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# Metal-Resistance Genetically Engineered Bacteria

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**Research Report No. 198**

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## ABSTRACT

Bacterial-based electrochemical and optical sensing systems that respond in a highly selective and sensitive manner to antimonite and arsenite have been developed. This was accomplished by using genetically engineered bacteria bearing one of two plasmids constructed for our studies. The first plasmid, pBGD23, contains the operator/promoter region (O/P) and the gene of the ArsR protein from the *ars* operon upstream from the β-galactosidase gene. In the absence of antimonite/arsenite, ArsR binds to the O/P site and prevents the transcription of the genes for ArsR and β-galactosidase, thus blocking expression of these proteins. When antimonite or arsenite is present in the sample, it binds to the ArsR protein, causing a conformational change in ArsR that leads to its release from the O/P site of the plasmid, thus allowing for the expression of β-galactosidase. Then, the amount of β-galactosidase expressed is quantified by using a substrate that produces a product that can be monitored electrochemically. In the second plasmid, pRLUX, the gene for ArsR is upstream from the reporter gene, *luxAB*, that encodes for the enzyme luciferase, whose activity can be monitored by bioluminescence. These bacterial sensing systems have excellent detection limits, respond selectively to arsenite and antimonite, and show no significant response to phosphate, sulfate, nitrate, and carbonate.



**FOCUS CATEGORY:** METHODS, TOXIC SUBSTANCES, WATER QUALITY

**KEYWORDS:** Bacteria, Biomonitoring, Biotechnology, Pollution Control, Toxic Substances, Water Quality

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# CHAPTER I - INTRODUCTION

In order to survive in unfavorable environments, microorganisms have evolved and developed intrinsic defense mechanisms. Bacteria have been isolated from unfavorable environments, such as hot springs or vents in oceans[1, 2] and from environments contaminated with toxic levels of metals.[3, 4]

Metals can be subdivided into three categories depending on their effect on the metabolism of bacteria: Essential (e.g., iron); essential, but only in trace amounts (e.g., copper, zinc); and non-essential and toxic (e.g., mercury, arsenic). Some of these metals can accumulate inside a bacterium cell through relative non-specific uptake pathways that are an inherent part of the transport mechanism of the cell; e.g., $AsO_4^{3-}$ can enter cells through the $PO_4^{3-}$ uptake pathway. However, under conditions of environmental stress that lead to high build-up in the intracellular concentration of a toxic metal, a bacterial response that reduces the toxicity to the bacteria is being triggered. Five different mechanisms have been proposed for heavy metal resistance in bacteria and other microorganisms.[5, 6] These mechanisms include: (a) exclusion of the metal by a permeability barrier; (b) exclusion by active export of metal from the cell; (c) intracellular sequestration by binding proteins; (d) extracellular sequestration; and (e) detoxification by chemical modification of the metal to reduce its toxicity. Depending on the metal any one of the above detoxification models or a combination of them can exist in bacteria.[6]

The intrinsic defense mechanisms of bacteria to certain metals and environmental toxic organic compounds are often plasmid-borne. Plasmids are small circular forms of DNA that, depending on the genes they carry, can endow bacteria with special properties such as resistance to antibiotics or toxic metal compounds. Below is a review of some of the better understood metal resistance systems found in bacteria.

**Mercury Resistance**

One of the most widely studied metal resistance systems in bacteria is the resistance to mercury compounds.[7-12] There is special interest in this system because the resistance mechanism results in the transformation of mercury from the toxic form, Hg (II) to the less toxic elemental form, Hg (0). The resistance system, which is coded for by a set of genes collectively known as the *mer* operon, exists in both gram-negative and gram-positive bacteria. Though the *mer* operons of the gram-negative and gram-positive bacteria are different, there is a clear relation between them. The mode of resistance to mercury compounds in gram-negative bacteria is discussed in more detail below.

The organization of the genes in the *mer* operon is as follows:


where O/P is the operator/promoter region of the operon and *merR*, *merT*, *merP*, *merC*, *merA*, *merB*, and *merD* are the genes that encode for the MerR, MerT, MerP, MerC, MerA, MerB, and MerD proteins, respectively. The transport and reduction of mercury are carried out by the MerT, MerP, MerA and MerB proteins. Mercury is extremely toxic and can bind to the cell surface proteins. To prevent such random binding and inactivation of enzymes, the bacteria transport the mercury in a protein-bound form.[10] The first step in the transportation process is the binding of mercury to the MerP protein, which is present in the bacterial periplasm. MerP has two cysteine residues in a region that is homologous to one found in the MerA protein. It has been proposed that these two cysteines sequester Hg (II) and transfer it to the thiol groups of the mercuric reductase, MerA. A mediator in the transfer process is the highly hydrophobic transmembrane protein MerT. MerT contains two pairs of cysteines, one pair located close to the outside of the membrane in a hydrophobic region, and a second pair

located on the inner side of the cell membrane. MerT is believed to transport the Hg (II) through the cell membrane into the cell.

The mercuric reductase, MerA, is a highly specific enzyme for mercuric and mercurous ions. The amino acid sequence of MerA is similar to the amino acid sequences of other flavin-containing oxidoreductases. MerA is a homodimer with one active site per subunit that contains a pair of cysteines. It is interesting to note that in all the mercuric reductases that have been studied, the active site contains 15 amino acid residues that are highly conserved. One of the proposed models for the reaction mechanism of MerA involves the transfer of electrons from NADPH to enzyme-bound FAD in order to reduce Hg (II) to Hg (0) .[10] Once this has occurred, Hg (0) can leave the cell through evaporation.

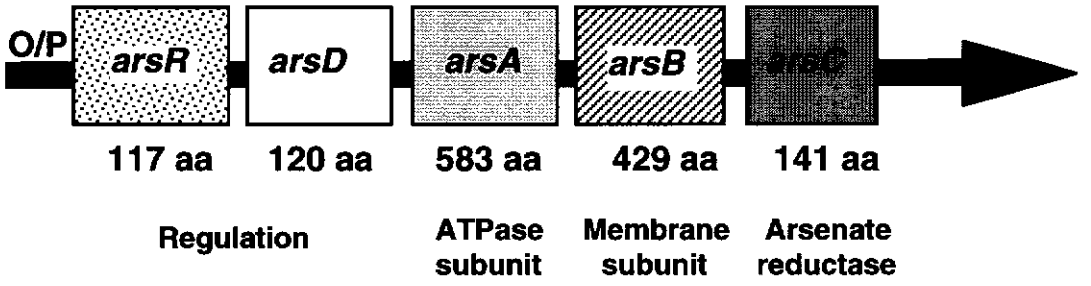
The MerB protein is an organomercurial lyase, whose enzymatic function is to reduce organic mercury. MerB is a monomer and has broad substrate specificity for primary, secondary, and tertiary alkyl mercuric halides, as well as for allyl, vinyl and aryl mercuric halides. In MerB, there are three cysteine residues that have been proposed to be involved in the organomercurial lyase reaction.[10] The MerB enzyme associates with an organomercurial thiolate complex, RHgSR', which binds to the -SH group of one of the cysteines. Then, a histidine residue transfers a proton to break the Hg-R bond.

The regulation of this system has been studied in detail.[13] MerR and MerD are two regulatory proteins involved in the controlled expression of the *mer* operon. The MerR protein has two physically distinct regions involved in binding to DNA and binding to Hg (II). The *merR* gene is under the control of a promoter that is different from the promoter for the rest of the *mer* operon. The regulation of the *mer* operon by the MerR involves the binding of the MerR protein to the promoter region preventing transcription of the *mer* operon. The MerD protein is expressed at low levels and is also believed to be a DNA-binding protein that binds to the O/P region with much lower affinity than MerR.[10,13,14]

**Arsenic and Antimonite Resistance**

The mechanism of detoxification for mercury by removal of the metal after a reduction step through evaporation is not the only one employed by bacteria. Other mechanisms have evolved, where the toxic metal ions are actively effluxed out of the bacterial cell. Examples of such mechanisms are the arsenic/antimonite resistance systems. These resistance systems are plasmid-born[15] and chromosomal[16] in *E. coli* and plasmid-borne in certain *Staphylococcus* strains.[17]

The *ars* operon of plasmid pR773 in *E. coli* consists of five genes: *arsR*, *arsD*, *arsA*, *arsB* and *arsC* , as shown below:




The proteins ArsA, ArsB and ArsC form a protein pump,[18, 19] which transports arsenite, antimonite and arsenate (after being reduced to arsenite) from the cytoplasm to the periplasm across the cell wall (see Figure 1). This is facilitated by ArsB, which is a transmembrane protein.[20] ArsA encodes for an ATPase subunit and is anchored on the ArsB protein.[22] The active form of ArsA is presumed to be a homodimer, with each ArsA having two ATP-binding sites.[23] ArsA is an arsenite-and antimonite-stimulated ATPase, which seems to undergo a conformational change upon the addition of ATP and the oxyanions.[24] It has been observed in systems that contain only the ArsB protein (e.g., in *Staphylococcus*) that expression of the ArsA protein from plasmid pR773 along with ArsB increases the transport of the metal ions drastically.[21]

The *arsC* gene encodes for the arsenate reductase enzyme.[25, 26] Though arsenite is more toxic than arsenate, ArsC reduces arsenate to arsenite in order for the

transmembrane proteins to be able to bind to the oxyanion. ArsC is coupled with another protein, glutaredoxin in the case of pR773 (thioredoxin in the case of *Staphylococcus aureus*) and is inactive in the absence of this coupling protein.[27] A mechanism of action has been proposed for the ArsC of *Staphylococcus aureus* that involves two of the four cysteines that are present in ArsC.[12, 19] The first step in the reduction of arsenate to arsenite involves the formation of hydrogen bonds between the arsenate oxyanion and certain amino acids in the active site of ArsC. In addition, a thioether bond with one of the two cysteine groups is formed. This is followed by a two-electron transfer from the cysteine to the arsenate, releasing arsenite. The oxidized cysteine then forms a dithiol with the free cysteine, thus, disabling it from binding to arsenite. Finally, the oxidized ArsC enzyme is reduced by a thioredoxin.

The *ars* operon is under the control of the a trans-acting regulatory protein, ArsR.[28, 29] ArsR is encoded by the *arsR* gene that is downstream from the operator/promoter in the *ars* operon. In the absence of the inducer (arsenite or antimonite), the ArsR protein forms a dimer and binds to a short DNA region upstream from the *ars* mRNA initiation site, preventing the transcription of the genes of the *ars* operon.[30] It has been shown that ArsR is released from the DNA region after addition of antimonite or arsenite. Arsenite/antimonite binds to a specific binding site on the ArsR protein that contains two cysteine residues and that is physically different from the DNA binding domain of the ArsR.[31] While ArsR controls the basal expression of the *ars* operon, the protein ArsD is believed to control the maximal expression.[32] ArsD has been shown to be an inducer independent trans-acting protein. It is interesting to note that both *arsD* and *arsA* are absent from the *Staphylococcus* plasmids and from the *E. coli* chromosomal DNA.

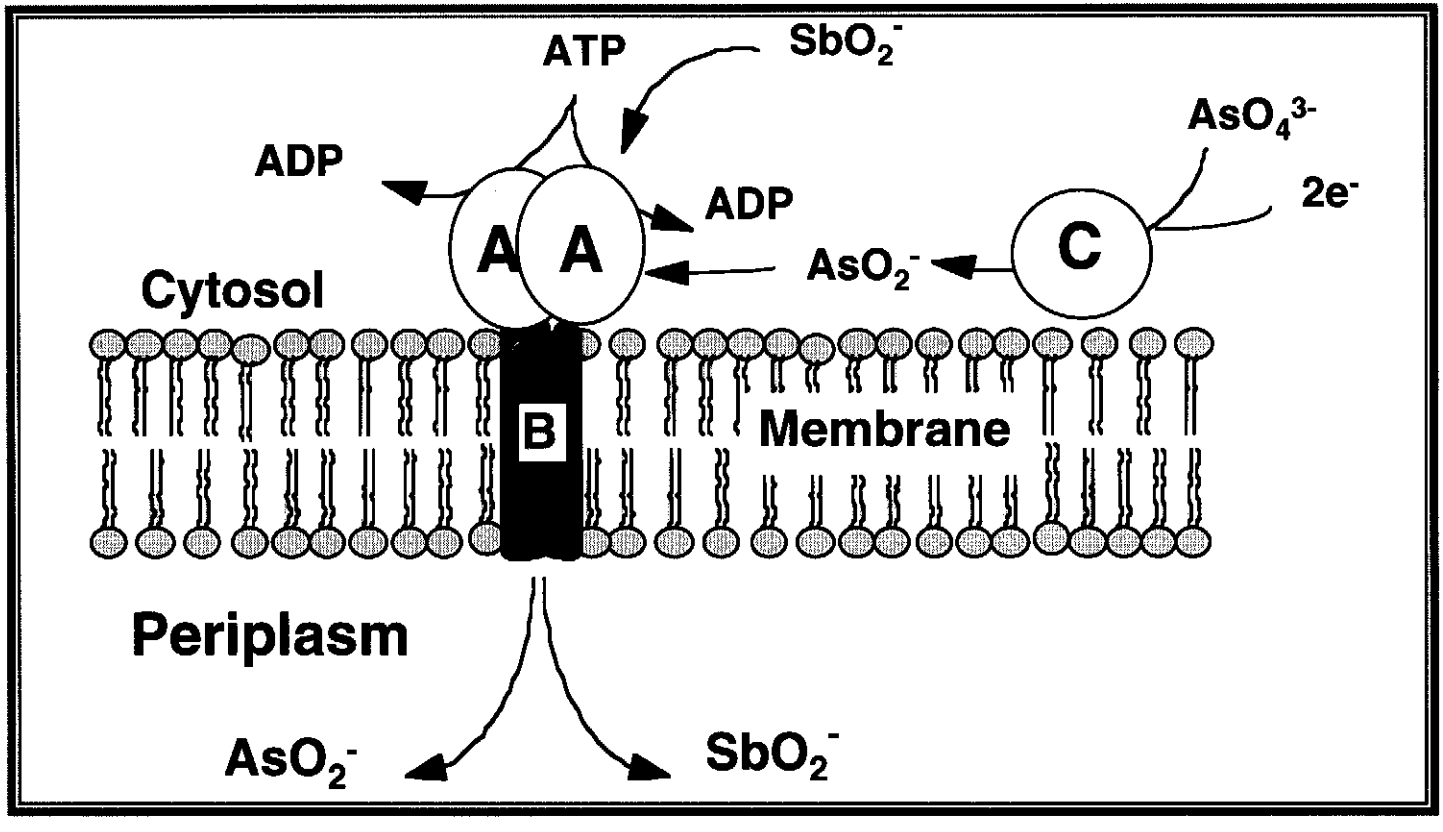



Figure 1. Schematic of the protein pump encoded by the *ars* operon. ArsA and ArsB are the proteins responsible for the effuxing of antimonite and arsenite from the cell. ArsC is the reductase that reduces arsenate to arsenite.

**Cadmium and Zinc Resistance**

Cadmium is an extremely toxic ion for the cell and, therefore, its presence is not desirable in the cytoplasm. In the case of zinc, although high concentrations of this metal are toxic to the cell, the cell needs zinc in trace amounts. The cadmium resistance in gram-positive bacteria (i.e., *Bacillus*[33], *Listeria*[34], and *Staphylococcus*[11]) is a result of a P-type ATPase, while in gram-negative bacteria (i.e., *Alcaligenes eutrophus*) is a result of a three-protein non-ATPase efflux system.[35]

The CadA protein in the case of *Staphylococcus* is a 727-amino acid long P-type ATPase that is expressed when the cells are exposed to $Cd^{2+}$ or $Zn^{2+}$. The CadA protein forms a pump that effluxes the cations out of the cell. It has been proposed that CadA has six $\alpha$-helical regions, two of which may be part of the actual $Cd^{2+}$ transport channel.[11, 12] There are three large cytoplasmic domains believed to be involved in $Cd^{2+}$ binding and in the initial transport from the cytoplasm to the cell membrane. The third cytoplasmic domain contains an ATP-binding motif and an aspartyl kinase, which is highly conserved in all P-type ATPases. The ATP phosphorylates the aspartic acid residue 415, which is subsequently dephosphorylated by the phosphatase in the second cytoplasmic domain. In this process of transferring the phosphate, it is believed that the protein goes through a high energy transition state.[35] The regulation of the cadmium pump occurs through another protein, CadC. The amino acid sequence of CadC is homologous to that of the regulatory protein in the arsenic system, ArsR.[36]

In the case of the gram-negative bacteria *Alcaligenes eutrophus* CH34, two mega-plasmids, pMOL28 and pMOL30, have been isolated.[37] The plasmid pMOL30 encodes for the resistance to $Cd^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Pb^{2+}$ and $Hg^{2+}$ ions. The plasmid pMOL28 encodes for resistance to $Co^{2+}$, $Ni^{2+}$, $Hg^{2+}$ and $CrO_4^{2-}$. The Czc system found in pMOL30 confers resistance to cadmium, zinc and cobalt, while the Cnr system found in pMOL28 confers resistance to cobalt and nickel. The Czc system is the

one studied in more detail and it functions as a cation/proton antiporter, which pumps the cations from the cells.

The elucidation of the genes of the Czc system and the function of the gene products is an issue of continuing debate. Mergeay and coworkers have proposed a model where CzcA functions as a pump driven by a proton gradient.[38] It has been hypothesized that CzcB could facilitate the export of ions without any release in the periplasm. The CzcC protein is an outer-membrane factor required in the release of ions in the extracellular medium. Another protein, CzcN, is believed to be a transmembrane protein, but its role in the transfer mechanism has not been elucidated yet. The CzcI protein is suspected to play a role in the regulatory mechanism of the operon, but there is no experimental evidence that proves this. The *czcD* gene, located downstream from the *czcCBA* genes, is believed to activate the high level efflux system. The role of CzcD has not been clearly defined.

Nies and coworkers have shown that CzcA and CzcB catalyze a highly effective efflux of $Zn^{2+}$.[36, 39] The CzcC subunit is needed to modify the substrate specificity of the system so as to include cadmium and cobalt, though it does not have any metal binding regions. It has been observed that the protein CzcA alone catalyzes the efflux of $Co^{2+}$. CzcA is proposed to have two hydrophobic domains, which are involved in the efflux of the divalent metal cations. The CzcB protein has a highly hydrophobic amino terminus, and is a membrane-bound protein bound in the cytoplasmic face of the membrane. It has two homologous histidine-rich fragments, which are proposed to be $Zn^{2+}$ binding sites. A two-component regulatory system involving a membrane bound CzcD and a soluble CzcR has been proposed.[40] The order of induction potential for the transcription of the *czc* operon is $Zn^{2+} > Cd^{2+} > Co^{2+}$.

## Copper Resistance

Copper is an essential trace metal that is required in the synthesis of metalloproteins like oxygenases and electron transport proteins. In *E. coli*, several chromosomal genes that are involved in the normal metabolism of copper have been identified, in addition to the plasmid-encoded resistance systems.[41] The copper uptake and transport operon regulates the concentration of copper in the cell. This copper uptake and transport system includes the CutA and CutB proteins, which are involved in the uptake of copper and are encoded by the *cutA* and *cutB* genes. The CutE and CutF proteins (expressed from the *cutE* and *cutF* genes) are involved in the transport and storage of copper in the cell. The export of the copper that is in excess of cellular requirements is through CutC and CutD. In addition to the chromosomal genes of *E. coli*, there is a plasmid-encoded copper resistance, *pco*, that has been described. This is similar to the plasmid-borne resistance found in *Pseudomonas*, *cop*, and to a chromosomal-based resistance existing in some *Xanthomonas* strains.[12, 42] Three of the *cop* gene products have been isolated. Two of these gene products are CopA and CopC, which are periplasmic copper-containing proteins with eleven binding sites for $Cu^{2+}$ in CopA and one in CopC. CopB is a transmembrane protein located in the outer membrane that also binds to $Cu^{2+}$. In the *cop* operon-mediated resistance to copper there is a high accumulation of copper ions in the periplasm. It has been proposed that an increase in the copper concentration in the medium, initiates the transport of $Cu^{2+}$ ions to the periplasm where it is trapped by the CopA protein.

## Other Metal Resistance Systems

Plasmid-determined bacterial resistance to chromate has been found in several bacteria such as *Streptococcus*,[45] *Pseudomonas*,[46] and *Alcaligenes*.[12] Chromate is transported into the cell through the sulfate uptake pathway. In the *Alcaligenes* chromate-resistance plasmid system, one of the genes encodes for a hydrophobic

protein called ChrA. The ChrA protein is similar to other transport-related proteins (e.g., ArsB) and is believed to be involved with either the reduced uptake of chromate or the efflux of chromate from the cell. The other gene from the chromate resistance system codes for ChrB, which seems to be involved in the induction of the *chr* operon. Bacterial resistance systems to silver, germanium[47] and tellurite,[12, 48] among other metals, have also been identified. However, there is still much work that needs to be done in order to characterize these systems and understand their resistance mechanism.

**Environmental Analysis Applications of Bacterial Resistance Systems**

In recent years, increased emphasis has been placed on employing the inherent chemical recognition properties of biological systems in the development of highly selective and sensitive bioanalytical techniques. In particular, antibodies, enzymes, receptors, binding proteins, lectins, etc., have found wide applications in techniques such as immunoassays,[49-51] biosensing,[51-53] and chemical separations.[54,55] Additional efforts have been devoted to mimicking the binding characteristics of these biomolecules by preparing synthetic receptors based on either host-guest principles[56,57] or molecular imprinting.[58,59] Although relatively unexplored, one of the most promising approaches in bioanalytical chemistry involves the control of gene expression by regulatory proteins.[60-64] To this end, we have investigated the feasibility of using bacterial sensing systems that contain genetically engineered plasmids to detect antimonite and arsenite. The bacteria used in these studies couple a selective response to antimonite/arsenite to the expression of the enzyme β-galactosidase.

The plasmid used in this study is derived from the naturally occurring plasmid R773 that confers resistance to oxoanions of antimony and arsenic in *E. coli*,[18,28-30] although plasmid-mediated resistance to these anions is not unique to *E. coli* has been found in several other bacteria.[11] As explained earlier, the specific DNA region of the native plasmid conferring this type of resistance is known as the *ars* operon. The *ars*

operon of *E. coli* plasmid R773 contains genes for five proteins, namely, ArsR, ArsD, ArsA, ArsB, and ArsC. ArsR and ArsD are regulatory proteins; ArsR controls the basal level of protein expression, while ArsD controls the maximal level of protein expression for the *ars* operon. ArsA and ArsB form a pump to remove antimonite/arsenite from the cytosol to the periplasm of the bacteria. ArsA is an ATPase that provides the energy necessary for this process, and ArsB is a transmembrane protein. ArsC reduces arsenate to arsenite, and the latter is subsequently effluxed by the pump. In the absence of antimonite/arsenite, the ArsR protein binds to the operator/promoter region of the plasmid repressing expression of the genes of the *ars* operon. When antimonite/arsenite enters the cell, it binds to ArsR and causes a conformational change in the protein resulting in the release of ArsR from the operator/promoter region of the plasmid.[65] In the absence of bound ArsR, RNA polymerase is able to transcribe the *ars* operon.

Since the native bacteria do not produce an analytically useful signal, we have designed bacteria that contain an engineered plasmid capable of generating a measurable analytical signal. The engineered plasmid, pBGD23 (shown bellow ),

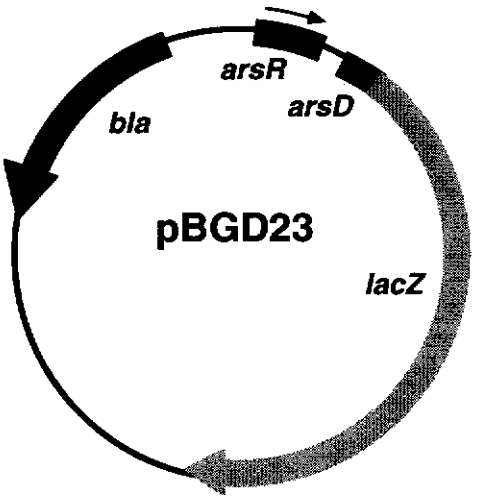



Figure 2. Plasmid pBGD23 containing the gene for ArsR upstream from the gene for β-galactosidase.

was constructed by removing the genes that encode for ArsA, ArsB, and ArsC and part of the gene that encodes for ArsD, and inserting the gene for the enzyme β-galactosidase, *lacZ*.[65] When bacteria containing the modified plasmid are exposed to antimonite/arsenite, ArsR is released from the plasmid and β-galactosidase is expressed. Thus, the β-galactosidase gene acts as a reporter gene in our system. The activity of expressed β-galactosidase is determined using *p*-aminophenyl-β-D-galactopyranoside (PAPG) as a substrate. The concentration of the product of this reaction, *p*-aminophenol (PAP), can be determined electrochemically by the following electrode reaction:


This electrochemical reaction has been used previously in the determination of β-galactosidase in flow injection immunoassays.[66-70] Under the proper conditions, the observed electrochemical response is proportional to the amount of β-galactosidase produced by the bacteria which, in turn, is related to the concentration of antimonite/arsenite to which the bacteria were exposed. To the best of our knowledge, this is the first time that electrochemical detection has been coupled to reporter gene technology for the development of sensing systems for ions.

In a separate study, an optical sensing system for antimonite and arsenite based on genetically designed bacteria was developed. Given the specificity of the *ars* operon for antimonite and arsenite, an even more sensitive and selective sensing system for these ions could emerge if part or the entire *ars* operon is coupled to a sensitive detection method like bioluminescence.[74] One such bioluminescent protein is bacterial luciferase, an enzyme that catalyzes the oxidation of $FMNH_2$ and a long chain aldehyde to FMN and the corresponding carboxylic acid; this is accompanied by the emission of

light at 490 nm.[75,76] This enzyme has been used in a number of bioanalytical methods, including the monitoring of protein expression.[74] Bacterial luciferase is a heterodimeric enzyme consisting of two subunits, LuxA and LuxB, that are coded for by the *luxA* and *luxB* genes of the *lux* operon. The long chain aldehyde is synthesized *in vivo*, through proteins encoded by the *luxC, luxD,* and *luxE* genes of the *lux* operon. If the aldehyde substrate (typically decanal) is introduced externally, then there is no need for the presence of the entire *lux* operon in the bacteria in order to produce bioluminescence. Indeed, the *luxA* and *luxB* genes are sufficient for the expression of the enzyme luciferase, and therefore, for the generation of the bioluminescence reaction.

In this study, bacteria were designed so that the genes that code for bacterial luciferase are fused to the *arsD* gene, which is downstream of the *arsR* gene. In this manner, the *arsR* gene controls the expression of bacterial luciferase. In our system, the *arsR* gene acts as the sensing element and luciferase acts as the reporter/transducer of a conventional sensor. When the bacteria is exposed to varying concentrations of antimonite or arsenite in a sample, varying amounts of luciferase are expressed. The activity of luciferase can be monitored by adding the substrate, decanal, and measuring the light emitted at 490 nm. The antimonite level present in the sample can be determined by relating it to the light emitted from the bacteria.

## CHAPTER II - RESEARCH PROCEDURES

### BACTERIAL SENSING SYSTEMS BASED ON ELECTROCHEMICAL DETECTION

**Reagents.** Potassium antimonyl tartrate was obtained from Aldrich (Milwaukee, WI). Sodium arsenate and sodium arsenite of the highest purity available, as well as bovine serum albumin (BSA), Triton X-100, *p*-aminophenyl-β-D-galactopyranoside (PAPG), agar, sodium phosphate (monobasic), and potassium chloride were purchased from Sigma (St. Louis, MO). All chemicals were at least reagent grade and were used as received. Luria Bertani (LB) broth was obtained from BIO101 (Vista, CA). All solutions were prepared using deionized (Milli-Q Water Purification System, Millipore, Bedford, MA) distilled water. Unless otherwise stated, all solutions were prepared using 0.100 M phosphate, pH 7.0, containing 1.0 mg/mL BSA (abbreviated as buffer/BSA throughout the text).

**Apparatus.** Electrochemical experiments were performed using a PAR 273 potentiostat and Model 270 software (EG&G Princeton Applied Research, Princeton, NJ). Electrochemical data were obtained using 200 μL of solution in a cell with a bottom-mounted working electrode (gold foil, geometrical area of 0.32 $cm^2$). The working electrode was cleaned by exposure to 6 M HCl for 5 min, followed by thorough rinsing first with 0.100 M phosphate buffer, pH 7.0, and then with buffer/BSA. Buffer/BSA was then placed in the cell, and the potential cycled in the working range (-0.100 to +0.400 V) for 35 cycles or until a stable background signal was obtained. The counter electrode was a platinum wire. The reference electrode was a Ag/AgCl wire in 1.0 M KCl. All potentials are reported vs Ag/AgCl (+0.23 V vs NHE). All voltammograms were obtained using a scan rate of 100 mV/s. For the analytical determinations, the buffer background was subtracted from each voltammogram.

Procedures were performed at room temperature (20 ± 3 °C) unless otherwise stated. Reported currents are the average of three values unless otherwise stated.

**Calibration Plot for *p*-Aminophenol (PAP).** Calibration plots for *p*-aminophenol were obtained using peak oxidation currents. A single measurement was made at each concentration in increasing order from 10 μM to 5 mM. This procedure was repeated two more times to give a total of three measurements at each concentration.

**Enzyme-Substrate Kinetics.** β-Galactosidase, PAPG in buffer/BSA, and buffer/BSA were placed in 1.7-mL centrifuge tubes and mixed to give a final concentration of 31 pM β-galactosidase and the desired final concentration of PAPG. The total volume for each sample was 1.0 mL, and the mixture was incubated at 37 °C. A volume of 200 μL of each sample was removed at several time points and the concentration of PAP produced was determined electrochemically. These data were used to determine the initial rate of the enzymatic reaction at different substrate concentrations.

**Calibration Plot for β-Galactosidase.** Appropriate aliquots of β-galactosidase, PAPG in buffer/BSA, and buffer/BSA were placed in 1.7-mL centrifuge tubes and mixed to give a final concentration of 8.0 mM of PAPG and the desired concentration of β-galactosidase. The total volume for each sample was 1.0 mL. After incubation at 37 °C for 2 h, an aliquot of each sample was removed and the concentration of PAP was determined electrochemically.

**Genetically Engineered Bacteria.** Plasmid pBGD23 was prepared by inserting an EcoRI-BclI fragment containing the *arsR* gene and part of the *arsD* gene into the EcoRI-BamHI site of vector pMLB1034.[65] The resulting plasmid, shown in Figure 2, has the *lacZ* gene fused in frame to the *arsD* fragment in such a way that a chimeric

protein in which residue 9 of β-galactosidase is fused to residue 23 of ArsD is produced. pBGD23 was transformed into *E. coli* strain JM109 cells using conventional protocols.[71]

**Calibration Plots for Antimonite and Other Anions.** For each series of experiments, a single colony of bacteria bearing the pBGD23 plasmid was selected and grown at 37 °C overnight in Luria Bertani medium containing 50 μg/mL ampicillin until the bacteria grew to a cell density corresponding to an absorbance of 0.8 at 600 nm. For each sample, 100 μL of bacterial solution and 100 μL of standard solution containing antimonite (or the other oxoanions tested) were placed in a 1.7-mL centrifuge tube and vortexed. Standard solutions were prepared by serial dilution starting with a $1.00 \times 10^{-3}$ M solution. Samples were incubated for a fixed time at 37 °C (induction step). Next, the samples were centrifuged at 5000 rpm for 10 min. The supernatant was removed and a lysis solution (5% (w/v) Triton X-100 in 0.100 M phosphate, pH 7.0, containing 1.0 mg/mL BSA) was added to the pellet of bacterial cells. Samples were vortexed for 15 s or until a pellet was no longer visible and placed on a shaker at room temperature. After shaking for sufficient amount of time to cause cell lysis, the samples were centrifuged for 5 min at 5000 rpm. The supernatant was removed from the tubes and placed in separate vials. Equal amounts (100 μL) of supernatant and 16 mM PAPG were placed in centrifuge tubes and mixed. The samples were incubated at 37 °C for 2 h. After this time, samples were removed from the incubator and the amount of PAP in each sample was determined electrochemically. All the analyses were performed in triplicate.

**BACTERIAL SENSING SYSTEMS BASED ON BIOLUMINESCENCE DETECTION**

**Reagents.** The restriction enzymes HindIII and Pvu II were purchased from New England Biolabs (Beverly, MA). Potassium antimonyl tartarate was purchased from Aldrich (Milwaukee, WI). n-Decyl aldehyde (decanal), bovine serum albumin (BSA), agar, tris[hydroxymethyl]aminomethane (Tris), sodium salt of EDTA, sodium arsenite,

magnesium sulfate, dithiothreitol (DTT), sodium phosphate (monobasic, and dibasic), potassium chloride, magnesium sulfate and all other reagents were obtained from Sigma (St. Louis, MO). Luria Bertani (LB) broth was from BIO101 (Vista, CA). All chemicals were reagent grade or better and were used as received. All solutions were prepared using deionized (Milli-Q Water Purification system, Millipore, Bedford, MA) distilled water.

**Apparatus.** Bioluminescence measurements were made on an Optocomp I luminometer from GEM Biomedical (Carrboro, NC) using a 100-μL fixed volume injector. All experiments were conducted at room temperature unless specified otherwise. All luminescence intensities reported are the average of a minimum of three replicates.

**Preparation of pRLUX plasmid.** The pRLUX vector (Figure 3) was prepared by inserting a HindIII-Pvu II fragment of pQF70 containing the *luxA* and *luxB* genes between the HindIII and Pvu II sites of plasmid pWSU1. The resulting plasmid retains the O/P region, *arsR* and part of the *arsD* gene of the *ars* operon. The *luxA* gene is fused in frame to the arsD fragmentent in such a way that a chimeric protein in which

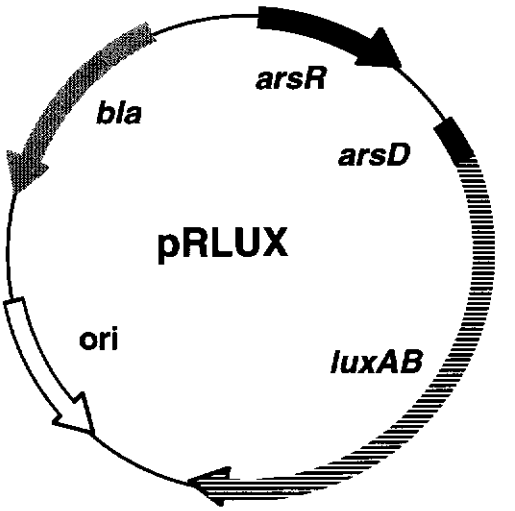



Figure 3. Schematic of the pRLUX plasmid.

the subunit A of luciferase is fused to ArsD. A detailed description of pWSU1 from which the *arsR* and *arsD* were isolated, and pQF70 from which *luxA* and *luxB* were isolated are found in references 77 and 78, respectively. The pRLUX vector, which also contains the gene for ampicillin resistance, was transformed into *E. coli* (strain JM109) using conventional protocols.[71]

**Bioluminescence study and calibration curve for decanal.** A single colony of the transformed bacteria was selected and grown overnight in 5 mL of LB broth with an ampicillin concentration of 50 μg/mL to a cell density that corresponds to an absorbance of 0.8 at 600 nm. A volume of 100 μL of the bacterial suspension was centrifuged at 5000 rpm for 5 min at 4 °C, and the supernatant was discarded. The bacterial pellet was resuspended in 200 μL of a 10 mM Tris-HCl, 1mM EDTA, 1 mM DTT, pH 8.0 (Tris-EDTA). A volume of 50 μL of this bacterial solution was mixed with 250 μL of Tris-EDTA buffer and was placed in the luminometer. Then, the bioluminescence reaction was triggered by the injection of 100 μL of decanal and the bioluminescence signal generated was measured for a period of 1 min at 1-s intervals. The decanal solutions used were serially diluted from a stock solution of 6.3 mM of decanal in methanol with the Tris-EDTA buffer. Decanal concentrations of 630 μM, 63 μM, 6.3 μM, 0.63 μM, 0.063 μM were used.

**Calibration curves for antimonite and arsenite.** A single colony of the transformed bacteria was selected and grown in 5 mL of LB media containing 50 μg/mL ampicillin overnight until the bacteria grew to a cell density corresponding to an absorbance of 0.8 at 600 nm. Then, 100 μL of the bacterial suspension was incubated with 100 μL of the inducer (antimonite or arsenite). Antimonite and arsenite solutions were prepared by serial dilution using deionized water containing 1 mg/mL of bovine serum albumin from a $1 \times 10^{-3}$ M stock solution. These bacteria were induced with antimonite for 2 h and

with arsenite for 3 h. After the induction time, the bacterial solutions were centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was discarded, and the bacteria were resuspended in 200 μL of the Tris-EDTA buffer. All the bacterial solutions were kept in an ice bath until their bioluminescence was measured. A volume of 50 μL of this bacterial solution was mixed with a volume of 250 μL of the Tris-EDTA buffer, followed by injection of 100 μL of 6.3 μM decanal. After a delay time of 10 s, the light emitted was collected for a period of 3 s.

Bacteria containing the pQF70 plasmid, which does not have the *arsR* gene, were used to prepare a calibration curve in order to evaluate the response of the bacteria in the absence of ArsR.

## CHAPTER III - DATA AND RESULTS

### BACTERIAL SENSING SYSTEMS BASED ON ELECTROCHEMICAL DETECTION

The bacteria used in the first study were genetically engineered to produce β-galactosidase upon exposure to antimonite and arsenite. This was accomplished by using bacteria bearing the plasmid pBGD23 that contains the operator/promoter region (O/P) and the gene of the ArsR protein from the *ars* operon upstream from the β-galactosidase gene. In the absence of antimonite/arsenite, ArsR binds to the O/P site and prevents the transcription of the genes for ArsR and β-galactosidase, thus blocking expression of these proteins. When antimonite or arsenite is present in the cell, it binds to the ArsR protein, causing a conformational change in ArsR that leads to its release from the O/P site of the plasmid, thus allowing for the expression of β-galactosidase. In the sensing system described in this report, the amount of β-galactosidase expressed was quantified by using the substrate *p*-aminophenyl-β-D-galactopyranoside and monitoring the product *p*-aminophenol electrochemically. It should be noted that in the designed plasmid the gene for β-galactosidase, *lacZ*, is fused in frame to *arsD* in such a

way that the resulting fusion protein contains the first 23 amino acids of ArsD fused to the ninth amino acid residue at the N-terminal domain of β-galactosidase. This fusion protein does not retain the function of the full ArsD, but maintains β-galactosidase activity.[65]

Before initiation of studies using the complete bacterial sensing system, the electrochemical behavior of *p*-aminophenol (PAP) was examined. Over a period of 1 h (expected maximal time to test the PAP content in a series of actual samples), the peak oxidation currents obtained using a 1.0 mM PAP solution did not change significantly, and the relative standard deviation (RSD) for eleven replicates was 2.0%.

The electrochemical response to varying PAP concentrations was also examined. As shown in Figure 4, the oxidation peak heights of the staircase voltammograms increase with increasing concentrations of PAP. Figure 5 shows a log-log calibration plot relating the observed anodic peak current ($I_{pa}$ ) to PAP concentrations ranging from 10 μM to 5 mM. A decade change in signal is observed per decade change in concentration, which indicates a linear relationship between the PAP concentration and $I_{pa}$. It should be noted that the data shown in this Figure include the range of currents observed during the actual assays (*vide infra*).

Although the rates of β-galactosidase-catalyzed reactions are faster at 37 °C than at room temperature, the kinetics of this enzyme with PAPG have only been reported at room temperature.[67] For this reason, the β-galactosidase-PAPG kinetics were examined at 37 °C in order to determine the best substrate concentration for the assay. The initial rates of this reaction (as $\Delta I_{pa}/\Delta t$) at different PAPG concentrations were used to construct the Hanes plot shown in Figure 6. The value of the $K_M$ was found to be 300 ± 34 μM and the maximum velocity 35.1± 0.3 μA/h. From the maximum velocity data (after converting currents to concentrations) and the β-galactosidase concentration of 31 pM the turnover number (TN) was calculated to be 1690 ± 14 $s^{-1}$. (Cf., $K_M$ = 179 mM and TN = 94 $s^{-1}$ for the same substrate at room temperature.)[67] On the basis of these

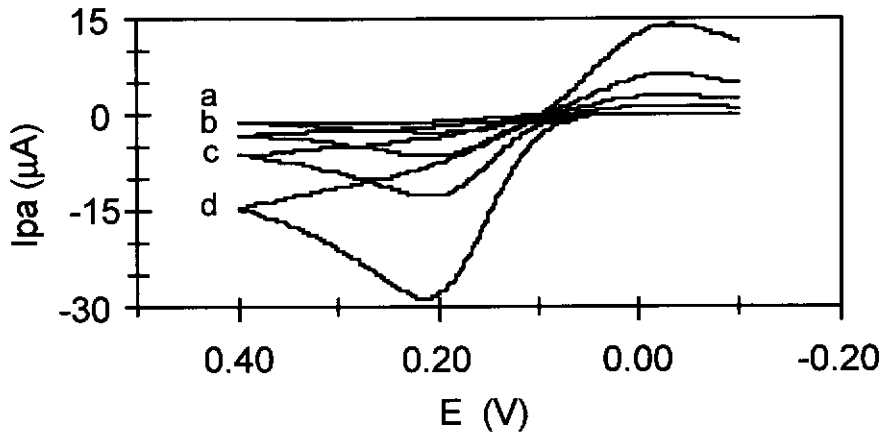



Figure 4. Cyclic staircase voltammograms of PAP in 0.100 M phosphate buffer, pH 7.0, containing 1.0 mg/mL BSA. Scan rate = 100 mV/s. (a) 10 μM PAP ; (b) 20 μM PAP; (c) 50 μM PAP; and (d) 100 μM PAP.

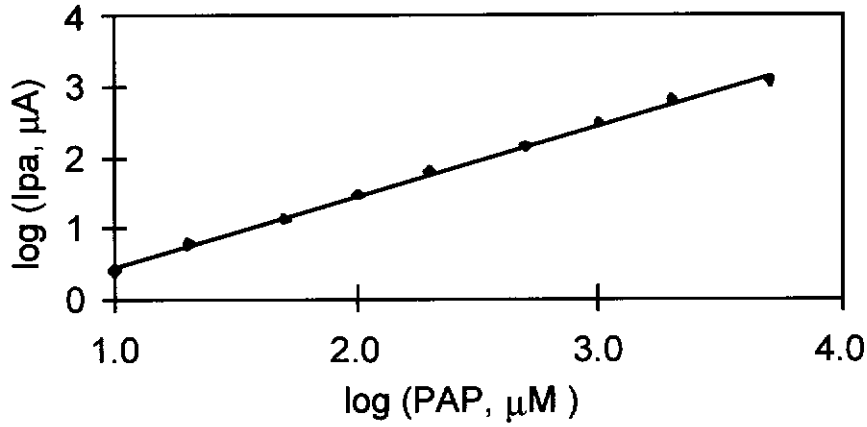



Figure 5. Calibration plot for PAP using peak currents ($I_{pa}$) from cyclic staircase voltammograms. Conditions as in Figure 2. Each point is the average of 3 determinations. The average relative standard deviation is 4.8%.

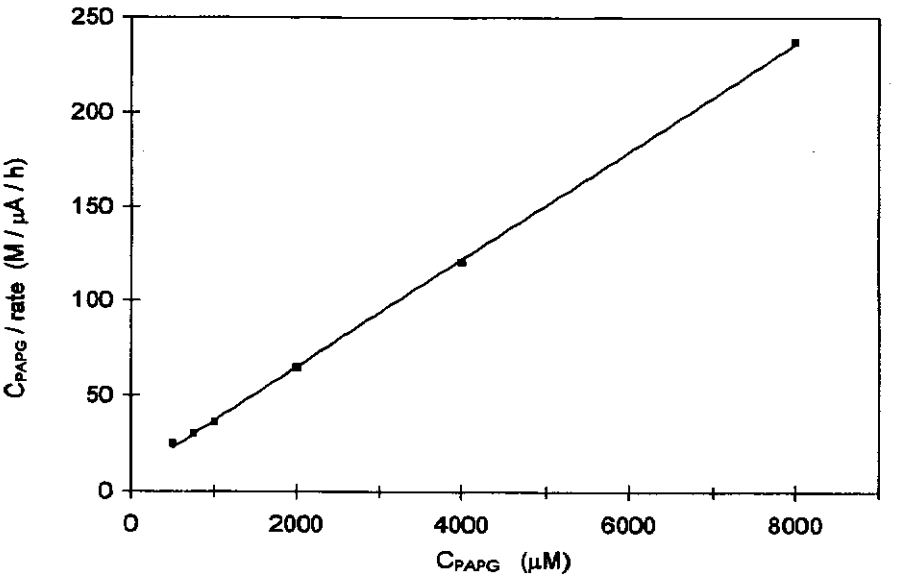



Figure 6. Hanes plot for the β-galactosidase-PAPG system at 37 °C in 0.100 M phosphate buffer, pH 7.0, containing 1.0 mg/mL BSA. A β-galactosidase concentration of 31 pM was used. The slope of the line is 28472 h $\mu A^{-1}$ and the standard error of the slope is 255 h $\mu A^{-1}$.

results, incubation at 37 °C using 8.0 mM PAPG was chosen for use in all subsequent studies. This PAPG concentration ensures that the enzymatic reaction proceeds at maximum velocity, which is determined by the amount of enzyme present, and that the rate of product formation is independent of substrate concentration.

A calibration plot for β-galactosidase was constructed to determine the range of enzyme concentrations that give rise to a linear relationship between the current generated and the amount of β-galactosidase present in the sample. For that, varying enzyme concentrations were incubated with 8 mM PAPG for 2 h. A linear response was

observed from approximately 5 pM to 250 pM of β-galactosidase, while the detection limit was 2.5 pM.

In order to investigate the response of the genetically designed bacterial sensing system, several parameters needed to be examined for their compatibility with the electrochemical detection scheme employed. For example, bacterial lysis procedures described in the literature (see reference 72) include dithiothreitol (DTT) in the lysis buffer. However, DTT passivates the electrode, and therefore, was not included in the lysis buffer employed in our studies. In addition to DTT, most common lysis procedures incorporate a 0.2% (w/v) Triton X-100 in the lysis solution. Better reproducibility and increasing signals were observed as the concentration of Triton X-100 in the lysis solution was increased from 0.2 to 5% (in 0.100 M phosphate, pH 7.0, containing 1.0 mg/mL BSA), but signals decreased at a concentration of 10%. Thus, 5% (w/v) was chosen as the Triton X-100 concentration for further studies. Longer lysis times also gave more reproducible results, so, for most of the studies, bacterial cells were allowed to lyse for 2 h. It was also observed that assay blanks containing no bacteria but treated with this lysis solution for up to 2 h gave signals that were not significantly different from those of the buffer background. Thus, the lysis buffer does not interfere with the electrochemical detection of PAP.

With lysis conditions optimized, the induction time in the presence of antimonite/arsenite and the incubation time of the expressed β-galactosidase with PAPG were varied to determine their effect on the response of the bacterial sensing system. Under our experimental conditions, the current response was found to be linear with time for the β-galactosidase/PAPG incubation step. A time of 2 h was chosen since it produced relatively large signals even when induction times of 0.5 h were used.

Two calibration plots using background-subtracted PAP oxidation peak heights plotted against the antimonite concentrations in the standards are shown in Figure 7. The data were obtained by incubating the antimonite with bacteria for either 0.5 or 17 h

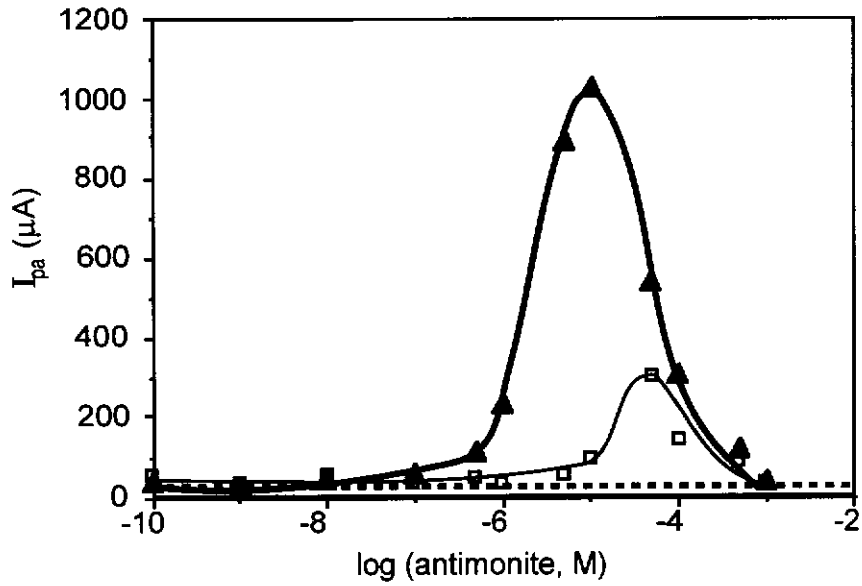



Figure 7. Calibration plots for antimonite at induction times of 0.5 h (❑) and 17 h (Δ). The broken line corresponds to the basal level expression of β-galactosidase. The average RSD was 20% and 9% (n = 3) at 0.5 h and 17 h induction times, respectively.

(induction time). Blanks containing bacteria which were not exposed to antimonite gave signals (indicated by the broken line in Figure 7) that were significantly different from buffer backgrounds. This is a result of basal-level expression of β-galactosidase from the genomic DNA of *E. coli*; i.e., β-galactosidase is expressed not only by induction of the reporter gene in the pBGD23 but also by bacterial genomic DNA. The larger signal observed was ~30-fold higher than the basal level. The reproducibility of replicates performed on a single day gave an average RSD of 20% (n = 3) when 0.5 h induction times between the antimonite and bacteria were used. Longer induction times of 17 h improved the reproducibility and gave an average RSD of 9%. The lowest RSD value (1.7%) was observed at 1 x $10^{-5}$ M. At the completion of a series of experiments, selected concentrations of antimonite were retested to verify that no significant change had occurred in the sample or the electrochemical response.

The observed responses are the result of several identifiable factors. Antimonite-induced expression of β-galactosidase generates a signal that increases with concentration up to antimonite concentrations of $10^{-5}$ M at 17 h induction times. Death of the bacteria caused by the toxicity of higher levels of antimonite results in a decrease in the induced signal at concentrations higher than $10^{-5}$ M. This is not surprising given that the designed plasmid does not encode for ArsA and ArsB, the proteins from the *ars* operon that form the protein pump responsible for effluxing antimonite from the bacterial cells. The genes that encode for these proteins were intentionally omitted in order to maximize the induction efficiency of the reporter gene in the presence of antimonite. The absence of the ArsA and ArsB ensures that antimonite cannot be effluxed from the cytoplasm of the cell and, thus, higher concentrations of cytosolic antimonite are obtained with lower concentrations of extracellular antimonite. This implies that the detection limit for antimonite should be better when the genes for ArsA and ArsB are absent.

In Figure 7, several differences are evident between the calibration plot obtained with a 0.5-h and a 17-h induction time. Figures of merit for these calibration plots and of a third calibration plot obtained with a 2-h induction time are summarized in Table 1 (see below) and show several trends. First, as one might expect, longer induction times lead

**Table 1. Figures of Merit from Antimonite Calibration Plots**

| Induction time (h) | Limit of detection[a] (M) | Observed maximum $I_{pa}$ (μA) |
|---|---|---|
| 0.50 | $1 \times 10^{-5}$ | 308 |
| 2.0 | $5 \times 10^{-6}$ | 794 |
| 17.0 | $1 \times 10^{-7}$ | 1028 |

[a] Reported value is the lowest experimental point that is significantly different from the blank (using t-test to compare the results of 3 blank determinations and 3 sample determinations; 95% confidence level)

to production of more β-galactosidase, which leads to more product formation and larger $I_{pa}$). Second, as shown in Figure 7, the peak in the calibration plot shifted to lower concentrations with longer induction times (probably because the longer the induction time, the lower the concentration of antimonite that can be tolerated by the bacteria).

Finally, the limit of detection was improved with increasing induction time. This may be explained by considering that at longer induction times lower concentrations of antimonite can induce sufficient β-galactosidase expression to be detected electrochemically above the blank.

As mentioned earlier, a conformational change in ArsR can be induced either by antimonite or arsenite, and either of these two anions can induce the *ars* operon. Therefore, it was expected that arsenite should have a similar effect as antimonite in the induction of the β-galactosidase reporter gene in bacteria bearing the plasmid pBGD23. To that effect, studies similar to those described above were performed with arsenite, and a calibration plot for this anion is shown in Figure 8. As shown in this Figure, the detection limit for arsenite is in the same order of magnitude as that for antimonite.

It has been reported that arsenate does not bind to ArsR in vitro,[30] and therefore, one may expect to see no response to this anion. However, some response to this anion was observed (detection limit of $1 \times 10^{-6}$ M after 17 h induction time with arsenate, and $1 \times 10^{-5}$ M after 2 h induction time). This in vivo response is due to intracellular conversion of arsenate to arsenite by the chromosomal arsenate reductase.[73]

The response of the bacterial sensing system to bismuthate ($BiO^+$) was also tested since it has been reported that it binds weakly to ArsR.[30] Using a 17-h induction time, the limit of detection for this ion was only $10^{-4}$ M, and the signal was increasing at least until the highest concentration of bismuthate tested, $1 \times 10^{-3}$ M. Higher concentrations of bismuthate could not be tested because of solubility considerations. It should also be noted that these studies were performed in water instead of phosphate buffer, also because of solubility limitations.

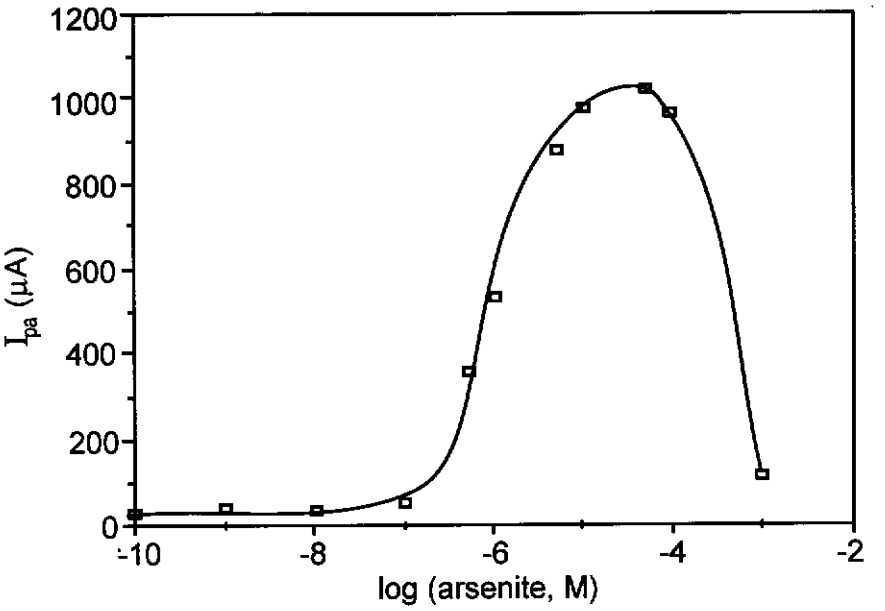



Figure 8. Calibration plot for arsenite using a 17-h induction time. Each point is the average of 3 determinations. The average relative standard deviation is 15% (n = 3).

The selectivity of the bacterial sensing system to some common oxoanions was also investigated, given that arsenite and antimonite are oxoanions. As shown in Table 2, the response signals generated after induction with nitrate, sulfate, phosphate, and carbonate are at least one hundred-fold smaller than the response obtained from antimonite. The response to these anions is, in fact, not significantly different from the blank bacterial response. This is not surprising given that these anions do not induce the *ars* operon as demonstrated by Wu and Rosen.[30] Indeed, to elicit a response in the bacterial sensing system, an analyte must be capable of both binding to the ArsR

**Table 2. Selectivity of the Bacterial Sensing System**

| Anion | $I_{pa}$ (μA)[a] | RSD (%) |
|---|---|---|
| Blank[b] | 35.4 | 6.1 |
| Arsenite | 1104 | 1.1 |
| Antimonite | 911.3 | 1.7 |
| Phosphate | 34.8 | 23.8 |
| Carbonate | 40.5 | 10.3 |
| Nitrate | 48.8 | 5.1 |
| Sulfate | 40.7 | 5.6 |

[a] From staircase voltammograms obtained after induction of the bacteria for 17-h with 1 x $10^{-5}$ M of the corresponding anions with the exception of phosphate, whose concentration was 0.1 M. Values are averages of triplicate determinations.

[b] Refers to bacteria that were not exposed to antimonite/arsenite.

protein and causing its release from the O/P site of the plasmid. Antimonite and arsenite are the most effective anions capable of accomplishing this function, thus they generate the highest response and can be detected at lower concentrations. It is interesting to note that phosphate, a common interference in many arsenic determination methods, does not pose a significant interference because it does not induce the *ars* operon.

## BACTERIAL SENSING SYSTEMS BASED ON BIOLUMINESCENCE DETECTION

In order to couple the selectivity conferred to bacteria by the *ars* operon with the sensitivity associated with bioluminescence detection, we have designed a plasmid that incorporates the genes of bacterial luciferase with the *arsR* gene of the *ars* operon. This plasmid contains the O/P region and the gene for the ArsR protein from the *ars*

operon upstream from the genes that code for luciferase, *luxA* and *luxB* (for simplicity abbreviated as *luxAB*) (Figure 3). In the constructed plasmid pRLUX the *luxA* gene is fused in frame to *arsD* in such a way that the resulting fusion protein contains part of the ArsD fused to the N-terminal domain of subunit A of luciferase. It has been shown previously that fusion proteins with truncated ArsD do not retain the regulatory function of the full ArsD.[65] Likewise, it has been demonstrated previously that fusions at the N-terminal domain of luciferase result in the formation of active enzyme.[81,82] As it will be demonstrated in this report, the fusion of truncated *arsD* with *luxA* yields a fusion protein that maintains luciferase activity, and whose expression is regulated by the ArsR protein.

The pRLUX plasmid was introduced into *E. coli* to yield bacteria that bioluminescence in the presence of antimonite and arsenite. The regulation of the expression of luciferase in this bacteria is as follows. In the absence of antimonite/arsenite, the ArsR protein binds to the O/P site of the plasmid preventing the transcription of the genes for ArsR and luciferase. When the inducer (antimonite or arsenite) is introduced it binds to ArsR causing a conformational change in the ArsR protein that results in its release from the O/P site of the plasmid. Consequently, protein expression of the bacterial luciferase can now commence.

A glow-type of bioluminescence emission is characteristic of a reaction catalyzed by the native bacterial luciferase. In order to determine whether the designed bacteria are capable of the glow-type emission characteristics of native luciferase, a bioluminescence emission study was performed. For that, the bioluminescence signal was measured for a period of 60 s after the injection of a given concentration of decanal. A typical bioluminescence emission is shown in Figure 9. This bioluminescence emission profile demonstrates a glow-type of bioluminescence, which is characteristic of native luciferase systems.[74] On the basis of this Figure and similarly

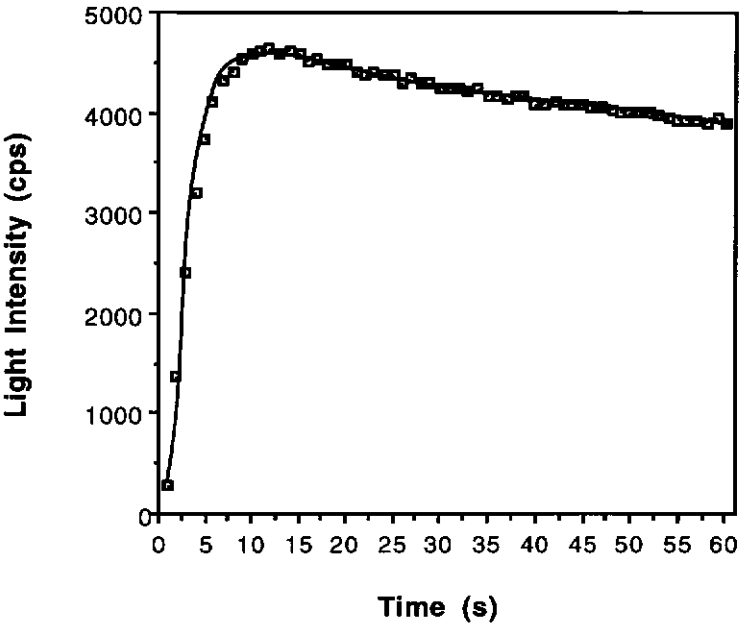



Figure 9. The bioluminescence emission of bacteria with pRLUX plasmid when 100 µL of 6.3 µM decanal was injected to a solution containing 50 µL of bacteria in 250 µL Tris-EDTA buffer.

obtained curves at other decanal concentrations, it was decided to integrate the intensity of the emitted for 3 s, after a 10 s delay from the time of injection of decanal.

The reaction catalyzed by native bacterial luciferase employs three substrates namely decanal, $FMNH_2$, and $O_2$. In order to control the kinetics of the reaction, it is necessary to control the concentration of the substrates. The concentration of $O_2$ available is in excess because the reaction is carried out in air and the concentration of $FMNH_2$ is dependent on the amount present inside the bacteria. Therefore, the concentration of externally added decanal has to be optimized in order to control the kinetics of the luciferase reaction in our system. If decanal were present in excess compared to $FMNH_2$ then all the intracellular $FMNH_2$ available would be consumed, thus stopping the luciferase reaction. In this case, the light emission observed follows a flash-type of kinetics instead of the characteristic glow-type observed with native

luciferase. Therefore, in order to obtain a glow-type bioluminescence emission the amount of decanal added needs to be optimized.

A calibration plot was constructed to determine the optimum concentration of the substrate decanal that is required for bioluminescence emission (Figure 10). The plot obtained has a good dynamic range for decanal from $10^{-4}$ M to $10^{-7}$ M. The working concentration of decanal was chosen after considering the intensity of the emitted light. A concentration of 6.3 μM solution of decanal, which is in the middle of the dynamic range, provides high enough signal while maintaining glow-type bioluminescence, and was used for all subsequent experiments.

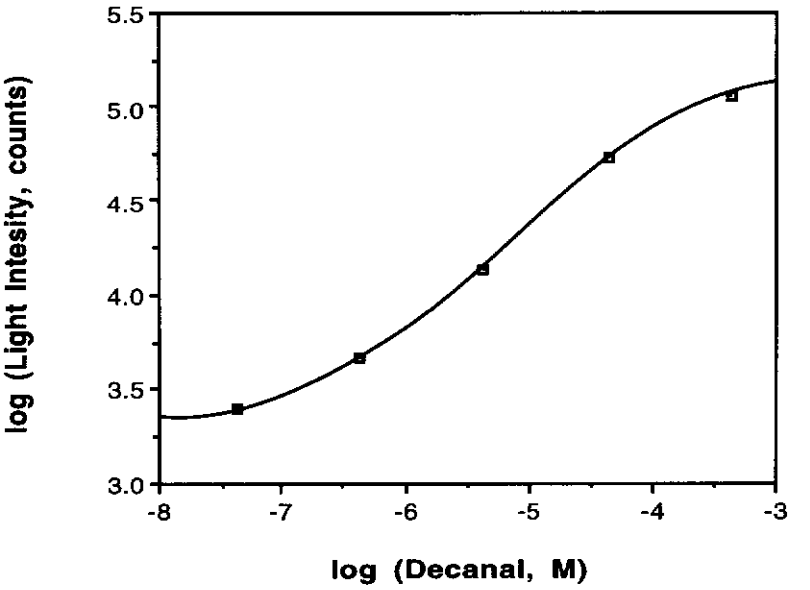



Figure 10. Calibration curve for decanal, obtained by injecting 100 μL of decanal (630 μM, 63 μM, 6.3 μM, 0.63 μM, 63 nM) to a solution containing 50 μL of bacteria with pRLUX plasmid in 250 μL of Tris-EDTA buffer. Light emitted was measured for 3 s.

The induction of luciferase expression is time dependant. Based on the design of the pRLUX vector. As the time of induction increases, there is an increase in the

bioluminescence measured. A time of induction of 2 h was chosen because it yields large enough bioluminescence signal while the assay time remains within acceptable limits.

The pRLUX plasmid is designed so that the ArsR protein regulates the expression of bacterial luciferase. As mentioned earlier, in our design all the genes of the *ars* operon that code for the protein pump have been deleted, and the *luxAB* genes have been inserted. Based on this design, the bacteria with the pRLUX plasmid can only sense antimonite but cannot pump the antimonite out of the bacterial cell. When the bacteria are exposed to antimonite two different events occur simultaneously. These are cell death and induction of luciferase expression. Since antimonite is toxic to the bacterial cell, when the accumulation of antimonite reaches certain levels, it kills the bacteria. However, as the concentration of antimonite increases, the concentration of the luciferase induced also increases. Therefore, we decided to study the effect of increasing the concentration of antimonite in bacteria containing the *arsR* and *luxAB* genes and in bacteria containing only the luciferase genes and not the *arsR* gene.

Bacteria containing the pQF70 plasmid, where the expression of luciferase is not regulated by ArsR, were used to develop a calibration curve for antimonite (Figure 11). It can be observed that as the antimonite concentration increases, there is a decrease in the bioluminescence signal emitted. It should also be noted that the intensity of the light that corresponds to the various concentrations of antimonite, is lower than the light emitted by bacteria that have not been exposed to antimonite. As the concentration of the antimonite increases, more bacterial cells die as demonstrated by the decrease in the bioluminescence emitted. Although this curve could be used as a calibration curve for antimonite, it should be stressed that the bacterial response is not specific for antimonite but rather the response should be similar for any toxic compound present in the sample. On the contrary, we postulated that the sensing system based on bacteria containing the pRLUX plasmid should be highly specific and that this specificity is confered by the ArsR regulatory protein. Therefore, we decided to study the effect of

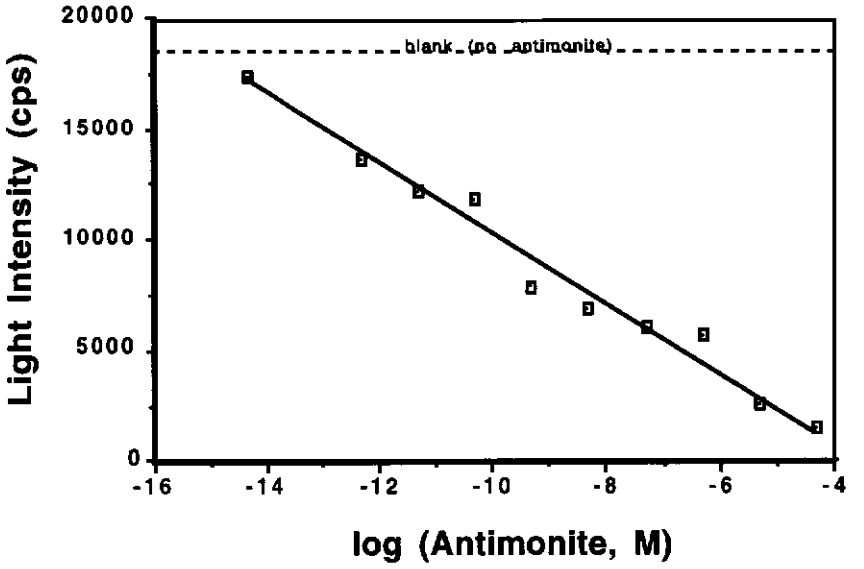



Figure 11. Calibration curve for antimonite performed after the bacteria with pQF70 plasmid were incubated with potassium antimonyl tartarate stock solutions for 2 h. A volume of 100 µL of 6.3 µM decanal was injected to 50 µL of bacteria in 250 µL of Tris-EDTA buffer. The bioluminescence signal was collected over a period of 3 s.

increasing the concentration of antimonite on the bacteria containing the pRLUX plasmid, where the expression of luciferase is regulated by the ArsR protein. When luciferase expression is induced in these bacteria both cell death and induction of luciferase would be expected to occur.

Our initial data (the system is still being optimized) are shown in Figure 12. This calibration curve can be broadly divided into two regions, analytical region I and analytical region II. Analytical region I is the portion of the curve, corresponding to "living light", where the intensity of the bioluminescence signal increases as the concentration of the antimonite increases. This portion of the curve is under the control of the *arsR* gene. In this region, the intensity of the bioluminescence signal emitted

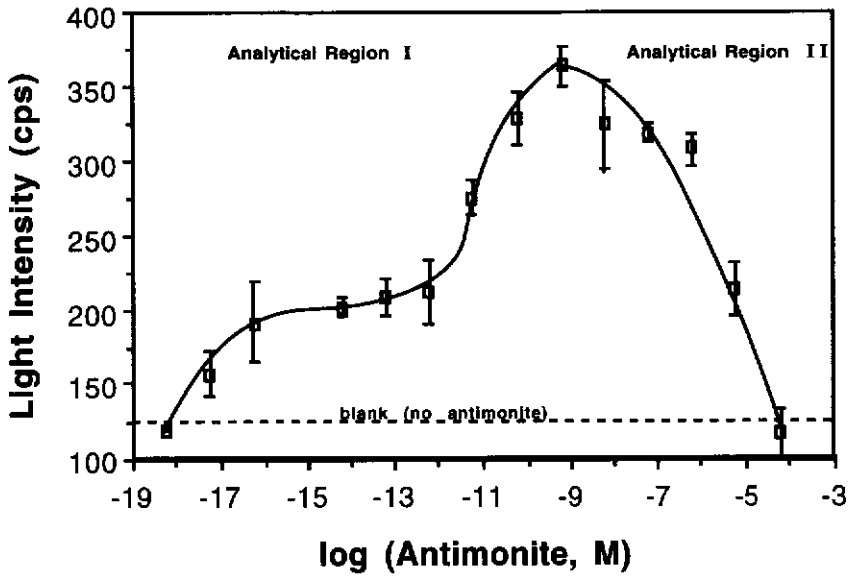



Figure 12. Calibration curve for antimonite performed after the bacteria with the pRLUX plasmid were incubated with potassium antimonyl tartarate stock solutions for 2 h. A volume of 100 µL of 6.3 µM decanal was injected to 50 µL of bacteria in 250 µL of Tris-EDTA buffer. The bioluminescence signal was collected over a period of 3 s.

increases with the concentration of antimonite in the sample. It can be seen that this portion of the curve is different from the calibration curve for antimonite obtained without the *arsR* regulation (Figure 11). In the calibration curve shown in Figure 11 the bioluminescence emitted decreases with increasing concentrations of antimonite. Moreover, the intensity of the light that corresponds to any antimonite concentration was lesser than the intensity of light emitted by bacteria grown in samples with no antimonite present (blank). In contrast, in bacteria containing the pRLUX plasmid the intensity of the bioluminescence signal is greater than the intensity of bioluminescence signal obtained for the blank. This indicates that the addition of antimonite to the sample

induces the expression of luciferase, which is controlled by the ArsR protein. In that respect, the ArsR protein acts like a switch that "turns on" the expression of luciferase when antimonite is present in the sample.

Analytical region II corresponds to a region of cell death. The bacteria are killed due to the toxicity of the antimonite at high concentrations of antimonite. At concentrations greater than $10^{-9}$ M, cell death, the other competing event becomes dominant. This portion of the curve is similar to the calibration curve obtained for a bacterial system without the ArsR regulation (Figure 11).

Next, we studied the response of the ArsR protein towards arsenite. Both arsenite and antimonite bind selectively to the ArsR protein. The arsenite calibration curve is similar to the calibration curve obtained for antimonite. The two regions, analytical region I and analytical region II are also present. This indicates that both antimonite and arsenite behave similarly and can induce the same response from ArsR.

The ArsR protein has a binding site that is very specific towards antimonite or arsenite and it cannot recognize other species such as phosphate or sulfate. None of these three anions induced expression of luciferase above the levels corresponding to the blank, even at concentrations a billion-fold higher than those of antimonite or arsenite.

## CHAPTER IV - SUMMARY AND CONCLUSIONS

In conclusion, bacterial sensing systems that respond selectively to antimonite and arsenite have been developed. These systems couple a plasmid-borne bacterial antimonite/arsenite resistance mechanism to a reporter gene. The reporter genes used in these studies were β-galactosidase (*lacZ*), an enzyme whose activity can be monitored electrochemically, and luciferase, an enzyme that produces bioluminescece. Although some β-galactosidase activity is naturally present in *E. coli* (from genomic DNA), the levels of induced β-galactosidase from plasmid pBGD23 by antimonite/arsenite are many-fold higher leading to detection limits as low as $1 \times 10^{-7}$ M.

Better detection limits should be obtained by using a reporter gene that, unlike *lacZ*, is not present in the *E. coli* genome because the background signal should be lower. In addition, reporter genes whose activity can be monitored by using more sensitive detection systems should yield methods with improved detection limits. In that respect, we coupled the selectivity of the ArsR protein to the sensitivity provided by bioluminescence detection in order to develop a highly sensitive and selective system for the determination of antimonite and arsenite. This optical sensing system employs genetically engineered bacteria containing a single plasmid that incorporates the regulatory gene of the efflux system, *arsR* and the genes of bacterial luciferase, *luxA* and *luxB*. In the designed plasmid, ArsR regulates the expression of bacterial luciferase in a manner that is dependent on the concentration of antimonite and arsenite in the sample. Thus, the bioluminescence emitted by the enzyme luciferase can be related to the concentration of antimonite and arsenite in the sample. Concentrations for antimonite and arsenite as low as $10^{-17}$ M and $10^{-15}$ M can be detected, respectively. This bacterial-based sensing system is at least a billion-fold more selective for antimonite and arsenite than for other anions such as phosphate and sulfate.

The two bacterial-based detection systems described in this report can form the basis for the development of highly selective and sensitive biosensors for the environmentally important species, arsenite and antimonite. The coupling of these bacteria with fiber optic technologies should allow the remote sensing of these species in groundwater and industrial effluents. These bacterial sensing systems can also be used to study the profiles of antimonite and arsenite in contaminated soils.

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