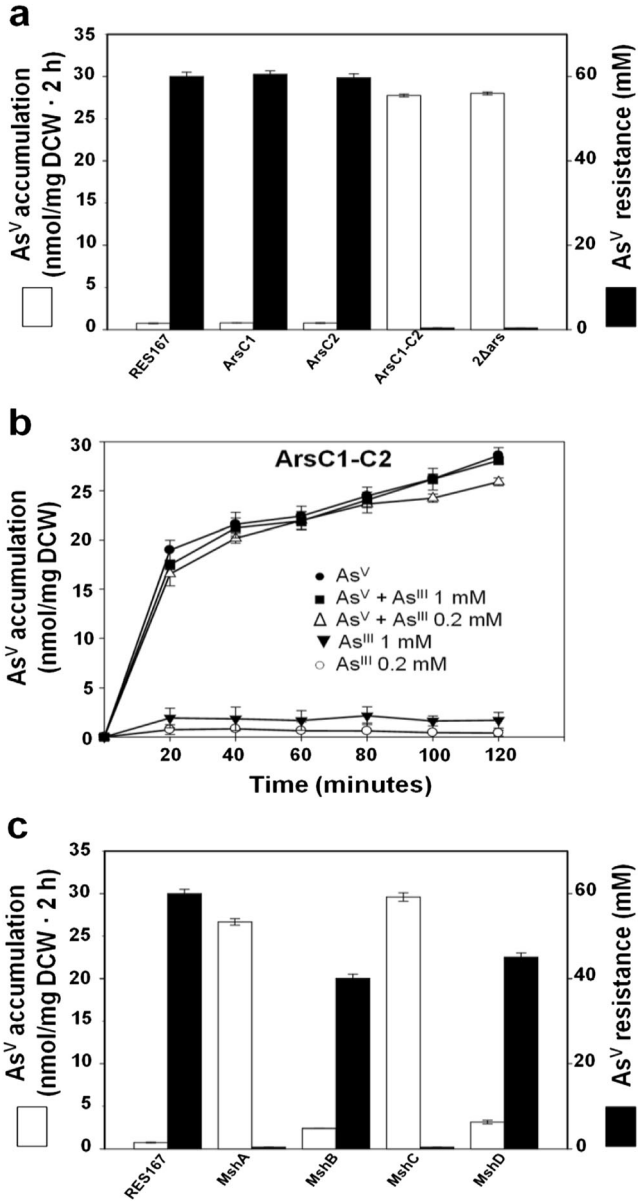

Fig. 1 Arsenate accumulation/resistance of *C. glutamicum ars* and *msh* mutant strains. The As^V contents (*white columns*) of *C. glutamicum* RES167 (control) and the **(a)** *arsC* and **(c)** *msh* mutants were determined 2 h after the cells were incubated with As^V (0.1 mM). As^V resistance (*black columns*) was assayed by culturing the strains in MMCLP medium for 16 h at the indicated As^V concentrations. *Error bars* indicate the standard deviation from three independent assays. RES167 *C. glutamicum* RES167; ArsC1 *C. glutamicum* ArsC1; ArsC2 *C. glutamicum* ArsC2; ArsC1-C2 *C. glutamicum* ArsC1-C2; 2Δars *C. glutamicum* 2Δars; MshA *C. glutamicum* MshA; MshB *C. glutamicum* MshB; MshC *C. glutamicum* MshC; MshD *C. glutamicum* MshD. **(b)** Time course of the As^V accumulated by *C. glutamicum* ArsC1-C2 exposed to the following: As^V 0.1 mM (*black circle*), As^{III} 0.2 mM (*white circle*), As^{III} 1 mM (*inverted black triangle*), or combinations of As^V (0.1 mM) and As^{III} [0.2 mM (*white triangle*) or 1 mM (*black square*)]

To investigate whether the mutant *C. glutamicum* ArsC1-C2 can be used for arsenic speciation, the time-dependent accumulation of arsenic was analyzed in the presence of

As^{III}, either alone or in combination with As^{V}. In the ArsC1-C2 mutant, the presence of As^{III} did not affect As^{V} accumulation, as even when the concentration of As^{III} was 10-fold higher than that of As^{V}, the amount of retained arsenic was the same as in the absence of As^{III} (Fig. 1b). This result suggested that incorporated As^{III} is rapidly removed from the cell by functional arsenite permeases (Acr3-1/Acr3-2), whereas As^{V} accumulates because of the absence of functional arsenate reductases (Villadangos et al. 2011; Ordoñez et al. 2009). Similar results were obtained in a study of *Arabidopsis thaliana*, in which silencing of the arsenate reductase gene *Atacr2* led to As^{V} hyperaccumulation (Dhankher et al. 2006).

Msh mutants accumulate arsenate

Mycothiol (MSH) is a glutathione surrogate present at millimolar concentrations specifically within actinobacteria (Ordoñez et al. 2009). It plays a critical role in cellular protection against oxidative stress and, as indicated above, in providing electrons for As^{V} reduction by some arsenate reductases. Therefore, we asked whether As^{V} accumulation could be improved under conditions of MSH depletion. Because MSH biosynthesis involves the genes *CgmshA*, *CgmshB*, *CgmshC*, and *CgmshD* (Newton et al. 2008), we analyzed As^{V} resistance and accumulation in the respective mutant strains, all of which showed increased As^{V} sensitivity. The MshA and MshC mutants were the most sensitive of the *msh* mutant strains, with sensitivities similar to that of the ArsC1-C2 double mutant (0.05 mM; Fig. 1 and Supplemental Table S1), whereas the As^{V} sensitivities of the MshB and MshD mutants were lower (40–45 mM; Fig. 1c). These findings are in agreement with previous results showing that MSH is a cofactor of the *C. glutamicum* arsenate reductases ArsC1 and ArsC2 (Ordoñez et al. 2008).

In the accumulation analyses, the highest retention values were likewise those of the MshA and MshC mutant strains: 27.50 and 30.00 nmol/mg, respectively (Fig. 1c), whereas retention by MshB or MshD was not significantly different than by the control strain RES167 (Fig. 1c).Our results are in agreement with those of Newton et al. (2008), in their study of MSH mutants in *Mycobacterium smegmatis*: In those strains, MshA and MshC activities were shown to be essential for MSH production, in contrast to the "leaky" behaviors of the MshB and MshD mutants.

Polyphosphate accumulation has no effect on arsenate accumulation or resistance

One of the mechanisms for metal tolerance is the sequestration of metal cations by long polymers of poly-phosphate (poly-P) (Pan-Hou et al. 2002), whose presence seems to alleviate cell stress (Alcántara et al. 2014). Since *C. glutamicum* has been shown to synthesize poly-P granules (Lindner et al. 2007), we examined whether they are involved in As^{V} accumulation and/or resistance.

C. glutamicum contains two enzymes involved in poly-P biosynthesis, PPK2A and PPK2B (Lindner et al. 2007). Thus, we engineered *C. glutamicum* strains to express the *Cgppk2A* or *Cgppk2B* gene in multicopy plasmids (pEppk2A and pEppk2B; Table 1) that were then transferred to the As^{V}-accumulating strains ArsC1-C2 and 2Δars. An analysis of As^{V} resistance and retention by these *Cgppk2*-carrying strains failed to show any differences compared to the parent strains (Supplemental Fig. S1), which suggests that the overexpression of poly-P biosynthesis genes in *C. glutamicum* is not involved in As^{V} resistance or accumulation. To verify that As^{V} has an effect on polyphosphate granule structure, ArsC1-C2 and 2Δars mutant strains with or without pEppk2A and pEppk2B plasmids were grown in the presence of different As^{V} concentrations. Engineered *C. glutamicum* cells were stained using the poly-P specific Neisser technique and then observed under the microscope. Mutant strains overexpressing the *Cgppk2* genes contained large poly-P granules that clearly disaggregated in proportion to the presence of As^{V} (data not shown). These results showed that in *C. glutamicum*, poly-P granules do not contribute to arsenic resistance or accumulation.

Arsenite accumulates in arsenite permease mutant strains

C. glutamicum RES167 is highly tolerant of As^{III}, as shown by its resistance to concentrations up to 12.00 mM (Fig. 2a). This is likely due to the following: (i) the presence of two functional arsenite Acr3 permeases, Acr3-1 and Acr3-2, which actively extrude As^{III} from *C. glutamicum* cells (Villadangos et al. 2012), or (ii) the apparent lack of genes used for As^{III} uptake. To determine whether, in the first case, disrupting this efflux mechanism would increase the amount of intracellular As^{III}, we analyzed As^{III} accumulation in *C. glutamicum* RES167 (control) and in the 2Acr3 and 2Δars mutants (Table 1). As^{III} accumulation was assayed using resting cells, either by (i) As^{III} addition (1 mM) to the reaction mix or (ii) As^{V} addition (0.1 mM) only to the reaction mix used to assay the mutant 2Acr3, which contains functional arsenate reductases and therefore should accumulate As^{III}.

The results showed that the resistance of the 2Acr3 and 2Δars mutants to As^{III} (0.40 and 0.10 mM, respectively; Fig. 2a) was very different from that of the control strain RES167 (12.00 mM). However, they retained significantly more As^{III}, 3.60 and 1.94 nmol/mg, respectively (7.2- and 4-fold) than the control RES167 (0.50 nmol/mg; Fig. 2a). When strain 2Acr3 was assayed in the presence of As^{V}, the As^{III} retention values were even higher, 7.44 nmol/mg (15-fold vs strain RES167; Fig. 2b). These results are in agreement with those reported for growing cells of *C. glutamicum* strain 2Acr3 (Feo et al. 2007), which mostly accumulated the As^{III}

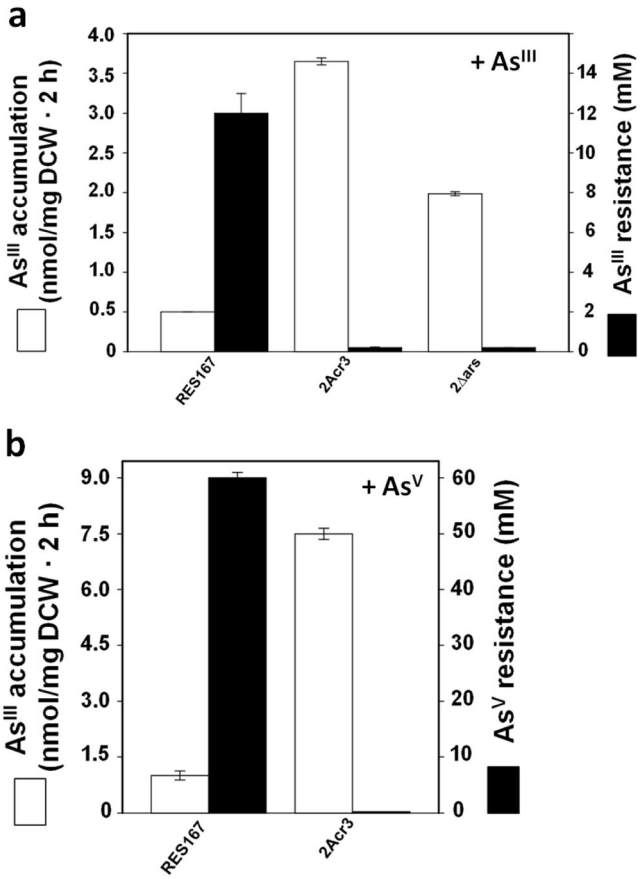

Fig. 2 Arsenite accumulation/resistance of *C. glutamicum acr3* mutant strains. The As^{III} contents (*white columns*) of *C. glutamicum* RES167 and the 2Acr3 and 2Δars mutants were determined after 2 h of incubation with As^{III} (1 mM) or As^{V} (0.1 mM). As^{III} (**a**) and As^{V} (**b**) resistance (*black columns*) was assayed by culturing the strains for 16 h in, respectively, TSB or MMCLP medium containing different As^{III} or As^{V} concentrations. *Error bars* indicate the standard deviation from three independent experiments. RES167 *C. glutamicum* RES167; 2Acr3 *C. glutamicum* 2Acr3; 2Δars *C. glutamicum* 2Δars

form, independent of the arsenic species (As^{III} or As^{V}) present in the medium. This increased accumulation of As^{III} by strain 2Acr3 when assayed in the presence of As^{V} was shown to be due to the facilitated entry of the latter species (in the absence of phosphate) in cells containing functional ArsC1 and ArsC2 arsenate reductases.

Overexpression of arsenite uptake porins, but not chelators, improves arsenic accumulation

In aqueous solution and at neutral pH, As^{III} usually enters bacterial cells through aquaglyceroporins (GlpF). This reflects the fact that As^{III} is for the most part protonated to form arsenic hydroxide [$As(OH)_3$], which structurally resembles glycerol [$C_3H_5(OH)_3$] (Meng et al. 2004), the latter the main compound taken by GlpF.

Since no *glpF* homologs were found in the *C. glutamicum* genome, we attempted to increase cellular As^{III} uptake/accumulation by the heterologous expression in *C. glutamicum* of *glpF* genes from two actinobacteria: *C. diphtheriae* NCTC 13129 (*CdglpF*) and *S. coelicolor* A3(2) (*ScglpF*). The As^{III} resistance of cells overexpressing *CdglpF* or *ScglpF* decreased (6.00 and 8.00 mM, respectively) compared to the control strains (12.00 mM; Fig. 3a). An analysis of the As^{III} uptake rate in these strains showed that the expression of *CdglpF* and *ScglpF* in *C. glutamicum* RES167 improved As^{III} uptake by 2.6- and 2.1-fold, respectively (Fig. 3a). This result provides evidence for the involvement of GlpF in As^{III} uptake, as observed in other bacteria and eukaryotes (Liu et al. 2002; Meng et al. 2004).

Next, we asked whether the expression of *glpF* led to additional As^{III} accumulation in the 2Acr3 and 2Δars strains. Indeed, strains 2Acr3 and 2Δars expressing *CdglpF* or *ScglpF* accumulated between 1.5- and 2-fold more arsenic than the background strains, with the expression of *CdglpF* more effective than that of *ScglpF* in terms of arsenic accumulation (Fig. 3b). As^{III} accumulation was highest (11-fold vs the RES167 control strain) in strain *C. glutamicum* 2Acr3 harboring plasmid pEglpCd (Figs. 2 and 3). Cellular extracts from RES167 and the recombinant strains 2Δars and 2Acr3, each containing plasmids pECM2 (control), pEglpCd and pEglpSc, were analyzed by SDS-PAGE in order to visualize the possible incremental expression of GlpF proteins. In all cases, extracts from the strains containing recombinant plasmids yielded an additional band (related to controls) of around 25 kDa, very close to the expected size of CdGlpF (25.6 kDa) or ScGlpF (26.8 kDa). The band was more intense when strain 2Δars was used as the background (see Supplemental Fig. S2).

An additional strategy to increase As^{III} accumulation in *C. glutamicum* is the cellular expression of chelating proteins (acting as possible biosorbents) containing thiol groups such as those present in cysteines. A similar affinity has been shown between metals such as As^{III} and Sb^{III} and certain cellular metallorepresors, which regulate downstream gene expression depending on their presence. In *E. coli*, there are two well-known proteins capable of binding As^{III}: the regulator ArsR and the metallo-chaperone ArsD. Therefore, we examined whether As^{III} retention was enhanced when strain *C. glutamicum* 2Δars (used as background) contained either additional copies of the genes *CgarsR1*/*CgarsR2* or expressed the functional fragment of the protein encoded by the *EcarsD* gene; the latter protein is a truncated version of native *E. coli* ArsD but has the same affinity for As^{III} (Yang et al. 2011). None of the recombinant strains increased As^{III} accumulation (Fig. 3c) nor was there an apparent change in the protein expression pattern, as determined by SDS-PAGE of the recombinant strains (see Supplemental Fig. S2). In our system, the overexpression of C*garsR1/CgarsR2* genes in *C. glutamicum* 2Δars was apparently toxic, as the growth of this strain was delayed in the presence of As^{III}; however, we

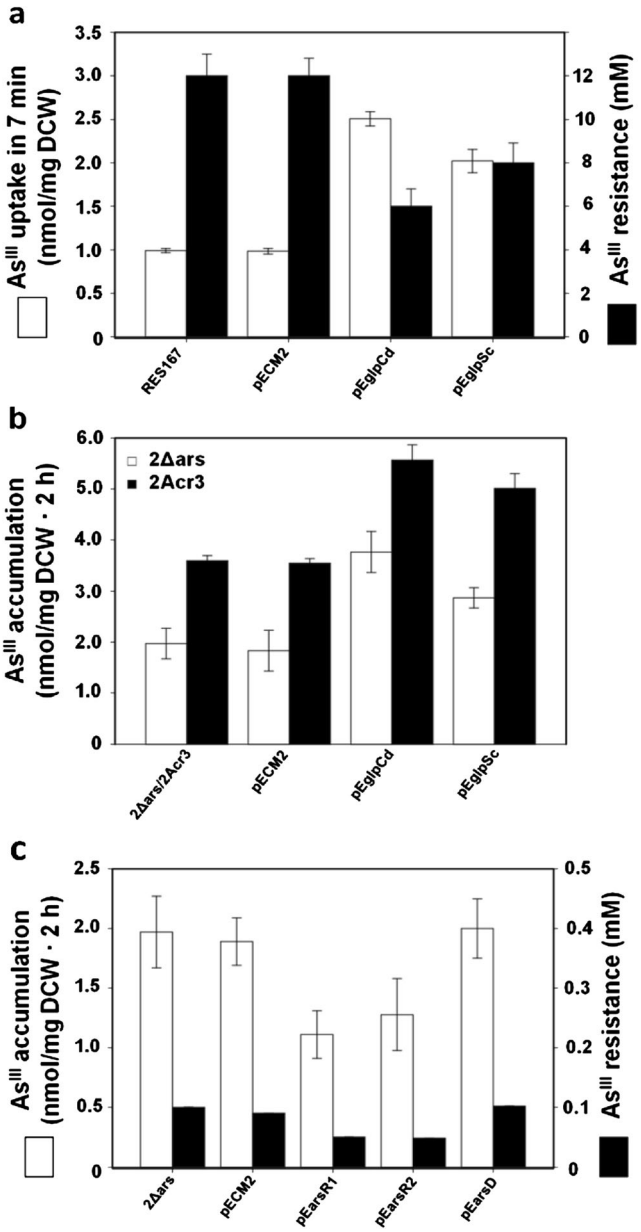

Fig. 3 Arsenite uptake, accumulation, and resistance assays in *C. glutamicum* mutants. As^{III} accumulation was evaluated in cultures grown in the presence of 1 mM As^{III} for **(a)** short (7 min; uptake) or **(b), (c)** long (2 h) time periods. Resistance was determined in cultures grown in TSB medium with increasing As^{III} concentrations. **(a)** As^{III} uptake (*white columns*) and resistance (*black columns*) assays using *C. glutamicum* RES167 as the following: (i) the control strain; (ii) the background strain containing the multicopy control (pECM2) or recombinant plasmids containing the *glpF* genes: pEglpCd or pEglpSc. **(b)** As^{III} accumulation assays using *C. glutamicum* 2Δars (*white columns*) or 2Acr3 (*black columns*) as (i) control strains; (ii) the background strain for plasmids pECM2, pEglpCd, or pEglpSc. **(c)** As^{III} accumulation (*white columns*) and resistance (*black columns*) assays using *C. glutamicum* 2Δars as (i) the control strain; (ii) the background strain for plasmids pECM2, pEarsR1, pEarsR2, or pEarsD. *Error bars* indicate the standard deviation

also cannot rule out that the cause was problems with the expression (transcription or translation) of plasmids containing metalloregulatory genes.

Discussion

The abundance of arsenic in the Earth's crust and the toxicity of this metal for all forms of life have highlighted the urgent need for bioremediation measures (Drewniak and Sklodowska 2013). However, despite the growing interest in bacteria as effective tools for bioremediation, thus far, there have been very few such efforts with respect to arsenic bioremediation (Tsai et al. 2009).

Among the many possible arsenic bioremediation processes, the preferred method has been intracellular accumulation by bacteria or yeasts, either exponentially growing cells (Singh et al. 2010; Dhankher et al. 2006) or resting cells (Singh et al. 2008b; Kostal et al. 2004). In growing cells, exposure to high concentrations of arsenic can hamper growth, leading to suboptimal accumulation (Shah et al. 2010). Therefore, in this work, we examined arsenic accumulation in resting cells suspended in TAB containing the appropriate arsenic concentration. This system is more readily reproducible, and the results are more homogeneous between replicates. The chemical and physical conditions used to assess arsenic accumulation in *C. glutamicum* were established in two previously published studies (Feo et al. 2007; Villadangos et al. 2010). An As^{V} concentration of 0.1 mM was shown to be the adequate to perform accumulation assays in the absence of phosphate, whereas for As^{III} a 10-fold higher concentration (1 mM) is needed for accumulation, even though the cellular toxicity of As^{III} is much higher than that of As^{V} (Tsai et al. 2009). This is likely due to the lack of GlpF in *C. glutamicum*, as in most organisms, this protein is involved in both glycerol and As^{III} uptake (Meng et al. 2004). In agreement with this line of reasoning is the recent observation that *C. glutamicum* is unable to grow on glycerol as the unique carbon source (Meiswinkel et al. 2013).

The aim of this study was to design genetically modified *C. glutamicum* strains able to accumulate and/or speciate different arsenic species (As^{III} or As^{V}), for their eventual use as specific biocontainers. Our efforts to improve arsenic accumulation in the GRAS bacterium *C. glutamicum* met with varying degrees of success. Thus, whereas arsenic retention was improved by the removal of the arsenic reduction (ArsC) or efflux (Acr3) systems or by the expression of cloned genes for arsenic uptake proteins (GlpFs), this was not the case for the expression of biosorbents, in contrast to previous work on the homologous overexpression of *E. coli EcarsR* or the expression of the *F. vesiculosus* metallothionein (Kostal et al. 2004; Singh et al. 2008b).

The prevalence of As^{V} in contaminated water reservoirs (more than 50 % of total arsenic; Shen et al. 2012) makes As^{V} an important target of bioremediation. Efficient As^{V} biocontainers in *C. glutamicum* were obtained by the removal of either both arsenate reductase genes (*CgarsC1* and *CgarsC2;* ArsC1-C2 mutant) or the genes involved in MSH

synthesis (*CgmshA* or *CgmshC*; MshA and MshC mutants). None of these mutant strains were able to reduce As^{V} to As^{III}, and they accumulated As^{V} in amounts (27.50–30.00 nmol/mg) that were 28- to 30-fold higher than accumulated by the RES167 control strain (Supplemental Table S1). Furthermore, our mutants are the most efficient bacterial As^{V} biocontainers described to date: at working concentrations (10 μM of As^{V}) equivalent to those used to study an *E. coli* arsenate reductase mutant (strain AW10; Singh et al. 2008b), *C. glutamicum* mutants accumulated 9- to 10-fold more As^{V} (5.8 nmol/mg; data not shown). Finally, these strains are specific for As^{V} accumulation, since they did not accumulate additional As^{III} (Fig. 1b).

In addition, by removing the systems that allow As^{III} extrusion (*Cgacr3-1* and *Cgacr3-2* genes), we obtained effective As^{III} biocontainers. The expression of genes involved in As^{III} uptake and transport also increased As^{III} accumulation, with overexpression of the arsenite transporters *CdglpF* and *ScglpF* in these As^{III} biocontainers having a significant effect on As^{III} retention. The expression of *CdglpF* in the 2Acr3 mutant led to improved As^{III} bioaccumulation, with 2- and 11-fold increases over the amount retained by the background 2Acr3 and the control strain RES167, respectively (Supplemental Table S1). However, the amount of As^{III} retained cannot be directly compared with that achieved using other microbial biocontainers because the absence of "optimal" As^{III} uptake systems makes wild-type corynebacteria very resistant to this arsenic species. How As^{III} is incorporated by *C. glutamicum* is unclear, but the absence of *glpF* genes may constitute an evolutionary survival mechanism for this saprophytic bacterium. In assays in which *E. coli* recombinant strains were used as biocontainers, the As^{III} working concentrations were much lower (10–100 μM; Singh et al. 2010; Kostal et al. 2004) than those used in this study with corynebacteria (1 mM) and would have been insufficient concentrations to achieve significant accumulation rates. In *E. coli* recombinant strains overexpressing the genes *arsR*, *PCS*, *glpF*, or *MT*, As^{III} accumulation increased by 10- to 50-fold (Sauge-Merle et al. 2003; Singh et al. 2010).

In summary, our engineered *C. glutamicum* strains have the potential to serve as bioremediation tools, by accumulating large amounts of arsenic. In addition, since these strains differentially accumulate As^{V} and As^{III}, a bacterial consortium based on *C. glutamicum* mutant strains and bacteria able to oxidize As^{III} to As^{V}, such as *Ochrobactrum tritici* (Branco et al. 2009), would offer a novel and efficient bio-tool for arsenic bioremediation using a two-step system for arsenic detoxification. In the first step, As^{III} would be oxidized to As^{V} by *O. tritici* (Fig. 4a); in the second, As^{V} and the remaining As^{III} would be accumulated by engineered *C. glutamicum* strains (mutants ArsC1-C2, Msh or Acr3-1/2; Fig. 4b). *C. glutamicum* mutant strains are therefore a promising and safe approach to arsenic bioremediation in contaminated environments, given that, with appropriate controls, they can be safely released into the environment.

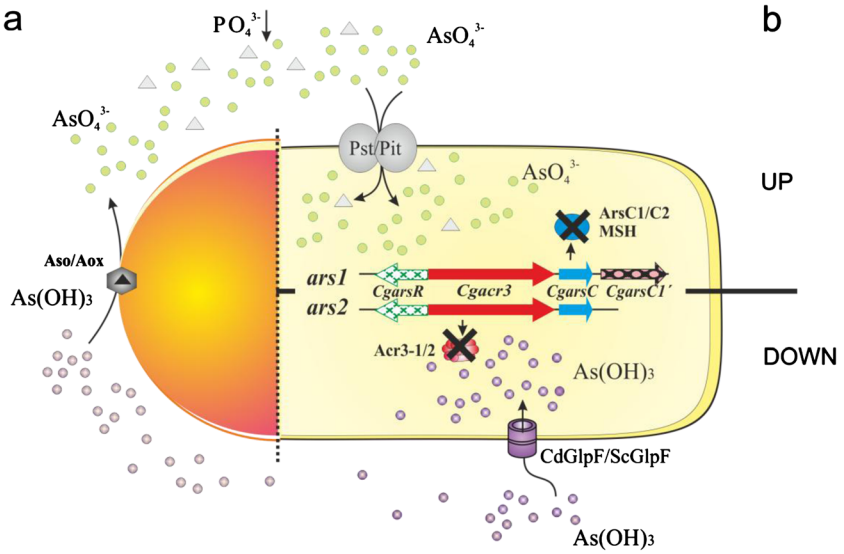

Fig. 4 Proposed microbial consortium system for the bioremediation of inorganic arsenic species. **(a)** In aquatic environments and at neutral pH, some species of bacteria (e.g., *Ochrobactrum tritici*) use periplasmic arsenite oxidases (Aso/Aox proteins) to oxidize arsenite [$As(OH)_3$; As^{III}] to arsenate (AsO_4^{3-}; As^{V}), which provides an energy source. **(b)** When phosphate levels (PO_4^{3-}) in the environment are low, engineered *C. glutamicum* strains can use the Pst/Pit system to incorporate As^{V}. Strains lacking functional arsenate reductases (ArsC1/C2; involved in biochemical conversion of As^{V} to As^{III}) or unable to synthesize MSH (UP) accumulate As^{V}; mutant strains lacking arsenite permeases activities (Acr3-1/2; involved in cellular extrusion of As^{III}) and expressing heterologous GlpF proteins (CdGlpF/ScGlpF) (DOWN) incorporate both arsenic species, but only As^{III} is finally accumulated. The gene structure of the *ars1* and *ars2* operons of *C. glutamicum* is indicated: *CgarsRs* encode the repressor proteins; *CgarsC1'* encodes the thioredoxin-dependent arsenate reductase

Acknowledgements AFV and EO were recipients of fellowships from the Junta de Castilla y León (Spain). This work was financially supported by grants from Junta de Castilla y León, Spain (ref. 2014/211330), and the HOA project of the Vrije Universiteit Brussel (Brussels). We thank Professor De Xoyse (H.P.A., London, UK), for providing *C. diphtheriae* DNA.

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