Development of a Set of Simple Bacterial Biosensors for Quantitative and Rapid Measurements of Arsenite and Arsenate in Potable Water

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Testing for arsenic pollution is commonly performed with chemical test kits of unsatisfying accuracy. Bacterial biosensors are an interesting alternative as they are easily produced, simple, and highly accurate devices. Here, we describe the development of a set of bacterial biosensors based on a nonpathogenic laboratory strain of *Escherichia coli*, the natural resistance mechanism of *E. coli* against arsenite and arsenate, and three reporter proteins: bacterial luciferase, β-galactosidase and Green Fluorescent Protein (GFP). The biosensors were genetically optimized to reduce background expression in the absence of arsenic. In calibration experiments with the biosensors and arsenite-amended potable water, arsenite concentrations at 4 μg of As/L (0.05 μM) were routinely and accurately measured. The currently most quantitative system expressed the bacterial luciferase as reporter protein, responding proportional with a concentration range between 8 and 80 μg of As/L. Sensor cells could be stored as frozen batches, resuspended in plain media, and exposed to the aqueous test sample, and light emission was measured after 30-min incubation. Field testing for arsenite was achieved with a system that contained β-galactosidase, producing a visible blue color at arsenite concentrations above 8 μg/L. For this sensor, a protocol was developed in which the sensor cells were dried on a paper strip and placed in the aqueous test solution for 30 min after which time color development was allowed to take place. The GFP sensor showed good potential for continuous rather than end point measurements. In all cases, growth of the biosensors and production of the strip test was achieved by very simple means with common growth media, and quality control of the sensors was performed by isolating the respective plasmids with the genetic constructs according to simple standard genetic technologies. Therefore, the biosensor cells and protocols may offer a realistic alternative for measuring arsenic contamination in potable water.

Introduction

Large populations in various parts of the world are using potable water contaminated with arsenic concentrations much higher than the permissible levels (for most European countries and the United States, 10 μg/L; elsewhere, 50 μg/L) (*1*). Arsenic, along with fluoride, is now recognized by the WHO as the most serious inorganic contaminant in drinking water. Systemic and chronic exposure to arsenic is known to lead to serious disorders, such as vascular diseases (Blackfoot disease and hypertension) and irritations of the skin and mucous membranes as well as dermatitis, keratosis, and melanosis. Inorganic arsenic is a human carcinogen, and ingestion of inorganic arsenic increases the risk of developing cancer of the bladder, liver, kidney, and skin (*2*). The clinical manifestations of chronic arsenic intoxication are referred to as arsenicosis. At present, there is no effective therapy for arsenicosis, and consequently, treatment involves reducing arsenic exposure and providing specific drugs for recovery and/or averting disease progression.

Most frequently, arsenic contamination is of natural origin since arsenic is a common element in the earth's crust. It occurs as a trace constituent in more than 200 minerals with an average concentration of 1.5−2 mg of As/kg (*3*). Currently, the largest case of arsenic poisoning takes place in Bangladesh (*4, 5*). In the 1970s, between 2.5 and 5 million tubewells (hand pumps) for domestic water supply and 5−6 million wells for irrigation use were installed in Bangladesh. Between 1987 and 1992, the first cases of arsenic poisoning were reported in Bangladesh. Today, it is estimated that out of 4 million tubewells, 1.12 million are affected by arsenic contamination and that between 20 and 30 million people (15−25% of the population of Bangladesh) are exposed to arsenic levels above 50 μg/L (*6*). The regional distribution of the high-arsenic waters in Bangladesh is extremely patchy and shows seasonal variation (*7*). Because of this high degree of variability, all wells must be tested separately and repeatedly for arsenic (*8*). The identification of safe tubewells is an important mitigation strategy. In Bangladesh, wells are tested and painted green if waters contain arsenic concentrations below 50 μg/L and painted red if arsenic concentrations are above 50 μg/L (*8*).

The testing of the millions of tubewells is most practically done by field test kits. Most of the field test kits currently used are based on the Gutzeit method. This involves the reduction of As(V) and As(III) by zinc to give arsine gas, which stains mercuric bromide paper (*2*). The color development on the paper disk is compared with a color scale to determine the arsenic level in the water sample. At present, there is no field test kit available that accurately measures arsenic concentrations at levels below 70 μg As/L (*8*). Recent statistical testing of several field test kits, which are all based on the mercuric bromide stain method, showed that 33% of the measurements would have to be considered false-negative, especially in the range below 70 μg As/L (*8*).

Here we describe and propose an alternative measuring strategy for arsenic detection in potable water based on whole-cell bacterial biosensors. The concept of using living bacterial cells to assess the chemical quality of water is about

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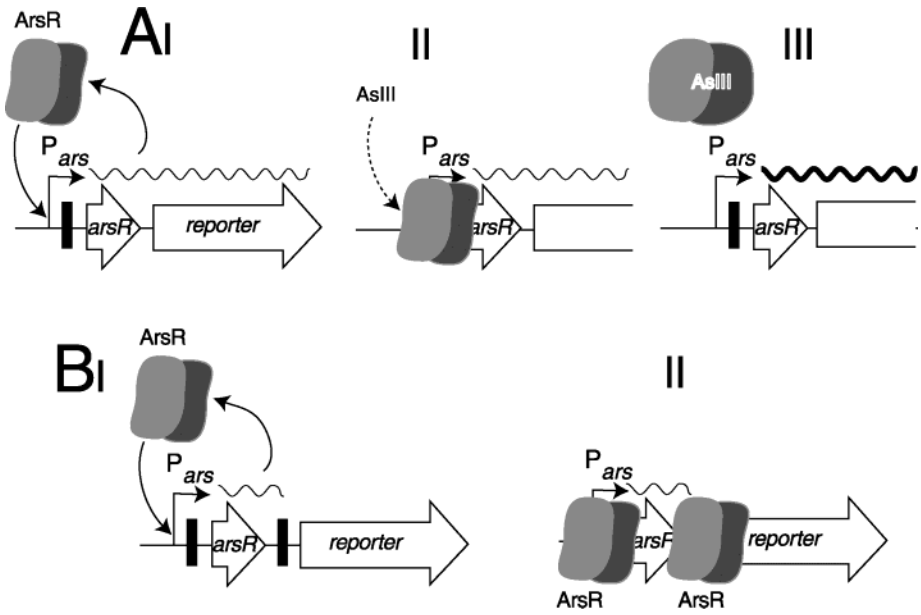

FIGURE 1. Principle of the arsenic biosensors. The *arsR* gene controls expression of the reporter gene (symbolized by arrows). In the situation with one ArsR binding site (black vertical bar), some background expression occurs from the *arsR* promoter (P_{ars}), symbolized as a wave line, which leads to the synthesis of ArsR itself (symbolized as dimer protein) and of the reporter protein. ArsR binds to its binding site (panel A_{II}) but in the presence of arsenite (As_{III}) will lose its affinity for its binding site on the DNA (panel A_{III}). In this case, repression will be relieved and transcription from the *arsR* promoter will be very high (thick wave line). In case of a secondary ArsR binding site downstream of *arsR* (panel B), only the *arsR* mRNA will be produced as background. ArsR will bind to both DNA binding sites and prevent RNA polymerase from reading the reporter gene (panel BII). In the presence of arsenite, ArsR will again lose its affinity, and expression from the reporter gene will be initiated (as in panel AIII).

10 years old but is not often applied outside research laboratories. The central measuring machinery in whole-cell living bacterial biosensors is comprised of regulatory proteins, which act as the recognition element. The sensor bacteria are genetically engineered in order to produce specific marker proteins upon recognition of the target chemical (*9*). The principle of the bacterial biosensor is rather simple, and sensor production only requires (self-sustained) growth of the microorganisms. Whole-cell living bacterial biosensors for arsenite have been described in the literature (*10*−*13*). Their detection system is mostly based on the natural resistance mechanism of bacteria against arsenate, arsenite, and antimonite. One such relatively well-studied resistance mechanism is that encoded by the *ars* operon found on the *Escherichia coli* plasmid R773 (*14*). The *ars* operon consists of a set of structural genes (*arsA*, *arsB*, and *arsC*) and two regulatory genes (*arsR* and *arsD*). ArsR controls the basal level of protein expression, and ArsD controls the maximal level of protein expression (*15*). In the absence of arsenite, the ArsR repressor binds to its operator/promotor site within the *ars* operon and prevents further expression of itself and the downstream *ars* genes (Figure 1). When arsenite enters the cell, it interacts with the ArsR repressor leading to a conformational change and dissociation of the ArsR protein from its operator. Subsequently, the *ars* genes become more highly expressed (*16*). Native bacteria containing the *ars* operon do not produce an analytical useful signal. Whole-cell living bacterial biosensors for arsenite usually contain the *arsR* gene plus the *arsR* responsive promoter fused to a marker gene coding for an easily measurable protein, like luciferase or β-galactosidase (*12*, *13*, *17*). Arsenite biosensors based on *E. coli arsR* are rather sensitive and react to arsenite concentrations at around 1 μg of As/L (*11*, *17*). One important aspect of the native ArsR regulatory system, however, hampers further development of a simple colorimetric field test, which could be most useful for field applications in developing countries. Since the promoter and operator site for ArsR-dependent gene expression are upstream of *arsR* itself, a basal level of *arsR* expression is required for the system to function or otherwise no ArsR protein would be produced. For this reason, gene fusions placed downstream of *arsR* will be subject to considerable background expression of the marker gene, making colorimetric methods unfeasible. Here we describe a simple and effective reduction of background expression by introducing a second binding site for ArsR (Figure 1). By doing so, several simple biosensor tests for the lower arsenite concentration range (5−100 μg As/L) were developed, among which was a paper strip colorimetric test.

Experimental Section

Molecular Biological Methods. All procedures for cloning, DNA manipulations, PCR, and DNA sequencing were performed according to well-described protocols (*18*).

Construction of Arsenite Biosensors. Arsenite biosensors were constructed in *E. coli* DH5α, a nonpathogenic laboratory strain used worldwide (*18*). The sensing part of all sensors was derived from the *arsR* gene from plasmid pBGD23, which contains a cloned fragment of the arsenic resistance operon from *E. coli* (R773) (*12*). By using the polymerase chain reaction, we amplified the *arsR* gene from plasmid pBGD23, excluding the *arsD* part, which is on pBGD23, but including the *arsR* promoter. As primers for the PCR, we used ArsRfor640 (5′ ccc ttt cgt ctt c*aa cgt t*tc caag 3′; 129 bp upstream of the start of *arsR* and introducing a *Hin*dIII site) and ArsRrev1120 (5′ aac ata *tga att c*ag gca aat ttt ttag 3′, covering the stop codon of *arsR* and introducing a unique *Eco*RI site). This fragment was cloned into pGEM-T-Easy (Promega Corp., Catalys AG, Wallisellen, Switzerland). For producing ArsR regulatable expression of the β-galactosidase gene (*lacZ*), the *arsR* fragment was cut out from the vector pGEM-T-Easy by digestion with *Hin*dIII and *Eco*RI and ligated with the *lacZ*-containing vector pMV132 (*19*). After transformation into *E. coli*, this produced plasmid pMV-arsR (Figure 2). To produce a system in which arsenite would lead to bioluminescence, the *arsR*-containing fragment was recovered from

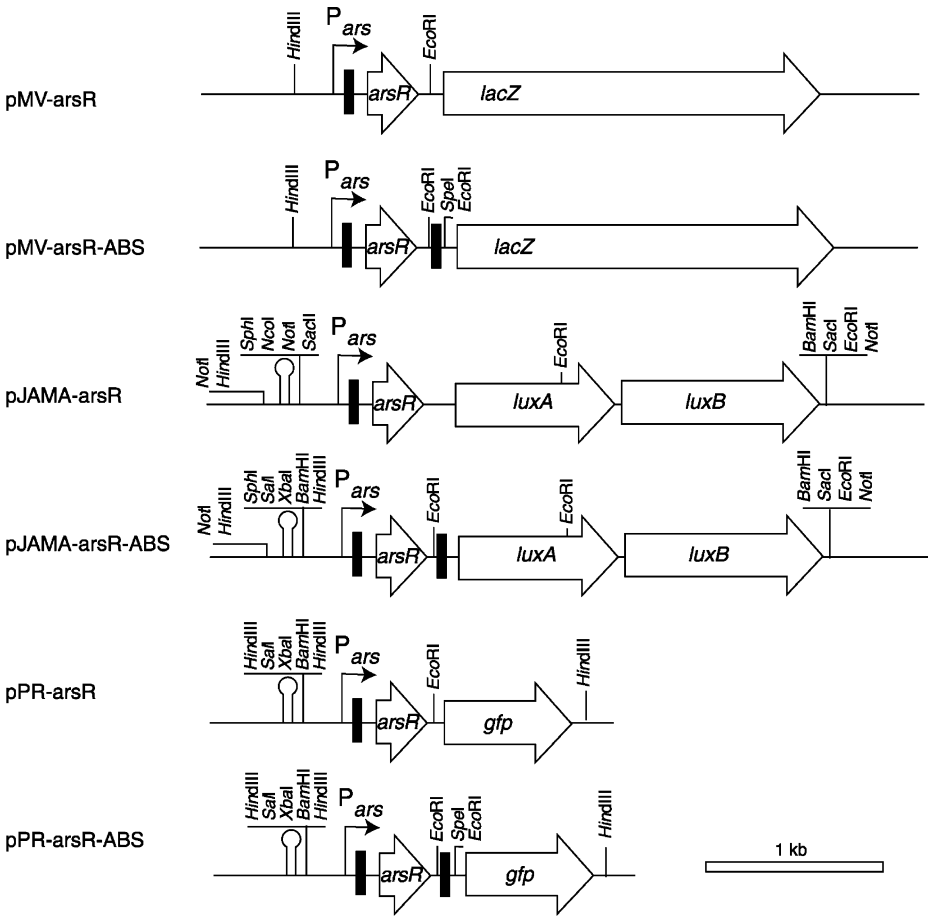

FIGURE 2. Schematic drawing of all the plasmids developed in this study. Genes are represented as arrows, drawn to proportion. Relevant restriction sites for cloning are shown in their usual abbreviations. The black vertical bars symbolize the ArsR binding sites and the "loop" structure points to a transcriptional terminator.

pGEM-T-Easy by cutting with *Sph*I and *Spe*I. The *arsR* fragment was then inserted into pJAMA8 (*6*), cut with *Sph*I and *Xba*I (yielding plasmid pJAMAarsR). pJAMA8 is a plasmid containing the promoterless luciferase genes from *Vibrio harveyi* (*20*). Finally, a plasmid was constructed in which *arsR* was fused to the gene for the Green Fluorescent Protein (*gfp*). This was done by again using the *Hin*dIII−*Eco*RI fragment with *arsR* and ligating this to the *gfp*-containing vector pPROBE (*21*) (resulting in plasmid pPR-arsR).

To reduce background expression from the *arsR* promoter, we placed a second copy of the ArsR DNA binding site between *arsR* and the respective reporter gene (Figure 2). Hereto, the ArsR binding site was amplified by the PCR with primers 001126 (5′ gaa ttc caa gtt atc tca cct acc 3′, 124 bp upstream of the start of *arsR*) and 010834 (5′ aat tca cat aac caa aaa cgc ata tga tg 3′, 52 bp upstream of *arsR*) and first cloned into pGEM-T-Easy. The 72-bp DNA fragment was then recovered by cutting with *Eco*RI. This fragment was inserted into plasmid pMV-arsR, digested with *Eco*RI fragment (yielding pMV-arsR-ABS), and inserted into pPR-arsR (yielding pPR-arsR-ABS). pJAMA-arsR-ABS was constructed by cutting pJAMA8 with *Sph*I and *Xba*I, recovering the *arsR*-ABS fragment from plasmid pPR-arsR-ABS by cutting with *Sph*I and *Spe*I and ligating both. Sequences of the *arsR*-ABS gene region are available on request.

Arsenic Measurements with the *E. coli* Luciferase Biosensors. Arsenite and arsenate in aqueous solution were measured with cell suspensions of *E. coli* (pJAMA-arsR) or *E. coli* (pJAMA-arsR-ABS). Cell stocks were prepared by inoculating one colony from a freshly grown culture on Luria-Broth (LB) agar plates with 50 μg/mL ampicillin in a 5-mL liquid LB culture with 50 μg/mL ampicillin overnight at 37 °C. The next morning, 1 mL of this culture was transferred into 50 mL of LB medium. The cells were incubated at 37 °C until a turbidity at 600 nm of between 0.5 and 0.6 was reached. The culture was then placed on ice for 15 min, and 10 mL of ice-cold sterile glycerol (87% (v/v)) was added and mixed. The mixture was kept on ice while it was divided into 0.5-mL portions in 1.5 mL-Eppendorf tubes, which were frozen in a dry ice/ethanol bath for 1 min and stored at −80 °C. For assays, a frozen portion was thawed in a water bath at 25 °C for 2 min right before starting the assay and 10-fold diluted in fresh LB medium. Assay mixtures containing 100 μL of diluted cell suspension and 100 μL of sample (or arsenite stock solution) were prepared directly in 96-well plates (Microlite1, Dynatech Industries, Inc., McLean, VA). Calibration series contained the following: 0, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 μM arsenite (end concentration). Arsenite solutions were prepared by dilution from a 1 g of As/L (13 mM) commercial stock (J. T. Baker, Deventer, The Netherlands) in tap water at pH 7. Arsenate solutions were prepared by dilution from a 1 g of As(V)/L (13 mM) stock solution ($Na_2HAsO_4 \cdot 7H_2O$, reagent grade, Sigma-Aldrich Co., St Louis, MO) in tap water at pH 7. Since the sensor cells still contain a chromosomally encoded arsenate reductase (*22*), they also react to arsenate. Use of phosphate-containing buffers was avoided since these were found to contain trace quantities of arsenic. The composition of tap water at our institute is described by Hug et al. (*23*). The 96-well plates with the assay mixtures were covered with a lid, taped with Parafilm, and incubated at 30 °C in a rotary shaker (190 rpm) for at least 30 min. Longer incubation times (1−1.5 h) resulted in stronger

light emission and slightly better determination of arsenite at low concentrations (at 0.1 μM and below).

After incubation, 25 μL of a decanal substrate solution (2 mM in 50% v/v ethanol in water) was added, mixed, and incubated for 3 min, after which the light emission was measured at 30 °C (Microlumat LB960 luminometer, Berthold). Light emission was integrated during 10 s. Arsenite concentrations in unknown samples were interpolated from the calibration curve.

Arsenite Measurements with the GFP Biosensors. *E. coli* DH5α (pPR-*arsR*) or *E. coli* DH5α (pPR-*arsR*-ABS) were freshly plated on LB agar plus 50 μg/mL kanamycin sulfate. One colony was inoculated in a 5-mL LB culture with 50 μg/mL kanamycin sulfate for 16 h at 37 °C at 200 rpm. The overnight culture was diluted 1:50 in sterile LB with kanamycin.

Assay mixtures contained 0.5 mL of diluted cell suspension and 0.5 mL of sample (or arsenite stock solution, containing 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.5, or 5.0 μM arsenite). Assays were incubated at 30 °C in a rotary shaker (190 rpm). For GFP measurements, culture samples of 200 μL (after 1 h) and 100 μL (after 2−5 h) were centrifuged for 1 min at 15000*g*, and the supernatant decanted. The cell pellet was resuspended in an appropriate amount of M9 minimal medium (between 10 and 50 μL, depending on the amount of cells). One microliter of cell suspension was transferred to a microscope slide, and the fluorescence of individual cells was determined by epifluorescence microscopy, as described elsewhere (*24*).

Arsenite Measurements with the *lacZ* Biosensors. The biosensor strains *E. coli* (pMV-arsR) and *E. coli* (pMV-arsR-ABS) were mainly used for developing a semiquantitative strip test. However, fully quantitative measurements can be performed with the same strains using spectrophotometric, chemiluminescence, or electrochemical detection of β-galactosidase activity (*12*, *17*). For use on paper strips, the cell cultures were cultivated from one colony from a freshly grown LB plate plus 100 μg/mL ampicillin in 25 mL of LB plus 100 μg/mL ampicillin for 16 h at 37 °C with shaking at 200 rpm. Cells in the culture were collected by centrifugation at 3500*g* and room temperature for 6 min. All traces of supernatant were removed, and the cells were carefully resuspended in 1 mL of sterile preheated (37 °C) drying protectant solution (i.e., 0.5% (w/v) peptone, 0.3% meat extract, 10% gelatine, 1% sodium ascorbate, 5% raffinose, and 5% sodium glutamate, dissolved in tap water) according to Malik (*25*). Pieces of 0.5 × 4 cm 3M paper were cut, and 5 μL of the cell suspension was placed at spots premarked with a pencil. Strips were dried for 5 min in a laminar flow cabinet, placed with up to 10 paper strips in a sterile 12-mL polypropylene tube (uncapped), and then subsequently dried at controlled temperature (20 °C) and vacuum in a lyophilizer (Alpha1-4, Christ AG, Germany). The following drying program was applied: 4 mbar for 2 h, 0.4 mbar for 2 h, 0.04 mbar for 2 h. Tests were performed by placing a strip in a 10-mL polypropylene vial with 1 mL of test solution or arsenite stock solution (end concentrations 0, 0.05, 0.1, 0.2, and 0.5 μM; diluted from a 1 g/L stock in tap water). Incubation was done for 30 min at 30 °C without shaking. Afterward, the strips were taken out and kept between plastic wrap to prevent drying, and 5 μL of X-gal substrate solution (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 1 mg/mL in H_2O) was pipetted on the bacterial spot. Color development was carried out for 30 min or longer, and the blue color was compared to that of spots from a simultaneous incubation with a series of known arsenite concentrations.

Results and Discussion

Quantitative Measurements with the Luciferase Sensor. As described by others, good and reproducible arsenite measurements can be performed with genetically engineered bacterial cells, which produce a marker protein under control of the ArsR regulatable promoter (*10*, *12*, *17*). Here we reengineered the plasmid and the bacterial strain to remove undesired parts of the *arsR* operon and to avoid any background expression of the reporter gene. For laboratory use, cells were cultivated on a common, rich growth medium (LB) with ampicillin, aliquoted after mixing with glycerol, and stored at −80 °C. Biosensor stocks prepared under these conditions stayed viable for at least 2 yr without severe loss of activity. At this point, we decided to use a plasmid with the genetic construct in *E. coli* because of the ease of

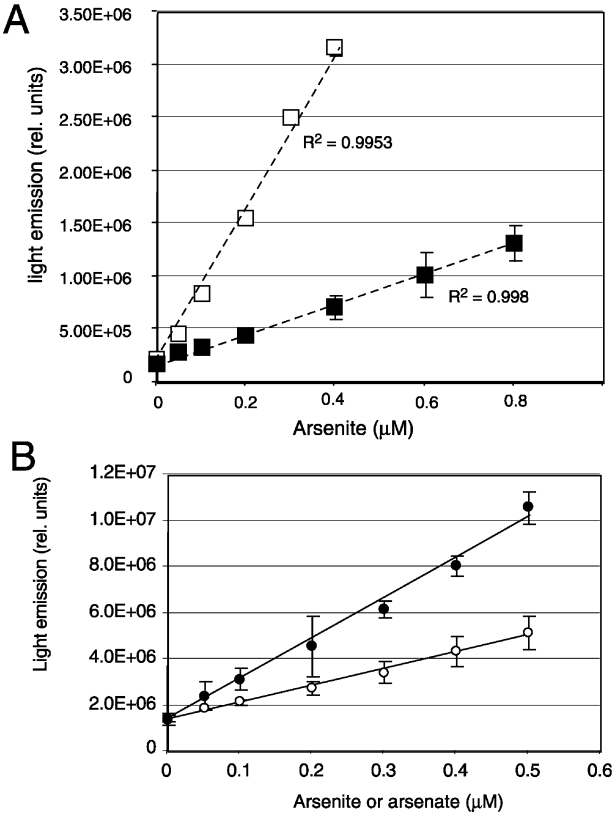

FIGURE 3. (A) Light emission from the *E. coli* (pJAMA-arsR) (open squares) and *E. coli* (pJAMA-arsR-ABS) (closed squares) strains. Contact time and incubation conditions were 1 h at 30 °C in tap water with diluted LB (see Experimental Section). R^2 values result from linear interpolation of the averages from three replicates. Error bars not visible were smaller than the size of the used symbol. (B) Light emission from the *E. coli* (pJAMA-arsR) biosensor in the presence of the same concentrations of arsenite (closed circles) or arsenate (open circles). Contact time and incubation conditions were 1 h at 30 °C. Data points represent the average of three replicates.

TABLE 1. Average Deviation of Linearly Interpolated Arsenite Concentrations from Experimentally Established Concentrations in the Luciferase Assay with *E. coli* (pJAMA8-arsR)

exptl arsenite concn (μM)	calcd according to trendline (μM)	deviation (%)	avg deviation in measurement (%)
0	0.014	nv[a]	3.0
0.05	0.044	12	0.6
0.10	0.095	5	8.5
0.20	0.188	6	1.7
0.30	0.313	4.3	1.0
0.40	0.40	0	4.5

[a] nv, no value.

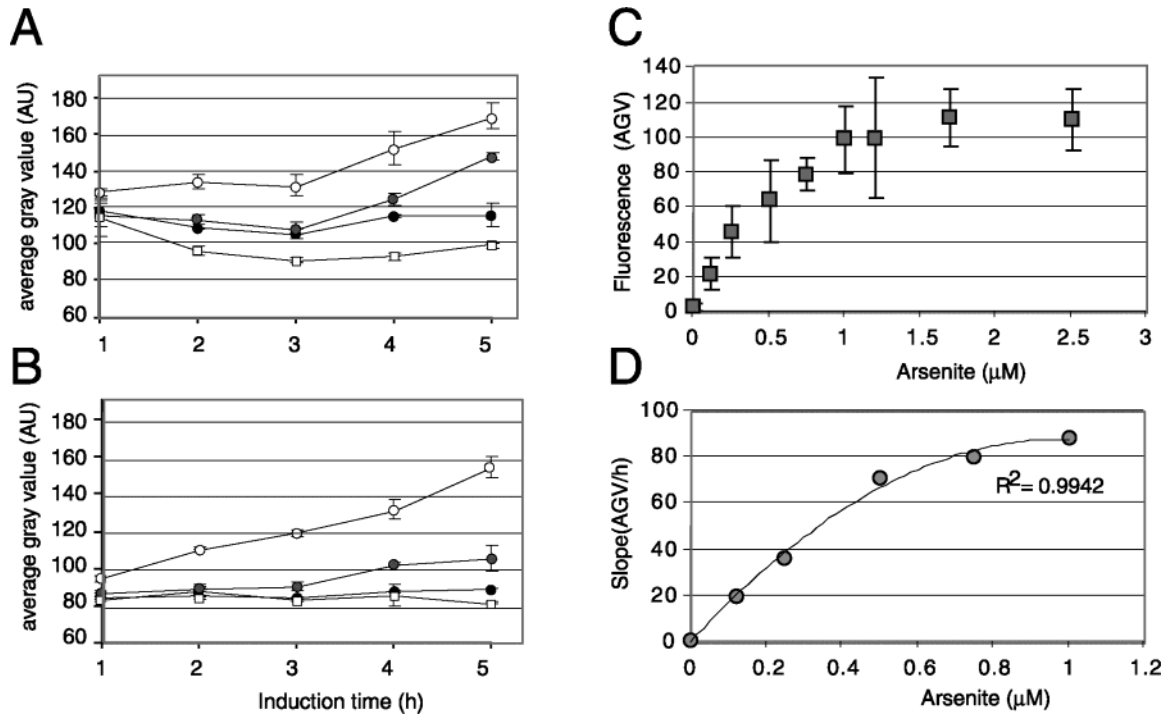

FIGURE 4. Arsenite measurements with the *E. coli* (pPR-arsR) (A) and *E. coli* (pPR-arsR-ABS) strains (B). Shown are the average fluorescence values (here represented as average gray value, the value given by the camera system) of populations of single cells in time and at different arsenite end concentrations in the assay (open squares, 0 μM; black circles, 0.25 μM; gray circles, 0.5 μM; open circles, 2.5 μM). An average gray value of 80 corresponds to dark cells and is an arbitrary value given by the camera system. Exposure time of fluorescence detection in panels A and B is 100 ms. (C) Average fluorescence of cells of *E. coli* (pPR-arsR-ABS) incubated with different arsenite concentrations after a 1-h incubation time at 30 °C and a 1-s exposure time. In this case, the background value of the camera system (at 1-s exposure, 90 units) was subtracted from the gray values of the cells. Error bars indicate the calculated average deviation from the average gray value for the whole measured population of cells (at least 1000 cells). Experiments were repeated three times with similar results. (D) Fluorescence increase rate of GFP expression under control of the *arsR* system in *E. coli* (pPR-arsR-ABS) at different arsenite concentrations. Graph data correspond to those presented in Table 2 and derive from linear regression of the average fluorescence values at each particular arsenite concentration sampled at 1−5 h of incubation. The fluorescence increase rate can be represented by a hyperbolic curve with the parameters: slope = $-90.856 \cdot [\text{arsenite}]^2 + 178.69 \cdot [\text{arsenite}] + 0.0278$.

determining the correctness of the genetic construction. However, more advanced biosensors may be engineered that carry the genetic constructs on their chromosome to increase genetic stability of the biosensor cells and to avoid incorporation of an antibiotic resistance gene.

Biosensor assays with the luciferase sensor cells were carried out directly in 96-well plates for use in a luminometer. The use of 96-well plates is desirable since standard calibrations with known arsenite concentrations can be incubated simultaneously. A minimum incubation time of the cells with the arsenite samples of 30 min was necessary in order to obtain a reproducible signal. However, any time up to 2 h can be chosen (data not shown). It should be noted that since we are working with living systems, continuing incubation of the cells with the sample will lead to increased gene expression, and thus, absolute light output values (or fluorescence and β-galactosidase values) are meaningless descriptors for arsenite concentration. Values produced by biosensors can only be compared to standards with known arsenic concentrations carried out under the same conditions. Therefore, either standardized incubations should be performed or at least two arsenite concentrations from a calibration curve should be measured along with the samples.

At an induction time of 1 h at 30 °C, a good linear response of the sensor cells to arsenite (in tap water) occurred between 0 and 0.5 μM (0 and 39 μg of As/L) (Figure 3). The lowest routinely measured concentration with 2-fold higher light output than that of the blank was 0.05 μM (4 μg of As/L). Since the samples in our assays are diluted 2-fold (due to addition of the cells), the effective concentration range for measuring arsenite was between 8 and 78 μg of As/L. At higher concentrations than 0.8 μM, the light output no longer increased linearly, and the sample will have to be diluted. For samples with unknown arsenite content, it is therefore advisable to prepare a 10−100-fold dilution. Engineering the second ArsR binding site in front of the *luxAB* genes resulted in a slightly lower (not significant in a pairwise Student's *t*-test) background luciferase activity and a 3−5-fold lower response to the same arsenite concentration (Figure 3a, closed squares). The standard error produced by using a linear trend line for fitting the luciferase measurements at arsenite concentrations around 0.05 μM was on the order of 10% (Table 1). For this particular calibration experiment, the average deviation of the luciferase output at the 0.05 μM

TABLE 2. Arsenite Measurements Using Increase of Fluorescence Rates by Cells of *E. coli* (pPR-arsR-ABS)[a]

exptl arsenite concn (μM)	slope (AGV/h)	R^2	calcd concn (μM)
0	0.66	nv	0.005
0.12	20.1	0.92	0.12
0.25	36.4	0.998	0.23
0.50	71	0.917	0.55
0.75	79.8	0.887	0.68
1.0	88.7	0.983	1

[a] Slope linearly interpolated from averages from triplicate incubations, for every hour during 5 h. Concentration calculated from hyperbolic slope vs arsenite concentration curve ($y = -90.856x^2 + 178.69x + 0.0278$; $R^2 = 0.9942$) obtained with SigmaPlot. nv, no value.

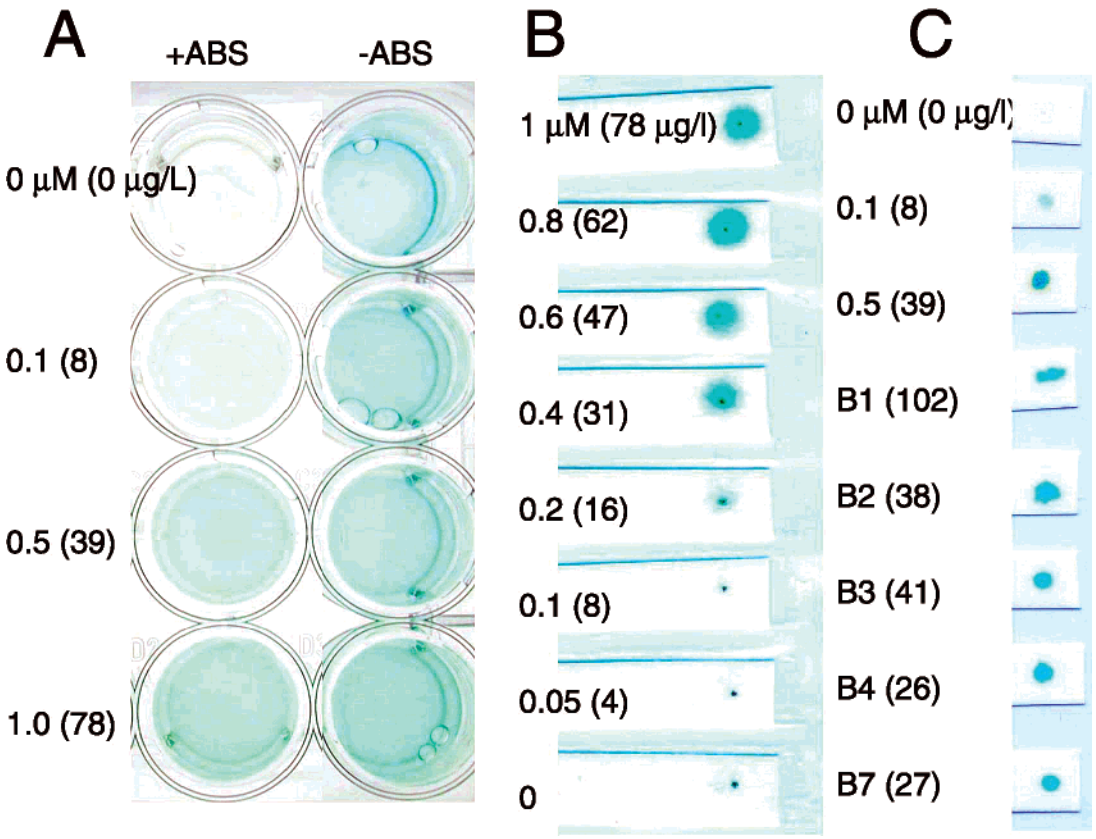

FIGURE 5. Illustrations of decolorization of X-Gal by the *E. coli* (pMV-arsR) and *E. coli* (pMV-arsR-ABS) sensor cells incubated with different arsenite concentrations. (a) Titerplate assay with resuspended cells of both β-galactosidase expressing sensors *E. coli* (pMV-arsR) (without the extra ArsR binding site) or *E. coli* (pMV-arsR-ABS) (with the second ArsR binding site). Cells were incubated for 30 min at 30 °C with the arsenite. Color development with X-Gal proceeded for 30 min, after which the digital image was taken. (B) Decolorization of X-Gal by dried and incubated cells of *E. coli* (pMV-arsR-ABS) on paper strips at different arsenite concentrations. Paper strips (stored for 2 weeks at 4 °C) were incubated for 30 min at 30 °C with the arsenite-containing solution, after which X-Gal was added, and the color was allowed to develop for another 30 min at room temperature (20 °C). (C) Paper strip tests with arsenite standards and five samples from Bangladesh groundwater were transported to our laboratory. Total arsenic concentration (μg of As/L) of the Bangladesh samples measured by atomic fluorescence spectroscopy (Stephan Hug, personal communication) is indicated in parentheses.

concentration was 0.6%. However, for the other concentrations tested, the standard error introduced by using a linear trend line was on the order of the average deviation from the mean calculated from triplicate measurements (5−10%).

Since the biosensor cells contain the arsenate reductase enzyme, they also respond to arsenate. However, when the same batch of cells was tested with arsenite and arsenate in the same concentration range, the cells responded with an approximately 2-fold lower light output for arsenate than for arsenite (Figure 3b). The reason for this difference may be that the rate of arsenite production from arsenate by the arsenate reductase does not follow the same kinetics as the interaction of arsenite with ArsR or the efflux rate.

Arsenite Measurements with the GFP Biosensor. The GFP biosensor was developed in order to avoid the use of substrates that are needed for measuring the activities of the luciferase or β-galactosidase. The GFP needs no cofactors or substrates for its fluorescence, and exposure to oxygen leads to spontaneous maturation of the fluorophore in the cell. Despite our relatively time-consuming analysis of the GFP fluorescence levels in single cells of *E. coli* (pPR-arsR) or *E. coli* (pPR-arsR-ABS), an increasing fluorescence of the cells was observed in response to increasing levels of arsenite in solution (Figure 4). The sensitivity of the GFP biosensor to arsenite was less than that of the luciferase sensor, with a higher level of detection of 0.1 μM. At arsenite concentrations above 1 μM, no further increase of the fluorescence level of individual cells was observed. However, by varying the camera exposure time and induction time, this upper range of detection could be increased to 2.5 μM (not shown). Introduction of the second ArsR binding site in front of the *gfp* gene clearly reduced the background level of the cells (a level of 81 units corresponds to dark cells) and improved the induction factors observed at the different concentrations (Figure 4a,b). Measuring with GFP fluorescence theoretically allows the use of rate of fluorescence increase per cell as a valid determinant for the arsenite concentration rather than the "end point" fluorescence level after a fixed incubation period. This was demonstrated by measuring cellular GFP fluorescence levels at different incubation periods and arsenite concentrations. The rate of fluorescence increase between 0 and 5 h could be approximated by linear interpolation (Table 2). When these rates of fluorescence increase were subsequently plotted against arsenite concentration (Figure 4d), an approximate linear range was found between 0.1 and 0.6 μM and an overall fitting by a hyperbolic curve (Table 2). When using the measured fluorescence increase rates in the hyperbolic equation, arsenite concentrations within 10% of the experimentally added level were predicted by the biosensor response, which is within the acceptable limits for analytical measurements.

Development of a Colorimetric Semiquantitative Paper Strip Biosensor Test. Arsenite measurements performed with bacterial biosensors that employ β-galatosidase have been reporter using chemiluminescence and electrochemical detection (*12*, *13*). However, both systems require the use of expensive instrumentation, jampering field applications. At a much simpler form, the *E. coli* arsenite biosensor cells could

be used to produce a color in response to arsenite. For this purpose, β-galactosidase would be a useful reporter protein since many different substrates are available for this enzyme, which will be converted to insoluble colored products. Unfortunately, also the arsenite sensor using *arsR* transcriptionally fused to *lacZ* produced too high background levels of β-galactosidase in the absence of arsenite (Figure 5a). Therefore, we again engineered a second binding site for the ArsR protein downstream of *arsR* and in front of *lacZ*. As for the GFP sensor, the addition of a second binding site in front of *lacZ* effectively reduced the background expression of β-galactosidase (Figure 5a). This allowed the use of the substrate X-Gal to assess β-galactosidase levels produced intracellularly by the sensor cells. As can be seen from Figure 5a, increasing shades of blue arise at increasing arsenite concentrations to which the cells have been exposed. Although the intensity of the blue color can be measured spectrophotometrically, producing similar calibration curves as shown before for luciferase or GFP, the biosensor cells expressing β-galactosidase could also be used on a solid medium for a qualitative strip test. Hereto small batches, containing approximately 10^7 cells, were dried on a paper matrix. When the paper strips were placed for 30 min in a tube with 1 mL of aqueous sample containing arsenite, then taken out, and the substrate X-Gal added, blue spots appeared, the intensity of which was proportional to the arsenite concentration. At the amount of biosensor cells used here and after an incubation time of 30 min, the blue color was barely visible at an arsenite concentration around 10 μg/L. This was designed in order to create the subjective interpretation of a "negative" test outcome at arsenite levels at or below the current drinking water limits of 10 μg of As/L. However, at any case, two defined arsenite concentrations should be taken along in each series of measurements (Figure 5c). As expected, the qualitative paper strip under these measuring conditions could not differentiate arsenite concentrations higher than 0.3 or 0.4 μM (Figure 5b). When tested with five water samples transported from Bangladesh to our institute, the paper strip correctly scored all five as "above" the 10 μg/L standard.

In our protocol of paper strip production, we were able to store the dried cells for 2 months at −20, 4, or 30 °C without noticeable loss of immediate β-galactosidase induction in the assay. On the other hand, since the cells were only dried on the paper matrix but not immobilized, a proportion of the cells resuspended from the strip into the test solution. Further development would be needed to completely immobilize the cells or contain them on the test strip (*26*).

Our work has shown that it is possible to develop very simple but effective biological tools to quantitatively and qualitatively measure arsenite and arsenate in aqueous solutions, even at concentrations below the current drinking water limit of 10 μg of As/L. Analyses with bacterial biosensors are different from regular analytical tools in the sense that the biosensors only give a meaningful signal within a limited concentration range, that the absolute signal value with unknown aqueous samples can only be related to values derived from simultaneously run standard series, and that the biosensors can be disturbed by the presence of toxic compounds. Despite this, when standardized protocols for induction time and amounts of cells per assay are followed, the measurements and even absolute response values are quite reliable. Quantitative measurements of arsenite can be made with the luciferase-based biosensors in the range that is most significant for environmental purposes (5−100 μg of As/L). The high sensitivity of the biosensor cells for arsenite has the additional advantage that unknown samples can be diluted, thereby also diluting possible inhibitory compounds. Qualitative measurements can be performed with the paper strip method, which could facilitate faster identification of samples containing arsenic concentrations substantially above drinking water standards and which subsequently could be analyzed in more detail. Furthermore, the paper strip method could also be used to test the effectiveness of local treatment methods, for instance those targeting the precipitation of arsenic with iron (*23*).

Despite their potential (*9*), bacterial whole cell biosensor methods have not received great interest for practical applications until now and have remained mostly research laboratory-based methods. Mainly, this is because the physiological activity and maintenance of the microorganisms can be somewhat difficult to control. On the other hand, reproduction of the biological part of the sensor device is self-sustained and, therefore, relatively easy to achieve. We have shown here that a nonpathogenic biosensor strain of *E. coli* can be controlled and maintained without great problems. These systems could be the basis for either larger scale production of such sensor devices as the paper strips or for local maintenance and production in quality control laboratories and hospitals, especially in developing countries, where the need to measure arsenic is most urgent.

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