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**Internal arsenite bioassay calibration using multiple bioreporter cell lines**

**Published in:**

Microbial Biotechnology (2008) 1(2), 149–157, doi:10.1111/j.1751-7915.2007.00011.x

Final submitted and revised version.

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Running title: Bioassay calibration with multiple cell lines

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## 1 **Abstract:**

2 Bioassays with bioreporter bacteria are usually calibrated with analyte solutions of known  
3 concentrations that are analyzed along with the samples of interest. This is done since  
4 bioreporter output (the intensity of light, fluorescence or colour) does not only depend on the  
5 target concentration, but also on the incubation time and physiological activity of the cells in  
6 the assay. Comparing the bioreporter output with standardized colour tables in the field seems  
7 rather difficult and error-prone. A new approach to control assay variations and improve  
8 application ease could be an internal calibration based on the use of multiple bioreporter cell  
9 lines with drastically different reporter protein outputs at a given analyte concentration. To  
10 test this concept, different *Escherichia coli*-based bioreporter strains expressing either  
11 cytochrome c peroxidase (CCP, or CCP mutants) or  $\beta$ -galactosidase upon induction with  
12 arsenite were constructed. The reporter strains differed either in the catalytic activity of the  
13 reporter protein (for cytochrome c peroxidase) or in the rates of reporter protein synthesis (for  
14 beta-galactosidase), which, indeed resulted in output signals with different intensities at the  
15 same arsenite concentration. Hence, it was possible to use combinations of these cell lines to  
16 define arsenite concentration ranges at which none, one or more cell lines gave qualitative  
17 (yes/no) visible signals that were relatively independent from incubation time or bioreporter  
18 activity. The discriminated concentration ranges would fit very well with the current  
19 permissive (e.g. WHO) levels of arsenite in drinking water (10  $\mu\text{g/L}$ ).  
20

## 21 **Introduction**

22 In the recent years, new tools for environmental monitoring have been developed on the basis  
23 of so-called bioreporter organisms. Bioreporters are often microbial strains which are  
24 genetically modified to produce easily detectable output in response to the presence of  
25 specific analytes. Several reviews have been dedicated to this rapidly developing field,  
26 exposing details of genetic constructions, type of reporters or analyte detection (Daunert et al.  
27 2000; Leveau & Lindow 2002; Belkin 2003; van der Meer et al. 2004; Harms et al. 2006).  
28 The detection system of bioreporter microorganisms is in most cases composed of a simple  
29 genetic circuitry, comprising a regulatory system (i.e. regulatory gene plus its cognate  
30 promoter) and one or more reporter genes. Commonly used reporter proteins are the  
31 chromogenic  $\beta$ -galactosidase (*lacZ*) or  $\beta$ -glucuronidase (*gusA*), luminescent luciferases (*luc* or  
32 *luxAB*) or autofluorescent proteins (GFP and variants) (Ivask et al. 2002; Paitan et al. 2004;  
33 Werlen et al. 2004; DeAngelis et al. 2005).  
34 For quantitative measurements, bioreporter assays need to be calibrated with analyte solutions  
35 of known concentrations, which are typically analyzed along with the samples of interest  
36 (Hakkila et al. 2004; Harms et al. 2005). The reason for this is that the bioreporter output  
37 (colour, luminescence or fluorescence) does not only depend on analyte concentration, but  
38 also on assay incubation time and the numbers and physiological activities of the cells in the  
39 assay (Jansson 2003; Stocker et al. 2003; van der Meer et al. 2004; Wells et al. 2005). Simple

1 applications like bioreporter field test kits or paper test strips are hampered by the difficulty to  
2 compare the resulting colour signals with standardized colour tables. Therefore, a robust and  
3 reliable system for internal calibration would be highly desirable.

4 The problem of variable incubation time and uncertain cellular activity could be overcome by  
5 the simultaneous use of multiple bioreporter cell constructs, each giving qualitative  
6 information about the presence or absence of analyte in a certain concentration range.  
7 Bioreporters could be designed and combined in such a way that not the actual individual  
8 output but the combination of reacting cell lines indicates the analyte concentration range in  
9 unknown samples without the need to measure exact rates or endpoints of colour formation by  
10 individual bioreporters (Fig. 1). Although this method would not report exact concentrations,  
11 the identification of concentration ranges should be sufficient for many applications, where,  
12 for instance, only a distinction of concentrations below and above a predefined permissible  
13 level is desired. If in combination with a chromogenic reporter protein this would allow signal  
14 interpretation by eye without instrumentation. In the case of arsenic in potable water, a  
15 distinction between concentrations below and above the WHO drinking water standard of 10  
16  $\mu\text{g/L}$  or the local standard of 50  $\mu\text{g/L}$  in the most arsenic-threatened countries of South-East-  
17 Asia would be most desirable.

18 To test this concept, we used two reporter genes to redesign bioreporter bacteria that had  
19 previously been developed for the detection of arsenite in drinking water, exploiting the ArsR  
20 sensor transcriptional repressor of the natural arsenic resistance of *Escherichia coli* (Stocker  
21 et al. 2003). In the presence of arsenite, ArsR loses affinity for its binding site on the DNA the  
22 result of which is depression of the *arsR* promoter. Equally effective in derepressing the *arsR*  
23 promoter via ArsR is antimonite. In cells which carry the arsenate reductase ArsC, arsenate  
24 (at 25% efficiency) and trimethylarsine oxide (at 10% efficiency) will also lead to  
25 derepression (Baumann & van der Meer, 2007). In one series of cell lines, the *arsR* promoter  
26 was combined with *lacZ* for  $\beta$ -galactosidase. Beta-galactosidase has many advantages and  
27 various chromo- and fluorogenic substrates exist to assay LacZ activity (Daunert et al. 2000;  
28 Lei et al. 2006). In a second series of cell lines we applied the *ccp* gene. Its product, the  
29 cytochrome c peroxidase (CCP) has not frequently been utilised in whole cell bioreporter  
30 bacteria so far, but a large variety of *ccp* mutants has been developed which display different  
31 catalytic activity for one of its substrates guaiacol (Iffland et al. 2000). The signal outputs of  
32 both reporters were thus varied in different ways: for LacZ bioreporters we modified the  
33 intervening sequence between *arsR* and *lacZ* in order to modulate *lacZ* expression. For *ccp*  
34 we maintained the same reporter synthesis rate, but hypothesized that because of different

1 catalytic activity of the reporters we would get variable output of the cells at the same arsenite  
2 concentration. Our research questions therefore were (i) if both types of reporter constructions  
3 would result in different levels of reaction sensitivity to arsenite and (ii) if combinations of  
4 such reporter cell lines with variable signal outputs would be suitable to infer arsenite  
5 concentration ranges without external calibration and at the required drinking water standards.  
6

## 7 8 **Results**

9  **$\beta$ -Galactosidase-based bioreporter assay.** Bioassays were performed in 96-well plates to  
10 allow spectrophotometrical measurements (Fig. 2) as well as visual signal detection by digital  
11 imaging (Fig. 3). For each bioreporter strain the bioassay was performed in triplicate with 15  
12 different arsenite concentrations ranging from 0.2 to 100  $\mu\text{g/L}$ . Strain 2245 was very sensitive  
13 and produced visible colour from the lowest arsenite concentration (0.2  $\mu\text{g/L}$ ) with a linear  
14 signal increase up to 6  $\mu\text{g/L}$  and a plateau above 10  $\mu\text{g/L}$  (Figs. 2 & 3). Colour development  
15 by strain 1595 began at approximately 10  $\mu\text{g/L}$  arsenite and linearly increased at higher  
16 concentrations. Both strains had a relatively low background activity in the absence of  
17 arsenite. In contrast, strain 1580 was characterized by a high background and little further  
18 signal increase toward higher concentrations. Strain 2066 was the least sensitive and gave  
19 visible signals only at very high concentrations (around 50  $\mu\text{g/L}$ ). Visual signal differentiation  
20 for neighbouring concentrations was difficult with all strains. The combination of qualitative  
21 (yes/no) signals of all four strains appeared suitable for the definition of three arsenite  
22 concentration ranges:  $<6 \mu\text{g/L}$ ,  $10\text{-}50 \mu\text{g/L}$  and  $>50 \mu\text{g/L}$  (Tab. 1A). The quality of the  
23 responses (visible colour or not) was independent from incubation time in a window between  
24 4 and 8 hours after induction. They were also independent from cell density for final cell  
25 culture turbidities in the assays of between  $\text{OD}_{600}$  of 0.1 and 1.0 (data not shown).

26 **Cytochrome c peroxidase-based bioreporter assay.** In this case, the assays were carried out  
27 with colonies exposed to arsenite on agar plates and transferred to paper in order to obtain  
28 optimal visual signal interpretation. For each strain the bioassay was performed in four  
29 replicates with 10 different arsenite concentrations ranging from 1  $\mu\text{g/L}$  to 5  $\text{mg/L}$ . The  
30 bioreporter strains 1971, 1981 and 1982 displayed relatively similar responses and produced  
31 clearly visible, concentration-dependent signals between 4 and 30  $\mu\text{g/L}$  followed by a plateau  
32 (Fig. 4). For the strains 1971 and 1982 a further signal increase could be detected after  
33 incubation with 1000  $\mu\text{g/L}$ . The background activity in the absence of arsenite was high

1 enough to result in visible spots. Strain 2031 showed no background activity without arsenite,  
2 while colour production was clearly visible at 4  $\mu\text{g/L}$  and increased strongly until 20  $\mu\text{g/L}$ .  
3 Strains 2332 and 2340 did not respond to low arsenite concentrations (1-10  $\mu\text{g/L}$ ) and  
4 produced slightly increasing signals between 20  $\mu\text{g/L}$  and 5 mg/L arsenite. The combination  
5 of the qualitative signals of these strains appeared to enable the distinction of arsenite free  
6 controls from the concentrations ranges 1-10  $\mu\text{g/L}$  and >20  $\mu\text{g/L}$  (Tab. 1B). Similar results  
7 were obtained with either twice as many cells per spot or shorter (2 h) and longer incubation  
8 (16 h) (Figure S1).

9 **mRNA content.** Reporter mRNA contents of cells exposed to three arsenite concentrations  
10 were quantified in order to determine whether differences in signal intensities for the reporters  
11 at identical arsenite concentrations were the result of differential transcription or of enzyme  
12 synthesis and enzyme activity. Levels of the *ccp* mRNA in the different CCP bioreporter  
13 strains were similar after incubation with the same arsenite concentration, but increased as a  
14 function of arsenite concentration (Fig. 5A). An exception was strain 1982, which for  
15 unknown reasons contained significantly ( $p < 0.05$ ) less *ccp* mRNA at the highest arsenite  
16 concentration. The level of *lacZ* mRNA in the different reporter strains generally increased as  
17 a function of arsenite concentration, but varied between all strains. At 100  $\mu\text{g As/L}$  the levels  
18 of *lacZ* mRNA differed significantly ( $p < 0.05$ ) between all strains, while at 10  $\mu\text{g As/l}$  the  
19 difference was only significant between strain 1580 and the other strains ( $p < 0.05$ ). This  
20 indicates that the change of the intergenic region between *arsR* and *lacZ* in the different  
21 constructs, and the use of two different plasmid vectors for cloning (pMV for 1580 and 1595;  
22 pPROBE for 2245 and 2066) did affect the amount of *lacZ* mRNA produced. Notably, *lacZ*  
23 expression in strain 1580 without and at 10  $\mu\text{g As/L}$  did not differ, which is the result of the  
24 leaky *arsR* promoter and read-through (Fig. 5B). Basal level of *arsR* expression is required  
25 for the natural resistance system to function because of the nature of the *arsR* transcriptional  
26 organization. Since ArsR is a transcriptional repressor which binds within its own promoter  
27 DNA, no *arsR* transcription would take place without leakiness.

28

## 29 **Discussion**

30 The aim of this study was to simplify the application of bioreporter bacteria by developing an  
31 internal calibration method. The idea was to infer analyte concentrations from combinations  
32 of qualitative (yes/no) signals provided by a series of cell lines with different sensitivities for  
33 the analyte. The quantitative response of a single cell line used so far in bioreporter assays is  
34 replaced by a series of complementary qualitative responses. The advantage of such biosensor

1 arrays is their relative independence from incubation times and bioreporter activities. We used  
2 two different ways to produce such variable reporter cell lines, i) by influencing the rate of  
3 reporter enzyme synthesis (*LacZ*) or ii) by influencing the reporter enzyme specific activity  
4 (for CCP). Various other ways of obtaining similar effects can be imagined, for instance, by  
5 using different stabilities of reporter proteins (Andersen et al. 1998). We focused on an  
6 arsenite detecting bioreporter, for which a simple qualitative assay could be useful in field  
7 campaigns notably in Southeast Asia (Smith et al. 2000; British Geological Survey 2001).

8 **LacZ-Bioreporters.** Arsenite measurements have been performed before with *LacZ*  
9 bioreporters under control of the *ArsR* promoter using classical spectrophotometric,  
10 chemiluminescence and electrochemical detection (Scott et al. 1997; Ramanathan et al. 1998),  
11 or by chromogenic visualization using X-gal (Stocker et al. 2003). We previously showed that  
12 leaky expression from the *arsR* promoter must be reduced to obtain gradually intensifying  
13 blue colour formation from X-gal as a function of arsenite concentration. This was  
14 accomplished by placing a second *ArsR* DNA binding site downstream of *arsR* (Stocker et al.  
15 2003). Upon long incubation times, however, cells with this bioreporter construct (i.e., strain  
16 1595) will intensify their blue colour at all arsenite concentrations, reducing the visibility of a  
17 good colour scale. We therefore produced one variant of strain 1595 in which the background  
18 is further reduced (strain 2066) by altering the ribosome binding site in front of *lacZ* (Fig. S1).  
19 Indeed, production of *LacZ* activity in strain 2066 is very low compared to the others, which  
20 was useful for the concept of the multi-strain assay, because the 2066 bioreporter only  
21 produces visible blue colour from X-gal at arsenite concentrations above 50 µg/L. At the  
22 engineered unique restriction sites in front of *lacZ* we then replaced fragments of various  
23 length originating from the wild-type *lacZ* promoter in *E. coli*, one of which (construct in  
24 strain 2245, Fig. S1) appeared to be suitable for the arsenite concentration range below 5  
25 µg/L. Interestingly, strain 2245 reacted with visibly different blue colour formation from X-  
26 gal already at 0.2 µg/L arsenite, compared to the negative control. Thus, the responses of  
27 strains 2245 and 1595 complement each other very well in the low (0.2-5 µg/L) and medium  
28 (5-50 µg/L) concentration ranges. Although strain 1580 is not directly useful in showing  
29 colour differences at varying arsenite concentrations, it can be used as positive control for the  
30 quality of the test.

31 By probing *lacZ* mRNA levels in the four strains, we could provide evidence that the different  
32 *LacZ* activities in the reporter strains as a function of arsenite is due to both differential  
33 transcription efficiency or mRNA stability and translational effects. Two transcriptional  
34 effects were clearly seen: First, the inclusion of the secondary *ArsR* binding site in all

1 constructs except 1580, which reduced mRNA formation in the absence of arsenite and  
2 secondly, the lower *lacZ* mRNA abundance from the constructs based on plasmid pPROBE  
3 (i.e., 2066 and 2245), which might be due to a different plasmid copy number. However, the  
4 strong LacZ activity difference between 2066 and 2245 was not apparent from mRNA levels  
5 (Fig. 5B), but the result from the absence (2066) and presence (2245) of a good ribosome  
6 binding site. One can conclude that our intention to control the bioreporter sensitivity on the  
7 level of reporter enzyme synthesis was successful in that it led to a range of bioreporter strains  
8 of quite complementary sensitivities including a nearly constitutive control strain.

9 **CCP-Bioreporters.** The use of cytochrome c peroxidase as reporter gene resulted in a useful  
10 colouration as well, although the assay was a bit less robust than that for  $\beta$ -galactosidase. The  
11 main advantage of *ccp* was the availability of a well characterized set of mutants with variable  
12 activity against the classical peroxidase substrate guaiacol (Iffland et al. 2000; Iffland et al.  
13 2001). In this case mRNA probing showed that all mutant *ccp* genes were transcribed with  
14 similar efficiencies, and as a function of arsenite concentration (except one, Fig. 5A).  
15 However, the colouration resulted in very different intensities at the same arsenite  
16 concentration for the various reporters. Those strains containing CCP with improved specific  
17 activity (1971, 1981, 1982) compared to the wild type (2031) gave high signals at very low  
18 arsenite concentrations, but with a relatively high background in the absence of arsenite. Their  
19 increased activity confirms previous findings of Wilming et al., who observed turnover  
20 numbers of 102 and 26 s<sup>-1</sup> of strain 1971 and 1982, respectively, compared with 0.35 s<sup>-1</sup> for  
21 the wild type enzyme (Wilming et al. 2002). The *ccp* gene in strain 1981 contains multiple  
22 mutations (Y39H-H60R-N184D-D217G-D224Y) and although the substrate turnover was  
23 reported to be only slightly higher [0.8 s<sup>-1</sup>(Iffland et al. 2001)] than that of the wild type, a  
24 colouration similar to that of 1971 and 1982 for arsenite concentrations up to 100  $\mu$ g/L was  
25 observed. The CCP of the bioreporter strains with the lowest observed activity and sensitivity  
26 in our tests (2332, 2340) had higher reported turnover numbers (13 s<sup>-1</sup> and 5 s<sup>-1</sup>, respectively)  
27 than the wild type enzyme (Wilming et al. 2002). The reasons for these discrepancies between  
28 reported enzyme activities and signals obtained in our experiments with whole cells are  
29 unknown. Measurements of enzyme activities in cell extracts were in accordance with the  
30 reported substrate turnover (supplementary Figure S1), which might be an indication for  
31 different enzyme activity in extracts and whole cells, or differences in substrate accessibility  
32 or transport in whole cells. Our results, however, show that it is possible to receive a set of  
33 bioreporter bacteria with varying sensitivity by modifying the catalytic activity of the reporter  
34 protein and to use combination of them for internal calibration.

1 **Conclusions.** We demonstrated that bioreporter bacteria of various sensitivities can be  
2 tailored by modifying different steps of the detection-signalling chain, i.e. the rate of reporter  
3 enzyme synthesis and the catalytic activity of the reporter protein. It appears thus possible to  
4 construct sets of bioreporter bacteria that, when combined, cover ranges of target  
5 concentrations that could not be covered by a single bioreporter organism due to restrictions  
6 of e.g. the induction factor, limitations of spectroscopic signal detection or the insensitivity of  
7 visual interpretation. Furthermore our results demonstrate the potential of reporter protein  
8 engineering as opposed to the former focus on analyte sensing and gene regulation for  
9 bioreporter improvement (van der Meer et al. 2004).

10 The accomplished modifications altogether resulted in two sets of bioreporter bacteria that  
11 can be used to infer analyte concentration ranges without external calibration. This  
12 quantitative information originates from combinations of robust qualitative signals of the  
13 individual bioreporter strains. The hypothesized working principle was thus shown to  
14 function. A further important result is that permissive concentrations of arsenic could be  
15 identified with both bioreporter sets: (i) below the WHO limit only two of the four LacZ  
16 bioreporter bacteria produced a visible signal, above 10 µg/L three or at concentrations above  
17 local arsenic standards (50 µg/L) all four strains were responding with clear signals; (ii) the  
18 CCP bioreporter bacteria indicate a concentration below the WHO limit when four out of six  
19 strains show signals. This set could also be reduced to four strains, since three strains gave  
20 redundant information.

21 Our approach promises to simplify bioreporter application by layman since it eliminates time  
22 consuming and technically demanding calibration. Especially for simple field detection  
23 devices such as paper strips (Stocker et al. 2003) our method would be of great value. Besides  
24 its higher robustness it has the advantage of the much easier interpretation of sets of  
25 qualitative sub-signals than of gradual colour changes.

26

## 27 **Experimental Procedures**

### 28 **Construction of ArsR plasmids.**

29 The following reporter plasmids were constructed (Fig. 6) in which ArsR controls expression  
30 of either the *lacZ* gene or the *ccp* gene [for cytochrome *c* peroxidase from *Saccharomyces*  
31 *cerevisiae* (Iffland et al. 2000)]. In plasmid pMV-arsR [strain number 1580, (Stocker et al.  
32 2003)] the *arsR* gene is placed directly upstream of *lacZ* and in the same direction of  
33 transcription, so that expression of *arsR* and *lacZ* are under control of the ArsR-regulated



1 promoter in front of *arsR* itself ( $P_{ars}$ , Fig. 6). To reduce background *lacZ* expression from the  
2 leaky  $P_{ars}$  promoter, a second ArsR DNA binding site (ABS) was introduced downstream of  
3 *arsR* in plasmid pMV-arsR-ABS [strain number 1595, Fig. 6, (Stocker et al. 2003)]. To  
4 change the sequence and ribosome binding site upstream of *lacZ*, we amplified the entire *lacZ*  
5 gene from the *E. coli* K12 chromosome while introducing HindIII and NheI restriction sites at  
6 the gene extremities. This *lacZ* DNA was then used to replace the *gfp* gene in the broad host  
7 range vector pPROBE' (Miller et al. 2000). The *arsR* gene and the downstream secondary  
8 ArsR binding site were recovered on a BamHI-SpeI fragment and inserted into pPROBE'-  
9 *lacZ* in front of *lacZ* to make pPR-ArsR-ABS-LacZ (strain 2066, Fig. 6). The ribosome  
10 binding site (RBS) of the original *lacZ* gene of *E. coli* was amplified together with the entire  
11 *lacZ* gene by PCR by using primer LacZ Rev NheI (5' gctagctattttgacaccagaccaactgg 3') and  
12 primer LacZ RBS For (5' gcttgtgagcggataacaatttcacacagg 3') and introduced in pPR-ArsR-  
13 ABS-LacZ digested by HindIII and NheI (plasmid pPR-arsR-ABS-RBS-LacZ, strain 2245,  
14 Fig. 6). Sequences of the *arsR* – *lacZ* intergenic regions are presented in supplementary figure  
15 S2.

16 The *ccp* reporter plasmids were all constructed by inserting a BamHI-SpeI fragment  
17 containing *arsR* and the second ArsR binding site from pPR-arsR-ABS (Stocker et al. 2003)  
18 in a pET15-based plasmid containing the *ccp* gene or its variants (Iffland et al. 2000; Iffland  
19 et al. 2001), pre-digested with BglII and XbaI (during which the T7 promoter is removed).  
20 The *arsR* gene is thus expressed from  $P_{ars}$  and on its turn regulates *ccp* expression (Fig. 6). In  
21 this manner we produced *E. coli* strains with plasmids pArsR-CCP-WT (strain number 2031,  
22 wild-type *ccp*), pArsR-CCP-R48I (strain 2332, R48I mutation in CCP), pArsR-CCP-R48T  
23 (strain 2340), pArsR-CCP-R48Q (strain 1971), pArsR-CCP-K2.4 (strain 1981, Y39H-H60R-  
24 N184D-D217G-D224Y mutations) and pArsR-CCP-PT1 (strain 1982, R48H mutation).

25 Arsenite bioreporters were constructed in *E. coli* Dh5 $\alpha$ .

26 **Cell preculturing.** Arsenite in aqueous solution was measured with cell suspensions of *E.*  
27 *coli* strains (Tab. 2). Bioreporter strains were plated from -80°C stock cultures on fresh Luria-  
28 Broth (LB) plates with appropriate antibiotic selection (ampicillin 100  $\mu$ g/mL or kanamycin  
29 50  $\mu$ g/mL) and grown overnight at 37°C. Five mL liquid LB medium plus antibiotic was  
30 inoculated with a single colony and incubated for 16 h at 37°C. Cultures were diluted in fresh  
31 preheated LB medium without antibiotics at a 1:50 ratio and incubated until an optical density  
32 at 600 nm of 0.5 was reached. With this culture triplicate bioassays were performed as  
33 described below.

1 **Arsenite assays with *E. coli*  $\beta$ -galactosidase (LacZ) bioreporters.** Assay mixtures  
2 containing 100  $\mu$ L cell suspension (OD<sub>600</sub> of 0.5, 1:5 diluted in water), 90  $\mu$ L arsenite stock  
3 solution and 10  $\mu$ l X-gal substrate solution (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactosidase,  
4 Serva, 0.2 mg/mL in dimethylformamid) were prepared directly in 96-well plates. Cells were  
5 exposed to different final arsenite concentrations (0, 0.05, 0.2, 0.5, 2, 4, 6, 10, 20, 25, 30, 40,  
6 50, 60, 70, 80 and 100  $\mu$ g/L). Arsenite solutions were prepared by dilution from a 50 mM (6.5  
7 g/L) sodium arsenite solution commercial stock (Merck) in tap water. The 96-well plates with  
8 the assay mixtures were covered with a lid and incubated at 30°C in a rotary shaker (700 rpm)  
9 for 22 hours. In order to detect the blue colour formed from X-gal conversion, the optical  
10 density was measured in a microplate reader (Spectramax 250, Molecular Devices) at 595 nm  
11 after 0, 1, 2, 3, 4, 5, 6, 7, 8 and 22 h. The turbidity resulting from the cell suspension itself  
12 was subtracted from the measured signal by measuring triplicate assay mixtures without X-  
13 gal.

14 **Arsenite measurements with *E. coli* cytochrome c peroxidase (CCP) bioreporters.** The  
15 CCP bioreporters were cultured as above for the LacZ reporters in small precultures for 16 h  
16 at 37°C after which cells were diluted fiftyfold and again grown until a culture turbidity of  $\approx$   
17 0.5 was reached. Cells were then concentrated by centrifugation to achieve a turbidity at 600  
18 nm of  $\approx$  6. Ten  $\mu$ l of these cell suspensions were then spotted on agar plates containing  
19 different concentrations of arsenite (0, 1, 4, 10, 20, 30, 50, 100, 200, 1000 and 5000  $\mu$ g/L) and  
20 briefly dried. After an incubation of four hours at 37°C the cell spots were transferred to paper  
21 by gently pressing a paper sheet (3M chromatography paper, Whatman) of the right diameter  
22 directly on the agar surface. Peroxidase activity of the cells was detected by incubating the  
23 paper sheets on filter paper which was wetted with 50 mM potassium phosphate buffer (pH  
24 6.0) containing freshly added guaiacol (final concentration 105 mM, Sigma) and hydrogen  
25 peroxide (final concentration 170  $\mu$ M, Merck) (Iffland et al. 2000). Colour development was  
26 allowed to proceed until clearly visible, after which the paper sheets were digitally  
27 photographed.

28 **Determination of mRNA contents.** Reporter strain cultures grown similarly were exposed to  
29 0, 10 or 100  $\mu$ g/L arsenite for 4 h at 37°C, after which total RNA was isolated from 5 ml  
30 culture using a Macherey Nagel Nucleo Spin Kit. Twenty  $\mu$ L of 120  $\mu$ l total RNA extract  
31 from each sample was blotted on a positively charged nylon membrane (Roche) using a  
32 Milliblot system (Millipore). The total RNA on the membrane was fixed with UV (1 min at  
33 120 mJ/cm<sup>2</sup>) and stored at room temperature until hybridization. DIG labelled DNA probes  
34 were synthesized using random primed labelling (DIG DNA labelling kit, Roche) from PCR

1 products generated with specific primers (Tab. 3) for *lacZ* and *ccp*, respectively.  
2 Hybridization was performed for 16 h at 57°C for the *ccp*- and 53°C for the *lacZ*-probe using  
3 DIG Easy Hyb solution (Roche) and 100 ng/μL probe. Subsequent washing was performed  
4 according to the manufacturer's protocol (Roche). DIG-DNA-RNA hybrids were detected  
5 with the NBT-CSIB (Roche) approach as described by the manufacturer. Dot blots were  
6 photographed and quantitatively analyzed using Image J, in which the signal intensities (i.e.  
7 the colour intensity of the dots) were expressed as average grey values.

8

9 **Acknowledgements.** The authors thank Kai Johnsson for the generous gift of the *ccp*  
10 variants. Alexandra Baumeyer is thanked for her help in *arsR-ccp* cloning and preliminary  
11 tests with cytochrome c peroxidase.

12

1 Table 1: Arsenite concentration ranges distinguished with combinations of LacZ-strains (A)  
 2 and CCP-strains (B). Strains that give a signal in the respective concentration range are  
 3 marked with x.

4	A	Concentration range	1580	1595	2245	2066		
5		< 6 µg/L	x		x			
6		10 – 50 µg/L	x	x	x			
7		> 50 µg/L	x	x	x	x		
8								
9	B	Concentration range	1971	1981	1982	2031	2332	2340
10		0 µg/L	x	x	x			
11		1 – 10 µg/L	x	x	x	x		
12		> 20 µg/L	x	x	x	x	x	x
13								

1 Table 2: Identification and characteristics of *E. coli* bioreporter constructs (ABS: ArsR  
2 binding site, RBS: ribosome binding site).

3	Strain (LacZ)	Plasmid
4	1580	pMV-arsR
5	1595	pMV-arsR-ABS
6	2245	pPR-arsR-ABS-RBS-lacZ
7	2066	pPR-arsR-ABS-lacZ
8	Strain (CCP)	
9	1971	pArsR-ABS-CCP-R48Q
10	1981	pArsR-ABS-CCP-K2.4
11	1982	pArsR-ABS-CCP-PT1
12	2031	pArsR-ABS-CCP-WT
13	2332	pArsR-ABS-CCP-R48I
14	2340	pArsR-ABS-CCP-R48T
15		

1 Table 3: Primers for the synthesis of probes for dot blot hybridization.

2 Primer name Target Sequence

3 sfi\_for *ccp* 5'-GCC ATG GCC AGC ACG GCC ACA CC-3'

4 R48Trev *ccp* 5'-GTG CCA AGC AAG AGT GAC TAA TAC GGG-3'

5 LacZfor *lacZ* 5'-TCG GTT ACG GCC AGG ACA GT-3'

6 LacZrev *lacZ* 5'-CAT CAT TAA AGC GAG TGG CAA CAT-3'

7

1 **Figure Captions**

2 Figure 1: Principle of the internal calibration approach. A – D represent bioreporter cell lines  
3 differing in their sensitivity to arsenite.

4

5 Figure 2: Colour development after 480 min by four bioreporter strains expressing LacZ  
6 measured as absorbance at 595 nm. Data points represent the average of three replicates.

7

8 Figure 3: Colour development from X-gal by four LacZ-based bioreporter strains as response  
9 to different arsenite concentrations. A) Wide concentration range overview of assay carried  
10 out in 96-well plates. The digital image was taken after 8 hours, whereas colour development  
11 proceeded for 22 hours. B) Colour detail in assays performed in 24-well plates. Image taken  
12 after 4 hours incubation time at 30°C.

13

14 Figure 4: Colour development in the six bioreporter strains expressing CCP as a function of  
15 arsenite concentration in the assay. Colonies were spotted on paper after 4 hour incubation  
16 with arsenite on LB-agar plates at 37°C. Digital image was taken 10 min after adding guaiacol  
17 and hydrogen peroxide to develop colour.

18

19 Figure 5: Comparison of signal intensities after dot blot hybridization of mRNA in the six  
20 bioreporter strains expressing CCP (A) and in four bioreporter strains expressing LacZ (B)  
21 induced with three different arsenite concentrations for four hours.

22

23 Fig. 6: Schematic picture of the plasmids developed in this study. Genes are represented as  
24 arrows, drawn to proportion. Relevant restriction sites for cloning are shown in their usual  
25 abbreviations. The black vertical bars symbolize the ArsR binding sites (ABS).

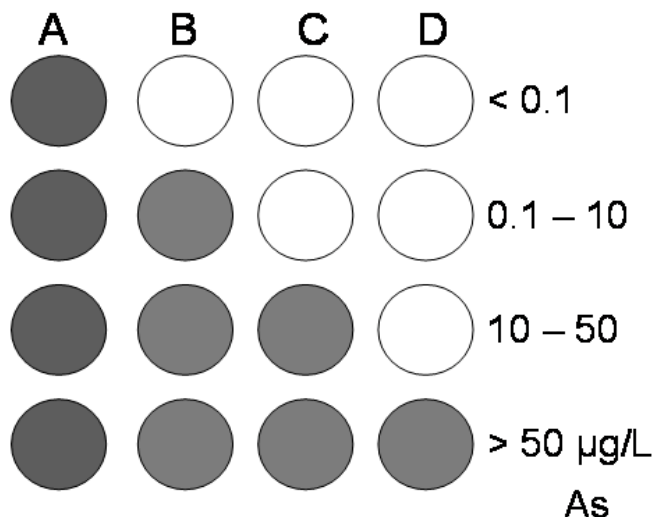
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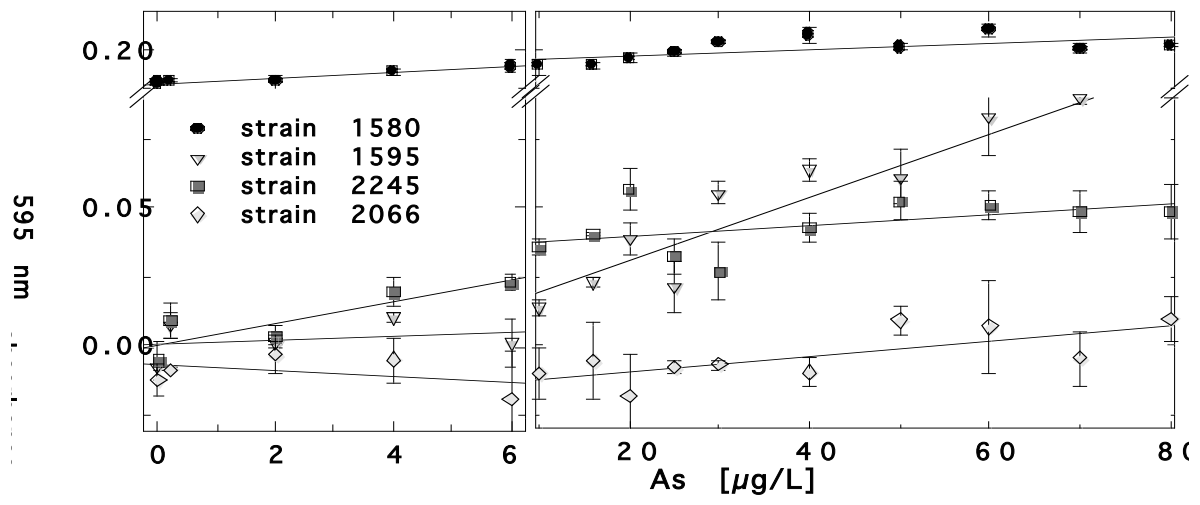


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4

Fig. 1



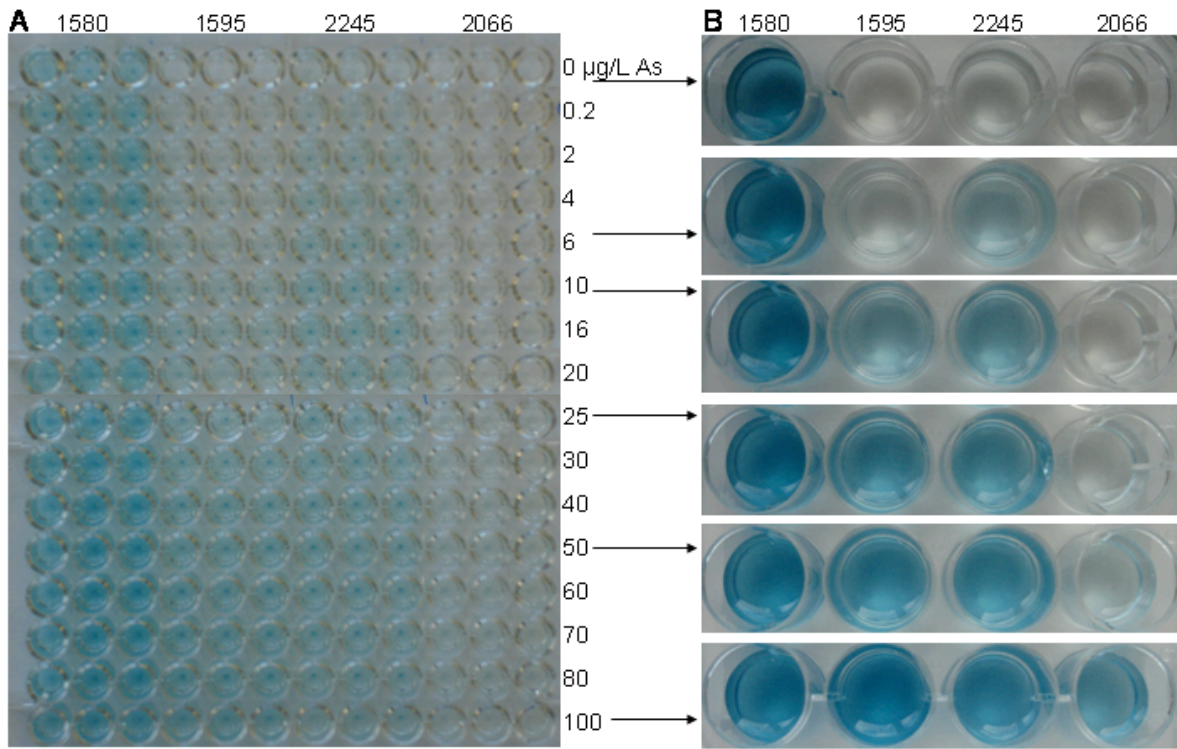
1 Figure 2



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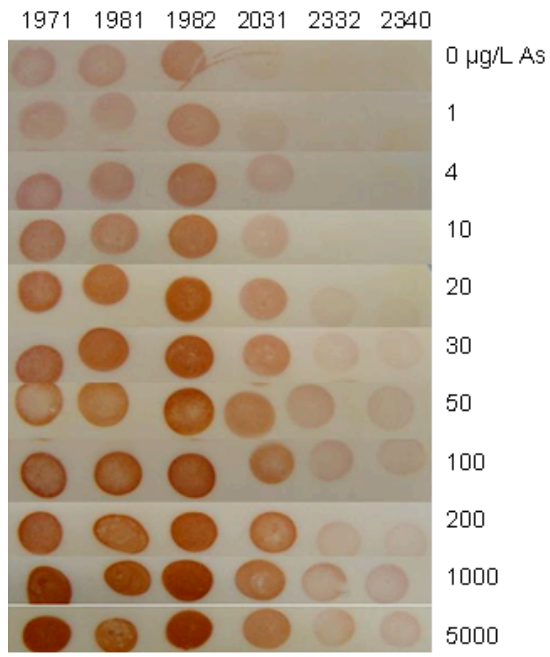
1 Figure 3



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3

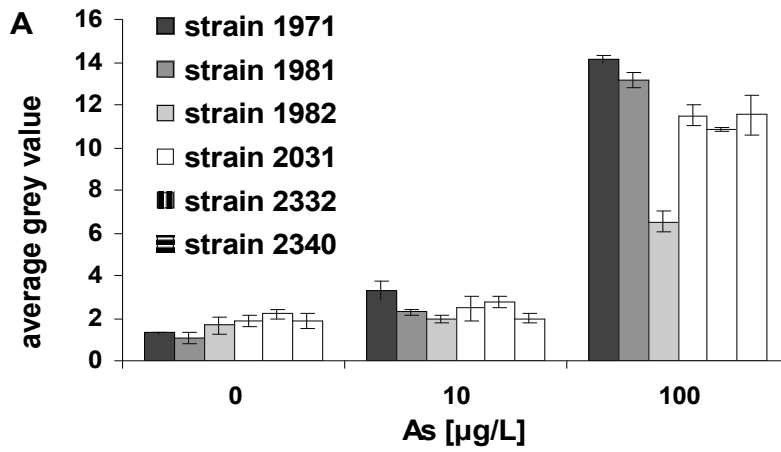
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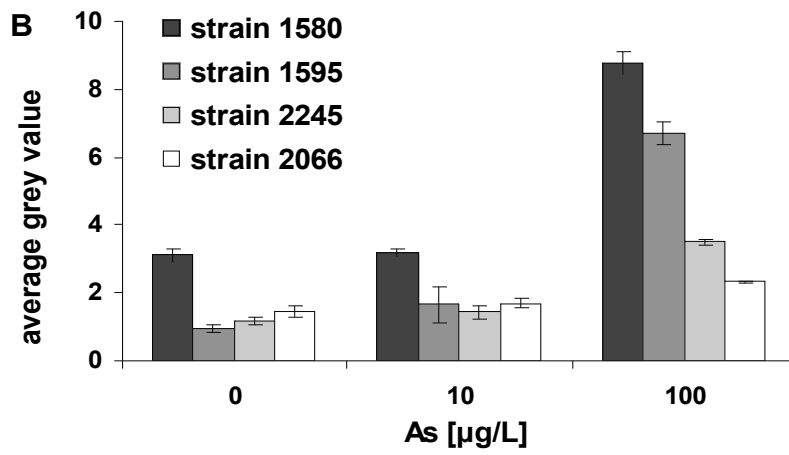
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1 Figure 5



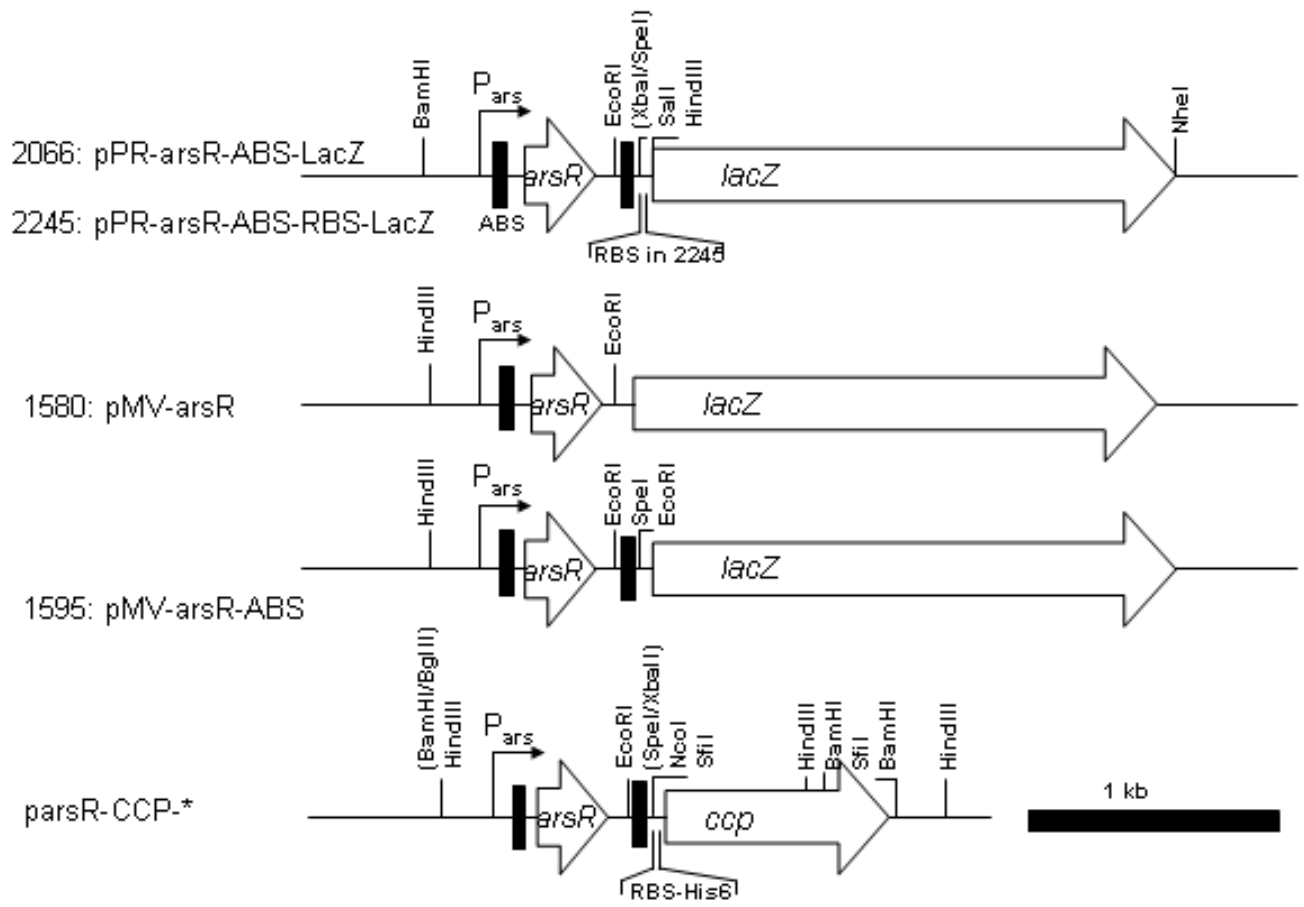
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1 Figure 6



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19  
20  
21

# 1 **Supplementary Material**

## 2 **Experimental Procedures**

3

4 **Measurement of cytochrome c peroxidase (CCP) activity in induced arsenite**  
5 **bioreporters.** The CCP bioreporters were cultured in 2 mL LB medium plus antibiotic for 16  
6 h at 37°C, diluted fifty fold and grown again until OD<sub>600</sub> of 0.5 was reached. The cultures  
7 were then incubated for 4 hours at 37°C with arsenite concentrations of 0, 10, 30, 50 and 80  
8 µg/L. After centrifugation of 1.5 ml cell culture at 4500 rpm for 5 min, cell pellets were  
9 resuspended in 1 ml of potassium phosphate buffer (50 mM, pH 6.0) and stored on ice until  
10 cell lysis. Cell lysis using sonication was performed with 30 W at intervals of 0.5 sec for 4  
11 min (Labsonic U, B. Braun Biotech International GmbH). 100 µl of this cell extract were  
12 mixed with 100 µl potassium phosphate buffer in a well of a 96-well plate. The substrate  
13 guaiacol (10 mM) and hydrogen peroxide (1.4 mM) were added and the enzyme kinetic was  
14 measured in a plate reader at 470 nm for 4 min. Measurements were conducted in triplicates.  
15 Protein content of the samples was established using the Bradford method.

16



1 **Figure Captions**

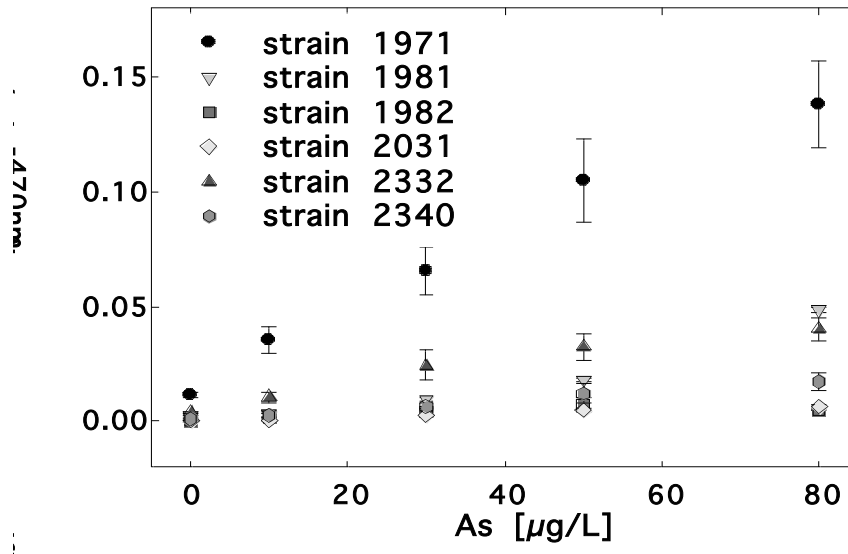
2

3 Figure S1: Specific activity of CCP in cell extracts from CCP bioreporter bacteria incubated  
4 for 4 h with five different arsenite concentrations.

5

6 Figure S2: Intervening sequences in the *arsR-lacZ* constructions between the end of *arsR* (*taa*,  
7 underlined) and the beginning of *lacZ* (*atg*, underlined). Relevant restriction sites used for  
8 cloning are indicated. The *ArsR* binding site motif is shaded in grey background. RBS,  
9 ribosome binding site. Plasmid pBGD23 is the original construct used by Scott et al, 1997.

1 Figure S1



2

1 Figure S2

2 pBGD23  
3 taaaaaatttgcctgaacatatatgttttatcaaatgcgaggatatttaagatgaaaacgtaatggatattgacccggcgatgtgttgc  
4 stop arsR rbs start arsD  
5 agcaccggcgctctgcggtacagatgttgatcagg  
6 fusion to 9<sup>th</sup> codon of lacZ BclI  
7  
8 pMV-ArsR basis pMV132 (1580)  
9 taaaaaatttgcctgaattccgatccgacaaccgatgaaagcggcgacgcgcagttaatcccacagccgccagttccgctggcggcatt  
10 stop arsR EcoRI  
11 ttaactttctttatcacacaggaacagct.atg  
12 rbs start lacZ  
13  
14 pMV-ArsR-ABS basis pMV132 (1595)  
15 taaaaaatttgcctgaattccaagttatctcacctaccttaaggtaatagtgatgattcatcatatgcggttttggttatgtgaattaat  
16 stop arsR EcoRI ArsR binding site motif  
17 cactagtgaattccgatccgacaaccgatgaaagcggcgacgcgcagttaatcccacagccgccagttccgctggcggcattttaactt  
18 SpeI EcoRI  
19 tctttatcacacaggaacagct.atg  
20 rbs start lacZ  
21  
22 pPR-ArsR-ABS-lacZ (2066)  
23 taaaaaatttgcctgaattccaagttatctcacctaccttaaggtaatagtgatgattcatcatatgcggttttggttatgtgaattaat  
24 stop arsR EcoRI ArsR binding site motif  
25 ctctagtgtcgacggaagctt.atg.acc.atg  
26 (SpeI/XbaI)SalI HindIII start lacZ  
27  
28 pPROBE/arsR-ABS-RBS-LacZ (2245)  
29 taaaaaatttgcctgaattccaagttatctcacctaccttaaggtaatagtgatgattcatcatatgcggttttggttatgtgaattaat  
30 stop arsR EcoRI ArsR binding site motif  
31 cactagagtgcactgcagaagcctgtgagcggataacaatttcacacaggaacagct.atg.acc.atg  
32 HindIII rbs lacZ start lacZ  
33