Bioluminescent bioreporter for assessment of arsenic contamination in water samples of India

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In the present study the most efficient *R*-factor controlling the *ars* operon was selected after screening of 39 *Escherichia coli* isolates by minimum inhibitory concentration test (MIC) studies from water samples of different geographical locations of India. Among all, strain isolated from Hooghly River (West Bengal) was found to have maximum tolerance towards arsenic and was further used for the development of bioreporter bacteria. Cloning of the *ars* regulatory element along with operator-promotor and *luxCDABE* from *Photobacteria* into expression vector has been accomplished by following recombinant DNA protocols. The bioreporter sensor system developed in this study can measure the estimated range of 0.74–60 μg of As/L and is both specific and selective for sensing bioavailable As. The constructed bacterial biosensor was further used for the determination of arsenic ion concentration in different environmental samples of India.

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1. Introduction

The first report of widespread environmental problems with arsenic (As) involved the leaching of the metal from mine tailings in Australia, Canada, Mexico, Thailand, the United Kingdom and the United States. Later, As-contaminated aquifers were reported in Argentina, Bangladesh, Cambodia, Chile, China, Ghana, Hungary, inner Mongolia, Mexico, Nepal, New Zealand, Philippines, Taiwan, the United States and Vietnam (Berg *et al.* 2001; Wilson 2002). Consumption of water from these naturally contaminated aquifers led to chronic As poisoning in many of these locations, with perhaps the worst cases in Bangladesh. The pandemic of arsenic pollution due to contaminated groundwater has also been reported from various parts of India like West Bengal, middle Ganga Plain in Bihar (Chakraborti *et al.* 2003), Uttar Pradesh, Jharkhand, several regions of Madhya Pradesh, Chhattisgarh and Assam.

Microorganisms particularly bacteria found in polluted waters and landfills have an array of resistance mechanism against the toxic effects of arsenic and its compounds by efflux, complexation or reduction, enzymatic conversion or intra- or extracellular sequestration and exclusion by a permeability barrier. The most widespread, arsenic resistance mechanism in microorganisms is the extrusion from the cells by the function of *ars* (arsenic resistance) gene system, popularly known as *ars* operon. *ars* operons are found in both gram-positive and gram-negative bacteria. The best studied and characterized arsenical system in bacteria is from the conjugative resistance factor R773 of *Escherichia coli.* It confers resistance against arsenate, arsenite and antimony with the expression of five gene system. The *ars* gene cluster consists of *arsR*-regulatory gene, *arsB* encoding for an arsenite-specific transmembrane pump and *arsC* encoding for an arsenate reductase (Jackson and Dugas 2003). The toxic effect of arsenic is reduced by the action of the ArsB, which is a membrane protein that pumps arsenite out of the cell; however, arsenate must be first reduced to arsenite by the soluble arsenate reductase ArsC. *arsR* codes for a repressor protein that regulates *ars* operon expression. ArsR controls the basal level of protein expression. 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. When arsenite enters the cell, it interacts with the ArsR repressor,

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leading to a conformational change and dissociation of the ArsR protein from its operator. Subsequently, the *ars* genes become more highly expressed (Xu *et al.* 1998). *arsA* produces an oxyanion-stimulated ATPase which couples ATP hydrolysis, thereby extruding arsenicals and antimonials by the action of ArsB protein; another regulatory protein ArsD is also identified in various arsenic-resistant species which are encoded by *arsD*. ArsD controls the upper/maximal level of protein expression by means of controlling the overexpression of ArsB, which itself is toxic to cells.

High toxicity and widespread occurrence has created a pressing need for effective monitoring and measurement of arsenic in soil and water. The detection of chemical species relies on classical methods such as atomic absorption spectrometry, cold vapour atomic fluorescence detection and inductively coupled plasma mass spectrometry due to their specificity and sensitivity. However, such methods might not distinguish pollutants that are available to biological systems from those that exist in the environment in the biologically inert forms. This is a particular concern with the toxic metals and metalloids. Recognition of this limitation has stimulated researchers for the use of *whole-cell bacterial biosensors,* consisting of genetically engineered bacteria containing a contaminant-sensing gene capable of detecting the presence of an analyte, coupled with a reporter gene capable of producing a detectable response gain of paramount significance. Numerous nonspecific microbial whole-cell sensors have been developed which react to nearly any kind of toxic substance (Karube and Nakanishi 1994). Sensor bacteria in which the promoter-reporter gene concept is operable have been developed to detect mercury (Selifonova *et al.* 1993; Virta *et al.* 1995), xenobiotic compounds (King *et al.* 1990; Applegate *et al.* 1998) and other heavy metals (Reither *et al.* 2001).

Luciferase genes are widely used reporter genes in prokaryotic as well as eukaryotic systems, because they provide the sensitive and simple detection of gene expression and regulation. The quantification of light emission, i.e. bioluminescence, is one of the most sensitive means of detection, and it can be measured with a liquid scintillation counter or a luminometer (Gordons *et al.* 1992), or even with X-ray film. The most commonly used luciferases are the firefly luciferase (*Photinus pyralis*) and the bacterial luciferases of *Vibrio harveyi* and *Vibrio fischeri*. The bacterial luciferases catalyse a reaction that involves the oxidation of a long-chain fatty aldehyde and flavin mononucleotide.

In the present study, we described the design of genetically modified bacteria and its use in quantification of bioavailable As ions, which contain the sensing element (*arsR*) along with operator/promotor of *ars* operon from an wild-type environmental *E. coli* isolate controlling the expression of structural genes of *lux* operon from *Vibrio fischeri*. The isolation of promoters with varying sensitivities could serve for development of bioreporters with improved detection capabilities because it might be possible that strongest promoter selected can detect the higher (above permissible limits, 50 ppb) as well as the lower concentration (below 10 ppb) of arsenic, so that effective removal measures can be adapted according to the severity of contamination. When these bacterial cells were exposed to varying concentrations of arsenic ions, the various proteins will be expressed producing bioluminescence. The emitted light can be monitored and correlated to the arsenic ions present in the water samples. Whole-cell biosensors can thus be very useful and efficient alternative for the detection and quantification of compounds not only of environmental importance but also of clinical and industrial relevance (Selifonova *et al.* 1993; Ramanathan *et al.* 1997).

2. Materials and methods

2.1 *Bacterial strains and vector*

Bacterial strains initially used in this study were the 39 wild-type, arsenic-resistant *E. coli* isolates were collected from different geographical regions of India as well as from Bangladesh, viz. Yamuna River, Hindon River and Hooghly River. *Vibrio fischeri* was a gift from Karen L Visick (Loyola University, Chicago, USA). DH5α (*E. coli*) strains were used for transformation studies. Expression vector pET 28a (Novagen) was used for cloning.

E. coli strains were grown on LA by streak culture method followed by incubation at 37°C for 18 h. Broth cultures were prepared by inoculating a single isolated colony in LB with above incubation conditions at 125 rpm. *Vibrio fischeri* was obtained as stab culture and was grown on Photobacterium agar (Sambrook and Russell 2001) with the incubation conditions of 18 h at 30°C. Liquid culture was grown on LB broth and Photobacterium broth supplemented with 25 µg/mL of chloramphenicol at same conditions with 125 rpm.

2.2 *Chemicals and reagents*

$NaAsO_2$ (sodium arsenite), Na_2HAsO_4 (sodium arsenate), pyruvic acid, NaH_2PO_4 , K_2HPO_4 , and $(NH_4)_2SO_4$ were obtained from Sigma-Aldrich (USA); growth mediums – LB Broth, LA, LB, Eosin Methylene Blue Agar (EMB), Photobacterium Agar, Photobacterium broth and enzyme proteinase K – were purchased from Himedia (India); Hi Assorted Biochemical Test Kit for gram-negative rods were procured from Himedia (India); DNA-modifying enzymes, Vent DNA polymerase and 1 kb DNA ruler were from New England Biolabs; glucose, isoamyl alcohol and chloroform, etc., were purchased from Qualigens (India). Low-range

DNA ruler marker bought from Banglore Genei, India. All chemicals were of analytical grade.

2.3 *Tolerance range of E. coli isolates towards arsenic*

The tolerance range of *E. coli* isolates towards arsenic was determined by growing the strains in different concentration of $NaAsO_2$ in liquid media. The minimal inhibitory concentration was determined as the lowest concentration that did not allow bacterial growth.

2.4 *DNA isolation*

Plasmid DNA of the highest arsenic-resistant strain isolated from Hooghly River water sample (namely, ARP-2) was isolated by the alkaline lysis method (Birnboim and Doly 1979) and was subjected to gel electrophoresis. Genomic DNA of *V. fischeri* was isolated by phenol chloroform isoamyl (PCI) method (Surzycki 2000).

2.5 *Construction of recombinant bacteria*

Plasmid pASPW2 is the expression vector constructed in which bacterial luciferase (*luxCDABE*) expression is controlled by the *ars* promoter from arsenic-resistant environmental *E. coli* isolate. Sensor plasmid was constructed by standard recombinant-DNA techniques as follows. The *arsR* gene and promoter/operator of the *ars* operon were isolated by PCR from the DNA isolated from ARP-2; gene-specific primers were designed according to already published and characterized gene sequence of plasmid R773 (accession number X16045), and the *Mlu*I and *Nco*I restriction sites were generated with the following oligonucleotide primers:

Forward: 5′-*CGA***CGCGT***GAATTCCAAGTTATCTCACCT*-3′
Reverse: 5′-*CATG***CCATGG***TTAGATGCAGACAGCCTTACT*-3′
(The *Mlu*I and *Nco*I restriction sites, respectively, are in bold-faced type, and the bases corresponding to the *arsR* gene are italicized).

PCR product (*op-arsR*) was taken and purified with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Both purified *op-arsR* amplicons and vector pET 28a were digested with the restriction enzymes *Mlu*I and *Nco*I. The resulting fragments were purified from an agarose gel by QIAquick gel extraction kit (Qiagen GmbH). The fragments were ligated and transformed into competent DH5α cells by electroporation (Sambrook and Russell 2001).

Next, the amplification of *luxCDABE* (5.7 kbp) was carried out by designing gene specific primers by NCBI tool (BLAST) after aligning with *Vibrio fischeri* (accession number AF170104). Restriction sites *Bam*HI and *Not*I was created in the forward and reverse primers respectively.

Forward: 5′-*CG***GGATCC***ATGAATAAATGTATTCCAATG*-3′
Reverse: 5′-*AAGGAAAAAA***GCGGCCGC***ATCCTTGATATTCTTTTGTATG*-3′
(The *BamH*I and *Not*I restriction sites, respectively, are in bold-faced type, and the bases corresponding to the *luxCDABE* genes are italicized).

After amplification, PCR product was purified. Now, both amplicon of *luxCDABE* and vectors containing *op-arsR* were digested with *Bam*HI and *Not*I. The vectors were then treated with calf intestinal phosphatase for preventing the self-ligation. Finally, both *luxCDABE* and vector were purified, ligated and transformed in to *DH5α* cells (Sambrook and Russell 2001).

2.6 *Cultivation of sensor cells*

Bacterial cells were cultivated in 50 mL of LB medium supplemented with 30 μg/mL of kanamycin in a shaker at 37°C until optical densities at 600 nm reached 0.5–0.6. Then the culture was divided into 500 μL aliquots in 1.5 mL eppendorf tubes and 500 μL of sterile glycerol (40%) was added and mixed. The eppendorf tubes were kept at –80°C for further use. Right before the assay, the cells were thawed and used with assay buffer.

2.7 *Luminescence measurements*

The different 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 μM (end concentrations) of the salt solutions $NaAsO_2$ and Na_2HAsO_4, were prepared and kept in dark bottles, and assay buffer containing (NaH_2PO_4, 33 mM; K_2HPO_4, 33 mM; $(NH_4)_2SO_4$, 0.09 mM, 5 mM glucose at pH 6.8 and 5 mM pyruvic acid) was prepared (Rasmussen *et al.* 1997). 100 μL of the thawed cells were mixed with the 1.9 mL of assay buffer, and these assay buffer treated cells were directly used for arsenic assay. Assay mixture containing 100 μL of these cells with 100 μL of different known concentrations of $NaAsO_2$ and Na_2HAsO_4 and of water samples were prepared directly in a 96-well white luminometer titre plates (Greiner Bio One). Plates were then covered with parafilm and incubated at 30° C with shaking at 125 rpm for time periods of 30, 60, 90 and 120 mins. Then the plate was moved to luminometer (Multi detection microplate reader, Synergy HT-BioTek, USA) and bioluminescence values as relative light units were noted immediately. The emitted relative light units were correlated with different concentration of arsenite As(III) and arsenate As(V) ions at standard incubation time by plotting calibration curves. All measurements were made in triplicates.

3. Results

3.1 *Initial screening and characterization of arsenic-resistant strains*

The water samples collected from the different locations in India as well as from Bangladesh had varying physical properties such as pH, temperature and turbidity. The initial screening of *E. coli* was done on Eosin Methyl Blue Agar (EMB) plates, which was confirmed by the various biochemical tests for *E. coli* (data not shown). A total of 39 *E. coli* strains were isolated from different geographical sites. Since the foremost aim was to analyse and characterize arsenic resistance, the strain exhibiting the highest level of tolerance to the sodium arsenite was selected from 39 arsenic-resistant *E. coli* strains for further use. The selected strain (ARP-2) showed the maximum tolerance value at 54 µg/mL. Screening for the presence of plasmid revealed a detectable plasmid of approximately 54 kb when DNA was subjected to gel electrophoresis (data not shown). PCR amplification of *op-arsR* genes from plasmid DNA was performed using gene-specific primers; the expected length of PCR products for *op-arsR* gene corresponding to 478 bp was obtained.

3.2 *Genomic DNA isolation and amplification of lux genes*

Genomic DNA of 4.2 Mbp was isolated from marine Photobacterium *Vibrio fischeri* and the PCR amplification of *luxCDABE* with gene-specific primers containing restriction sites yielded the expected size of 5.71 kbp when run on 0.8% agarose gel with 1 kb DNA ruler.

3.3 *Construction of the sensor system containing plasmid pASPW2*

By genetic engineering the non luminescent DH5α bacterial cells were altered in a way that they produce light when they come in contact with arsenic ions. The sensing part of the sensor system is the *arsR* gene isolated from arsenic resistant wild-type *E. coli*, which encodes the regulatory protein and the *ars* promoter.

Therefore, we linked together the *arsR* gene with its operator/promotor, the binding site for protein of *arsR* and reporter gene *luxCDABE* (promoterless) coding for bioluminescence. *luxAB* coding for light production enzyme luciferase and *luxCDE* coding for the substrate of the bioluminescent reaction (figure 1).

3.4 *Luminescence measurement assay*

Luminescence measurements were standardized to detect arsenic ions following simple measurement protocols described in the experimental section. Induction conditions like incubation temperature, amount of sensor bacteria per measurement and growth phase of bacteria, were kept constant throughout the experiment, but incubation time and amount of arsenic ions in sample were varied for measurement in known concentrations. Since the detection system is a

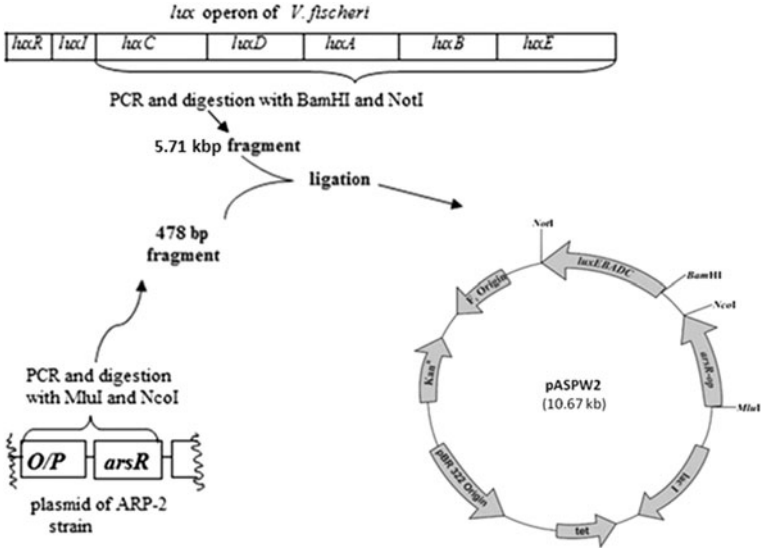

Figure 1. Construction of plasmid pASPW2 (10.67 kb). *luxCDABE* (5.7 kb) genes from genomic DNA of *V. fischeri* was amplified and digested by *BamH*I and *Not*I. A fragment of 478 bp was amplified from plasmid DNA of wild-type *E. coli* strain and digested with *Nco*I and *Mlu*I. Both amplified fragments were purified and ligated in pET28a.

biological one, the output of the biosensor cells is nonlinear. However, the accurate determination of As in unknown samples is possible by using calibrations with solutions of known arsenic concentrations.

Conditions which affected the results were the incubation temperature and the time used for incubation of bacteria with arsenic. When the sensor cells were incubated with the different concentrations of arsenite (0.05–0.8 μM), although the light emission started at 30 min was not good enough till the end of 90 mins for reproducible results to be obtained. The low luminescence signal measured at the beginning of experiment was significantly increased and the maximum light output was achieved only at 120 min, allowing a better R^2 coefficient of 0.9912 to be obtained as compared to 0.8503 at 90 min. (figure 2A). Light emission continued to increase after 120 min of incubation but there have not been much change in sensitivity (data not shown). At 150 min, the intensity of signal levelled off. Increase in concentration above 0.8 μM did not give linear light output and the samples were diluted.

Since the sensor cells still contain the chromosomally encoded arsenate reductase enzyme (Carlin *et al.* 1995), they also react to arsenate. Almost the same results were obtained in terms of regression values when the same sensor cells were exposed to arsenate concentrations (0.05–0.8 μM) but the relative light units emitted were approximately half that in the case of arsenite (figure 2B).

The genetically modified bacteria then was further used for assessment of As in different water samples; the actual concentration was calculated using calibrations of known As concentrations and are shown in table 1. In addition, parameters like cell density and measurement protocols have been reported to influence the sensitivity of the sensor and the light output (Reither *et al.* 2001). In our experiments also, late logarithmic and stationary growth phase of the bacteria affected the luminescence assays by decreasing the emission of light in the presence of arsenic ions. Cell density above 3×10^6 cells per mL decreased the intensity of light (data not shown) and luminescence production was highest when the cell density 3×10^6 cells per mL was used in the assay. Also,

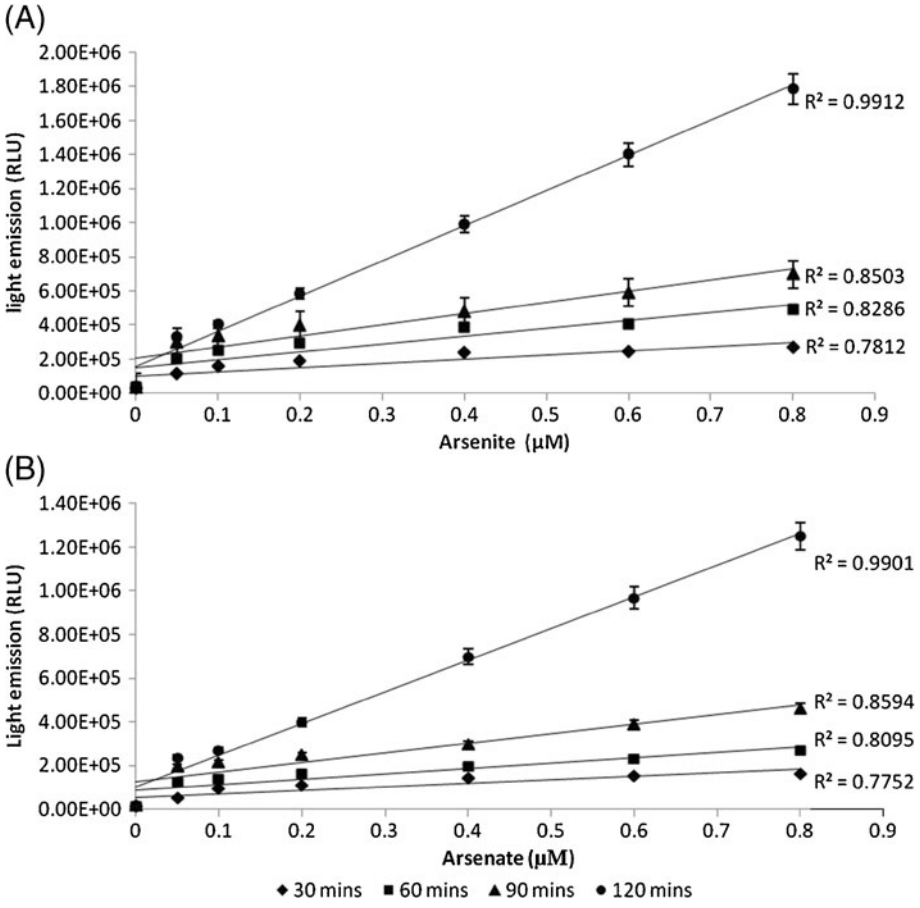

Figure 2. Light emission from the *E. coli* pASPW2 sensor when incubated with different concentration of (**A**) arsenite and (**B**) arsenate (0.05–1 μM) at different time intervals at 30°C, R^2 values result from linear interpolation of the three replicates. Error bars are shown when they are larger than the symbols used.

Table 1. Arsenic concentration (μM) found in various water samples collected

S. No.	Location for water sample collection	No. of samples collected	Range of arsenic concentration (μM)	Mean arsenic concentration (μM)
1.	Janak Puri*	2	0.41–0.44	0.425
2.	Satya Niketan*	2	0.52–0.54	0.53
3.	Vikas Puri*	3	0.44–0.46	0.45
4.	Patel Nagar#	1	0.6	0.60
5.	Model Town II*	1	0.29	0.29
6.	Malviya Nagar#	1	0.69	0.69
7.	Sarojini Nagar#	1	0.66	0.66
8.	Karol Bagh*	1	0.12	0.12
9.	Tuglakabad#	2	0.60	0.60
10.	Yamuna River	3	0.66–0.70	0.68
11.	Munirka*	3	0.65–0.70	0.675

*Tap water; # ground water; no symbol, river water.

the lower cell density below this resulted in low level of detectable signal.

3.5 *Induction of bacterial sensor pASPW2 with other metals*

Response of the constructed bacterial sensor with other metals like antimony, cadmium, zinc, mercury and lead along with arsenic salts was next examined. The sensor cells, when incubated with metal ions for 2 h at 30°C and luminescence measurement was done as described in the section on materials and methods, it was found that none of the metal other than antimonite induced the bacterial sensor at low and nonlinear fashion (figure 3). Also, the induction was not strong enough to either make any measurements nor any results can be derived from those observations. Thus, it can be said that the pASPW2 sensor is specific for the detection of arsenic ions.

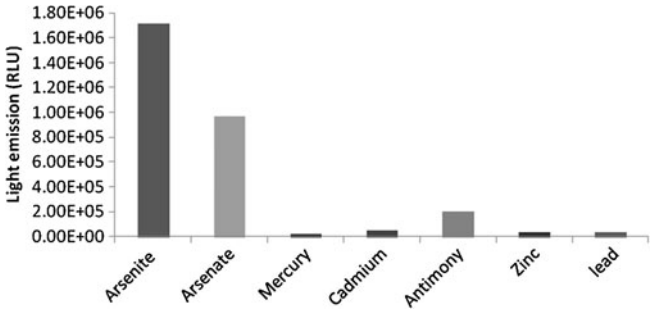

Figure 3. Response of the arsenic bacterial biosensor to various metals. When pASPW2 was incubated with 0.05 μM of As(III), As(V), Hg(II), Cd(II), Sb(II), Zn(II) and Pb(II). After incubation for 2 h at 30°C, the luminescence was measured as described in experimental section. All values given are mean values of three replicates.

4. Discussion

The present study offers a biological solution for the rectification of arsenic in water by using a bacterial resistance mechanism against arsenic coupled with the genetic engineering mechanisms, giving rise to bacterial bioreporter which is sensitive and specific to arsenic ions. This plasmid contains the operator-promoter region and gene coding for ArsR protein from the *ars* operon from wild-type *E. coli* strain isolated from environmental samples, fused with the *lux* structural genes associated with the production of bioluminescence. The genes of highly arsenic-resistant strain was used so that the sensor system constructed can successfully quantify the As amount until the higher toxicity causes reduction in light production giving nonlinear response (Bitton and Koopman 1992).

While performing the assay for unknown samples, the calibrations with known arsenic concentrations were incubated simultaneously with unknown samples in the 96-well plates for use in luminometer. In order to quantify arsenic ions with the bacterial biosensor, the approach of linear regression was used. Linear regressions were calculated by correlating the determined values of luminescence with arsenic concentrations at standard incubation time, as is usually reported (Selifonova *et al.* 1993; Barkay *et al*. 1997). The induction time of luciferase is time dependent; as the induction time increases, there is an increase in the bioluminescence emitted by the sensor bacteria containing pASPW2 plasmid. Although light emission started after 30 min of cell–arsenic contact, which was increased to several thousand folds on continuous incubation, but at the end of

90 min, the light output was not stable enough to get the reproducible results. Therefore, a standard incubation time of 2 h was chosen to get the stable and reliable results, also prolonged incubation was avoided as this is a living system and on continuous incubation gene expression will be higher producing strong signals, which will be meaningless. At this induction time of 2 h at 30°C the biosensor luminescence was stable and reliable and hence significantly correlated to arsenic concentrations. The estimated range of arsenic which can be measured by our sensor bacteria is between 0.05 and 0.8 μM (0.74 μg to 60 μg of As/L). At higher concentration of more than 0.8 μM the nonlinearity in light output was observed so the samples were diluted. Arsenic concentration in unknown water samples was interpolated from calibration curves obtained from samples with known arsenic concentrations.

The reason for the less light output when same sensor cells were incubated with arsenate could be the difference in rate of production of arsenite from arsenate by *arsC* and interaction of arsenite with *arsR* or the efflux rate. When the cell-density-dependent luminescence assays were performed, it was found that at the cell density of 3×10^6 cells per mL induced maximum light production. The higher cell density above this in the assay reduced the intensity of light output possibly because of the arsenic adsorption on the cell wall or light adsorption due to the turbidity of the cell suspension. Also, the specificity of the bacterial biosensor was checked by incubating these cells with various heavy metals like mercury, lead, antimony, cadmium and zinc. Among the metals tested, only antimony induced the sensor to a slight extent giving low light output. Earlier studies (Shi *et al.* 1994) showed that the ArsR protein of R773 is activated by both As^{3+} and Sb^{3+}.

Keeping in mind the severity of arsenic contamination, deleterious health effects and lack of effective removal technologies in poorer countries, we have demonstrated the use of biological sensing system that respond selectively to arsenic ions, where the expression of bacterial luciferase is controlled by the ArsR, the regulatory protein of the *ars* operon. Therefore, the work described here is also the implication of R-factor-mediated resistance mechanism of bacteria against arsenic in the development of a whole-cell bacterial sensor from the wild-type strains of *E. coli* which are uncharacterized and isolated from the arsenic polluted sites. As described by the others, good and reproducible results can be achieved by genetically engineered bacteria cells (Ramanathan *et al.* 1997; Petanen *et al.* 2001); here also the bacterial sensor constructed is simple but is an effective tool for measuring arsenic ions quantitatively from water samples. Our biosensor responded in the range 0.05–0.8 μM, which is even lower than the permissible limit (10 μg of As/L). In contrast to routine analysis of total arsenic content, the sensor bacteria described here primarily measures bioavailable arsenite, the most toxic form of inorganic arsenic, and thereby demonstrates its potential in complementing the traditional analytical method in quantification of biological available forms. A comparison of bioavailability of a metal with its total content allows the examination of an inert metal which can be helpful in the planning and controlling of the bioremediation process.

Despite the great potential of the bacterial sensors, there is still need to test them outside laboratory. As the maintenance of cultures is difficult to control outside the laboratory, which is the reason that the bacterial sensors developed till date have not received great interest. The development of portable biosensor kits will be necessary for biosensors to be used on site to monitor environmental pollution. The practical application of cell-array biosensors is likely to facilitate the comprehensive analysis of environmental pollution at specific sites. Thus, the successful integration of the powerful applications of these biosensors in pollution management may be one alternative to reverse the years of global environmental mistreatment.

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