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Pavlovian-Type Learning in Environmental Bacteria: Regulation of Herbicide Resistance by Arsenic in *Pseudomonas putida*

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ABSTRACT

The canonical *arsRBC* genes of the *ars1* operon in *Pseudomonas putida* KT2440, which confer tolerance to arsenate and arsenite, are followed by a series of additional ORFs culminating in *phoN1*. The phoN1 gene encodes an acetyltransferase that imparts resistance to the glutamine synthetase inhibitor herbicide phosphinothricin (PPT). The co-expression of *phoN1* and *ars* genes in response to environmental arsenic, along with the physiological effects, was analysed through transcriptomics of cells exposed to the oxyanion and phenotypic characterization of *P. putida* strains deficient in different components of the bifan motif governing arsenic resistance in this bacterium. Genetic separation of *arsRBC* and *phoN1* revealed that their associated phenotypes operate independently, indicating that their natural co-regulation is not functionally required for simultaneous response to the same signal. The data suggest a scenario of associative evolution, akin to Pavlovian conditioning, where two unrelated but frequently co-occurring signals result in one regulating the other's response – even if there is no functional link between the signal and the response. Such surrogate regulatory events may provide an efficient solution to complex regulatory challenges and serve as a genetic patch to address transient gaps in evolving regulatory networks.

1 | Introduction

The default view of how expression of bacterial operons is regulated by physicochemical and nutritional cues includes the action of transcriptional factors (TFs, either activators or repressors) that recognize specifically such signals and in turn either enable or curb transcription of often clustered genes whose products build the physiological response to the upstream inputs (Janga, Salgado, and Martínez-Antonio 2009; Dudek and Jahn 2021). This general scenario then splits in a large number of particular cases that diverge in significant ways from the rule. For instance, many TFs show a degree of side-promiscuity towards, for example, gratuitous inducers which make promoters to be turned on/off by a physiologically wrong signal (Abril et al. 1989; de Lorenzo and Pérez-Martín 1996). In other cases, the evolutionary history of the system makes regulation of a new operon to keep the TF of the earlier precursor and thus maintaining a faulty signal-response profile (de Las Heras, Chavarría, and de Lorenzo 2011). Finally, some genes are expressed upon signals which proxy the environment where the responses are to unfold but do not produce a direct response to the trigger input. This last scenario is most typical of virulence genes, which often recruit iron-starvation promoters controlled by the Fur repressor for effective expression in characteristically Fe-deprived

target niches, for example, animal tissues (Mekalanos 1992; Porcheron and Dozois 2015).

The growing availability of bacterial genomes and transcriptomes has revealed a wealth of naturally occurring regulatory architectures whose evolutionary logic is sometimes difficult to grasp (Stormo and Tan 2002). One conspicuous example is the regulation of the two co-existing As-resistance operons found in the genome of the Gram-negative soil bacterium and plant colonizer *Pseudomonas putida* KT2440 (Figure 1). Ars operons, which are widespread through the bacterial realm (Páez-Espino et al. 2009), enable tolerance to arsenate (AsV) through reduction to arsenite (AsIII) by a cytoplasmic arsenate reductase ArsC, followed by extrusion of the thereby reduced oxyanion by a cognate efflux pump (see Kruger et al. 2013, for a review). The first oddity of As resistance in *P. putida* is the redundance of the *ars* operons: both gene clusters separately deliver high levels of As tolerance, their duplication resulting in only a minor advantage in terms of enduring exposure to the oxyanion at different temperatures (Fernández et al. 2014; Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015). The second is the cross-regulation between their promoters *Pars1* and *Pars2* by their corresponding regulators ArsR1 and ArsR2 (Fernández et al. 2016), which originate an intriguing bifan motif (Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021) of uncertain functionality (Figure 1). Finally, each of the core *arsRDC* resistance genes of the two clusters is followed by and co-transcribed with additional ORFs that may or may not have any relationship with arsenic (Páez-Espino et al. 2009; Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015).

One of such genes is *phoN1* which is located 4 ORFs downstream of the last *arsC* in the canonical operon (Páez-Espino, Chavarría, and de Lorenzo 2015), but forming part of the same transcriptional unit (Fernández et al. 2016). *phoN1* encodes an acetylase which inactivates the commercial herbicide Glufosinate [phosphinothricin (PPT)], a structural analogue of glutamate that, similarly to antibiotic arsinothricin (Kuramata et al. 2016; Nadar et al. 2019), inhibits glutamine synthetase (Hoerlein 1994). PTT is naturally produced by several species of *Streptomyces* (Schwartz et al. 2004), but it started to be massively produced by chemical synthesis for selection of genetically modified crops (McElroy and Brettell 1994; Duke and Cerdeira 2010) implanted with bacterial *bar* or *pat* genes which endow resistance to the herbicide (Herouet et al. 2005). The location of *phoN1* downstream of an arsenic-response promoter and its plausible induction by the oxyanion is intriguing, as the only known effector of ArsR1 (and ArsR2) is arsenite (Fernández et al. 2014, 2016; Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015; Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021). Under this arrangement, one signal triggers responses to two separate and quite distinct environmental challenges, one directly related to the inducing signal (arsenic) and the other not (PPT). Note also that the regulatory cross-talk between the two As-resistance operons of *P. putida* makes mutual control of their respective promoters to shape a distinct bifan motif which is believed to provide a better temporal regulation of signal

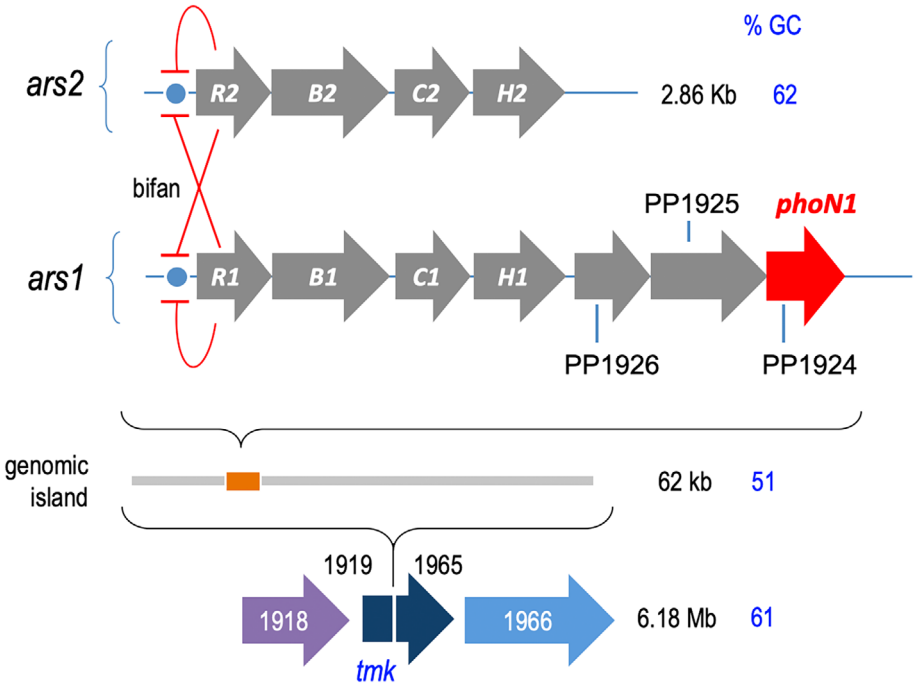

FIGURE 1 | Organization of the two arsenic resistance operons of *P. putida* KT2440 in respect to *phoN1* (PPT^R) and regulatory interplay between them. The upper part of the figure shows the arrangement of the 2.86 kbp chromosomal segment of *P. putida* KT2440 with the As-resistance *ars2* operon with canonical genes *R* (repressor), *B* (transporter), *C* (reductase) and *H* (NADPH-dependent flavin mononucleotide reductase). The GC contents accredit its complete assimilation to the rest of the genomic DNA of this bacterium. The arrangement of the ecoparalogous *ars2* cluster (Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015) is shown below aligned with *ars1*. The high amino acid sequence similarity through the *arsRBCH* genes (Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015) stops after the *H* cistron. In the case of *ars1*, H is followed by 3 co-transcribed genes, the last of which (*phoN1*) encodes an acetylase that endows resistance to the herbicide phosphinothricin (Páez-Espino, Chavarría, and de Lorenzo 2015). The *ars1* operon is included in a 62 kbp genomic island inserted in the *tmk* gene with a clearly different origin (GC content 51%). The distinct bifan motif that rules the regulatory interplay of *ars1* and *ars2* is sketched between the two schemes.

propagation and filtering noisy signal inputs (Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021). Still, the agency of this specific regulatory arrangement, in particular the predicted surrogate regulation of *phoN1* expression by environmental arsenic, remains puzzling.

In this work, we have genetically and physiologically dissected the interplay between arsenic tolerance and herbicide resistance in *P. putida* KT2440 and investigated how the two traits ended up being manifested simultaneously in response to only one of the two signals. For this, we document below the transcriptional association between their cognate genes and its consequences in vivo when having them expressed together or by separate under a heterologous expression system. The results hint at a case of heritable evolutionary memory (Casadesús and D'Ari 2002; Mitchell et al. 2009) embodied in the *ars* regulatory device. The resulting genetic patch is reminiscent of a Pavlovian-like learning process (Pearce and Hall 1980; Tagkopoulos, Liu, and Tavazoie 2008; Zhang et al. 2014) in which two simultaneous signals can end up eliciting a response to both of them even if one of the initial stimuli is later missing. The consequences of such patches in the evolution of regulatory networks are discussed.

2 | Results and Discussion

2.1 | Regulatory Architecture of the Ars Operons Clusters in *P. putida* KT2440

The high tolerance of *P. putida* KT2440 to arsenic oxyanions can be traced to the combined action of the products of two separate *ars* operons encoded in separate locations of the genome of this bacterium (Figure 1). The gene cluster named *ars2* consists of a streamlined canonical arsenic resistance module that includes the genes encoded by an arsenite transporter (*arsB2*) and an arsenate reductase (*arsC2*). These are preceded of *arsR2*, which encodes the As (III)-responsive repressor ArsR2. No other ORFs directly connected to arsenic can be spotted upstream or downstream of such a basic *arsRBC* cluster. Inspection of the GC contents of the region and tetranucleotide distribution (Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015) suggest that such a DNA segment has been assimilated to the core genetic complement of this bacterium for a long time (Figure 1). The genomic context and evolutionary roadmap of the other arsenic resistance gene cluster (*ars1*) looks however very different. Not only it maps in a location quire distant from *ars2* but also its sequence signatures indicate that it was acquired more recently. The *ars1* cluster forms part of a large (62 kb) genomic island inserted in the midst of the *tmk* gene, an occurrence absent in other *P. putida* isolates (Belda et al. 2016; Wirth et al. 2023). Furthermore, inspection of the genomic region nearby the core *arsR1B1C1* cluster reveals the presence of a number of other ORFs which could be co-expressed with the *ars* genes and thus suspect of being functionally related. Some of these have been annotated and their role predicted and experimentally verified – for example, *arsH* (Chen, Bhattacharjee, and Rosen 2015; Páez-Espino et al. 2020). The most intriguing of them is the one called *phoN1* also called *arsN1* (Nadar et al. 2019) which encodes an acetyltransferase able to inactivate the glutamine synthetase inhibitor PTT, commercially known as glufosinate. This is a synthetic, simplified derivative of the antibiotic bialaphos, which is naturally produced by some *Streptomyces* strains. PPT has been widely used as herbicide for the selection of transgenic plants engineered to express the cognate resistance gene *bar*. Note also that PPT has structural similarity to yet another unrelated antibiotic, arsinothricin, which is produced also by some rare *Burkholderia* isolates (Kuramata et al. 2016; Nadar et al. 2019). The question thus arises on how and why resistance to a modern herbicide appears together with an otherwise classical operon for arsenic resistance. Furthermore, the fact that both ArsR variants can regulate each other's promoters (Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021) could enter a physiological connection between two-tiered arsenic tolerance and herbicide resistance.

To clarify the regulatory scenario above, we started by inspecting in detail the expression of the *ars* gene clusters of wild-type *P. putida* in the presence or absence of their canonical inducer, arsenite. To this end, we blew up the regions of interest out of the Ars (III)-responsive transcriptome of *P. putida* (Figure A1). The data clearly show that the oxyanion induced *arsR2B2C2H2* transcription as an apparent stand-alone single multicistronic operon, with no significant effects on flanking or nearby genes (Figure A2a). In contrast, as shown in detail in Figure 2, a number of genes located downstream of the *ars1* core were also co-induced in response to As (III) in what looked like a single transcript encompassing *arsR1B1C1H1-PP1926-PP1925-phoN*. The predicted functions of the genes preceding *phoN* include a phosphatase and a mono-oxygenase, respectively (Yoshinaga, Cai, and Rosen 2011), but their actual encoded function in As resistance (or other tasks) remains uncertain. In any case, the data indicated that the mRNA that starts at the *Pars1* promoter reaches out and finishes at the end of the *phoN1* cistron (Figure 2a).

To test whether the transcriptional regulatory scenario shown in Figure 1 had its corresponding physiological counterpart, we also inspected resistance of *P. putida* to PPT in the presence or absence of arsenite. To this end, we grew cultures of the reference strain *P. putida* (wild-type, Table 1) in minimal M9 + citrate medium (supplemented with uracil) and added with combinations of the oxyanion and the herbicide ranging 0–10 mM As (III) and 0–10 mM PPT. As shown in Figure 3, bacteria hardly grew in the presence of the herbicide unless the culture was added with subinhibitory concentrations of As (III), maximum growth happening at 2–3 mM of the inducer. Note that higher levels of arsenite are toxic in any case and cells stop growing by As (III) concentrations of 6–7 mM. In contrast, an isogenic strain with a complete deletion of the *ars1* operon was sensitive to both PTT and concentrations of As (III) above 2 mM. The residual resistance to the oxyanion could be trailed to the action of the second As-resistance operon *ars2*, since deletion of both clusters made *P. putida* altogether very sensitive to arsenite concentrations above 0.2 mM (Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015).

2.2 | PPT Resistance and As Tolerance Are Biologically Independent Traits

To clarify the functional dependence (or lack of it thereof) between herbicide resistance and tolerance to As oxyanions, the genomic region encompassing the *phoN1* gene of *P. putida* wild type was deleted seamlessly from its site in the *ars1* operon, thereby generating strain *P. putida* Δ*phoN1* (Table 1 and

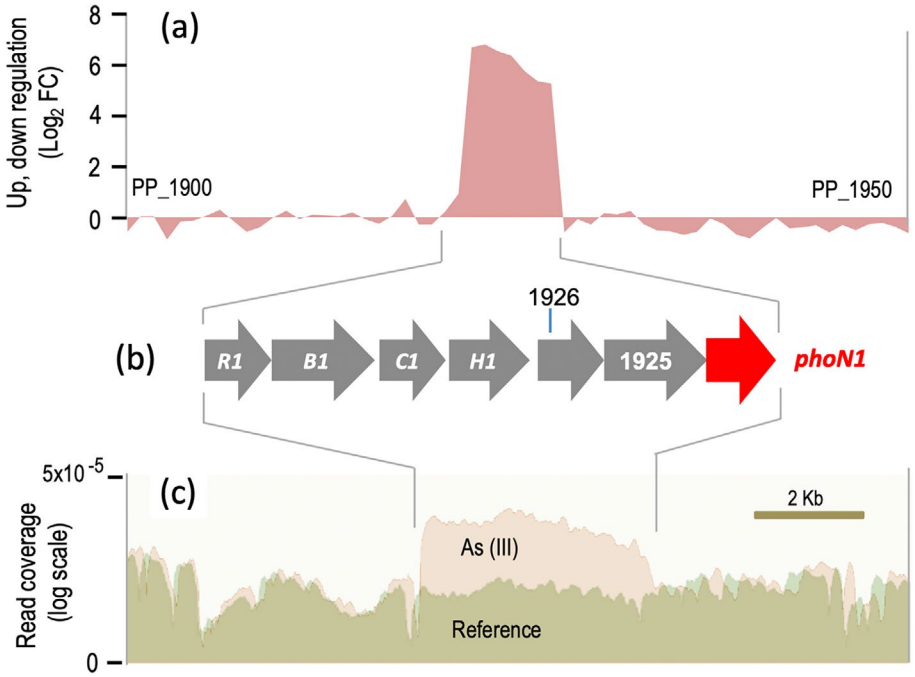

FIGURE 2 | Induction of *phoN1* by As (III) along with the rest of the ars1 operon of *P. putida* as revealed by transcriptomic analysis of genomic region PP_1900-PP_1950. (a) Sequence coverage plots from transcriptome profile of area between PP_1900 and PP_1950 of *P. putida* KT2440 genome, that encompasses the *ars1* operon. The plot shows relative gene expression intensity of *ars1* genes in *P. putida* KT2440 grown in LB and supplemented with 1 mM arsenite, using cells grown in the absence of the oxyanion as reference conditions. (b) Genomic organization of the ars1 operon in *P. putida* KT2440 constituted by *arsR1* (*R1*), *arsB1* (*B1*), *arsC1* (*C1*), *arsH1* (*H1*), two genes of unknown function (1925 and 1926) and *phoN1* (highlighted in red). (c) RNA sequencing-based transcriptome profile of *ars1* genes in *P. putida* KT2440. Total number of cDNA reads is annotated responding to the *ars* genes in the reference (pale green) or arsenite-exposed cells (pale orange). Geneious software was used for visualization.

Páez-Espino, Chavarría, and de Lorenzo 2015). As shown in Figure 4, using as a reference the concentrations of PPT and As (III) where clearer effects were seen in the experiments of Figure 3, it became clear that *phoN1* was responsible for resistance to the herbicide and that its expression to an effective level was elicited by As (III) by means of readthrough transcription from the upstream promoter *Pars1*. These results not only verified the known function of *phoN1* as a PPT resistance gene but also that its efficacy in vivo was dependent on arsenic and that this could be traced to its co-transcription under the same signals that elicited tolerance to arsenic. The ensuing question was whether the two resistances were physiologically linked, that is, phenotypic manifestation of PPT resistance inherently depends on the presence of arsenic and/or co-occurrence of *ars* products. To examine this, we used plasmid pVPTT1, in which transcription of the *phoN* gene was engineered under the control of an IPTG-inducible promoter (Table 1). pVPPT1 was then placed in *P. putida* Δ*phoN1* and the resulting strain grown in the presence of different As and PPT concentrations. As evidenced in the heat maps shown in Figure 5, the two phenotypes could be manifested separately by just splitting their regulatory devices. Furthermore, overexpression of PPT resistance upon addition of IPTG to the Δ*phoN1* (pVPTT1) strain regardless of As (III) led to cell overgrowth (Figure 5c), thereby ruling out participation of either arsenic itself or any of the other gene products of the *ars1* operon otherwise induced by the oxyanion.

The scenario that emerges from these data is that expression of the herbicide resistance has evolutionarily become subject to a regulatory mechanism that responds to a signal alien to the function of the *phoN* gene proper, that is, arsenic induces not only resistance to the oxyanion but also to an herbicide whose mechanism of action is completely different and thus lack any cross-protection or functional synergy. To shed some light on this apparent paradox, we inspected the in vivo consequences of such unusual regulatory setup.

2.3 | Arsenic Resistance and Herbicide Tolerance Are Physiologically Intermingled in *P. putida*

That As tolerance can be genetically and phenotypically separated from PPT resistance says little of the logic behind the regulatory takeover of *phoN1* expression by an As-dependent regulatory device. In this sense, it is worth noting that *Pars1* activity (which reaches out *phoN1*) is controlled not only by its cognate repressor ArsR1 but also cross-regulated by ArsR2, the equivalent regulator of the paralogous operon *ars2* (Figure 1). To sort out the role and plausible interplay of the two regulatory systems with the manifestation of the herbicide resistance phenotype, we built *P. putida* strains seamlessly deleting *arsR1* and *arsR2*—either together or separated—but keeping intact the *Pars1* and *Pars2* promoter sequences and their ArsR operators.

The resulting strains were then subject to separate sensitivity tests to either PPT or arsenite. The results of these assays are shown in Figure 6. Expectedly, in the absence of As (III), wild-type cells and those which had lost only one of the ArsR variants were sensitive to PPT (Figure 6a). However, the double mutant Δ*arsR1* Δ*arsR2*

TABLE 1 | Strains and plasmids.

	Relevant characteristics	Reference
Strains		
P. putida KT2440	Prototrophic, TOL plasmid-cured *P. putida* mt-2	(Belda et al. 2016)
P. putida TEC1	Rif^R, *P. putida* KT2440 with an internal deletion of *pyrF*	(Galvão and de Lorenzo 2005)
P. putida Δ*ars1*	TEC1 with a seamless deletion of the whole *ars1* gene cluster	(Páez-Espino, Chavarría, and de Lorenzo 2015)
P. putida Δ*phoN1*	TEC1 with a seamless Δ*phoN1* deletion	(Páez-Espino, Chavarría, and de Lorenzo 2015)
P. putida Δ*arsR1*	TEC1 with a seamless Δ*arsR1* deletion	This work
P. putida Δ*arsR2*	TEC1 with a seamless Δ*arsR2* deletion	This work
E. coli CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1* λ*pir* lysogen	Laboratory collection
E. coli HB101	Sm^r; *rpsL recA thi pro leu hsdR*$^-$*M*$^+$ (*E. coli* K-12/*E. coli* B. hybrid)	Laboratory collection
E. coli DH5α	F$^-$ F80d [*lacZ*ΔM15], Δ(*lacZYA-argF*), ΔU169, *recA1, endA1, hsdR17, R*$^-$*M*$^+$, *supE44, thil, gyrA, relA*	Laboratory collection
E. coli M15	K12, ΔM15 *lacZ* deletion for α complementation	Laboratory collection
Plasmids		
pTEC	Km^R, FOA^S, Ura^+, MCS-Km-MCS, *ori*R6K/ origin of transfer *mob*RK2 and *pyrF*$^+$	(Galvão and de Lorenzo 2005)
pTU•DR1	pTEC inserted with a *Not*I-*Sac*I 1.23 kb DNA segment composed of a 0.69 kb fragment upstream of the *arsR1* gene and 0.54 kb downstream, composed upon assembly of PCR products resulting from amplification of genomic DNA with oligonucleotide primers FWDR1Up/ RVSR1Up and FWDR1Down/RVSR1Down (Table A1).	This work
pTU•DR2	pTEC inserted with a *Not*I-*Sac*I 1.15 kb DNA segment composed of a 0.54 kb fragment upstream of the *arsR1* gene and 0.61 kb downstream, composed upon assembly of PCR products resulting from amplification of genomic DNA with oligonucleotide primers FWDR2Up/ RVSR2Up and FWDR2Down/RVSR2Down (Table A1).	This work
pVLT33	Km^R, RSF1010-*lacI*^{q}/P_{tac} hybrid broad-host-range expression vector, MCS of pUC18	(de Lorenzo et al. 1993)
pVPPT1	pVLT33 inserted with a 0.55-kb *Eco*RI-*Hin*dIII PCR fragment containing the *phoN1* gene of *P. putida*	(Páez-Espino, Chavarría, and de Lorenzo 2015)
pRK600	Cm^R, *ori*ColE1, *mob*RK2, *tra*RK2	(Kessler, de Lorenzo, and Timmis 1992)
pQE32	Ap^R, Cm^R His_6-tagging, T_5 *lac*-driven expression vector.	(Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021)
pQR32-ArsR1	pQE32 inserted with structural *arsR1* gene	(Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021)
pREP4	Km^R, oriV p15A, *lacI*^{q}, partner of pQE32 for IPTG-inducible expression	(Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021)
pSEVA225T	Km^R, low copy number vector for *'lacZ* translational fusions	(Martínez-García et al. 2023)

(Continues)

TABLE 1 | (Continued)

	Relevant characteristics	Reference
pSEVA225T•R1	pSEVA225T inserted with a 297 bp *Eco*RI-*Bam*HI fragment spanning −150 pb upstream of the transcription initiation site of P_{ars1} and 78 bp (26 leading amino acids) of the *arsR1* structural gene, amplified with PCR primers 5Pars1Eco and 3Pars1Bam RVSR2Down (Table A1).	This work

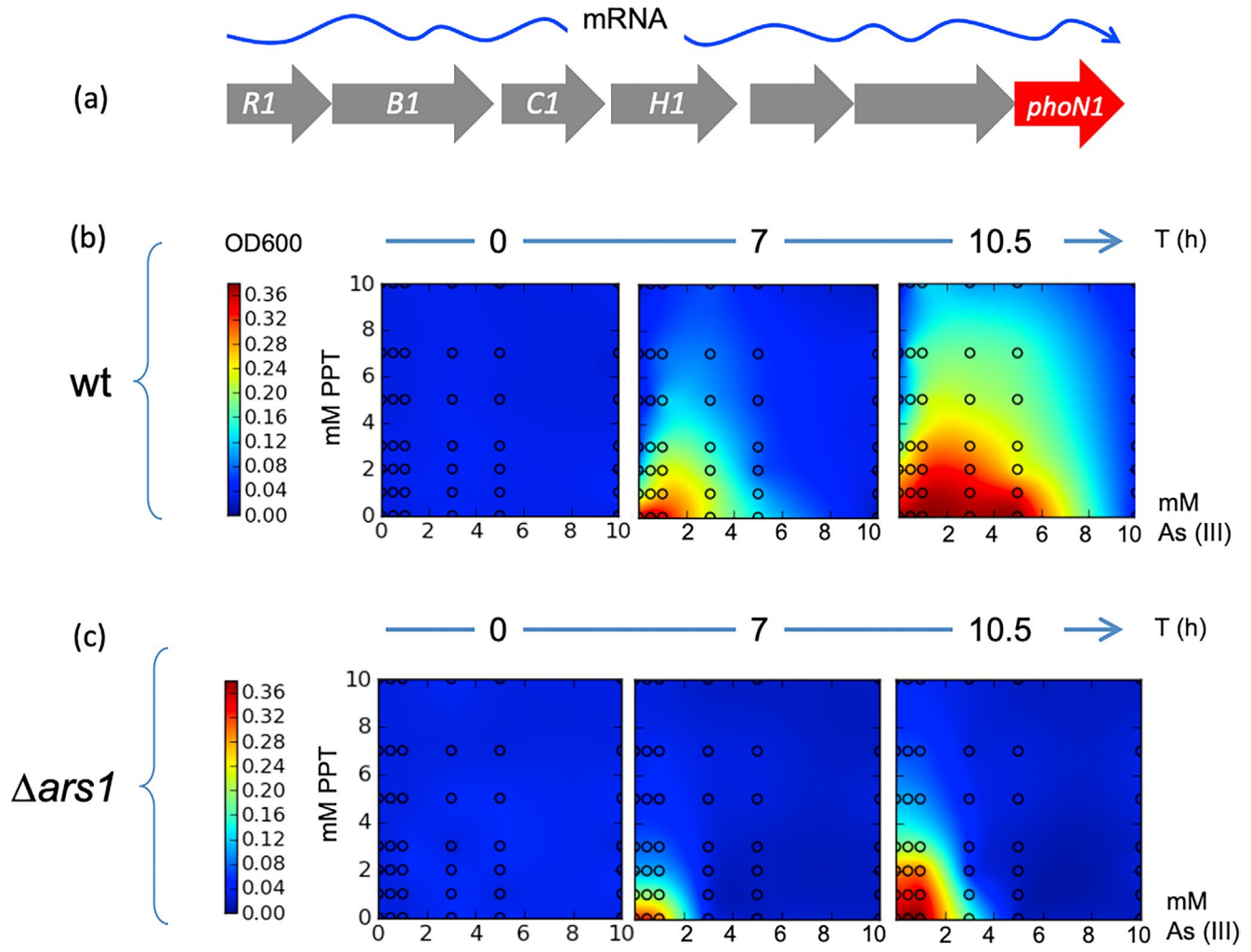

FIGURE 3 | The PPT^R phenotype of *P. putida* relies on induction of the *ars1* operon by As (III). (a) Sketch of the polycistronic mRNA starting in *arsR1* upon exposure of the cells to As (III) and reaching out the end of the transcript with the *phoN1* sequence. (b) Wild-type *P. putida* cells were inoculated in M9-citrate medium (supplemented with uracil, see Experimental Procedures) in microtitre plates and cultures at 30°C for the times indicated in the presence of the PPT and As (III) concentrations shown in each case. Growth was recorded as optical density (OD) at 600 nm and the values were interpolated using the function *stat_density2d* to generate density heat maps from the R package *ggplot2*. (c) Same analyses using a complete deletion of the *ars1* operon (Δ*ars1* strain).

was tolerant to the herbicide, plausibly due to the unrestrained expression of the whole *ars1* operon in the absence of any repressor (Figure 6a). However, the tests for assessing the resistance to As (III) of the same strains revealed that the loss of *arsR1* caused a considerable sensitivity to the oxyanion, which was only reverted in the double mutant Δ*arsR1* Δ*arsR2*. This was counterintuitive, as one would expect the loss of just one of the repressors to maintain the resistance level of the wild-type strain. Instead, the experiment of Figure 6b hinted at a separate role of the ArsR1 protein in As resistance different from its mere action as a transcriptional repressor. But what could that be?

2.4 | ArsR1 Contributes to As Tolerance Independently of arsBCH Genes

It has been reported before that overexpression of ArsR of various origins in *E. coli* allows intracellular accumulation of As

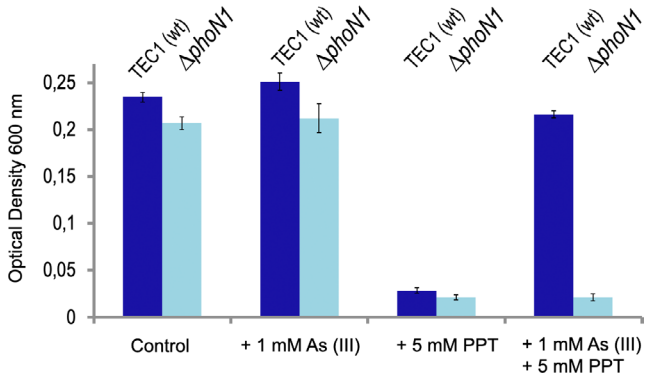

FIGURE 4 | Resistance of wild-type *P. putida* TEC1 and its Δ*phoN1* derivative to PPT in the presence or absence of As (III). The growth of each strain was followed in 96-well plates with M9-citrate (with uracil) and added with As (III) and PPT at the concentrations indicated in each case. The experiments were carried out with biological triplicates and technical duplicates at 30°C. OD_{600} measurements after 12 h of incubation are shown. Note that manifestation of the PPT^R phenotype requires *phoN1* and As (III).

and thus increased tolerance to the oxyanion (Kostal et al. 2004; Maleki and Shahpiri 2022). Since the fully-derepressed *Pars1* promoter is strong (Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021) and the ribosomal-binding site (RBS) sequence of the *arsR1* gene is also good (Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015), chances are that ArsR1 is produced at high levels in cells exposed to the oxyanion. To verify this, we built a translational fusion between the leading 26 triplets of *arsR1* and *lacZ*, including *Pars1* and preceded by −150 nt upstream of the transcription start site. The resulting plasmid (pSEVA225T•R1, Table 1) was then placed in *P. putida* cells. These were grown in LB with or without added 0–20 mM As and β-galactosidase measured after 5 h. (Figure A3), we hypothesized that the abundance of the regulator could provide an extra layer of protection due to its inherent ability to bind arsenite and thus act as trap for otherwise loose intracellular ions (Kostal et al. 2004; Maleki and Shahpiri 2022). To test this possibility as an inherent, host-independent biological activity, *arsR1* was cloned in vector pQE32, originating pQE32-ArsR1 (Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021), where a $LacI^q$-controlled *T5 lac* promoter drives expression of a His_6-tagged ArsR1 protein. Plasmids were then separately transformed in *E. coli* M15 strain, bearing compatible $LacI^{q+}$ plasmid pREP4 (Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021). Transformants were grown in LB medium with antibiotics for securing plasmid retention, induced with IPTG and then with various concentrations of As (III). As shown in Figure 6c, at low mM As (III), cells expectedly loaded with ArsR1 were more tolerant to the chemical stressor than the control without the expression device. ArsR1 thus endowed *E. coli* with a superior endurance to arsenic which was independent of its role as a transcriptional regulator. Therefore, it appears that ArsR1 can functionally behave as a sort of As metallothionein-like protein likely by binding the arsenite in the cytoplasm of the cell and improving tolerance to the oxyanion. Taken together, these results provide a rationale to the observation that the *arsR1* deletion of *P. putida* is more sensitive to arsenic than the wild-type strain while still exhibiting resistance to PPT. That the double mutant Δ*arsR1* Δ*arsR2* shows resistance to the oxyanion (Figure 6b) could be due to the unrestrained expression of the second operon *ars2* in cells lacking any regulation. One way or the other, the core *arsRBCH* modules of each cluster seems to have evolved for synergically merging the potential of each of the activities encoded, whereas *phoN1* appears as an add-on which benefits from the upstream transcriptional flow but without any apparent functional connection to the *ars* genes. But how could this come about?

2.5 | Surrogate Regulation: A Case of Pavlovian-Reminiscent Evolutionary Process

The genome of *P. putida* contains two recognizable operators for binding ArsR repressors (Figure A4), which correspond to gene clusters *ars1* and *ars2* (Figure 1). Also, the chromosome encodes two distinct genes for dealing each with two known and widely used herbicides. These are PPT (resistance encoded by *phoN1*) and methionine sulfoximine (MetSox), another glutamine synthetase inhibitor resistance encoded by *phoN2* (Páez-Espino, Chavarría, and de Lorenzo 2015). The evolutionary phylogeny of each of them is likely to stem from acetylases active on structurally related natural compounds produced by soil bacteria. Specifically, PPT is a structural analogue of yet another As-containing antibiotic (arsinothricin) produced by a rice rhizosphere-associated *Burkholderia* strain (Kuramata et al. 2016; Nadar et al. 2019), which may have acted as a primary driver of evolutionary *phoN1* emergence. Yet, while *phoN2* is expressed constitutively (Páez-Espino, Chavarría, and de Lorenzo 2015), *phoN1* is transcribed only upon exposure of cells to arsenic salts. It is also intriguing that *phoN1*-type PPT acetyl transferases may or may not appear in bacterial genomes associated with As-related genes (VanDrisse, Hentchel, and Escalante-Semerena 2016), thereby suggesting autonomous evolutionary trajectories. On these bases, we entertain that the connection between As resistance and PPT resistance observed in *P. putida* may stem from an earlier evolutionary contingency. The scenario could be one in which soil *Pseudomonads* bearing the *arsRBCH* cluster were simultaneously exposed to inorganic arsenic salts and either PPT (Schwartz et al. 2004) or arsinothricin (Kuramata et al. 2016; Nadar et al. 2019) in sites inhabited by *Streptomyces* and *Burkholderia* strains naturally producing one or both herbicides. Such a niche-specific pressure may have caused that the signal for triggering transcription of *ars* genes ends up being co-opted for *phoN1* expression. Once this association emerged, it is conceivable that it later propagated and further evolved upon intensive exposure to synthetic counterparts of the herbicides. This is not a mere speculation, as inorganic arsenicals have not only been used in agriculture as pesticides or defoliants for many years (Bencko and Yan Li Foong, 2017) but also contaminated soils as a result of fallout in mining operations (Walsh, Sumner, and Keeney 1977). It is plausible that such soils were later treated with chemically synthesized PPT, thereby creating an extra pressure for stabilization of the extant regulatory architecture that we see in *P. putida*, which could be later propagated through its incorporation to a mobile DNA segment (Figure 1).

The scenario for the development of the hereby described *surrogate regulation* phenomenon is reminiscent of an associative learning process in which a simultaneous response to two

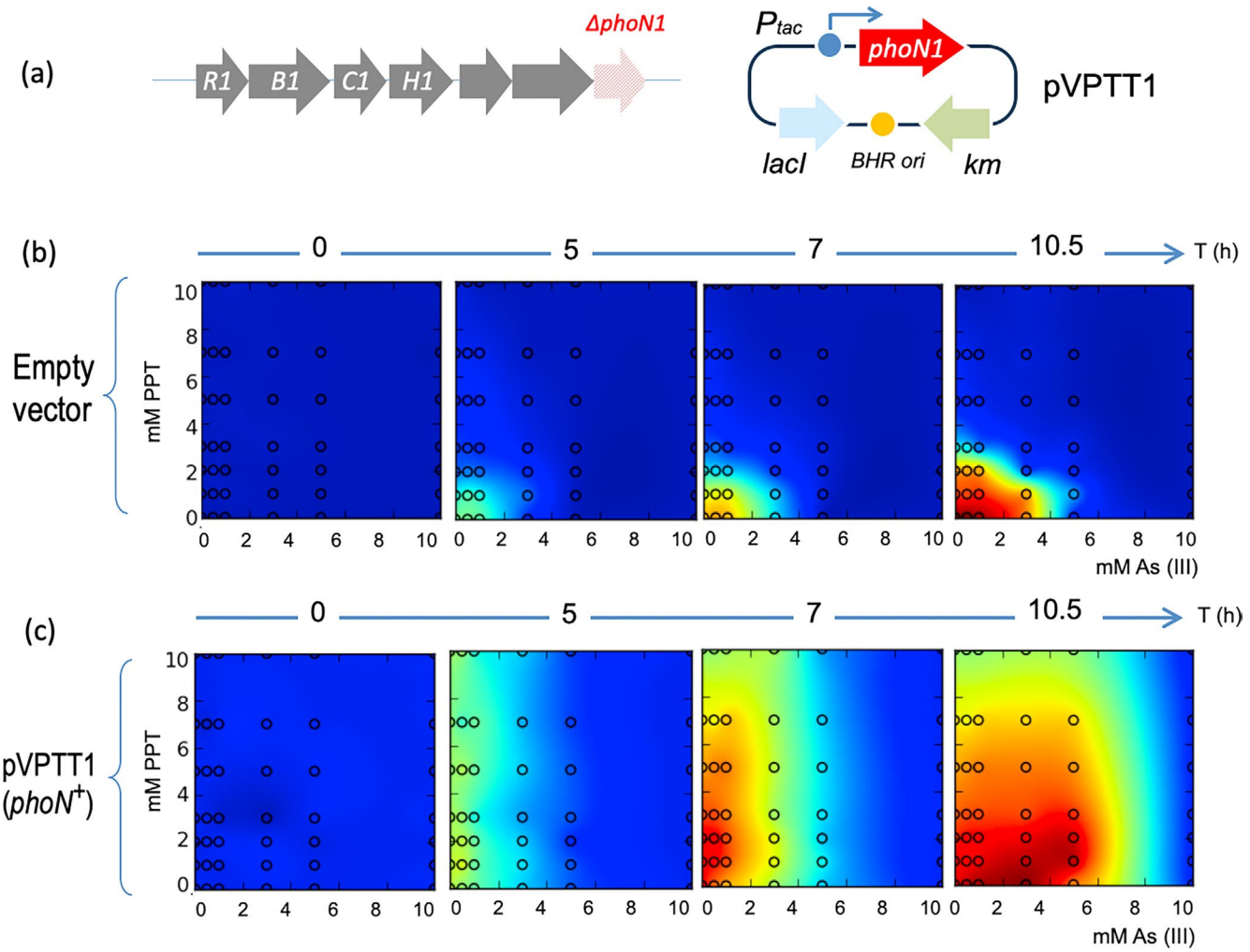

FIGURE 5 | PPT resistance and As (III) tolerance in strains with *arsR1B1C1H1* and *phoN1* expressed separately in *trans*. (a) Relevant genetic constructs in test strains. *P. putida* Δ*phoN1* is altogether identical to wild-type *P. putida* TEC1 except for the seamless deletion of the *phoN1* gene at the end of the *ars1* operon. This strain was endowed with either an empty IPTG-inducible expression vector pVLT33 (Table 1) or the same vector containing the inserted structural *phoN1* gene (plasmid pVPTT1 sketched to the right). (b) Growth of *P. putida* Δ*phoN1* (pVLT33) under various PPT and As (III) concentrations. The experiment was run as indicated in the legend to Figure 3, although the medium was added with 1 mM IPTG. (c) Same with strain *P. putida* Δ*phoN1* (pVTPP1). OD_{600} scale is the same as in Figure 3.

unrelated challenges are triggered by just one of the stimuli – if that happen to co-occur with the other. Following Pavlov's terminology, arsenic would be both an unconditional stimulus (because the logic reaction to it would be transcription of the As resistance genes) as well as a conditional stimulus for expression of PPT resistance, because response to herbicide is conditional upon the association between the herbicide and the As salt. Under this frame (Figure 7c,d), conditioning herbicide resistance to arsenic is entertained to result of a contingent concurrence in time and space between the As salts and PPT, thereby resulting in an evolutionary case of positive conditioning. Figure 7 summarizes this scenario, which by no means may be exclusive of the particular case at stake. It is plausible that new genes systematically leverage heterologous transcriptional signals for expression and can benefit from co-opting other triggering signals that act as proxies for the genuine target of the corresponding activity. If such co-occurrence persists, the regulatory device may be fixed as a surrogate control. In contrast, if they diverge, each function might evolve different regulatory architectures. Transcriptional networks, for example, those involved in virulence (Mekalanos 1992; Porcheron and Dozois 2015), reveal abundant control schemes in which responses to an environmental cue trigger expression of a very specific virulence factor. Surrogate regulation of the sort hereby described is thus one more evolutionary mechanism for solving multi-objective optimization challenges.

3 | Experimental Procedures

3.1 | Materials, Culture Conditions and General Procedures

The bacterial strains and the plasmids used in this work are listed in Table 1. *Pseudomonas putida* was grown at 30°C with vigorous shaking (170 rpm) in rich LB or M9 minimal medium (Sambrook and Rusell 2001) with 2 mM $MgSO_4$ and 0.2% (w/v) citrate as the sole carbon source. *E. coli* was grown in LB medium at 37°C. When required, antibiotics were added to the medium: kanamycin (Km, 50 μg/mL),

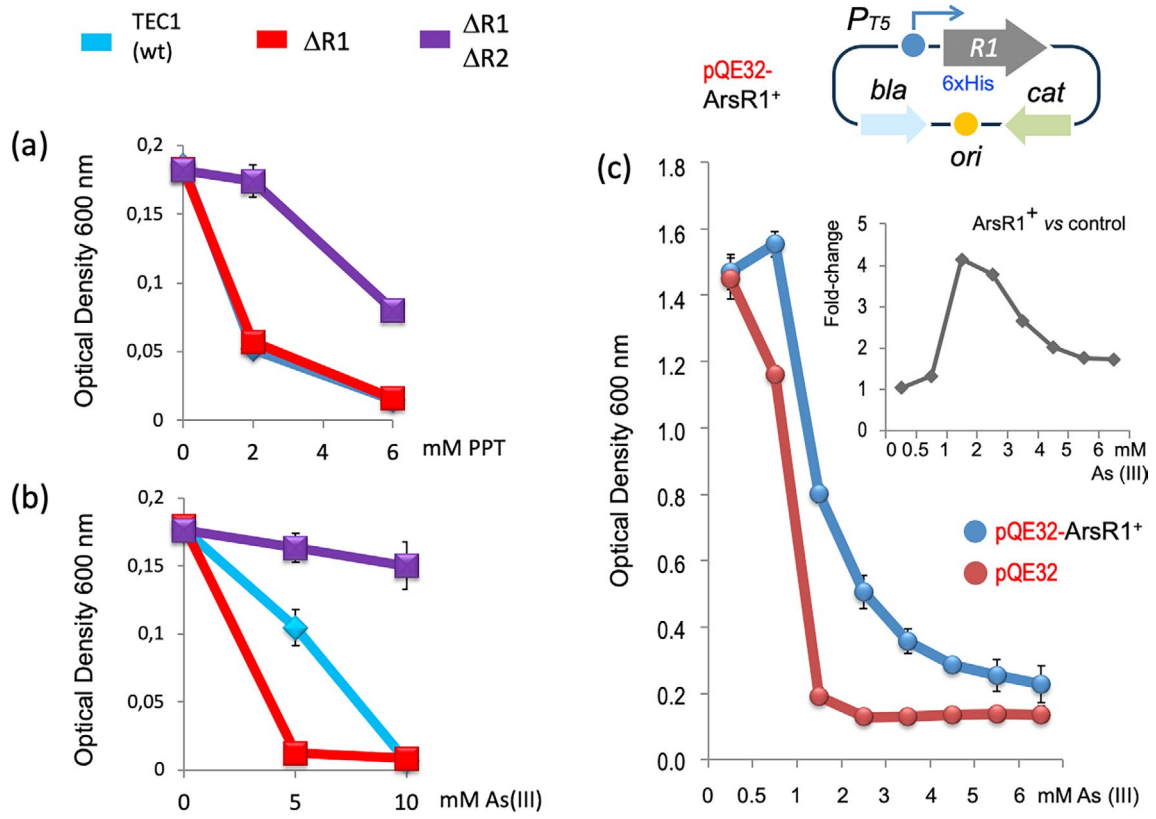

FIGURE 6 | Physiological effects of *phoN1* expression regulation by ArsR repressors. (a) Differential sensitivity to PPT of *P. putida* with or without repressors ArsR1, ArsR2 or both. Cultures inoculated with the strains indicated were grown for 7 h in M9 citrate + uracil medium in the presence of the PPT concentrations indicated. Growth was then recorded as OD_{600} in duplicated technical and biological replicas. (b) Same strains tested for sensitivity to growing concentrations of As (III). Cultures inoculated with the strains indicated were grown for 7 h in M9 citrate + uracil medium in the presence of the As (III) concentrations indicated. Growth was then recorded as OD_{600} in duplicated technical and biological replicas. (c) ArsR1 acts as an As metallothionein-like protein in *E. coli*. Triplicate cultures of *E. coli* M15 (pREP4) in LB carrying plasmids pQE32-Control (red line) or pQE32-ArsR1 (sketched on top blue line) were grown for 24 h at 37°C in the presence of 0.5 mM IPTG and the specified concentrations of arsenite from 0 to 6 mM. The OD_{600} was recorded at the end of the period. The insert shows the fold change between the growth of control versus the $ArsR1^+$ strains at the specified arsenite concentrations.

ampicillin (100 μg/mL) and chloramphenicol (Cm, 30 μg/mL). Isopropyl-1-thio-β-galactopyranoside (IPTG) was added at a concentration of 1 mM where indicated for activating the $lacI^q/P_{tac}$ expression system of plasmids derived from vector pVLT33 or pQE32 (Table 1). For growing the Δ*pyrF* reference strain *P. putida* TEC1 strain and its derivatives, uracil (Sigma Aldrich) was added to all media at 20 μg/mL. Where required, plasmids were transferred from *E. coli* donors to *P. putida* recipients as explained (Páez-Espino, Durante-Rodríguez, and de Lorenzo 2015). β-Galactosidase levels of *P. putida* cells bearing an *arsR1'-'lacZ* translational fusion were measured as indicated (Durante-Rodríguez, Páez-Espino, and de Lorenzo 2021).

3.2 | Directed Genomic Deletions

Seamless deletion mutants of the *arsR1* and *arsR2* genes of *P. putida* TEC1 were made with a reported method (Galvão and de Lorenzo 2005) with delivery plasmids pTU•DR1 and pTU•DR2, respectively the inserts of which bear the boundaries of the desired deletions. These plasmids were separately transferred from donor *E. coli* CC118λ*pir* to *P. putida* TEC1 strain (Table 1) by tripartite conjugation. Genomic co-integration, was followed by resolution of the co-integrates with fluoroorotic acid (FOA) as described (Galvão and de Lorenzo 2005). The accuracy of the resulting deletions was verified by PCR of the FOA^R *P. putida* clones with the upstream and downstream primers indicated in Appendix A. The double deleted strain *P. putida* Δ*arsR1* Δ*arsR2* was built by successive deletion of one gene after the other with the same method (Galvão and de Lorenzo 2005).

3.3 | Sensitivity Tests

Herbicide resistance experiments were done in M9 medium with citrate as sole carbon source, supplemented with uracil as mentioned above and added with the concentration the inhibitory compound indicated in each case. Tests were performed by growing strains under examination in 96-well plates and with biological triplicates and technical duplicates for each condition tested. Similarly, sodium arsenite ($NaAsO_2$ from Sigma Aldrich Chemicals) was added to the cultures in the same microtitre plate format at the levels marked for each experiment.

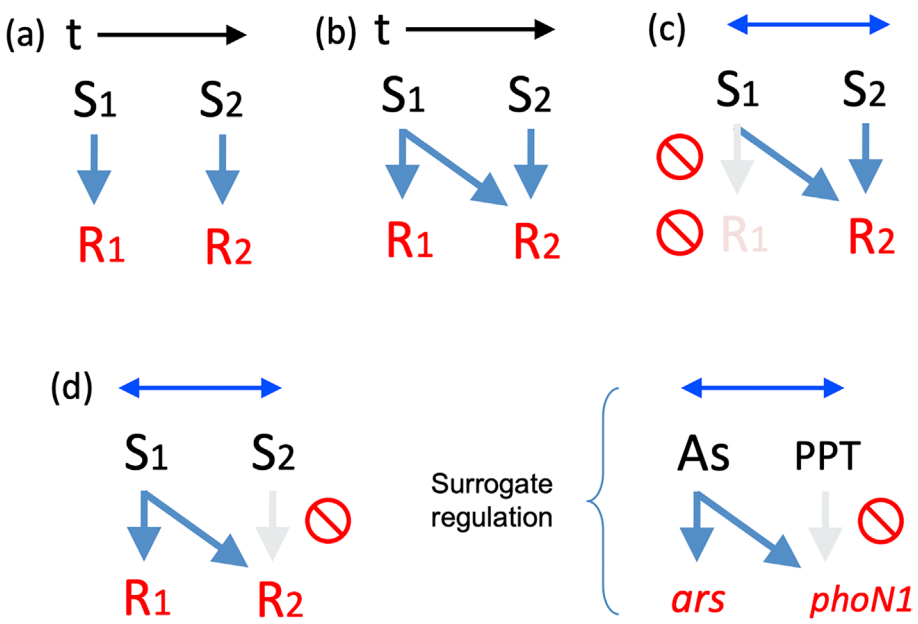

FIGURE 7 | Evolutionary scenarios for emergence of surrogate Pavlovian-type regulatory nodes. (a) Independent stimuli (S_1 S_2) at subsequent times generate unrelated responses (R_1 R_2). (b) Anticipatory regulation: The earlier stimulus activates simultaneously an early response for dealing with S_1 as well as another response R_2 for a stimulus that will come later, that is, the system embodies a memory device that anticipates S_2, even before S_2 materializes (Mitchell et al. 2009). (c) Associative Pavlovian response: Two simultaneous but unrelated stimuli trigger the same response to just one of them. (d) Surrogate Pavlovian-type response: one of the two simultaneous and unrelated stimuli trigger responses to both. This is the scenario that we entertain for the As (III)-dependent expression of *phoN1* in *P. putida*.

3.4 | Transcriptomics

P. putida KT2440 cells were grown at 30°C in LB medium (Sambrook and Rusell 2001). Overnight cultures were diluted with fresh media to OD_{600} of 0.05, in triplicate, and supplemented with 1 mM $NaAsO_2$(treated) or without arsenite (control) and incubated at 30°C with aeration until mid-exponential phase (OD_{600} of 0.6). Cells were harvested by centrifugation at 13000 × g for 5 min at 4°C. Total RNA was extracted from the cell pellets by using the High Pure Isolation Kit (Roche) following the protocol established by the manufacturer. RNA concentration was determined spectrophotometrically with a Nanodrop and genomic contamination was analysed by using the RNA as template in a PCR reaction. RNA final concentration and purity was confirmed by gel electrophoresis and by using an RNA 6000 Nano kit in an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. The integrity of the RNA was estimated at the Genomic Service of Complutense University (Madrid). After the optimal quality was determined as optimum (RNA integrity number [RIN] > 7), the triplicates of each conditions (control and treatment) were mixed and RNAseq was performed with total RNA obtained (2.3 μg for control sample and 2.7 μg for treatment sample) by Illumina platform at Macrogen (Seoul, South Korea), using their pipeline protocols that follow previously described techniques (Martin and Wang 2011). Briefly, after PCR-amplifying fragments from the obtained cDNA fragments with insert sizes between 200 and 400 bp were selected for paired-end sequencing. The cDNA sequences fragments obtained from were mapped using *P. putida* KT2440 genome as a reference (GCA_000007565.2).

3.5 | Data Analyses

Density heat maps of Figures 3 and 5 were generated using the *ggplot2* package in R. The *stat_density2d* function was used to visualize the density distribution of data points. Expression profiles were calculated for each sample and gene as read count. DEG (Differentially Expressed Genes) analysis was performed on four comparisons pairs as requested using edgeR software (Robinson, McCarthy, and Smyth 2010). The results showed 442 genes which satisfied $|FC| \geq 2$ and exactTest raw p value < 0.05 conditions in at least one of comparison pairs. To reduce biases in analysis, artefacts such as low-quality reads, adaptor sequence, contaminant DNA or PCR duplicates are removed. Transcript abundances were measured with mapped read count within gene region. In case of strand specific library, sense or anti-sense read counts are extracted by strand. Expression profiles are represented as read count and normalization value which is based on the transcript length and depth of coverage. The FPKM (fragments per kilobase of transcript per million mapped reads) values were used as a normalization value.

Author Contributions

David Paez-Espino: conceptualization, investigation, methodology, formal analysis, software. **Gonzalo Durante-Rodríguez:** methodology, investigation. **Elena Alonso Fernandes:** investigation, methodology. **Manuel Carmona:** investigation, methodology, software. **Victor de Lorenzo:** conceptualization, funding acquisition, investigation, writing – original draft, writing – review and editing, formal analysis, supervision, resources.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Transcriptomic data presented in the study are deposited in the NCBI repository, accession numbers SAMN41000924 and SAMN41000925; BioProject PRJNA1101689: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1101689.

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Appendix A

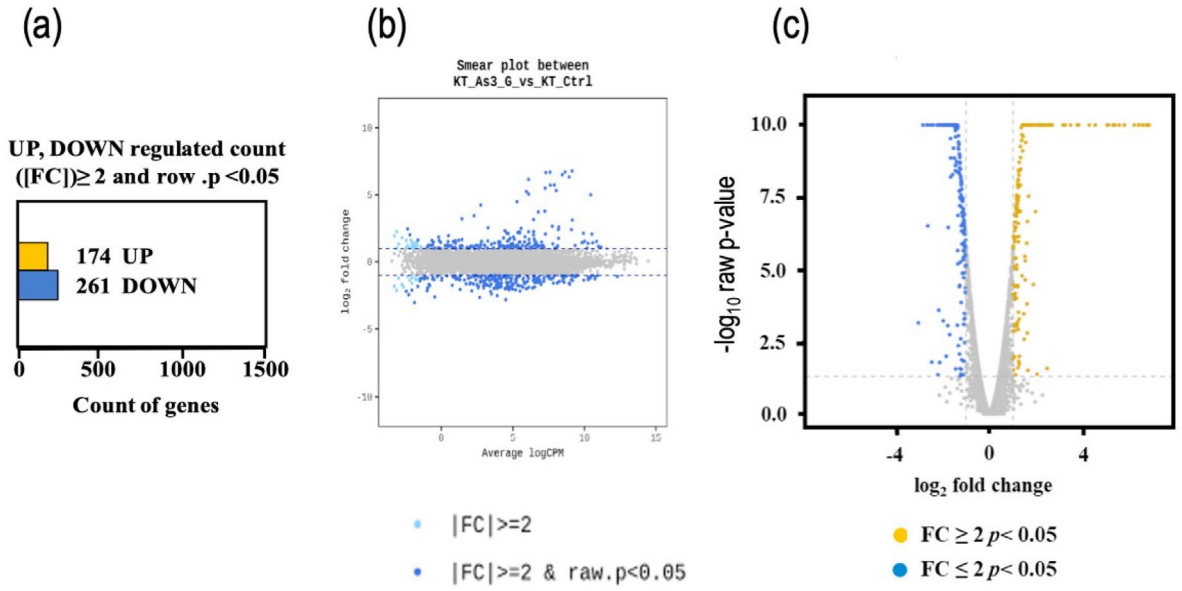

FIGURE A1 | Changes in the *P. putida* KT2440 transcriptome upon exposure to As (III). Representations of the number of genes regulated by arsenite in *P. putida* KT2440. (a) Overall number of up and down regulated genes based on fold change. (b) Smear plot of transcriptomic changes of *P. putida* KT2440 in response to arsenite. (c) Log2 fold change and p-value obtained from the comparison between two groups plotted as volcano representation. BioProject ID Reference: PRJNA1101689. BioSample accessions SAMN41000924, SAMN41000925.

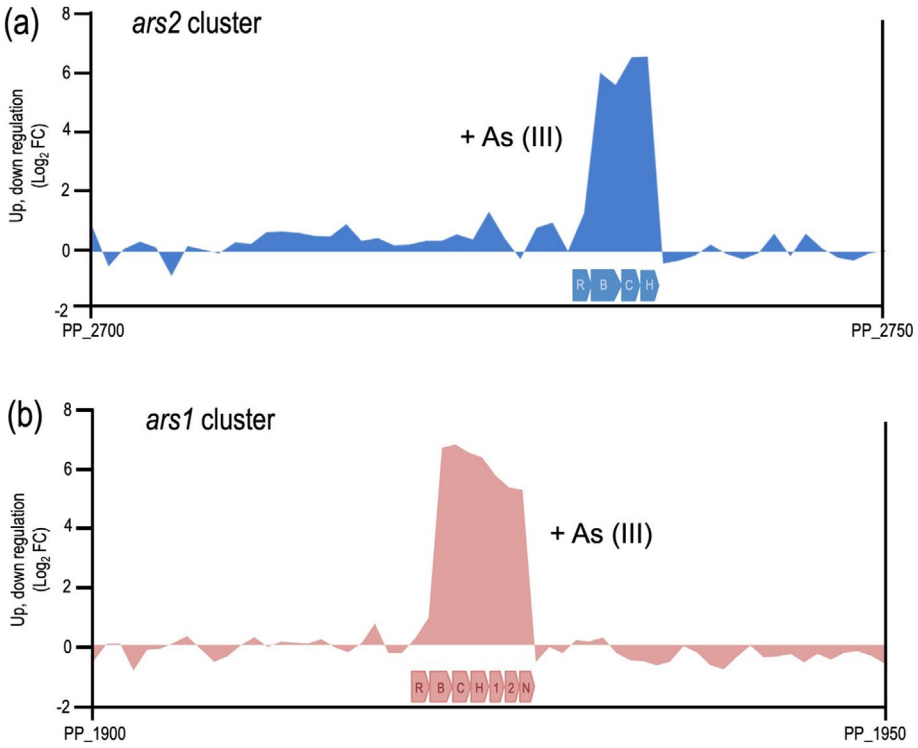

FIGURE A2 | Detail of the gene expression profile through the genomic segments of *P. putida* KT2440 encompassing the *ars2* and *ars1* gene clusters. Sequence coverage plots from transcriptome profile of *P. putida* KT2440 ars genes. (a) Relative gene expression intensity of ars2 genes in *P. putida* KT2440 grown in LB and supplemented with 1 mM As (III), using cells grown in the absence of As (III) as reference conditions. Blue boxes represent the *ars2* operon represented by *arsR2* (*R*), *arsB2* (*B*), *arsC2* (*C*) and *arsH2* (*H*) genes. (b) Relative gene expression intensity of *ars1* genes in *P. putida* KT2440 grown in LB and supplemented with 1 mM As (III), using cells grown in the absence of As (III) as reference conditions. Pale orange boxes represent the *ars1* operon represented by *arsR1* (*R*), *arsB1* (*B*), *arsC1* (*C*) and *arsH1* (*H*), two genes with unknown function (1 and 2), and *phoN* (N). Data replotted for comparison from Figure 2 of main text.

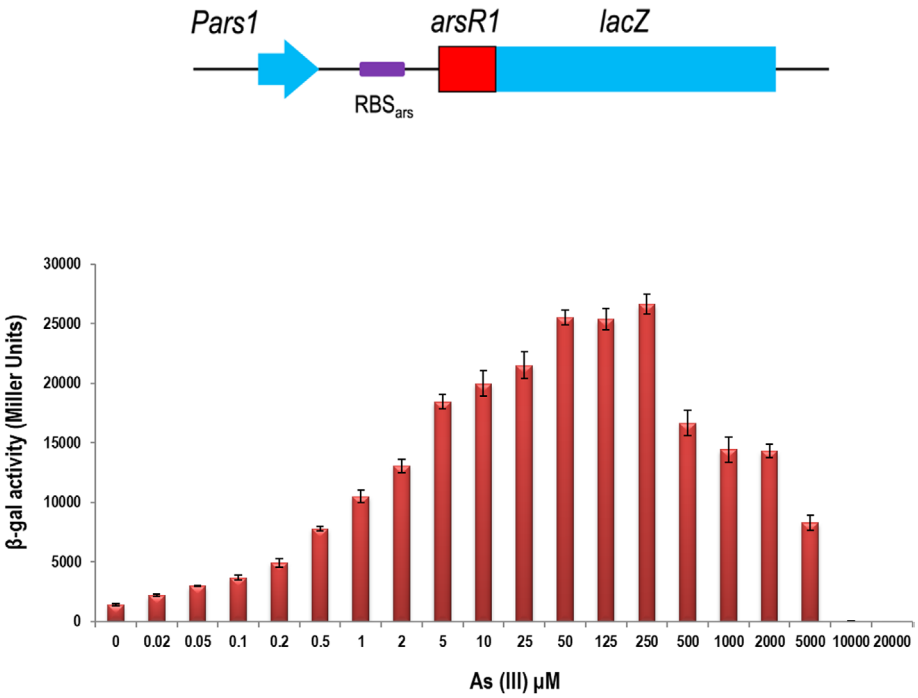

FIGURE A3 | Responsiveness of a low copy plasmid-borne translational *arsR1'-'lacZ* to As (III). *P. putida* KT2440 transformed with pSEVA225T•R1 (Table 1) bearing the insert sketched above was grown at 30°C in LB medium with increasing concentrations of arsenite as indicated until the mid- exponential culture phase, at which point β-galactosidase activity was measured and expressed in Miller units.

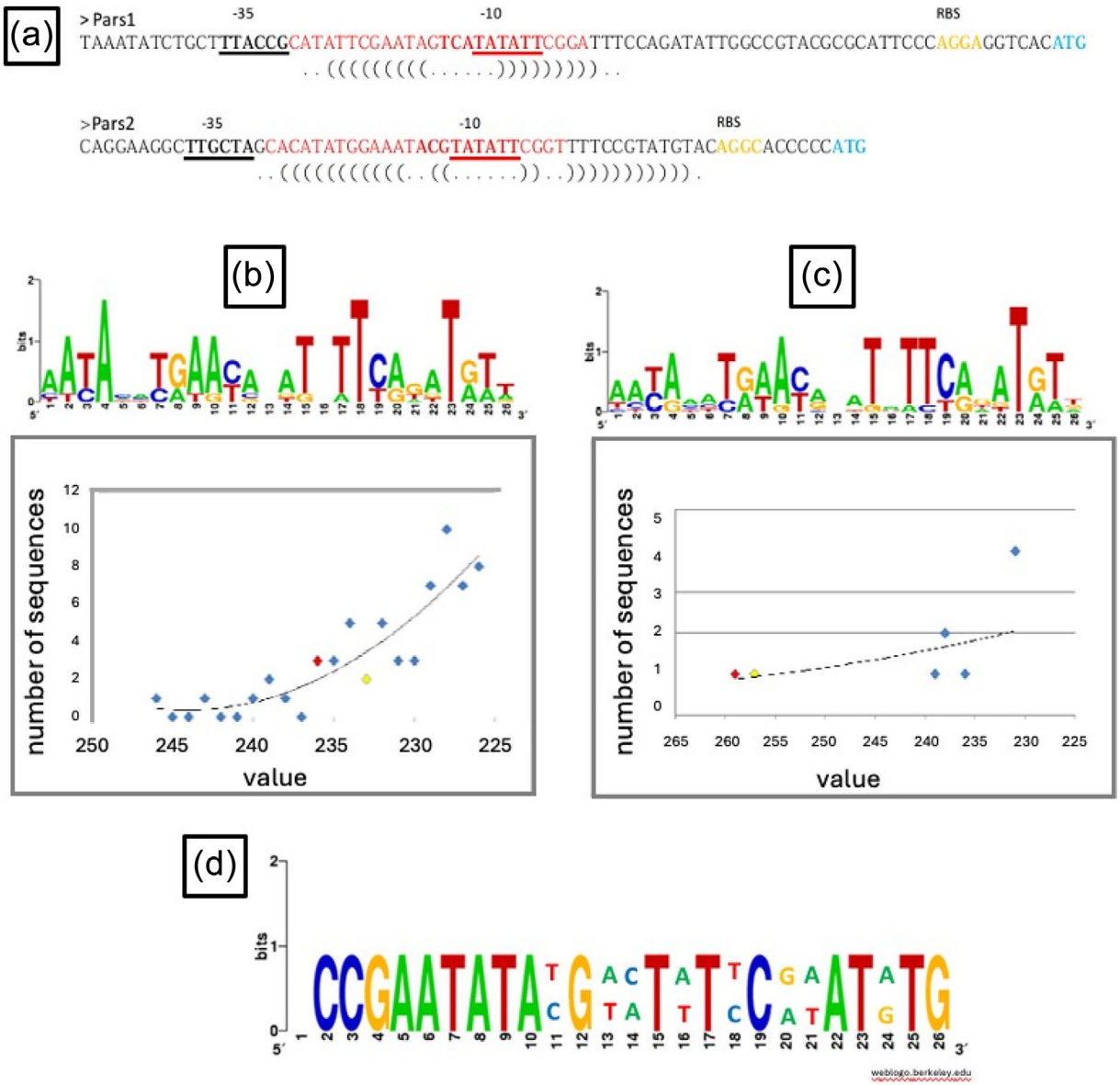

FIGURE A4 | In silico analysis of ArsR1- and ArsR2-binding sites to intergenic regions of the *P. putida* KT2440 genome. (a) Putative promoters P*ars*1 and P*ars*2. The regions corresponding to the −35 and −10 boxes (sigma-70 transcription factor-dependent promoters) for each case are underlined. The first amino acid (Met) of the *ars*R regulators is indicated in blue. The RBS regions are indicated in orange, and the operator region for each case, based on the consensus for the SmtB/ArsR family (tgtgATTTAATCATATG CG TTTTTGGTTATGtgtt) is shown in red. Predictions were made using the *BPROM* program from the *SoftBerry* package. Parentheses indicate the palindromic region using the *RNAfold* program. (b) DNA-binding region consensus using the 7 homologs of the SmtB/ArsR family. Consensus sequence representations were generated using *WebLogo* program (http://weblogo.berkeley.edu/). The graph below indicates the number of intergenic sequences (12.5% of the total genome) in *P. putida* KT2440 relative to the identical number of bases pairs to the consensus (in arbitrary units). The higher the value, the greater the number of bases identical to the consensus. The intergenic regions were extracted using the *coderet* application and the similarity matrix of the characterized homologs used to search in the non- coding genome of *P. putida* was generated using the *prophecy* application. The group including P*ars*1 is shown in red, and P*ars*2 in yellow. (c) P*ars*1 and P*ars*2 are included along with the 7 previous homologs from panel (b). The graph shows that both P*ars*1 and P*ars*2 have, by far, the highest values, indicating their specificity for their promoter regions. (d) Identity between *ars* operons in *P. putida* KT2440, consistent with the observed cross-regulation between the two *ars* systems.

TABLE A1 | Oligonucleotides used in this work.

Name	Sequence 5′-3′
FWDR1Up	CCACCAGCGGCCGCTCCTGGGACACCTGAGAACGAACTC
RVSR1Up	ACTACAACTCAATCAGCGAAGGGAAGTCGTGACCTCCTGGGAATGCGCGTA
FWDR1Down	GACTTCCCTTCGCTGATTGAGTTGTAGT
RVSR1Down	GTCCCGAGCTCCGTACTCGCTGAAACCGATGCCGAA
FWDR2Up	CCACCTGCGGCCGCACCGAATACACGGGTGAACTGCCG
RVSR2Up	GCAGCATGAAAATCTCGCTTGGTGATGAGGGGGTGCCTGTACATACGGAAAAC
FWDR2Down	TCATCACCAAGCGAGATTTTCATGCTGC
RVSR2Down	GTCCCGAGCTCTACAGGAATAGCACCAGCAGGGTGG
5 Pars1 Eco	GCGAATTCTGATCGGTACCAAGCAATCGG
3 Pars1 Bam	AGAGGATCCATCAGCAGGGTCATCCGGGC