

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31

Functional coexistence of twin arsenic resistance systems
in *Pseudomonas putida* KT2440

by

A. David Páez-Espino[§], Gonzalo Durante-Rodríguez and Víctor de Lorenzo^{*}
Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco, Madrid 28049, Spain

Keywords: *Pseudomonas putida*, arsenic, detoxification, phosphate, *tmk*

Running Title: The two *ars* operons of *Pseudomonas putida*

*Correspondence to: Víctor de Lorenzo
Centro Nacional de Biotecnología-CSIC
Campus de Cantoblanco, Madrid 28049, Spain
Tel: +34 91 585 45 36; Fax: +34 91 585 45 06
E-mail: vdlorenzo@cnb.csic.es

[§]Current address: DOE Joint Genome Institute, Walnut Creek, California, USA

1 ABSTRACT

2

3 The genome of the soil bacterium *Pseudomonas putida* KT2440 bears two virtually identical *arsRBCH*
4 operons putatively encoding resistance to inorganic arsenic species. Single and double chromosomal
5 deletions in each of these *ars* clusters of this bacterium were tested for arsenic sensitivity and found that
6 the contribution of each operon to the resistance to the metalloid was not additive, as either cluster
7 sufficed to endow cells with high-level resistance. However, otherwise identical traits linked to each of the
8 *ars* sites diverged when temperature was decreased. Growth of the various mutants at 15 °C (instead of
9 the standard 30 °C for *P. putida*) uncovered that *ars2* affords a much higher resistance to As (III) than the
10 *ars1* counterpart. RT-PCR of *arsB1* and *arsB2* genes and *lacZ* fusions to the *Pars1* and *Pars2* promoters
11 traced the difference to variations in transcription of the corresponding gene sets at each temperature.
12 Functional redundancy may thus be selected as a stable condition -rather than just as transient state- if it
13 affords one key activity to be expressed under a wider range of physicochemical settings. This seems to
14 provide a straightforward solution to regulatory problems in environmental bacteria that thrive under
15 changing scenarios.

16

17

18 INTRODUCTION

19

20 One of the most common stresses that environmental bacteria undergo is that of exposure to heavy
21 metals and metalloids (Giller *et al.*, 1998). In particular, inorganic arsenic has been pinpointed as one of
22 the earliest chemical stressors that primitive prokaryotes had to cope with (Tamaki and Frankenberger,
23 1992). Owing to its structural similarity to phosphate but endowed with a different chemical reactivity,
24 ancestral bacteria had to solve the problem of distinguishing essential phosphate from toxic arsenate
25 (Mukhopadhyay *et al.*, 2002). One clear hint of such an evolutionary challenge is the nearly universal
26 preservation of genes encoding arsenite (As^{III}) pumps along with arsenate (As^V) reductases in virtually all
27 bacteria where the issue has been examined (Paez-Espino *et al.*, 2009). Many *ars* operons have been
28 studied in great detail (Wu and Rosen, 1993; Rosen, 1995; Oremland and Stolz, 2003). They appear in
29 different genomic configurations depending on the specific bacterial strain (Paez-Espino *et al.*, 2009). The
30 core genes of the *ars* systems encode the transcriptional repressor ArsR, the arsenite efflux pump ArsB,
31 and the arsenate reductase ArsC (Xu *et al.*, 1998; see Supplemental material for an analysis of protein

1 families). Additional genes e.g. *arsA*, *arsD*, *arsH*, *arsN* or *arsO*, found in the vicinity of the *ars* operon
2 determine accessory functions known or suspect to be related to arsenic resistance (Paez-Espino *et al.*,
3 2009).

4
5 The soil bacterium *P. putida* strain KT2440 is an archetypal strain of ubiquitous saprophytic
6 microorganisms endowed not only with a remarkable adaptability to diverse environments and nutrients
7 (Nelson *et al.*, 2002), but also to tolerate a whole range of physicochemical insults (Nikel *et al.*, 2014).
8 Inspection of its genomic sequence reveals an unexpected capacity to tolerate heavy metals and
9 metalloids, the resistance genes for some of which are duplicated (Canovas *et al.*, 2003a). The most
10 conspicuous of them, which is the subject of the study below, is that of the two complete *arsRBCH*
11 operons for resistance to As^{III} and As^V, which map in non-adjacent regions of the genome of this
12 bacterium (Canovas *et al.*, 2003a). By using the multi-tiered genetic approach described below we show
13 that the paradoxical presence of virtually identical copies of the *ars* genes in the same chromosome can
14 be traced to their unlike optimality at different temperatures. That the two *arsRBCH* clusters allow their
15 cognate products to run the same function under different environmental parameters seems to be
16 instrumental for expanding their functional scope (Sanchez-Perez *et al.*, 2008).

17

18 RESULTS AND DISCUSSION

19

20 *The two ars operons of P. putida*

21

22 The starting point of this work is the earlier recognition by Canovas *et al.* (2003a) of two chromosomally-
23 born *ars* operons in the genome of *P. putida* KT2440, which have been denominated *ars1* and *ars2* (Fig.
24 1). Having two systems for resistance to arsenic is an exceptional circumstance not only for the genus
25 *Pseudomonas*, but for most bacteria which typically have just one of such operons (Achour *et al.*, 2007;
26 Paez-Espino *et al.*, 2009). Further scrutiny of some DNA sequence signatures (e.g., genomic context, CG
27 content, codon adaptation index; Sharp and Li, 1987) indicated that the *ars2* cluster was the
28 housekeeping, indigenous As resistance device of *P. putida* KT2440 which is shared with virtually all
29 members of the genus. On the contrary, the *ars1* operon is located in what appears to be a ~ 62 Kb
30 genomic island that is absent in all of the five closest *P. putida* strains which have been sequenced (DOT-
31 T1E, F1, GB-1, S16 and W619; Fig. 1). Such genomic island, that contains 65 genes (including the *ars1*

1 operon; Supplementary Table S1), interrupts a thymidylate kinase (*tmk*) gene, thereby dividing the
2 corresponding ORF in an upstream 5' sequence (ORF1919) and a downstream 3' portion (ORF1965; Fig.
3 1). Supplementary Fig. S1 provides a more detailed map of the DNA signatures of the insert found in the
4 *tmk* sequence. Since thymidylate kinase is an essential function, the split *tmk* gene may still provide an
5 active product through protein complementation, or *P. putida* KT2440 could have a thus far unidentified
6 functional backup. In any case, the long insert endows the strain with two very similar genomic DNA
7 sequences (i.e. *ars* clusters) that encode the same apparent biological task. Unlike metabolic genes,
8 resistance to inorganic arsenic species is an entirely dispensable function in the absence of the
9 corresponding oxyanions. We thus wondered about the functional meaning of the redundancy of the *ars1*
10 and *ars2* clusters, specifically the benefit, if any, of having two copies of equivalent genes in the same
11 cells.

12

13 *Organization and function of the ars1 and ars2 operons of P. putida* KT2440

14

15 Examination of the sequences of the two highly similar *ars* clusters of *P. putida* KT2440 (Fig. 1) predict
16 them to determine a co-transcribed operon composed of a self-repressed transcriptional regulator (*arsR*),
17 a membrane-bound transporter that extrudes As^{III} out of the cell (*arsB*), an arsenate reductase (*arsC*) for
18 transformation of As^V to As^{III} and an *arsH* gene of unknown function but also important for arsenic
19 resistance (Canovas *et al.*, 2003a). A more detailed analysis of the *ars* sequences in *P. putida* and their
20 regulatory regions can be found in the Supplemental Information. The degree of DNA sequence identity
21 between homologous genes of the two different operons varies 68-78 %, while the corresponding primary
22 proteins overlap by 73-87 %. To examine the genotype / phenotype relationships between these cluster
23 and actual resistance to arsenic species we first tested growth of *P. putida* KT2440 in solid and liquid LB
24 medium added with growing concentrations of either NaH₂AsO₃ (for As^{III}) or Na₂HAsO₄ (for As^V). As
25 shown in Fig. 2a, the strain was highly resistant to the more oxidized form of the metalloid, as we
26 detected significant growth on plates containing up to 300 mM arsenate. Under the same conditions, *E.*
27 *coli* W3110 (which also has a housekeeping chromosomally-encoded *ars* system; Carlin *et al.*, 1995)
28 stopped growth altogether above 50 mM Na₂HAsO₄ (Fig. 2b). As a control, *E. coli* AW3110Δ*ars* (Carlin *et*
29 *al.*, 1995) which lacks any way of resisting arsenic, could not grow at all even at the lowest concentrations
30 tested (Supplementary Fig. S2). This observed resistance of *P. putida* KT2440 to exogenous arsenate
31 levels in the range of 0.3 M places this strain among those microorganisms most vigorously curbing the

1 toxic effects of the oxyanion, only comparable to some *Corynebacteria* (Mateos *et al.*, 2006) and certain
2 fungal species (Canovas *et al.*, 2003b; Canovas *et al.*, 2004; Messens and Silver, 2006). In contrast, the
3 same strain was much more sensitive to arsenite, growth being nearly completely inhibited beyond 10
4 mM. Still, this inhibitory concentration was much higher than those that stopped *E. coli* W3110 (Fig. 2b)
5 and *E. coli* AW3110 Δ *ars* (Supplementary Fig. S2). Resistance to arsenite could be nearly doubled (from
6 10 mM to 20 mM) when cells were pre-exposed for 8-12 h to subinhibitory concentrations of NaAsO₂ (5
7 mM; Fig. 2c and 2d), thereby suggesting that the genes behind the phenotype were inducible. That *P.*
8 *putida* KT2440 was hyper-resistant to arsenic and that resistance was stimulated by arsenite immediately
9 indicated the phenomenon to reflect the action of the *ars* operons mentioned above.

10

11 *ars1* and *ars2* clusters contribute differentially to arsenic resistance

12

13 In order to establish the functional relationship between *ars* clusters and the resistance phenotype as well
14 as determining the relative contribution of each of the systems (*ars1* and *ars2*) to the high resistance (Fig.
15 2) we generated a number of entirely isogenic strains lacking one operon, the other or both. To this end,
16 we resorted to a genetic method for generating seamless and iterative genomic deletions in *P. putida*
17 based on the counterselection of the *pyrF* gene (the bacterial equivalent to the yeast URA3) caused by
18 fluoro-orotic acid (FOA) as a way to resolve intermediate deletion steps (Galvao and de Lorenzo, 2005;
19 Experimental procedures). Due to this method, the reference strain is the one called *P. putida* TEC1,
20 which is *P. putida* KT2440 deleted of the *pyrF* gene (Galvao and de Lorenzo, 2005; Table 1). On this
21 basis, each cluster was precisely excised i.e. from -110 nt upstream of the first ATG leading codon of
22 *arsR* –for maintaining the promoter- to the TGA stop codon of the corresponding downstream *arsH* gene.
23 The phenotypes of the strain set lacking *ars1*, *ars2* or both were then tested on LB plates amended with
24 uracil and containing high concentrations of As^{III} (5 mM) or As^V (250 mM). At the same time, the ability of
25 these strains to accumulate total arsenic in their biomass was examined through Plasma Mass
26 Spectrometry as explained in the Experimental procedures section. The outcome of this experiment (Fig.
27 3), exposed a number of salient features. First, the double Δ *ars1* Δ *ars2* strain was entirely sensitive to the
28 oxyanions added to the plates (Fig. 3a), what traced the hyper-resistance of the wild-type bacteria to the
29 products of these gene clusters. This ruled out any role of other genes annotated somewhere else in the
30 *P. putida* chromosome as *arsC* variant (e.g. PP1645) in the high resistance to arsenic species just
31 discussed. The experiment of Fig. 3a shows also that the *ars1* cluster accounts for much of the

1 phenotype at stake, while deletion of *ars2* has a lesser effect on resistance and contributes to the overall
2 resistance of the wild-type strain to a smaller extent. These results matched well the As accumulation
3 data. As shown in Fig. 3b, the loss of *ars1* made cells to retain more arsenic than the wild type bacteria –
4 plausibly because of a reduced ability to pump the oxyanion out. The lacking *ars2* resulted also in an
5 increase of accumulated arsenic, although the effect was not as pronounced as in the case of *ars1*. To
6 further verify the role of each of the clusters, strains bearing separate $\Delta ars1$ and $\Delta ars2$ deletions were
7 complemented with their corresponding *arsRBCH* operons. For this, each cluster was separately captured
8 from genomic DNA (see Experimental procedures for details) and cloned in broad host range vector
9 pVLT33 (de Lorenzo *et al.*, 1993). The thereby produced plasmids pVCI1 (*ars1*⁺) and pVCI2 (*ars2*⁺) were
10 then introduced respectively in $\Delta ars1$ and $\Delta ars2$ strains and the resistance to arsenic salts tested as
11 before. Supplementary Fig. S3 shows the results, which confirm the origin of the resistance phenotypes in
12 the corresponding operons. Finally, that the two clusters were separately functional and that *ars1*
13 provided a more vigorous resistance to arsenic was consistent with heterologous expression experiments.
14 In this case, the amplified *arsRBCH* operons were cloned in *E. coli* vector pGEM-T, originating pGCI1
15 (*ars1*⁺) and pGCI2 (*ars2*⁺). These were introduced in *E. coli* DH5 α and tested as before on plates
16 containing arsenic. Supplementary Fig. S4 shows that the heterologous host acquired a higher resistance
17 (2 to 5 fold) to the oxyanions than the recipient cells and that *ars1* was superior to *ars2* in that role. Table
18 2 compares and summarizes all these findings.

19

20 Taken together, the data above accredit the roles of *ars1* and *ars2* in bringing about hyper-resistance to
21 arsenic salts. Also, the acquired operon *ars1* seems to be major contributor to the phenomenon under the
22 conditions tested. However, when the inhibitory concentrations were tested by growth to end-point (Table
23 2), the differences between the two clusters observed in agar plates became less clear, as each cluster
24 appeared to endow cells, given enough time, with a high resistance to either compound. The differences
25 in this case were in growth rates, not in clear-cut sensitivity. This raised the question on why 2 very similar
26 operons are found in *P. putida* when having just one of them could suffice to cause the resistance
27 phenotype in its virtual entirety. The experiments below were then set up to answer such standing
28 question.

29

30 *Phenotypes endowed by the ars genes of P. putida KT2440 under different environmental conditions*

31

1 One evolutionary scenario that could favour stable maintenance of genes running the same function is
2 that the task is optimally deployed by each of them at different environmental conditions. The As
3 resistance demands of some niches can be met by just having different gene expression doses. But it is
4 also possible that some evolving functions may have not found yet a solution to the need of performing
5 under two dissimilar physicochemical situations with a single gene or gene cluster. In these cases, one
6 could entertain a solution consisting in having two or more variants of the same gene(s), each optimized
7 for a set of environmental parameters (Andam *et al.*, 2011). Since two major determinants of niche
8 specificity for environmental bacteria are osmotic pressure and temperature, we tested the performance
9 of each of the two *ars* systems of *P. putida* under low salt (LB, 0.1 M NaCl) and high salt (LB, 0.4 M salt)
10 as well as under low (15 °C) and high (30-37 °C) temperature. For this, we grew each of the strains under
11 the conditions specified and recorded growth along time. Supplementary Fig. S5 shows that osmotic
12 stress had no influence on the relative contribution of the *ars* cluster to arsenic resistance, whether As^{III} or
13 As^V. But the situation changed when the same experiments were run at different temperatures. In this
14 case, the efficiency of the two arsenic resistance systems varied significantly at 15 °C vs. 30-37 °C. As
15 shown in Fig. 4, the efficiency of the endogenous *ars2* system dramatically increased at low temperatures
16 (15 °C) whether the toxicant was NaAsO₂ or Na₂HAsO₄. In contrast, the *ars1* operon appeared to function
17 better at 30-37 °C specially when facing As^V (Fig. 4c and 4d). These data suggested specialization of
18 each of the *ars* clusters to high or low temperatures. The next obvious question was whether these
19 differences could be traced to disparities in the respective expression levels or they involved other
20 functional variations in each of the operons.

21

22 *Thermal adaptation of ars1 and ars2 expression reflects temperature-dependent activity of P_{ars1} and P_{ars2}*
23 *promoters*

24

25 The simplest explanation to account for the results of Fig. 4 is that each *ars* cluster is expressed at its
26 own optimal temperature, high or low. To have a gross indication of whether this could be the case, we
27 estimated changes in the transcription levels of the *arsB* genes at different temperatures by using RT-Q-
28 PCR. By taking advantage of the small differences between the DNA sequences of the two homologous
29 genes in either *ars* operon (see Experimental procedures section) and using the *rpoN* gene as an internal
30 control (Kohler *et al.*, 1994; Yuste *et al.*, 2006) we could differentiate expression of each of the clusters -
31 *arsB* being a proxy of the whole operon. As shown in Supplementary Fig. S6, when treated with 75 mM

1 As^V, the endogenous *ars2* cluster had significantly higher levels of transcription than the acquired *ars1*
2 operon, which seemed to be repressed at the lower temperature. In contrast, the differences in
3 transcription of the two clusters were less pronounced at 30 °C (Supplementary Fig. S6). While these
4 results were indicative of different thermal optima for *arsB* transcript levels in each system they did not
5 clarify whether they stemmed from initiation (i.e. promoter activity) or they could be due to differential
6 mRNA stability. To elucidate this, we assembled equivalent reporter gene fusions between the predicted
7 promoter regions of each of the *ars* clusters (Fig. 5a and 5b; see Experimental procedures) and a
8 promoterless *lacZ* reporter gene. For this, we cloned ~ 200 bp DNA fragments spanning from -182 to just
9 before the predicted RBS sequence of each *arsR* gene in low-copy, broad host range promoter-probe
10 vector pSEVA225 (Silva-Rocha *et al.*, 2013); Table 1), thereby producing plasmids pSEVA225-*P_{ars1}* and
11 pSEVA225-*P_{ars2}*. These were then separately passed to strains *P. putida* TEC1 (wt) and *P. putida* Δ *ars1*
12 Δ *ars2* and their β -galactosidase levels measured at 15 °C and 30 °C in media with and without sub-
13 inhibitory concentrations (0.2 mM) of As^{III}, the effector molecule that triggers the *ars* operon (Cai *et al.*,
14 1998). As shown in Fig. 5, at 30 °C, while the basal expression levels of non-induced *P_{ars1}* and *P_{ars2}* were
15 different (higher promoter escape in *P_{ars2}*), the β -galactosidase activity of each of the fusions in the wild-
16 type strain induced with arsenite were very similar and indistinguishable of those observed in the double
17 deletion strain Δ *ars1* Δ *ars2* (i.e. the reference for fully de-repressed promoter activity). In contrast, the
18 lower temperature noticeably inhibited the activity of *P_{ars1}* while it maintained comparatively high that of
19 *P_{ars2}*. These results were not only consistent with the As resistance of the Δ *ars1* and Δ *ars2* mutants (Fig.
20 4) and the RT-Q-PCR data (Supplementary Fig. S6) discussed above, but they also drew the origin of the
21 different phenotypes to the inherent temperature-dependent behaviour of *ars1* and *ars2* expression.
22 Although other adaptive changes could also be at play (e.g. temperature-dependent specialization of the
23 proteins encoded in the *arsRBCH* operons or a thermo-sensitive mRNA fold; Johansson *et al.*, 2002) the
24 performance of the *ars* promoters under different thermal regimes *in vivo* sufficed to account for the entire
25 phenomenon. On these bases, we argue that the evolutionary *raison d'être* of the maintenance of two
26 repeated *ars* operons in *P. putida* is that their combination widens the range of arsenic-polluted niches
27 (specifically regarding temperature) that this strain can thrive in as a free-living, generalist bacterium.

28

29 *Conclusion*

30

1 Although variants of a core *arsRBC(H)* operon are common in the genomes of a wide variety of bacteria
2 (Paez-Espino *et al.*, 2009) only a few cases, like the one discussed in this paper, have been found where
3 the same genetic arrangement appears twice in the same chromosome. As shown above, we have found
4 that the contribution of each *ars* operon to the phenotype of arsenic resistance of *P. putida* KT2440 was
5 not additive, as either cluster sufficed to ultimately endow cells with a high-level of resistance to the
6 metalloid. The key question is thus why this strain contains two *ars* operons to reach a resistance level
7 that could be grossly reached with only one? Examination of genomic signatures and the composition of
8 the 62 kb island where *ars1* is located (Supplementary Table S1) indicated that this cluster was added to
9 the indigenous genetic complement of the strain through HGT. The composition and gene distribution of
10 bacterial genomes usually reflect the capacity of adaptation to different ecological niches. It is usual to
11 find a direct link between the genetic complement of a given chromosome and the environment where it
12 operates. But inspection of genomic sequences often yields paradoxes difficult to explain, e.g. the
13 presence/absence of certain genes, regulatory associations and genetic redundancies with no obvious
14 logic. Current models of evolution suggest that co-existence of identical functions may not be stable
15 unless they endow new properties that can be positively selected from the beginning (Francino, 2005).
16 Should this not to be the case, there is a competitive evolution of the gene copies resulting in the
17 maintenance of the most efficient variant and pseudogenization and eventual loss of the remaining genes.
18 In *P. putida* KT2440 however we do not detect such pseudogenization of one of the two *ars* systems,
19 what makes us entertain that maintenance of the repeated gene clusters is ultimately beneficial. In this
20 case temperature seems to be the key factor that favours the coexistence of the two similar operons, as
21 reflected by the different patterns of expression and efficiency reported above. The abundance of
22 redundant genes in environmental bacteria can therefore be understood under an evolutionary
23 perspective, namely the tradeoff between genomic instability due to DNA sequence repetitions vs. the
24 benefit of expanding the scope of the encoded function to a wider range of physicochemical settings.

25

26 EXPERIMENTAL PROCEDURES

27

28 *Strains, culture conditions, and general procedures*

29

30 The bacterial strains and plasmids used in this work are listed in Table 1. *Pseudomonas putida* strains
31 and *Escherichia coli* cells were grown in Luria-Bertani (LB) rich medium (Sambrook *et al.*, 1989) at either

1 15 °C, 30 °C and 37 °C as indicated in each case. Experiments in Petri dishes were made with the same
2 media added with 1.5 % (w/v) agar. Antibiotics were used at the following concentrations: ampicillin (Ap)
3 150 µg/ml, kanamycin (Km) 50 µg/ml, chloramphenicol (Cm) 30 µg/ml. In order to grow *P. putida* Δ *pyrF*
4 strains, uracil (Sigma Aldrich Chemicals) was added to the plates at 20 µg/ml. 5-Fluoroorotic acid (FOA,
5 Zymo Research) was used at 250 µg/ml to counterselect maintenance of the *pyrF* activity (orotidine-5'-
6 phosphate decarboxylase, see below). Where needed, the medium was added with NaCl up to a
7 concentration of 0.4 M for increasing osmotic stress. Resistance to arsenic species was grossly tested by
8 plating serial dilutions of cultures of each strain onto agar plate containing filtered sodium arsenite
9 (NaH_2AsO_3) or sodium arsenate (NaH_2AsO_4) from Sigma Aldrich Chemicals, as necessary for the
10 experiment at stake. For a more precise determination of minimal inhibitory concentrations (MICs) and
11 monitoring growth, strains were cultured in 96-microwell plates and incubated at 30°C for 24 h with orbital
12 shaking, and OD_{600} being recorded each 60 min. For the pre-induction experiments we first pre-cultivated
13 cells for 8-12 h in LB medium with 5 mM As^{III} . Bacteria from such cultures were then diluted in fresh
14 media with other As concentrations to a final $\text{OD}_{600} = 0.05$ which was considered time = 0 of the
15 subsequent growth. All oligonucleotides employed for the assembly of the various constructs and strains
16 were synthesized by Sigma-Genosys. Plasmids and cloning procedures were handled following the
17 standard methods described in Sambrook *et al.* (1989). In order to construct the plasmids used in
18 complementation studies, each of the two operons was separately amplified with primers that added
19 *EcoRI* and *HindIII* sites upstream and downstream respectively, of the cognate *arsRBCH* clusters. To this
20 end we employed 5'CGGCAAGCTTGAGCGTATCCAGGC3' and 5'CGTCCCGGAATTCGAGGCGATTG3'
21 for the *ars1* operon, as well as 5'GGTGCAAGCTTTGGGC TGTCCATCG3' and
22 5'TCGACCGAATTCCGTGGCGACG3' for the *ars2* counterpart. The products generated in this manner
23 were cloned as *EcoRI-HindIII* fragments in broad host range vector pVLT33 (Silva-Rocha *et al.*, 2013)
24 Table 1), thereby producing plasmids pVCI1 (*ars1*⁺) and pVCI2 (*ars2*⁺), respectively. The same oligos
25 were used to generate PCR-derived DNA segments and then cloned also in *E. coli* vector pGEM-T
26 (Promega) as 3.13 kb and 3.11 kb *EcoRI/HindIII* inserts, resulting in plasmids pGCI1 (*ars1*⁺) and pGCI2
27 (*ars2*⁺), which were used in the heterologous expression experiments shown in Supplementary Fig. S4.

28

29 *Construction of seamless Δ ars *P. putida* strains*

30

1 Precise and scarless deletion mutants of the *ars1* and *ars2* operons of *P. putida* KT2440 (Fig. 1) were
2 made with the method of Galvao and de Lorenzo (2005), which exploits the positive and negative
3 selection endowed by the *pyrF* gene for forcing the occurrence of recombination between otherwise
4 distant homologous segments introduced with an specialized delivery vector. For deleting the *ars1* cluster,
5 an upstream 1.29-kb DNA segment spanning from 0.1 kb to 1.3 kb in respect to the first amino acid codon
6 (Met) of *arsR1* gene was amplified from genomic DNA with primers 5'CCGCGCTCTAGAT
7 CCTATACTGGGCGTCGATAACC3' and 5'GGTAGAGCTCAATGCTCTGCTCGATTTGCTG3', which leave
8 *XbaI* and *SacI* restriction sites respectively flanking the PCR product. By the same token, a downstream
9 1.24-kb fragment starting right after the stop codon of *arsH* was generated with primers
10 5'TCGAAGCGGCCGCACCACTGAGGGCACCATGAC3' and 5'CGGTCTATTCTAGATAATGAGCAT
11 GTCGCATCC3') that enter flanking *NotI* and *XbaI* sites. The two PCR products were then assembled as
12 a single *SacI-NotI* fragment in the *oriT⁺ pyrF⁺* vector pTEC, originating plasmid pTUD1. The insert of this
13 plasmid engineered in that way thus bears the boundaries of the desired deletion. To actually generate
14 the Δ *ars1* mutant, pTUD1 was transferred from donor *E. coli* CC118 λ *pir* to *P. putida* Δ *pyrF* cells (TEC1
15 strain, Table 1) by tripartite conjugation on membrane filters (0.45 μ m, Millipore) using *E. coli* HB101
16 (pRK600) as the helper for suicide mobilization (Table 1). After 6 to 8 h of incubation at 30 °C on LB agar
17 supplemented with uracil, the conjugation mixture was plated on M9 minimal selective medium
18 (Sambrook *et al.*, 1989) supplemented with citrate (0.2 %) and MgSO₄ (2 mM) for integration of the
19 delivery plasmid. Resolution of the resulting co-integrate was selected by separately re-plating individual
20 clones in minimal medium with FOA as restrictive condition. The accuracy of the genomic deletion was
21 verified by PCR of the DNA the resulting *P. putida* clones with the upstream and downstream primers
22 mentioned above. Generation of the Δ *ars2* strain followed the same method, excepting that the set of
23 primers to generate the homology fragments for the chromosomal deletion produced DNA segments of a
24 different size. Specifically, the oligos 5'TCTAGATGTGCTAGCAAGCCTTCCTGG3' and 5'-
25 GGTAGAGCTCAATGCTCTGCTCGATTTGCTGC-3' were used to amplify a 0.88-kb upstream region as
26 a *XbaI-ScaI* fragment, while 5'GCGGCCGCGGCGTGAGTTGGCTGACC3' and 5'-CGCTCCCCCATC
27 TAGATAAGCCAGTG-3' added *NotI* and *XbaI* sites to the ends of a 1.19-kb downstream sequence. As
28 before, the two amplified DNAs were assembled in vector pTEC to produce pTUD2 plasmid, which was
29 later delivered to *P. putida* TEC1 and the co-integrate resolved and verified as in the case of Δ *ars1*.
30 Plasmid pTUD2 was also delivered to the *P. putida* Δ *ars1* generated above and processed in the same
31 manner, to yield the double deleted strain *P. putida* Δ *ars1* Δ *ars2*.

1

2 *Determination of total intracellular arsenic*

3

4 To examine the sum of As^{III} and As^V associated to the bacterial biomass, each of the *P. putida* TEC1
5 derivatives under examination were grown in 20 ml of LB medium supplemented with uracil and added
6 with a final concentration of 100 mM sodium arsenate and the cultures let to grow until an OD₆₀₀ of 1.0.
7 Cells were then spun down (4 °C, 4000 rpm, 10 min) and the pellet resuspended in 1 ml of MilliQ water.
8 The samples were then transferred to a previously pre-dried (100°C, 24 h) and weighted (down to the 5-
9 decimal digit) Eppendorf tubes. These specimens were dried (100°C, 24 h) and the weight of the residues
10 determined in biological duplicates. Samples were subsequently treated with nitric acid (HNO₃) and the
11 level of total arsenic determined with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an
12 ICP-MS Elan 6000 Perkin-Elmer Scieix equipped with autosampler AS 91 technology.

13

14 *RT-Q-PCR*

15

16 In order to determine the relative levels of *arsB1* and *arsB2* transcripts under various temperatures,
17 cultures of *P. putida* KT2440 were grown at either 15 °C or 30 °C (OD₆₀₀ ~ 0.45) in LB medium until mid-
18 exponential phase, at which time they were added with 75 mM arsenate. 15 mins later, the biomass from
19 25 ml of each the cultures was recovered by centrifugation and the total RNA pool extracted using Trizol®
20 Reagent (Invitrogen) with the method provided by the supplier. 1.0 µg of each of the RNA samples were
21 then retrotranscribed with the iScript™ cDNA Synthesis Kit (BioRad). This strain-specific and
22 temperature-specific cDNA was the material employed to gross quantify the transcriptional signals of the
23 *arsB* genes as proxies of expression of the corresponding operons. To this end,
24 5'TGTGATTTCTACCCGCAAG3' and 5'GGCGAAAACATTGAGGATC3' primers, matching the *arsB1*
25 gene sequence were designed for reporting expression of the *ars1* operon, while 5'TCAACCC
26 GTCGCGTGCTG3' and 5'-GCTCACCCACCTGCTGGA-3' (matching *arsB2*) were used to follow *ars2*. As
27 an internal reference, we also employed primers 5'CAACGATGACGACGAATGG-3' and 5'-ATCA
28 GGGTCACGGCAATC-3', which amplify the cDNA of housekeeping gene *rpoN*, which is known not to
29 vary significantly through the whole growth curve (Kohler *et al.*, 1994; Yuste *et al.*, 2006). RT-Q-PCR
30 reactions were performed with two biological and technical replicates using the iCycler™ platform of
31 BioRad.

1

2 *P_{ars}-lacZ transcriptional fusions and β -galactosidase assays*

3

4 DNA segments containing all regulatory elements driving transcription of the *P_{ars1}* and *P_{ars2}* promoters
5 were prepared by first subjecting the regions upstream of each of the two *arsR* genes to a thorough
6 analysis in search of promoters and targets for transcriptional factors (Supplementary Information). As a
7 result, it was concluded that all sequences of interest were contained in either case in a 208-bp region
8 which spanned -182 to +26 nt in respect to the proposed transcription origin, ending just before the
9 predicted RBS sequence. Genomic DNA of *P. putida* KT2440 was then used to amplify the corresponding
10 sequences with pairs of primers 5'GCGAATTCTGATCGGTACCAAGC3' / 5'GGGAAGCTTGAATGC
11 GCGTACGGCCAATATCTG3' and 5'GCGAATTCATGTTGGCATCTCG3' / 5'GGGAAGCTTGTACATACG
12 GAAAACCGAATATACGTAT3'. These amplifications yield *EcoRI-HindIII* fragments that bear the
13 predicted regulatory sequences of *P_{ars1}* and *P_{ars2}*, respectively. These fragments were then ligated as
14 *EcoRI-HindIII* inserts to the corresponding sites of low-copy number, broad host range promoter-probe
15 vector pSEVA225 (Silva-Rocha *et al.*, 2013; Table 1). This generated transcriptional fusions between the
16 inserted promoter regions and a promoter-less, complete *lacZ* gene (including a vector-borne SD
17 sequence upstream of the leading ATG), thereby rendering plasmids pSEVA225-*P_{ars1}* and pSEVA225-
18 *P_{ars2}*, respectively. For determination of promoter activity, plasmids were passed to *P. putida* TEC1 (wt)
19 and *P. putida* Δ *ars1* Δ *ars2* as indicated above. Each transformed strain was grown at 15 °C and 30 °C in
20 LB medium added or not with sub-inhibitory concentrations of As^{III} 0.2 mM until the cultures reached the
21 stationary phase. At that point, β -galactosidase was measured in permeabilized cells (Miller, 1972).

22

23 *DNA sequence analyses*

24

25 For bioinformatic inspection of genes and regulatory regions of interest we employed the BioEdit
26 Sequence Alignment Editor (Hall, 1999) and the ApE-A plasmid Editor v.1.17 (Copyright© 2003-2008, M.
27 Wayne David). The ORF FINDER program (NCBI, <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was utilized
28 for default ORF searches on public genome databases. The BLAST platform (Altschul *et al.*, 1997) was
29 used for studying similarity/identity of sequences and, specifically TBLASTN for comparison of amino acid
30 primary sequences. Nucleotides and proteins alignments were done with ALIGN (Wilbur and Lipman,
31 1983) and CLUSTALW (Thompson *et al.*, 1994) respectively, in the BioEdit editor

1 (<http://www.mbio.ncsu.edu/bioedit/page2.html>). The promoters *Pars1* and *Pars2* were predicted by
2 applying the BPROM program of the SoftBerry package (<http://linux1.softberry.com/berry.phtml>) on the
3 regions under inspection.

4

5 ACKNOWLEDGMENTS

6

7 This study was supported by the BIO program of the Spanish Ministry of Economy and Competitiveness
8 (MINECO), the ST-FLOW and ARISYS Contracts of the EU, the ERANET-IB Program and the PROMT
9 Project of the Autonomous Community of Madrid. We thank Javier Tamames for help with DNA sequence
10 analyses, as well as Rafael Silva-Rocha and Max Chavarría for vectors and useful advice. The authors
11 declare no conflict of interest.

12

13 REFERENCES

14

- 15 Achour, A. R., Bauda, P. and Billard, P. (2007) Diversity of arsenite transporter genes from arsenic-
16 resistant soil bacteria. *Res Microbiol* **158**: 128-137.
- 17 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997)
18 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl*
19 *Acids Res* **25**: 3389-3402.
- 20 Andam, C. P., Fournier, G. P. and Gogarten, J. P. (2011) Multilevel populations and the evolution of
21 antibiotic resistance through horizontal gene transfer. *FEMS Microbiol Revs* **35**: 756-767.
- 22 Cai, J., Salmon, K. and DuBow, M. S. (1998) A chromosomal *ars* operon homologue of *Pseudomonas*
23 *aeruginosa* confers increased resistance to arsenic and antimony in *Escherichia coli*.
24 *Microbiology* **144**: 2705-2713.
- 25 Canovas, D., Cases, I. and de Lorenzo, V. (2003a) Heavy metal tolerance and metal homeostasis in
26 *Pseudomonas putida* as revealed by complete genome analysis. *Environ Microbiol* **5**: 1242-1256.
- 27 Canovas, D., Mukhopadhyay, R., Rosen, B. P. and de Lorenzo, V. (2003b) Arsenate transport and
28 reduction in the hyper-tolerant fungus *Aspergillus* sp. P37. *Environ Microbiol* **5**: 1087-1093.
- 29 Canovas, D., Vooijs, R., Schat, H. and de Lorenzo, V. (2004) The role of thiol species in the
30 hypertolerance of *Aspergillus* sp. P37 to arsenic. *J Biol Chem* **279**: 51234-51240.

- 1 Carlin, A., Shi, W., Dey, S. and Rosen, B. P. (1995) The *ars* operon of *Escherichia coli* confers arsenical
2 and antimonial resistance. *J Bacteriol* **177**: 981-986.
- 3 de Lorenzo, V., Eltis, L., Kessler, B. and Timmis, K. N. (1993) Analysis of *Pseudomonas* gene products
4 using *lacI^q/Ptrp-lac* plasmids and transposons that confer conditional phenotypes. *Gene* **123**: 17-
5 24.
- 6 Francino, M. P. (2005) An adaptive radiation model for the origin of new gene functions. *Nature Genet* **37**:
7 573-577.
- 8 Galvao, T. C. and de Lorenzo, V. (2005) Adaptation of the yeast URA3 selection system to gram-negative
9 bacteria and generation of a $\Delta betCDE$ *Pseudomonas putida* strain. *Appl Env Microbiol* **71**: 883-
10 892.
- 11 Giller, K. E., Witter, E. and McGrath, S. P. (1998) Toxicity of heavy metals to microorganisms and
12 microbial processes in agricultural soils: a review. *Soil Biol Biochem* **30**: 1389-1414.
- 13 Hall, A. H. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for
14 Windows 95/98/NT. *Nucl Acids Symp Ser* **41**: 95-98.
- 15 Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557-580.
- 16 Herrero, M., de Lorenzo, V. and Timmis, K. N. (1990) Transposon vectors containing non-antibiotic
17 resistance selection markers for cloning and stable chromosomal insertion of foreign genes in
18 gram-negative bacteria. *J Bacteriol* **172**: 6557-6567.
- 19 Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M. and Cossart, P. (2002) An RNA
20 thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* **110**: 551-
21 561.
- 22 Kessler, B., de Lorenzo, V. and Timmis, K. N. (1992) A general system to integrate *lacZ* fusions into the
23 chromosomes of gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid
24 studied with all controlling elements in monocopy. *Mol Gen Genet* **233**: 293-301.
- 25 Kohler, T., Alvarez, J. F. and Harayama, S. (1994) Regulation of the *rpoN*, ORF102 and ORF154 genes
26 in *Pseudomonas putida*. *FEMS Microbiol Lett* **115**: 177-184.
- 27 Manoil, C. and Beckwith, J. (1985) *TnphoA*: a transposon probe for protein export signals. *Proc. Natl.*
28 *Acad Sci USA* **82**: 8129-8133.
- 29 Mateos, L. M., Ordonez, E., Letek, M. and Gil, J. A. (2006) *Corynebacterium glutamicum* as a model
30 bacterium for the bioremediation of arsenic. *Int Microbiol* **9**: 207-215.

- 1 Messens, J. and Silver, S. (2006) Arsenate reduction: thiol cascade chemistry with convergent evolution.
2 *J Mol Biol* **362**: 1-17.
- 3 Miller, J. H. (1972) *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring
4 Harbor, N.Y.
- 5 Mukhopadhyay, R., Rosen, B. P., Phung, L. T. and Silver, S. (2002) Microbial arsenic: from geocycles to
6 genes and enzymes. *FEMS Microbiol Revs* **26**: 311-325.
- 7 Nelson, K. E., Weinl, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A., Fouts, D. E.,
8 Gill, S. R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R. T., Daugherty, S., Kolonay,
9 J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris Lee, P.,
10 Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D.,
11 Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C.,
12 Eisen, J. A., Timmis, K. N., Dusterhoft, A., Tumbler, B. and Fraser, C. M. (2002) Complete
13 genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida*
14 KT2440. *Env Microbiol* **4**: 799-808.
- 15 Nikel, P. I., Martínez-García, E. and de Lorenzo, V. (2014) Biotechnological domestication of
16 Pseudomonads through synthetic biology. *Nature Microbiol Revs* (In Press).
- 17 Oremland, R.S. and Stolz, J.F. (2003) The ecology of arsenic. *Science* **300**: 939-944.
- 18 Paez-Espino, D., Tamames, J., de Lorenzo, V. and Canovas, D. (2009) Microbial responses to
19 environmental arsenic. *Biometals* **22**: 117-130.
- 20 Rosen, B.P. (1995) Resistance mechanisms to arsenicals and antimonials. *J Basic Clin Physiol*
21 *Pharmacol* **6**: 251-263.
- 22 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular cloning : a laboratory manual*. Cold Spring
23 Harbor Laboratory Press, Cold Spring Harbor, New York.
- 24 Sanchez-Perez, G., Mira, A., Nyiro, G., Pasic, L. and Rodriguez-Valera, F. (2008) Adapting to
25 environmental changes using specialized paralogs. *Trends Genet* **24**: 154-158.
- 26 Sharp, P. M. and Li, W. H. (1987) The codon Adaptation Index--a measure of directional synonymous
27 codon usage bias, and its potential applications. *Nucl Acids Res* **15**: 1281-1295.
- 28 Silva-Rocha, R., Martinez-Garcia, E., Calles, B., Chavarria, M., Arce-Rodriguez, A., de Las Heras, A.,
29 Paez-Espino, A. D., Durante-Rodriguez, G., Kim, J., Nikel, P. I., Platero, R. and de Lorenzo, V.
30 (2013) The Standard European Vector Architecture (SEVA): a coherent platform for the analysis
31 and deployment of complex prokaryotic phenotypes. *Nucl Acids Res* **41**: D666-675.

- 1 Tamaki, S. and Frankenberger, W. T., Jr. (1992) Environmental Biochemistry of Arsenic. In *Reviews of*
2 *Environmental Contamination and Toxicology*. G. Ware (ed). Springer New York, pp. 79-110.
- 3 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of
4 progressive multiple sequence alignment through sequence weighting, position-specific gap
5 penalties and weight matrix choice. *Nucl Acids Res* **22**: 4673-4680.
- 6 Wilbur, W. J. and Lipman, D. J. (1983) Rapid similarity searches of nucleic acid and protein data banks.
7 *Proc Natl Acad Sci USA* **80**: 726-730.
- 8 Wu, J. and Rosen, B.P. (1993) Metalloregulated expression of the *ars* operon. *J Biol Chem* **268**: 52-58.
- 9 Xu, C., Zhou, T., Kuroda, M. and Rosen, B.P. (1998) Metalloid resistance mechanisms in prokaryotes. *J*
10 *Biochem* **123**: 16-23.
- 11 Yuste, L., Hervas, A. B., Canosa, I., Tobes, R., Jimenez, J. I., Nogales, J., Perez-Perez, M. M., Santero,
12 E., Diaz, E., Ramos, J. L., de Lorenzo, V. and Rojo, F. (2006) Growth phase-dependent
13 expression of the *Pseudomonas putida* KT2440 transcriptional machinery analysed with a
14 genome-wide DNA microarray. *Environ Microbiol* **8**: 165-177.
- 15 Zafra, O., Fraile, S., Gutierrez, C., Haro, A., Paez-Espino, A. D., Jimenez, J. I. and de Lorenzo, V. (2011)
16 Monitoring biodegradative enzymes with nanobodies raised in *Camelus dromedarius* with
17 mixtures of catabolic proteins. *Env Microbiol* **13**: 960-974.
- 18
19
20
21
22
23

1 TABLE 1. Strains and plasmids

2

Strains	Relevant characteristics	Reference
<i>P. putida</i> KT2440	<i>P. putida</i> mt-2 derivative cured of pWW0	Nelson <i>et al.</i> , 2002
<i>P. putida</i> TEC1	Rif ^R , Δ <i>pyrF</i> derivative of <i>P. putida</i> KT2440	Galvao and de Lorenzo, 2005
<i>P. putida</i> Δ <i>ars1</i>	Rif ^R , TEC1 deleted of the <i>ars1</i> operon by using pTUD1	This work
<i>P. putida</i> Δ <i>ars2</i>	Rif ^R , TEC1 deleted of the <i>ars2</i> operon by using pTUD2	This work
<i>P. putida</i> Δ <i>ars1</i> Δ <i>ars2</i>	Rif ^R , TEC1 deleted of <i>ars1</i> and <i>ars2</i> operons by sequential use of pTUD1 and pTUD2	This work
<i>E. coli</i> CC118	Δ (<i>ara-leu</i>) <i>araD lacX74 galE galK phoA20 thi-1 rpsE</i> <i>rpoB argE recA1</i>	Manoil and Beckwith, 1985
<i>E. coli</i> CC118 λ <i>pir</i>	<i>E. coli</i> CC118 lysogenized with λ <i>pir</i> phage	Herrero <i>et al.</i> , 1990
<i>E. coli</i> HB101	<i>E. coli</i> K12/ <i>E. coli</i> B hybrid, Sm ^R , <i>rpsL recA thi pro leu</i> <i>hsdR-M</i> ⁺	Sambrook <i>et al.</i> , 1989
<i>E. coli</i> DH5 α	F ⁻ Φ 80 Δ <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1</i> <i>endA1 hsdR17 R-M</i> ⁺ <i>supE44 thi gyrA relA</i>	Hanahan, 1983
<i>E. coli</i> W3110	K12 F ⁻ IN (<i>rrnD-rrnE</i>)	Strain collection
<i>E. coli</i> AW3110	K12 F ⁻ Δ <i>ars::cam</i> F ⁻ IN (<i>rrnD-rrnE</i>)	Carlin <i>et al.</i> , 1995

Plasmid

pGEM-T Easy	Ap ^R , cloning vector for PCR fragments	Promega
pGC1	Ap ^R , pGEM-T inserted with PCR fragment spanning complete <i>ars1</i> operon with its native promoter	This work
pGC2	Ap ^R , pGEM-T inserted with PCR fragment spanning complete <i>ars2</i> operon with its native promoter	This work
pTEC	Km ^R , FOA ^S , <i>pyrF</i> ⁺ (Ura ⁺), MCS, R6KoriV, origin of	Galvao and de

	transfer RK2 <i>oriT</i> , cloning vector for chromosomal integration by homologous recombination in <i>P. putida</i> TEC1	Lorenzo, 2005
pTUD1	Km ^R , pTEC inserted with a DNA segment composed of the 1.29 kb upstream and 1.24 downstream region of the <i>ars1</i> operon. Delivery vector for Δ <i>ars1</i> deletion	This work
pTUD2	Km ^R , pTEC inserted with a DNA segment composed of the 0.88 kb upstream upstream and 1.19 downstream region of the <i>ars2</i> operon. Delivery vector for Δ <i>ars2</i> deletion	This work
pVLT33	Km ^R , RSF1010 <i>oriV-lacI^R/Ptac</i> broad-host-range expression vector with pUC18 MCS	de Lorenzo <i>et al.</i> , 1993
pVCI1	Km ^R , pVLT33 inserted with complete <i>ars1</i> operon	Zafra <i>et al.</i> , 2011
pVCI2	Km ^R , pVLT33 inserted with complete <i>ars2</i> operon	This work
pRK600	Cm ^R , ColE1 <i>ori</i> , RK2 <i>mob</i> , RK2 <i>tra</i> , helper of conjugal transfer	Kessler <i>et al.</i> , 1992
pSEVA225	Km ^R , pBBR1 <i>oriV</i> , <i>lacZ</i> promoter probe broad host range vector	Silva-Rocha <i>et al.</i> , 2013
pSEVA225-Pars1	Km ^R , pSEVA225 inserted with <i>P_{ars1}</i> promoter	This work
pSEVA225-Pars2	Km ^R , pSEVA225 inserted with <i>P_{ars2}</i> promoter	This work

1

2

3

4

1 TABLE 2. Resistance of *P. putida* and *E. coli* strains to different As species
 2

Strain (plasmid)	RESISTANCE TO ARSENIC (mM) ^a			
	As ^V 15 °C	As ^V 30 °C	As ^{III} 15 °C	As ^{III} 30 °C
<i>P. putida</i> KT2440	300	300	8	10
<i>P. putida</i> TEC1	300	300	8	10
<i>P. putida</i> Δ <i>ars1</i>	200	200	3	3
<i>P. putida</i> Δ <i>ars2</i>	100	220	2	7
<i>P. putida</i> Δ <i>ars1</i> Δ <i>ars2</i>	2	2	0,1	0,2
<i>P. putida</i> Δ <i>ars1</i> (pVCI1)	350	350	10	12
<i>P. putida</i> Δ <i>ars2</i> (pVCI2)	350	350	10	12
<i>E. coli</i> W3110 ^b	-	2	-	0,5
<i>E. coli</i> AW3110	-	0,2	-	0,05
<i>E. coli</i> DH5 α (pGEM-T)	-	2	-	0,5
<i>E. coli</i> DH5 α (pGCI1)	-	7	-	7
<i>E. coli</i> DH5 α (pGCI2)	-	5	-	3

^a Plate growth assays.

^b *E. coli* strains do not grow optimally at 15 °C (max 48h)

3

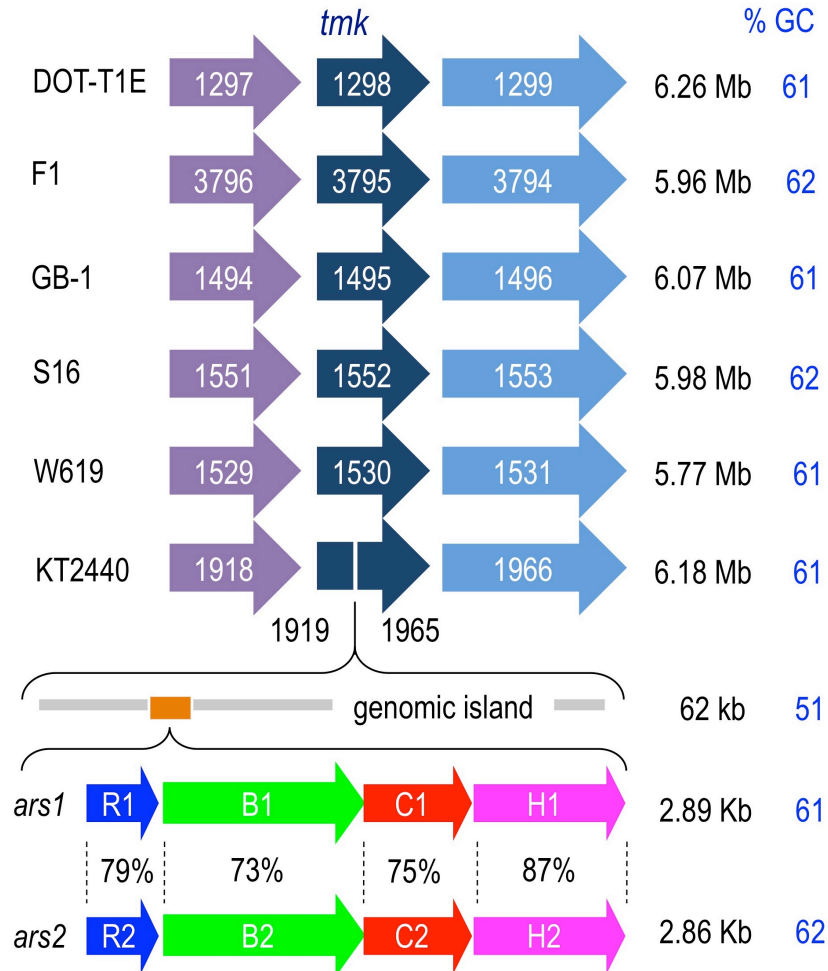
4

1 **FIGURES**

2

3 **Figure 1.** The *ars* operons of *P. putida* KT2440 and their connection to the *tmk* (thymidylate kinase) gene.

4



5

6

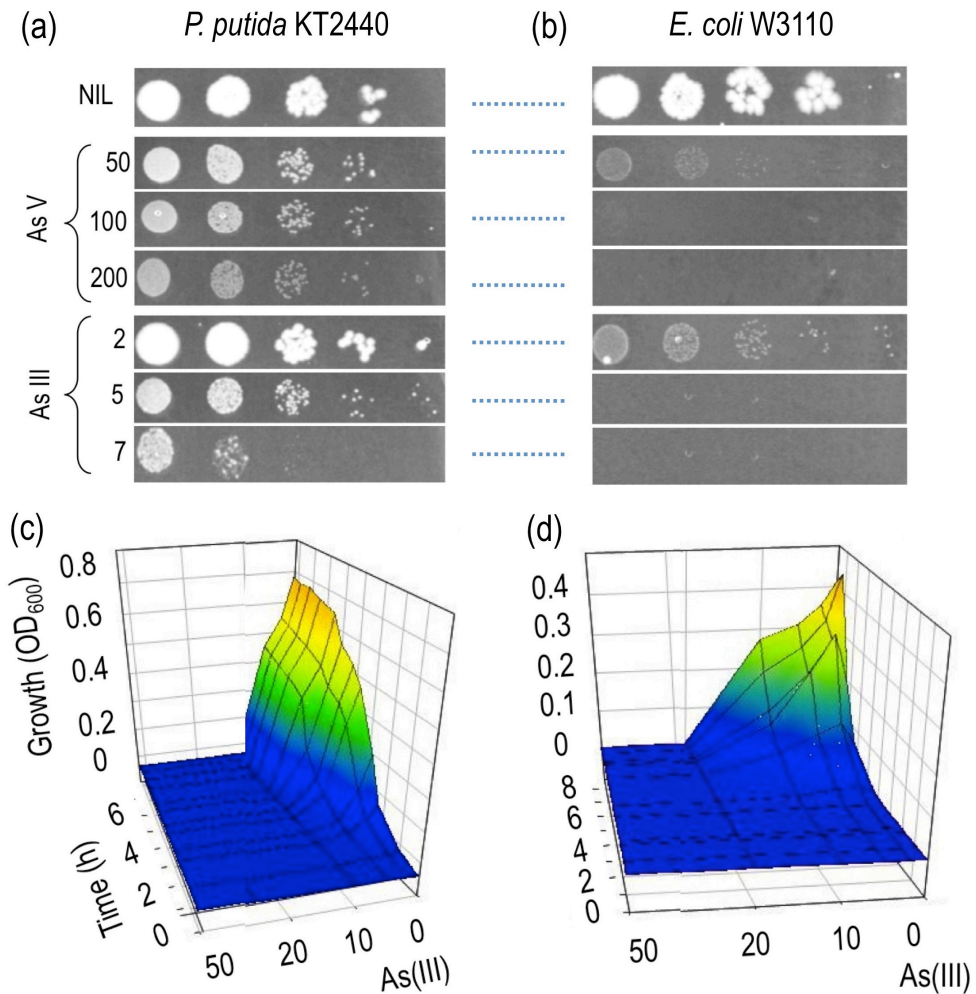
7 The upper part of the figure shows the genomic context of the 62 kb-length genomic island where *ars1*
8 operon is located (bottom) in respect to the same chromosomal location in other *P. putida* strains. The
9 CG contents of the corresponding segments are indicated along the size (Mb) of the respective full
10 genomes. See Supplementary Table S1 for a complete list of all genes contained in the genomic island.
11 The lower row of the figure shows the identity of amino acid sequences between the two *ars* operons (the
12 *ars2* cluster maps in a distant location of the *P. putida* KT2440 genome; Canovas *et al.*, 2003a). DNA
13 sequence identity through the homologous regions is 69 % (*arsR*), 74 % (*arsB*), 68 % (*arsC*) and 78 %
14 (*arsH*).

15

16

1 **Figure 2.** Resistance of *P. putida* KT2440 to arsenic salts.

2



3

4

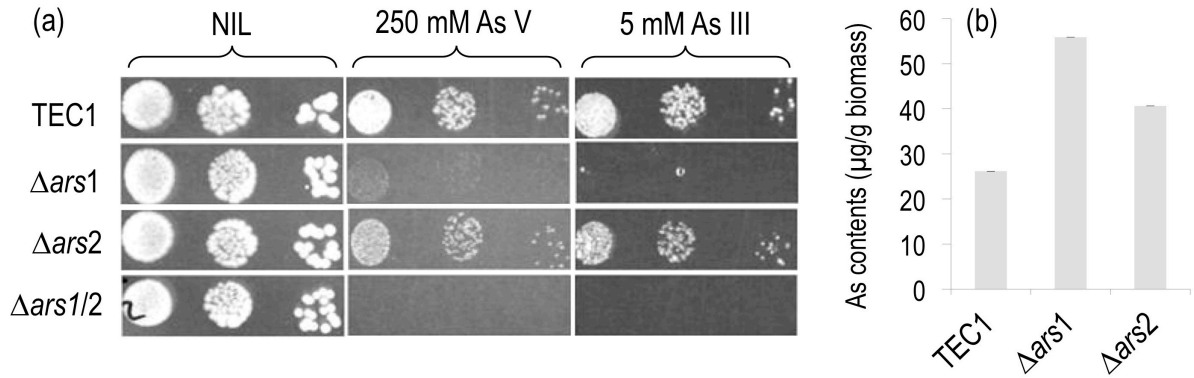
5 (Top) Plate assays. The resistance of *P. putida* KT2440 (a) and *E. coli* W3110 (b) to the indicated
 6 concentrations of As^V and As^{III} was tested by plating 8 μ l serial dilutions of cell suspensions (10^6 , 10^5 , 10^4 ,
 7 10^3 , and 10^2 cells/ml) for 22 h on LB agar with the indicated concentrations of the corresponding salts.
 8 (Bottom) Growth of *P. putida* KT2440 in liquid LB medium supplemented with different concentrations of
 9 As^{III}. The assays were performed using a non-induced pre-inoculum (c), or a starter pre-induced with 5
 10 mM As^{III} (d, see Experimental procedures). Growth differences were more pronounced through the As^{III}
 11 concentration range 5.0-20 mM. Equivalent growth assays using 100 mM As^V as a preinducer produced
 12 comparable patterns.

13

14

1 **Figure 3.** Arsenic resistance and accumulation by *P. putida* strains with or without and arsenic resistance
 2 operons.

3



4

5

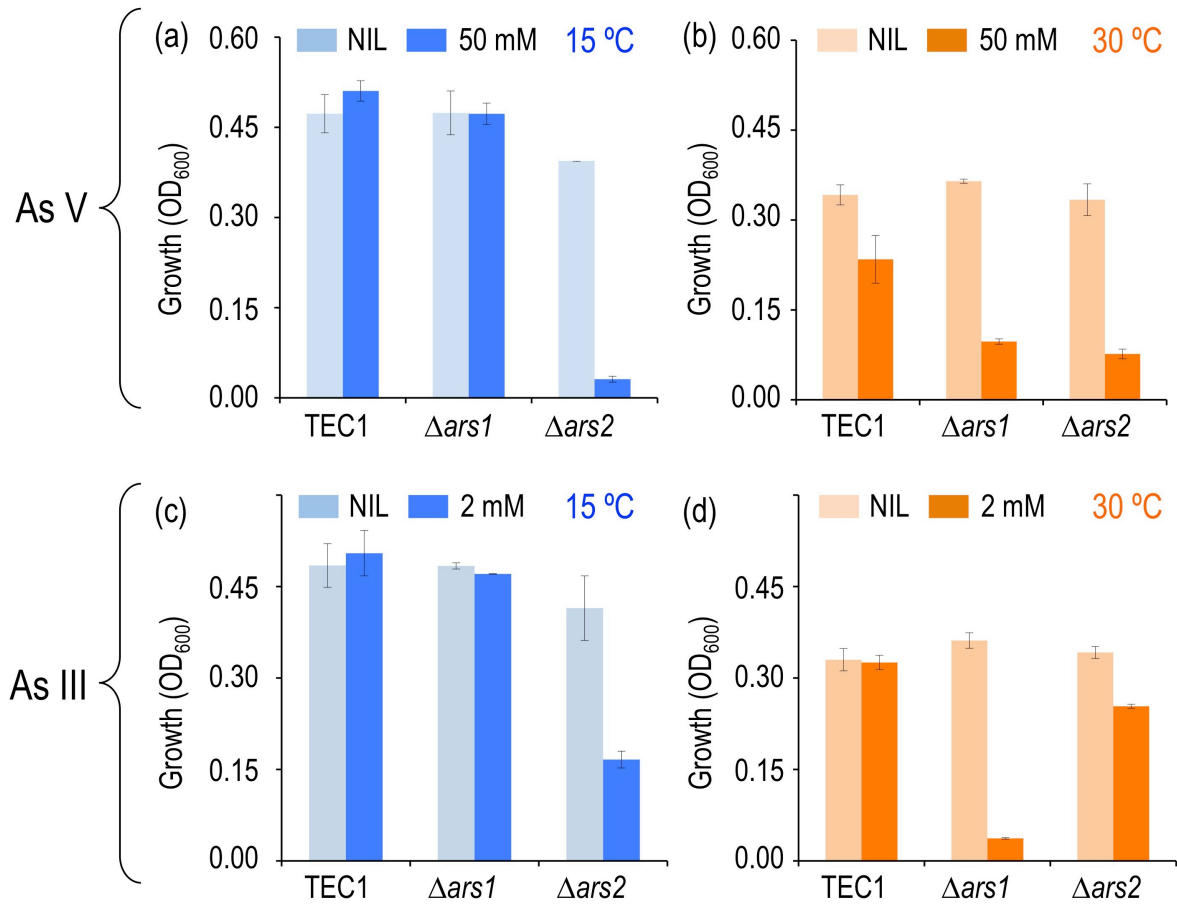
6 **(a)** Plate growth assays of *P. putida* TEC1 (wt) and its derivatives $\Delta ars1$, $\Delta ars2$ and $\Delta ars1 / \Delta ars2$ on
 7 agar-LB, 48 h. Serial dilutions represent 10^6 , 10^5 , and 10^4 cells/ml. **(b)** Total intracellular arsenic
 8 accumulation by *P. putida* TEC1 (wt) and its single-deletion derivatives $\Delta ars1$ and $\Delta ars2$ determined with
 9 Coupled Plasma Mass Spectrometry (see text).

10

11

1 **Figure 4.** Arsenic resistance of *P. putida* TEC1, $\Delta ars1$ and $\Delta ars2$ at different temperatures.

2



3

4

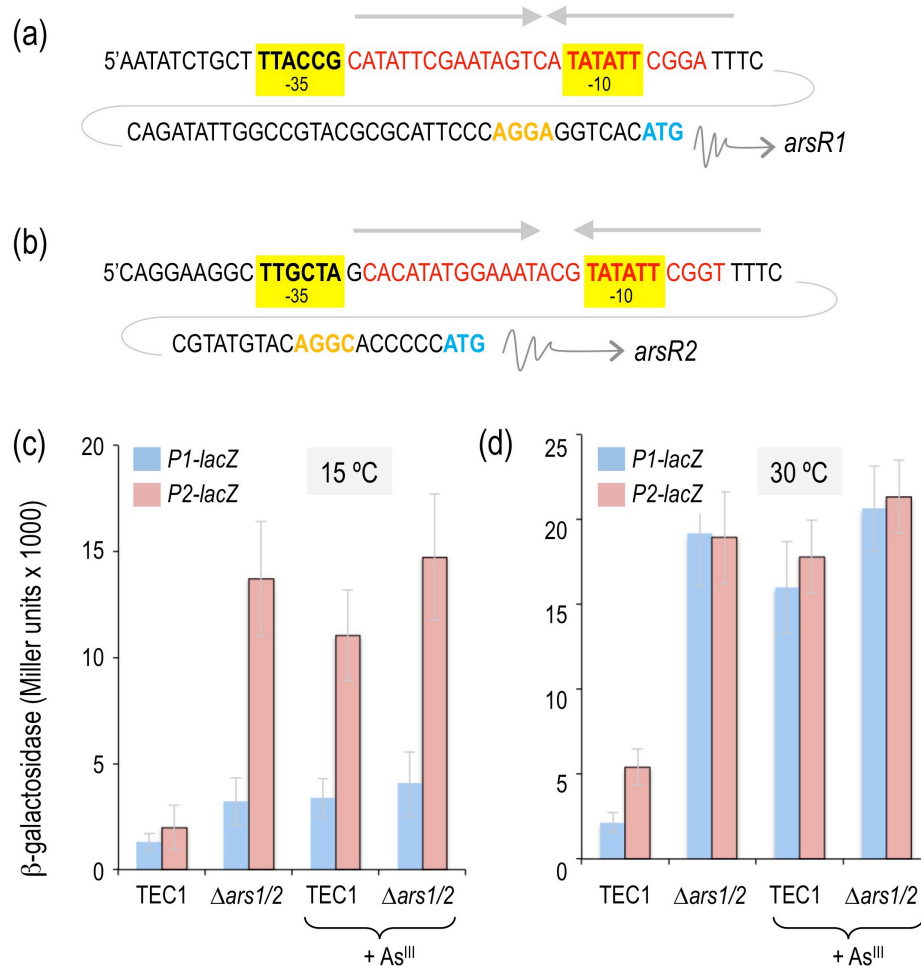
5 For the experiments at 15 °C (panels a, c) triplicate cultures of the strains indicated in each case were
 6 grown for 48 h in a 96-microwell-plate reader with orbital shaking in the presence of the concentrations of
 7 As^V or As^{III} specified for each condition and the OD₆₀₀ recorded at that point. The assays at higher
 8 temperature (30 °C, panels b, d) were done under identical conditions excepting for the incubation period,
 9 which was reduced to 8 h (similar results were obtained at 37 °C, not shown). In each case, the
 10 incubation times were those at which no further growth was observed in the cultures, what was
 11 operatively considered *stationary phase*.

12

13

1 **Figure 5.** Quantification of *in vivo* activity of the P_{ars1} and P_{ars2} promoters with transcriptional *lacZ*
 2 fusions.

3



4

5

6 Panels (a) and (b) show the organization of P_{ars1} and P_{ars2} , with the -35 and -10 boxes highlighted in
 7 yellow. The grey arrows indicate the quasi-palindromic DNA sequence predicted to be bound by each of
 8 the ArsR variants (in red). The RBSs are marked in orange while the leading ATG codon of each gene is
 9 signposted in blue. Panels (c) and (d) show the accumulation of β -galactosidase by *P. putida* TEC1 and
 10 its $\Delta ars1 \Delta ars2$ double deleted derivative harbouring transcriptional fusions P_{ars1} -*lacZ* (blue bars) and
 11 P_{ars2} -*lacZ* (pink bars). Each of the four strains were grown in LB medium at either 15°C or 30°C in
 12 presence or absence of 0.2 mM As^{III} as indicated until they reached stationary phase. The data show the
 13 average of 3 separate experiments.

14

15

SUPPLEMENTAL INFORMATION

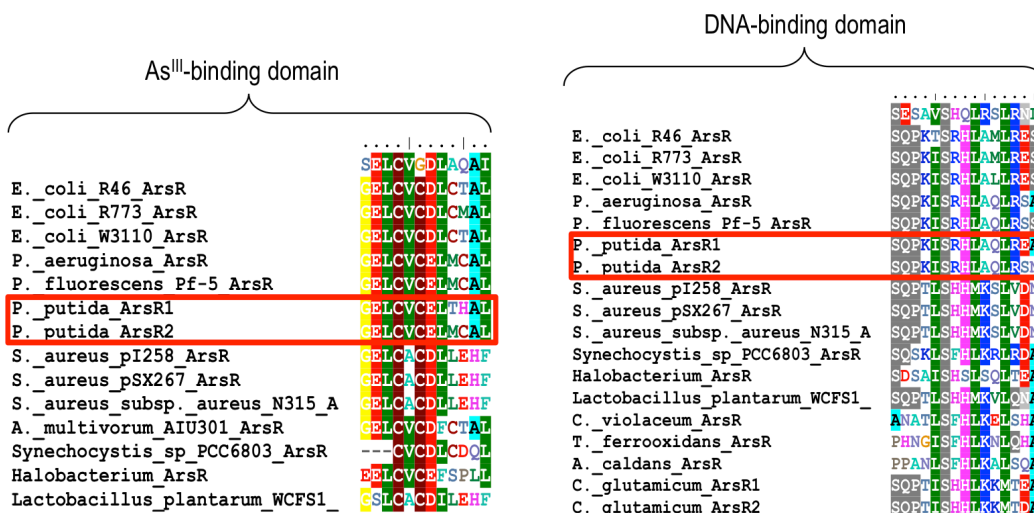
Genetic organization of the *ars1* and *ars2* clusters and their regulatory regions

The two *ars* clusters (i.e. the core *arsRBC* sequences) of *P. putida* KT2440 were analyzed using a set of bioinformatics approaches based on sequence alignments and phylogeny. The two *arsC* genes of *P. putida* determine thioredoxin-dependent arsenate reductases:

Ppu1928	MNRRYLMKVL	FMCTHNSCRS	ILSEALFNHL	APEGMEAVSS	GSFSPGKVNE
Ppu2716MRVL	FMCTANSCRS	ILSEAMFNHL	APPGFEAVSA	GSFPKGQVLP
Bsu2578	...MENKIIY	FLCTGNSCRS	QMAEGWAKQY	LGDEWKVYSA	GIEAHG.LNP
Ppu1928	RALKTLEAAG	IPTAGLSSKA	SDAFESSPPD	IVVTVCDRAA	GEACPFFGFP
Ppu2716	RSLSTLQQAN	ISTEGLSSKG	NDAFEGNPPD	IVITVCDKAA	GEACPVYFGP
Bsu2578	NAVKAMKEVG	IDISNQTSDI	IDS DILNNAD	LVVTLCDGAA	.DKCPMTPPH
Ppu1928	SLKAHWGLAD	PSAVTGSETE	IEEAFQTTLA	KIDERVRAFI	ALPFSQLSQD
Ppu2716	ALKSHWGLD	PSDVVGDEAT	VDAAFRATLA	RIESRCQAFF	ALPFDHLDRE
Bsu2578	VKREHWGFDD	PARAQGTEEE	KWAFFQVRD	EIGNRLKEFA	ETGK.....
Ppu1928	ELKAEFARIG	AL			
Ppu2716	QLKHALDRIG	SL			
Bsu2578			

Alignment of primary sequences of the active centers of bacterial As^V reductases. The enzyme catalyzes the reaction As^V → As^{III}. There are two main families of arsenate reductases based on their structure and reaction mechanism, based on the location of catalytic cysteine residues i.e. coupled to thioredoxin, or to glutaredoxin (Messens and Silver 2006; Paez-Espino et al. 2009). Ppu1928 and Ppu2716 correspond to the sequences of *P. putida* KT2440 *ArsC1* and *ArsC2*, respectively. *B. subtilis* *ArsC* (Bsu2568) was used as a thioredoxin-dependent protein of reference in the alignments. Note the perfect match of the active center (yellow residues), the conservation of 3 Cys residues at specific locations and the high identity of the amino acid sequences through much of the length of the reductases. The *P. putida* enzymes thus qualify as thioredoxin dependent reductases. Alignment of the same proteins with the glutaredoxin dependent *E. coli* *ArsC* exposed no significant identity in the active center (not shown).

the most common type of bacterial enzyme to that end. Finally, inspection of the *arsR1* and *arsR2* genes (Fig. below)



Conservation of business parts of ArsR proteins. The figure shows the specific sequences at stake in each of the *P. putida* *ArsR* repressors in comparison to other proteins of the same family. Note the very high similarity of both functional regions (Shi et al. 1996).

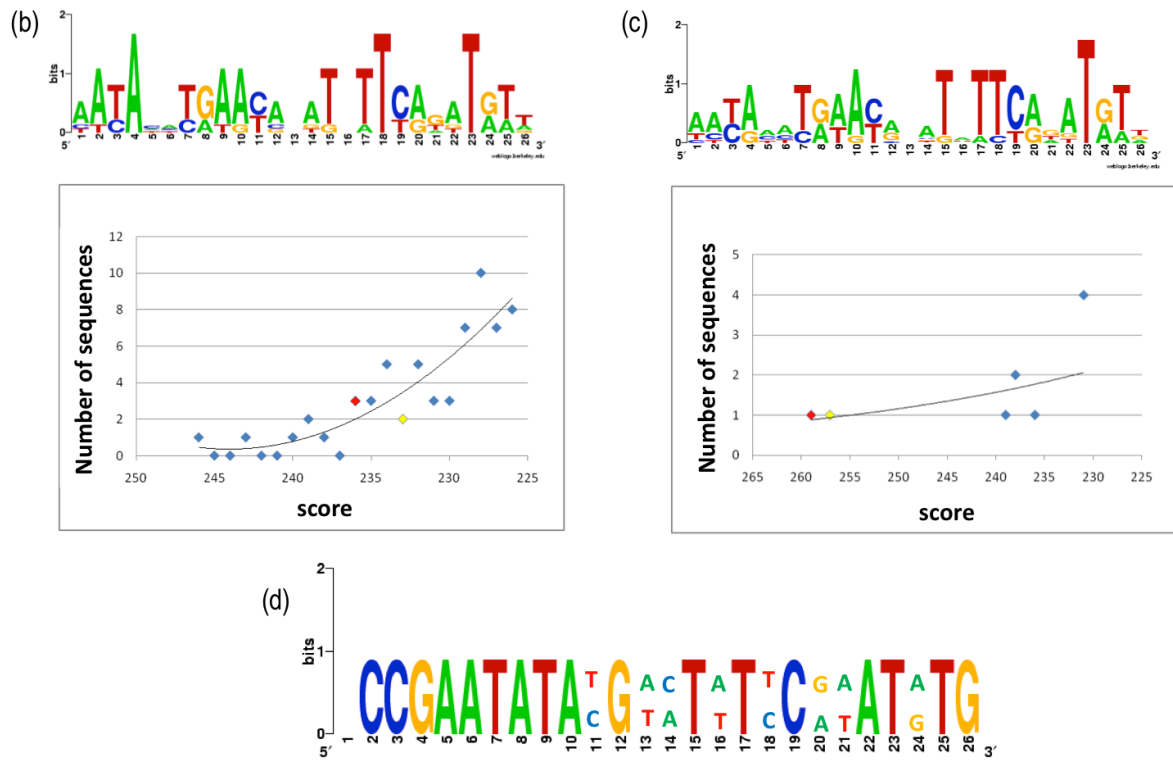
revealed that the corresponding proteins maintain both the characteristic DNA binding domain and the arsenite binding domain of the SmtB/ArsR family of repressor proteins (Busenlehner *et al.*, 2003). The promoter regions of each of the two *ars* clusters were examined in more detail with the *BPROM* program of the *SoftBerry* software.

```

> Pars1         -35                -10                RBS
TAAATATCTGCTTTTACCGCATATTCGAATAGTCATATATTCCGGATTTCCAGATATTGGCCGTACGCGCATTCCCAGGAGGTCACATG
..((( ((((((.....)))))))))...

> Pars2         -35                -10                RBS
CAGGAAGGCTTGCTAGACATATGGAAATACGTATATTTCGGTTTTCCGTATGTACAGGCACCCCATG
..((( ((((((.....))..)))))))))...

```



Analysis of the *P_{ars1}* and *P_{ars2}* promoter regions of *P. putida* and their ArsR-binding operators. (a) Recognition of promoter sequences at the upstream region of the *ars1* and *ars2* genomic regions. The -35 and -10 motifs typical of sigma-70 promoters are underlined. The sequences encompassing in each case the operator regions based on the consensus target sites for repressors of the SmtB/ArsR family (Busenlehner *et al.*, 2003) are indicated in red along with their cognate palindromic sequences. The ribosomal binding sites are marked in orange. The blue color identifies the leading codon of each *arsR* gene in either operon. (b) Consensus DNA binding motif of 7 SmtB/ArsR family homologs. The graph at the bottom shows the number of inter-genic sequences of *P. putida* KT2440 relative to the number of bases identical to the consensus. Red: group including *P_{ars1}*; yellow: group including *P_{ars2}*. (c) Same, but adding the putative operators of *P_{ars1}* and *P_{ars2}*. (d) Sequence identity between the ArsR operator regions of *P. putida* KT2440.

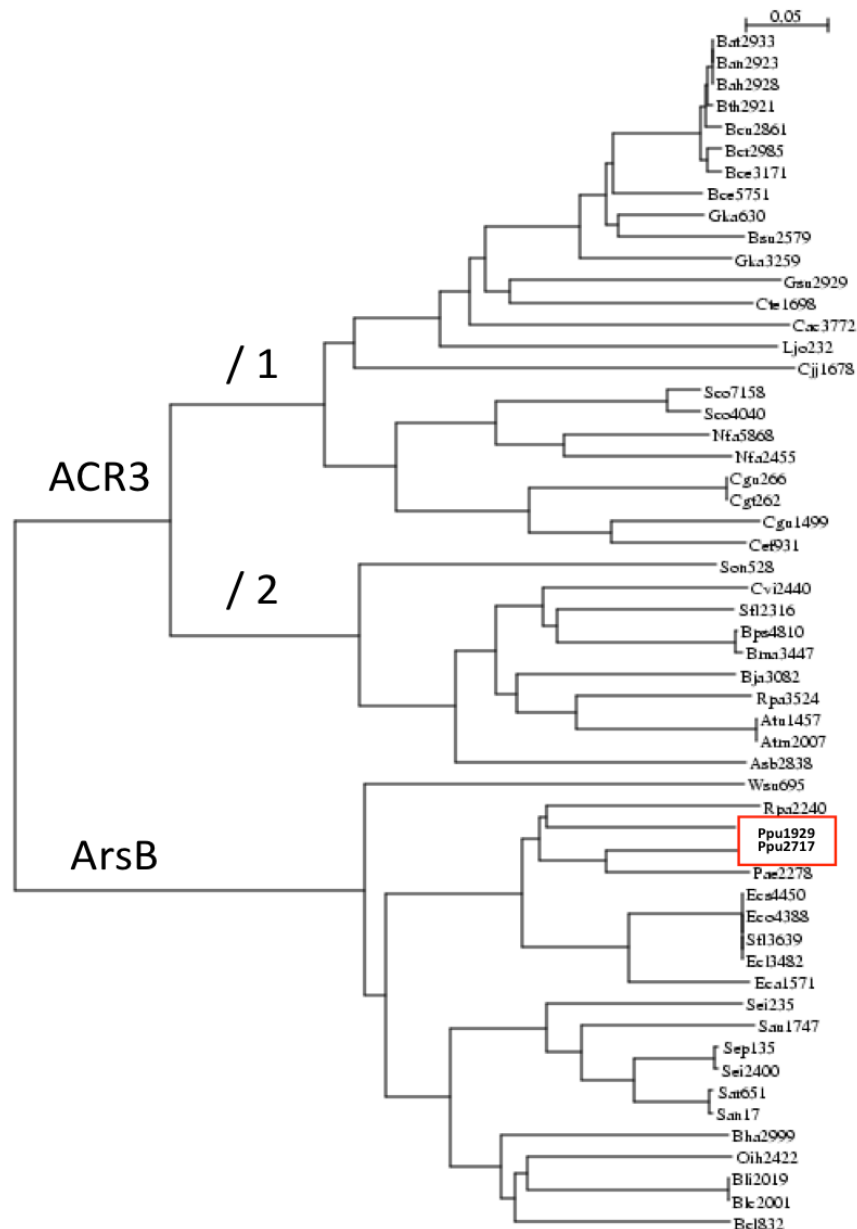
In either case, a palindromic region that coincides with the consensus target sequence of the transcriptional repressor family SmtB/ArsR (Busenlehner et al. 2003) was found overlapping the -10 and -35 boxes of typical sigma-70-dependent promoter regions of each operon (Figure a above). Further inspection of such putative ArsR operators within the *P_{ars1}* and *P_{ars2}* promoters in respect to other known binding sites (Figures b and c above) as well as to each other (Figure d above) make it very unlikely that these repressors bind intergenic regions of the *P. putida* genome other than their very operons at the *ars* promoters. This plausibly rules out any significant regulatory cross-talk of arsenic with genes other than those involved in the tolerance to the oxyanion.

6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28

1 *Phylogeny of bacterial arsenite pumps*

2

3 Both in the case of *ars1* and *ars2* (Fig. 1, main text) each of the corresponding *arsB* genes encodes an
 4 As^{III}-specific pump. Prokaryotes host two distinct families of arsenite efflux transporters, which are
 5 qualified as members of the so-called ArsB and the ACR3 protein families (Achour *et al.*, 2007; Paez-
 6 Espino *et al.*, 2009; see below). Inspection of the transporters encoded by the *arsB1* and *arsB2* genes of
 7 *P. putida* (Fig. below) clearly indicates that they belong to the ArsB-type i.e. the efflux pump of the type
 8 most represented among γ -proteobacteria (Rosen and Liu, 2009). The tree below was built with the
 9 *Clustalw* software applied to different representative groups of bacteria (Proteobacteria, Actinobacteria
 10 and Firmicutes).
 11



12

13

14

15 ACR3/1-type efflux pumps were dominant among *Bacillus* genera (Ban, *Bacillus anthracis*; Bce, *Bacillus*
 16 *cereus*; Bsu, *Bacillus subtilis*) and *Actinobacteria* (Cgu, *Corynebacterium glutamicum*). ACR3/2-type was
 17 mainly present in alpha-proteobacteria (Rpa, *Rhodopseudomonas palustris*; Atu, *Agrobacterium*

1 *tumefaciens*). Among the ArsB-type representatives we found gamma-proteobacteria (Eco, *Escherichia*
2 *coli*; Pae, *Pseudomonas aeruginosa*). The red box shows the two *arsB* genes of *P. putida* (Ppu).

3
4
5 REFERENCES

6
7 Achour AR, Bauda P , Billard P. 2007. Diversity of arsenite transporter genes from arsenic-resistant soil
8 bacteria. *Res Microbiol* **158**: 128-137.

9 Busenlehner LS, Pennella MA , Giedroc DP. 2003. The SmtB/ArsR family of metalloregulatory
10 transcriptional repressors: Structural insights into prokaryotic metal resistance. *FEMS Microbiol Revs*
11 **27**: 131-143.

12 Messens J , Silver S. 2006. Arsenate reduction: thiol cascade chemistry with convergent evolution. *J Mol*
13 *Biol* **362**: 1-17.

14 Paez-Espino D, Tamames J, de Lorenzo V , Canovas D. 2009. Microbial responses to environmental
15 arsenic. *Biomaterials* **22**: 117-130.

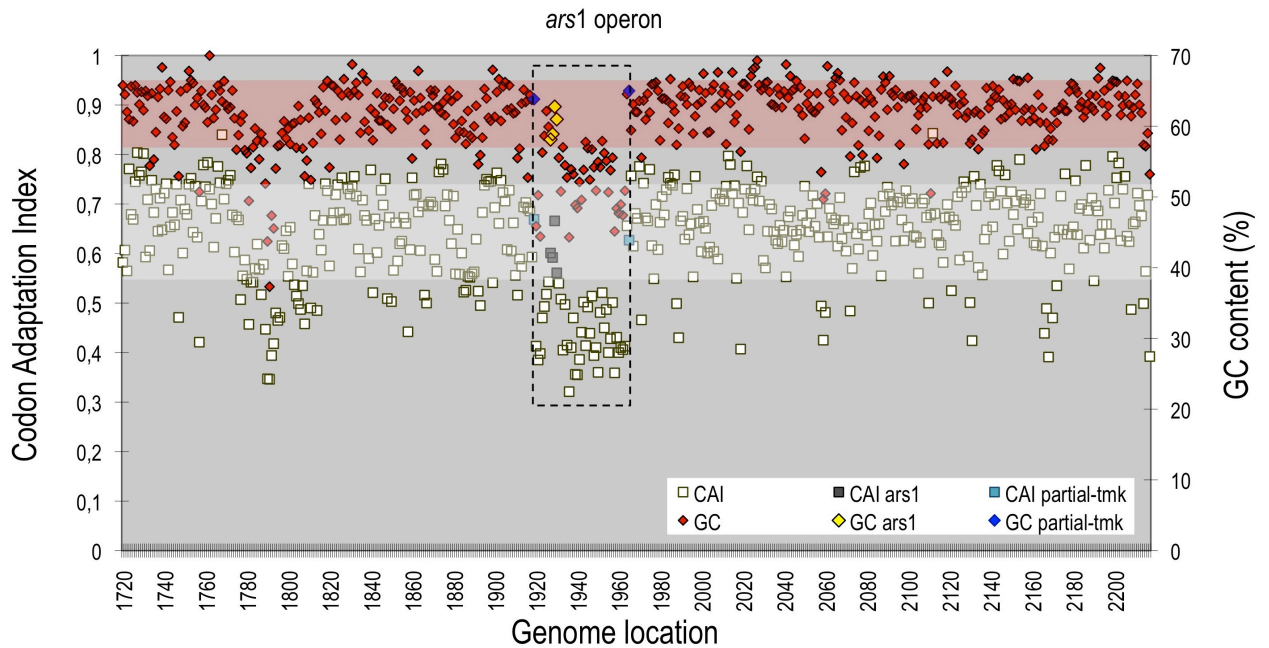
16 Rosen BP , Liu Z. 2009. Transport pathways for arsenic and selenium: a minireview. *Environ Int* **35**: 512-
17 515.

18 Shi W, Dong J, Scott RA, Ksenzenko MY , Rosen BP. 1996. The role of arsenic-thiol interactions in
19 metalloregulation of the *ars* operon. *J Bio Chem* **271**: 9291-9297.

20
21

SUPPLEMENTARY FIGURES

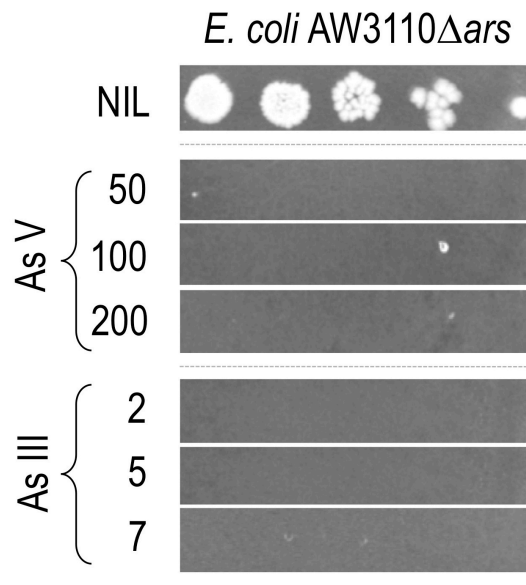
Supplementary Figure S1. Identification of the genomic island that carries the *ars1* operon of *P. putida* KT2440.



The criteria include the Codon Adaptation Index (CAI) and the GC content of the genomic segment under examination on the background of average values in the *P. putida* KT2440 genome. The picture shows the CAI (diamonds, yellow) and GC (squares, grey) of the chromosomal region containing the island (dashed box) where *ars1* operon is located. The CAI and GC values corresponding to the interrupted *tmk* gene are shown in light and dark blue, respectively. Red stripe, average distribution of GC content within this strain. White stripe, average variation of CAI.

1 **Supplementary Figure S2.** Sensitivity of Δars *E. coli* to arsenic salts.

2



3

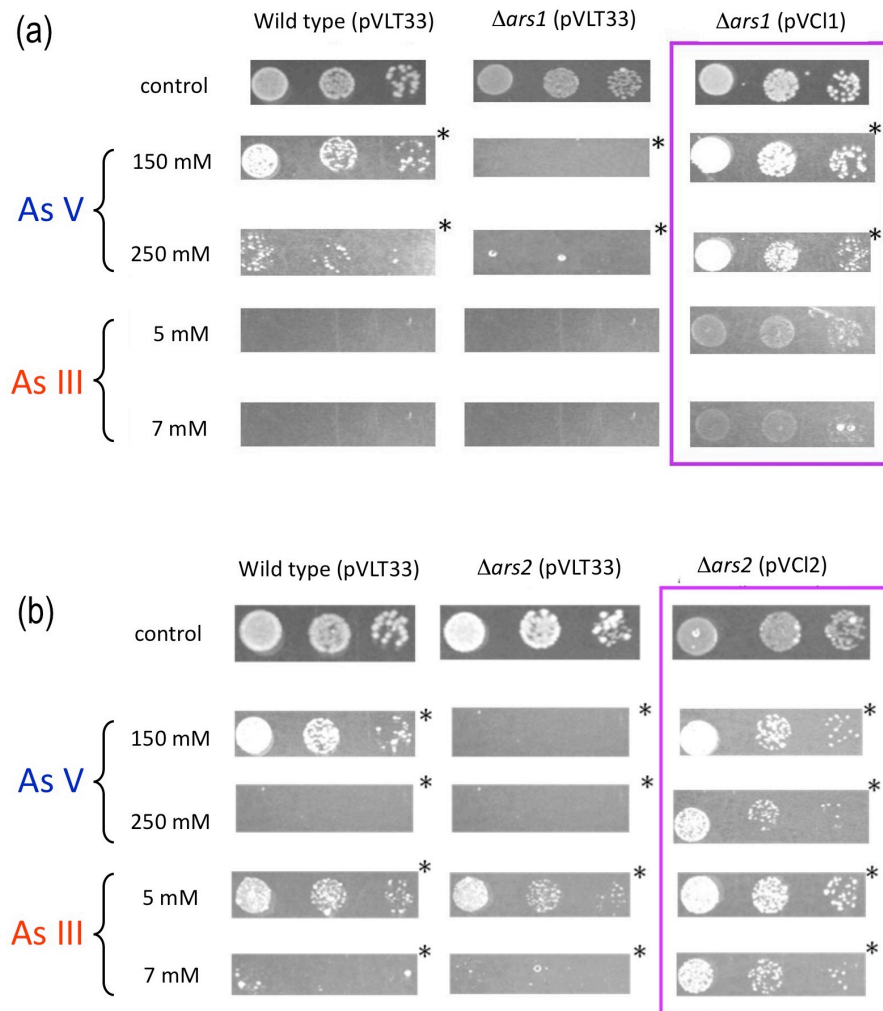
4

5 This is an additional control to the experiment shown in Fig. 2a of the main text. The tolerance of *E. coli*
6 AW3110 Δars , which lacks any function for tolerating As species was tested by plating 8 μ l serial dilutions
7 of cell suspensions (10^6 , 10^5 , 10^4 , 10^3 , and 10^2 cells/ml) for 22 h on LB agar with the concentrations of the
8 corresponding salts. Note complete lack of growth in the presence of any of the oxyanions.

9

10

1 **Supplementary Figure S3.** Plate complementation assays of Δars *P. putida* mutants with broad host
 2 range *ars*⁺ plasmids.
 3

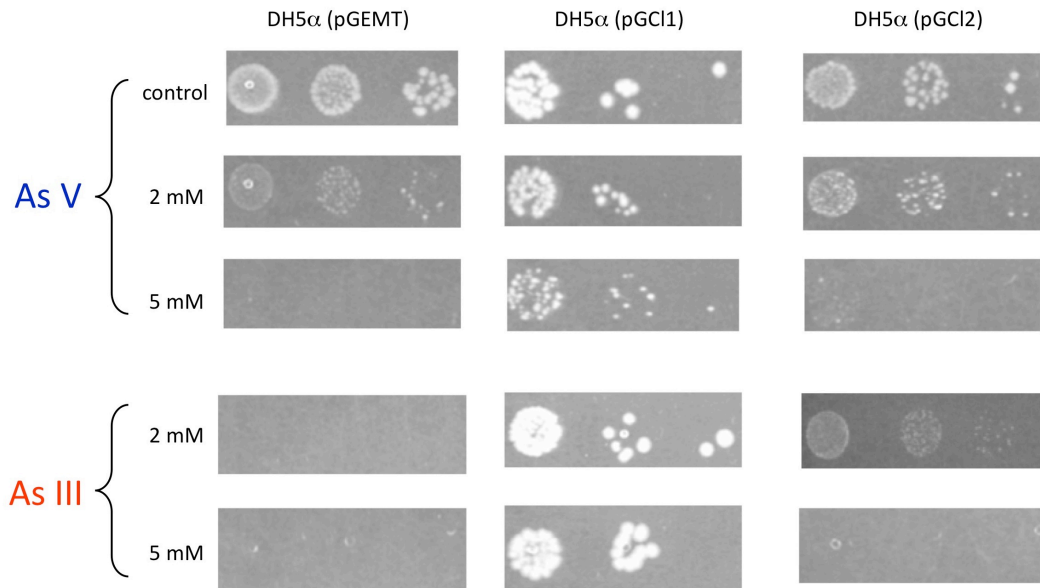


4
 5
 6
 7
 8
 9
 10
 11
 12
 13

(a) Complementation of $\Delta ars1$ deletion. Reference strain *P. putida* TEC1 and its $\Delta ars1$ derivative were transformed with either an empty vector (pVLT33) or with the *ars1*⁺ plasmid pVCI1 and 8 μ l serial dilutions of the transformants plated for 16 h or 38 h (indicated with an asterisk) on LB plates with the concentrations of arsenic species indicated in each case. The actually complemented strain is framed in magenta. (b) Complementation of $\Delta ars2$ deletion. Same but using the $\Delta ars2$ derivative of *P. putida* TEC1 as the recipient of the *ars2*⁺ plasmid pVCI2.

1 **Supplementary Figure S4.** Heterologous expression of *P. putida* ars clusters in *E. coli* DH5 α .

2



3

4

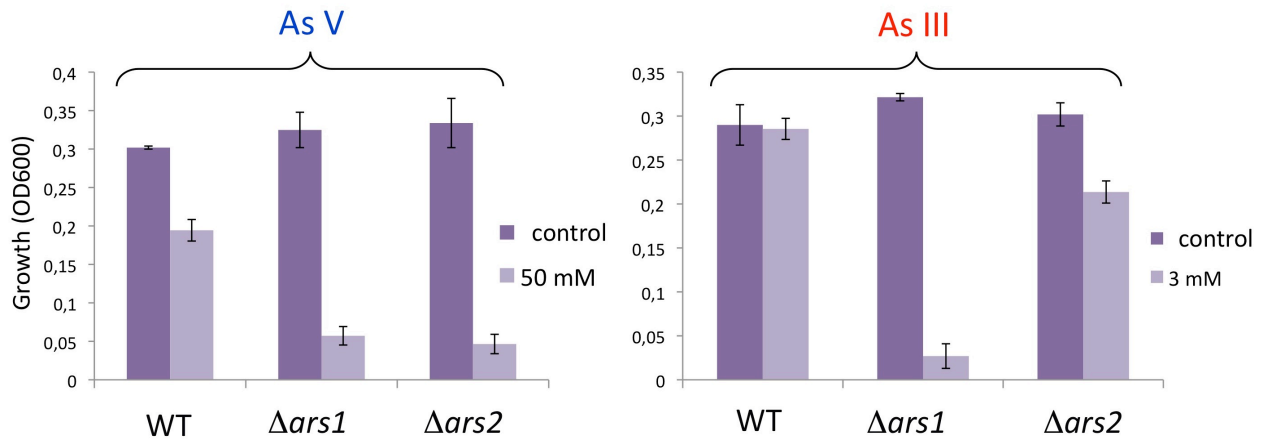
5 The *ars1*⁺ and *ars2*⁺ plasmids pGCI1 and pGCI2 (Table 1) were transformed, along with the empty vector
 6 pGEMT in *E. coli* DH5 α . Serial 8 μ l dilutions of the transformants were then plated on Petri dishes with
 7 LB, ampicillin and the concentrations of As^V and As^{III} indicated in each case. Pictures were taken after 48
 8 h at 30 °C.

9

10

1 **Supplementary Figure S5.** Arsenic resistance of *P. putida* TEC1 strain and its $\Delta ars1$ and $\Delta ars2$
2 derivatives under osmotic stress.

3



4

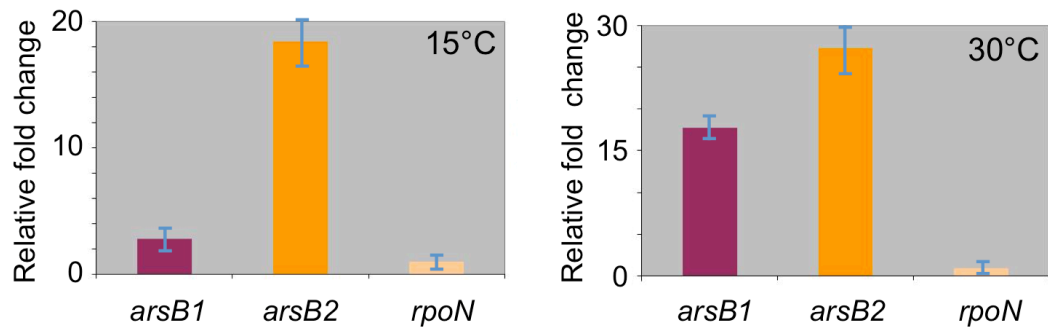
5

6 Each of the strains indicated were grown in biological and technical triplicates in LB medium amended
7 with uracil and added with 0.4 NaCl. The OD₆₀₀ of the cultures was recorded after 8 h of growth at 30°C
8 using a 96-microwell-plate reader (spectrophotometer Victor2 –Perkin Elmer) with orbital shaking. No
9 phenotypic effects were observed when compared to similar conditions without NaCl (compare with Fig.
10 4b and Fig. 4d of the main text)

11

1 **Supplementary Fig S6.** Relative expression of *P. putida* KT2440 *arsB* genes at different temperatures.

2



3

4

5 Transcription of each of the genes *arsB1* and *arsB2* at 15 °C or 30 °C was made with Q-PCR as
6 explained in the Methods section as proxies for expression of the cognate operons. The *rpoN* gene signal
7 is used as an endogenous non-changing control for the reaction. The data show the average values of
8 two technical and two biological duplicates of the same experiment.

9

10

11

1 **Supplementary Table S1.** Genes encoded in the 0.62 Mb genomic island inserted in the *tmk* gene of *P.*
 2 *putida*.

3

LOCUS	Gene	Predicted function
PP_1919	<i>tmk</i>	Thymidylate kinase, N-terminal interruption
PP_1920		Hypothetical protein
PP_1921		Hypothetical protein
PP_1922		Hypothetical protein
PP_1923		Hypothetical protein
PP_1924	<i>phoN1</i>	PPT-N-acetyltransferase, putative
PP_1925		Monooxygenase, putative
PP_1926		Phosphatase family protein, putative
PP_1927		Arsenical resistance protein ArsH, putative
PP_1928	<i>arsC1</i>	Arsenate reductase
PP_1929	<i>arsB1</i>	Arsenite efflux transporter
PP_1930	<i>arsR1</i>	Arsenic resistance transcriptional regulator
PP_1931		Conserved hypothetical protein
PP_1932		Hypothetical protein
PP_1933		Hypothetical protein
PP_1934		Hypothetical protein
PP_1935		Transcriptional regulator, Cro/C1 family
PP_1936		Hypothetical protein
PP_1937		Helicase, putative
PP_1938		Hypothetical protein
PP_1939		Formaldehyde dehydrogenase, truncation
PP_1940		Methyl-accepting chemotaxis transducer
PP_1941		Hypothetical protein
PP_1942		Transcriptional regulator, LysR family
PP_1943	<i>purU3</i>	Formyltetrahydrofolate deformylase
PP_1944		Aminomethyltransferase, putative
PP_1945	<i>folD1</i>	5,10-methylene-tetrahydrofolate dehydrogenase/cyclohydrolase
PP_1946		Oxidoreductase, short chain dehydrogenase/reductase family
PP_1947		Conserved hypothetical protein
PP_1948		Benzaldehyde dehydrogenase
PP_1949		Oxidoreductase, GMC family
PP_1950		Conserved hypothetical protein
PP_1951		Oxidoreductase, short chain dehydrogenase/reductase family
PP_1952		Metallo- β -lactamase family protein
PP_1953		Oxidoreductase, short chain dehydrogenase/reductase family
PP_1954		Conserved hypothetical protein
PP_1955		Cytochrome P450 family protein
PP_1956		Hypothetical protein
PP_1957		Oxidoreductase
PP_1958		Hypothetical protein
PP_1959		Hypothetical protein
PP_1960		Hypothetical protein
PP_1961		Hypothetical protein
PP_1962		Site-specific recombinase, phage integrase family

PP_1963		Hypothetical protein
PP_1964		Deoxynucleotide monophosphate kinase, putative
PP_1965	<i>tmk</i>	Thymidylate kinase, C-terminal interruption

1

2

3