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Construction of Spores for Portable Bacterial Whole-Cell Biosensing Systems

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Whole-cell sensing systems based on living genetically engineered bacteria are known to have high sensitivity, selectivity, and rapid response times. Although these systems have found applications in biomedical and environmental analyses, their limited shelf life and transportability still restrict their use for on-site monitoring of analytes. To that end, we have developed a new method for the long-term preservation, storage, and transport of whole-cell biosensing systems that is based on bacterial spores, a dormant form of life. Specifically, we have employed spore-forming bacteria such as *Bacillus subtilis* and *Bacillus megaterium* for development of luminescent sensing systems for two model analytes, namely, arsenic and zinc. These sensing cells were converted to spores, which can then be "revived" (germinated) at a later time to generate viable and metabolically active cells. Herein, we demonstrate that these spore-based sensing systems retained their analytical performance, in terms of detection limit, dynamic range, and reproducibility, after storage at room temperature for at least 6 and 8 months, respectively, as well as after three cycles where the cells alternated between being dormant or active, i.e., sporulation−germination cycles. The ability to cycle the sensing cells between active and dormant states prolongs the cell's lifetimes and increases their robustness and ruggedness, thus making them more amenable for field applications. In addition, the small size of spores allows for their easy transport and incorporation in miniaturized portable devices. Finally, we envision that this novel strategy could expand the use of whole-cell biosensors for on-site sensing not only in mild environments but also in harsh environments and locations where there is no easy access to a laboratory, e.g., in developing countries.

Whole-cell sensing systems employ genetically engineered living cells that contain biospecific recognition elements for the detection of analytes of interest. In bacterial operon-based whole-cell sensing systems, the sensing element is comprised of a regulatory gene encoding a regulatory protein and a specific operator/promoter (O/P) sequence of DNA. The regulatory protein is capable of recognizing the analyte and controlling expression of a reporter gene that is placed under transcriptional control of the O/P. Upon binding the target analyte, the regulatory protein activates gene transcription, with subsequent expression of the reporter protein leading to the generation of a detectable signal. The reporter gene is expressed in a concentration-dependent manner, and calibration plots can be constructed by relating the signal generated with the concentration of analyte. Whole-cell sensing systems have been employed in a variety of environmental bioassays, as well as in biotechnology, pharmacology, and clinical chemistry applications. Numerous whole-cell sensing systems have been developed for environmental monitoring purposes. These include biosensors for the detection of toxic compounds such as mercury, arsenic, cadmium, lead, and several organic pollutants present in different types of environmental samples.[1−3] Recently, they have also been used for monitoring the bioavailability of chemicals such as nitrogen, phosphorus, and carbon in soil.[4] Additionally, whole-cell sensing systems have been developed for the detection of analytes of biological interest, including sugars, drugs, and quorum sensing signal molecules.[5−7] Various aspects of the construction of genetically engineered microorganisms and their application as biosensors in various fields have been discussed in recent reviews.[1,3,8−10] It is important to note that these bacterial sensing systems provide an inexpensive and simple way to selectively, sensitively, and rapidly detect very low levels of analytes. Additionally, they supply important information about the bioavailability and activity of the analyzed compounds.

In the past decade, our laboratory and those of others have successfully developed a good number of whole-cell sensing systems that have found applications in a variety of fields.[3,5,7,11,12]

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The previous work we performed with whole-cell biosensors gave us sufficient insight into these systems to identify what their advantages as well as their limitations are, posing the interesting problem of how to enhance their viability and utility. Moreover, it prompted us to postulate that the utility of whole-cell sensing systems could further be enhanced by packaging them in an appropriate manner that increases their shelf life and improves their effective use for on-site monitoring. To that end, certain requirements need to be met. Whole-cell-based biosensors possess the characteristics required for field analysis in that they are sensitive, reproducible, robust, and easy to use.[13,14] Portability, preservation, and long-term storage of the sensor bacteria are also crucial features for on-site applications. Methods for incorporating the biosensing cells into transportable devices, such as sol−gel encapsulation, immobilization on microtiter plates, and attachment onto optic fiber tips, have been developed.[15−17] Moreover, various preservation techniques have been employed to maintain the bacterial sensing cells viable and active, which include freeze-drying, vacuum-drying, continuous cultivation, and immobilization in organic and inorganic biocompatible polymers.[18] Although these methods are promising, improvements are needed, especially for long-term storage in unfavorable conditions. Limitations include costly and complex procedures required for freeze-drying method, less well-proven performance record of vacuum-drying process, labor intensiveness of continuous cultivation process, and biodegradation of organic polymers where cells are embedded.[18] We now report an alternative method of preservation, storage, and transport of sensor bacteria based on the generation of bacterial spores. This method is simple, inexpensive, robust, and appropriate for long-term maintenance, thus circumventing the undesired characteristics of the methods described above.

Some bacteria, such as certain *Bacillus* and *Clostridium* species, can adapt to changing environments and harsh conditions by forming highly resistant spores. Spores are dry, naturally hardy, dormant cells that can survive most environmental challenges, e.g., extreme temperatures, desiccation, and exposure to solvents and noxious chemicals for long periods of time, up to millions of years.[19] Along with these unique features, spores are capable of regaining their full metabolic activities in favorable environmental conditions. In fact, despite their inert state, they can sense even small amounts of nutrients, such as sugars and amino acids, and respond to them by germinating to viable, growing, and metabolically active cells.[20] Indeed, the most important function of spores is to lock the bacterial DNA into a stable crystalline state and exclude any toxic molecule that may be present, thus preserving the microorganism's genetic material.[17] Relevant to our goal is that spore formation, i.e., sporulation, as well as spore germination can be achieved in the laboratory using simple and well-established protocols.

In this work, we have employed spore-forming bacteria such as *Bacillus* species for the development of sensing systems and have generated spores as a simple, inexpensive, stable, and resistant way of storage and transport of the developed biosensing systems. Although *Bacillus* strains have previously been employed in whole-cell sensing,[21,22] their spore-forming ability and spore features have not been exploited for preservation of such sensing systems. Specifically, we have employed *Bacillus subtilis* and *Bacillus megaterium* strains for the development of sensing systems for arsenic and zinc, respectively, as model analytes. Additionally, we have evaluated the biosensor analytical performance in terms of detection limit, dynamic range, reproducibility, and selectivity, both before spore formation and after storage of the spores for a period of time, as well as after several cycles where the cells are dormant and then "revived" to be active again, i.e., sporulation−germination cycles.

EXPERIMENTAL SECTION

Reagents. Potassium antimonyl tartrate, sodium arsenate, sodium phosphate (monobasic), sodium arsenite, zinc chloride, tetracycline, and erythromycin were purchased from Sigma-Aldrich (Milwaukee, WI). Luria Bertani (LB) agar, M9 minimal medium, and LB broth were obtained from Difco (Sparks, MD). DNA primers were purchased from Operon Biotechnologies (Huntsville, AL). All chemicals were reagent grade or better and were used as received. All solutions were prepared using deionized, distilled water (Milli-Q water purification system, Millipore, Bedford, MA). The chemiluminescent substrate for β-galactosidase, Galacton-light plus, and light emission accelerator-II, a luminescence enhancer reagent, were purchased from Tropix (Bedford, MA), and used as suggested by the manufacturer.

Plasmids and Bacterial Strains. *B. subtilis* strain ars-23 contains plasmid pMUTin-23 bearing a set of three genes that confer resistance to arsenic (*arsR*, *arsB*, *arsC*) along with the reporter gene *lac-Z* encoding β-galactosidase under control of the ArsR regulatory protein (Figure 1).[23] ArsR is a DNA-binding repressor protein that regulates the expression of the *ars* operon. Upon binding As(III), ArsR releases itself from its DNA binding site, thus permitting gene transcription. ArsB is a membrane protein that pumps As(III) out of the cell using the electrochemical gradient across the membrane as the energy source. ArsC is a small cytoplasmic protein that reduces As(V) to As(III). For more details on the detoxification system encoded by the *ars* operon we refer the reader to a review article by Daunert et al.[1]

The plasmid pSD202 (Figure 1) was prepared by inserting a *Kpn*I-*Sph*I fragment containing the e*gfp* and *smtB* genes between the *Kpn*I-*Sph*I sequences of plasmid pMM1522 (Mobitec, Boca Raton, FL). The e*gfp* gene, encoding the enhanced green fluorescent protein (EGFP), was isolated from plasmid pEGFP (Clontech, Mountain view, CA) using polymerase chain reaction (PCR). Similarly, the sequences of the O/P and *smtB* gene of the *Synechococcus* PCC7942 *smt* operon were isolated from plasmid

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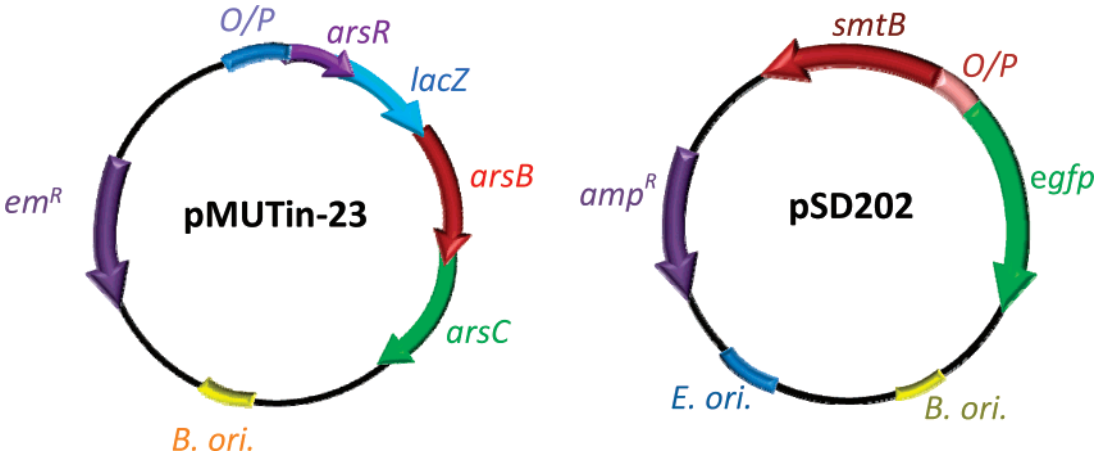

Figure 1. Schematic of the plasmids pMUTin-23 and pSD202 employed for arsenic and zinc sensing, respectively.

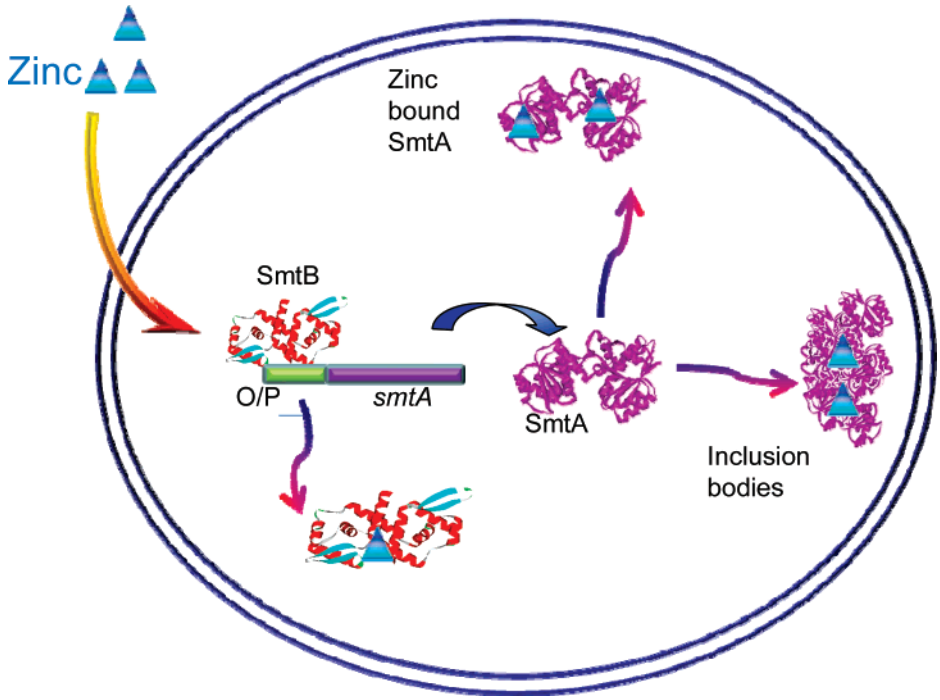

Figure 2. Machinery of the *smt* operon in *Synechococcus* PCC7942.

pJLE3C.[24] The *smtB* gene codes for a zinc-binding protein that regulates expression of the *smt* operon. SmtB is a repressor protein that, upon binding zinc, unbinds itself from the O/P region, thus allowing expression of the *smtA* gene, which is under the promoter control. The *smtA* gene codes for the SmtA protein, a metallothionein that binds zinc. The protein-bound metal is either transported within the cell for use by zinc-dependent enzymes or is sequestered in inclusion bodies.[25] A schematic of the *smt* operon machinery is shown in Figure 2. The overlap extension of the two gene fragments was carried out by PCR to create the *Kpn*I-*Sph*I fragment containing the e*gfp* and *smtB* genes. The resulting pSD202 vector contains the O/P region of the *smt* operon, the *smtB* gene, and the e*gfp* gene placed downstream under control of the *smt* operon promoter. It is worthy to note that the reporter gene in the plasmid vector replaces the *smtA* gene in the original operon. The construction of the pSD202 plasmid was verified by digesting the plasmid with *Kpn*I and *Sph*I and confirming the lengths of the digested fragments on a 1% agarose gel. The plasmid pSD202 was transformed into *B. megaterium* strain WH320 cells using conventional protocols (Mobitec, Boca Raton, FL).

Bacterial Spores. Spores of both types of bacterial sensing cells, i.e., the ars-23 *B. subtilis* strain and the *B. megaterium* strain for zinc constructed in this work, were prepared by using standard protocols and media, as described elsewhere.[26] Briefly, spores were generated by placing bacterial cells in sporulation medium at 37 °C with vigorous shaking for 4 days. All spores were harvested by centrifugation at 10 000*g* for 10 min at 4 °C, washed twice, and resuspended in deionized distilled water. Spore suspensions were then sonically vibrated for 5 min using the 550 sonic dismembrator from Fisher Scientific (Pittsburgh, PA) to release the endospores from the outer cell wall. Sonicated spore suspen-

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sions were centrifuged at 10 000*g* for 10 min at 4 °C. The supernatant was decanted to remove the outer cell wall debris, and the free spores were resuspended in deionized distilled water at an optical density of 0.8 at 600 nm (OD_{600}) and stored at room temperature for various periods of time.

Apparatus. Chemiluminescence and fluorescence measurements were made on a Polarstar Optima microplate luminometer from BMG Labtech (Durham, NC). All experiments were conducted at room temperature unless specified otherwise. All luminescence intensities reported are the average of a minimum of three replicates and are expressed in relative light units (RLU). Spores were observed at different magnifications (3000−8000×) under a Hitachi S-3200 scanning electron microscope at the University of Kentucky electron microscopy facility.

Dose−Response Curves for Antimonite, Arsenate, and Arsenite. A single colony of *B. subtilis* ars-23 strain or a volume of 20 μL of spore suspension was added to 300 mL of LB broth containing erythromycin (50 μg/mL). Bacterial cells from colony and spores were grown overnight at 37 °C, 250 rpm until the OD_{600} reached 0.6−0.7. Aliquots of 1 mL of bacterial suspension were then transferred into culture tubes. Analyte standard solutions of concentrations ranging from 1×10^{-4} to 1×10^{-9} M were prepared in deionized, distilled water by serial dilutions of freshly prepared 1×10^{-2} M stock solutions of potassium antimonyl tartrate, sodium arsenate, and sodium arsenite, respectively. A volume of 200 μL of each of these standard solutions and deionized, distilled water as blank was added in triplicate to the culture tubes containing the cell suspension. These culture tubes were then incubated at 37 °C at 250 rpm for 1 h. Next, bacterial suspensions were centrifuged at 8000*g* for 5 min at 4 °C. The pelleted cells were washed with 100 mM phosphate buffer, pH 7.4, then resuspended in 1 mL of the same buffer containing 0.2% (w/v) Triton-X-100, and placed in a shaker at 37 °C at 250 rpm for 1 h in order for the reporter enzyme β-galactosidase to be released into the medium after cell lysis. A 30 μL volume of cell lysate was added to 100 μL of chemiluminescent substrate solution and incubated in a 37 °C shaker at 250 rpm for 1 h. The chemiluminescence signal was triggered by injecting 100 μL of accelerator-II solution into the substrate−enzyme mixture. After a delay of 20 s necessary to reach maximal light emission, the chemiluminescent signal was collected over a period of time of 3 s on the microplate luminometer.

Dose−Response Curves for Zinc. A single colony or a volume of 20 μL of spore suspension of the *B. megaterium* strain bearing the reporter plasmid pSD202 was grown overnight at 37 °C in a culture tube containing 5 mL of LB broth and tetracycline (20 μg/mL). When the OD_{600} reached 0.7−0.8, the bacteria were transferred into a 500 mL flask containing 100 mL of LB broth and tetracycline (20 μg/mL) and grown at 37 °C until the OD_{600} reached 0.8. The bacterial cells were then centrifuged at 6000 rpm at 4 °C for 5 min, and the supernatant was discarded. The cells were washed twice with 10 mL of M9 minimal salt medium, then resuspended in M9 medium supplemented with $CaCl_2$ and $MgSO_4$ and 10% glucose until an OD_{600} of 0.8 was obtained. Aliquots of 1.8 mL of cell suspension were transferred into culture tubes. Zinc standard solutions ranging from 1×10^{-4} to 1×10^{-7} M were prepared in deionized, distilled water by serial dilution starting with a 1×10^{-2} M solution of zinc chloride. A volume of 200 μL of each of these standard solutions and deionized, distilled water as blank was added in triplicate to the culture tubes containing the cell suspension. After incubation for 1 h, the cells were harvested by centrifugation at 6000 rpm for 5 min at 4 °C. The supernatant was discarded, while the cells were washed twice with 100 mM phosphate buffer, pH 7.5, and resuspended in 500 μL of the same buffer. Subsequently, a volume of 100 μL of cell suspensions was diluted to 1 mL with the same buffer in glass tubes. A volume of 200 μL of these diluted cell suspensions was transferred in triplicate into a 96-well microtiter plate. The fluorescence measurements were carried out in the microplate luminometer with excitation wavelength set at 480 nm and emission wavelength at 510 nm.

Selectivity Studies. The selectivity of *B. subtilis* strain ars-23 was evaluated by incubating the sensing cells with solutions of potassium antimonyl tartrate, sodium arsenate, sodium arsenite, potassium ferricyanide, potassium dichromate, and sodium sulfate, in the concentration range of 1×10^{-4} to 1×10^{-8} M. Similarly, the selectivity of the *B. megaterium* strain for zinc developed in this work was evaluated by incubating the sensing cells with solutions of zinc chloride, cobalt chloride, nickel chloride, and cadmium chloride, in the concentration range of 1×10^{-4} to 1×10^{-6} M. In both cases, the assay was performed as described above.

RESULTS AND DISCUSSION

In the past 15 years, whole-cell sensing systems have been successfully employed for the detection of a variety of analytes. In fact, their selectivity/specificity and sensitivity characteristics have made them an attractive tool for a number of analytical applications. In order for these biosensing systems to find practical on-site applications, they need to be incorporated into devices. For that, in our work, we have developed a method based on the use of *Bacillus* spore-forming bacteria, which provides with a simple, reliable, and inexpensive means of maintenance, storage, and transport of the bacterial sensing systems and which could facilitate the integration of living cells into portable analytical devices. As models of the broad applicability of this spore-based approach, we developed two distinct sensing systems for the detection of arsenic and zinc, respectively, which employ different reporter genes and different *Bacillus* species as the host microorganism. Arsenic is a well-characterized environmental contaminant with toxic effects on humans that lead to vascular diseases, dermatitis, and cancer,[27] whereas zinc is an essential element whose deficiency is responsible for decreased growth rate and mental development in infants,[28] impaired wound healing and immune function, and reproductive failure, among others.[29] Specifically, the *B. subtilis* strain ars-23 contains a plasmid bearing *lac-Z*, the gene encoding for the reporter enzyme β-galactosidase, fused in-frame to part of the *ORF2* gene, downstream of the *arsR* gene of the *B. subtilis ars* operon.[19] The other genes of the *ars* operon, *arsB* and *arsC*, are located downstream of the *lac-Z* gene (Figure 1). The β-galactosidase reporter protein catalyzes the

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Table 1. Analytical Characteristics of the Spore-Based Whole-Cell Sensing System for Antimonite/Arsenate/Arsenite Evaluated before Sporulation and after Several Sporulation/Germination Cycles, as Well as after Long-Term Storage of the Spores[a]

analyte	before sporulation	cycle 1	cycle 2	cycle 3	after 6 months storage
			Detection Limit (M)		
antimonite	2.8×10^{-8}	1.6×10^{-8}	1.6×10^{-8}	2.8×10^{-8}	1.6×10^{-8}
arsenate	1.6×10^{-7}	1.6×10^{-8}	2.8×10^{-7}	1.6×10^{-7}	2.8×10^{-7}
arsenite	2.8×10^{-7}	1.6×10^{-7}	2.8×10^{-7}	2.8×10^{-7}	2.8×10^{-7}
			Dynamic Range (M)		
antimonite	1.6×10^{-5}–2.8×10^{-8}	1.6×10^{-5}–1.6×10^{-8}	1.6×10^{-5}–1.6×10^{-8}	1.6×10^{-5}–1.6×10^{-8}	2.8×10^{-5}–1.6×10^{-8}
arsenate	2.8×10^{-5}–1.6×10^{-7}	8.3×10^{-5}–1.6×10^{-7}	1.6×10^{-4}–2.8×10^{-7}	1.6×10^{-4}–2.8×10^{-7}	2.8×10^{-4}–2.8×10^{-7}
arsenite	1.6×10^{-5}–2.8×10^{-7}	1.6×10^{-4}–1.6×10^{-7}	1.6×10^{-4}–2.8×10^{-7}	1.6×10^{-4}–2.8×10^{-7}	1.6×10^{-5}–2.8×10^{-7}

[a] The limit of detection was defined as the analyte concentration that produced a signal equal to or higher than the average signal produced by the blank plus three standard deviations.

hydrolysis of β-galactosides. In this study, we chose to monitor the activity of the β-galactosidase enzyme by using a chemiluminescent substrate, a 1,2-dioxetane β-D-galactopyranoside derivative. The enzymatic reaction results in the emission of light at 463 nm in a manner that is directly proportional to the concentration of bioavailable analyte, in this case, arsenite or antimonite, present in the sample. The first step in the analytical characterization of this bacterial strain was to demonstrate that the *Bacillus* cells were capable of sensing. For that, we incubated the cells with varying concentrations of sodium arsenite and potassium antimonyl tartrate in the conditions described above. Since the genetic construct employed in this study includes *arsC*, a gene encoding the ArsC protein that functions as arsenate reductase to reduce arsenate to arsenite, we also incubated the sensing cells with varying concentrations of sodium arsenate. The β-galactosidase expressed by the bacteria in response to the analyte intracellular amount could be related to the extracellular concentration of analyte. Dose−response curves were constructed, thus showing that the *Bacillus* cells could sense all of the three analytes in a dose-dependent fashion. The ability of responding to both arsenite and arsenate is important for the analysis of real samples, where different species of arsenic could be present. The signal intensity increased until the analyte levels reached 1×10^{-4} M. At higher concentrations, a decrease in signal was observed due to cell death resulting from the toxicity associated with the analyte itself. At concentrations lower than 1×10^{-8} M for antimonite and lower than 1×10^{-7} M for arsenate and arsenite, no significant change in the light emission was observed. The detection limits for antimonite, arsenate, and arsenite were found to be 2.8×10^{-8}, 1.6×10^{-7}, and 2.8×10^{-7} M, respectively, with dynamic ranges over 2−3 orders of magnitude. The detection limit was defined as the analyte concentration that produced a signal equal to or higher than the average signal produced by the blank plus three standard deviations (SD). The intra- and interassay reproducibility was satisfactory, as shown by values of the coefficients of variation ranging 4−14%. Selectivity studies were also performed with the *B. subtilis* ars-23 strain. Cells were treated with solutions at different concentrations of sodium sulfate, potassium ferricyanide, potassium dichromate, and sodium arsenite, respectively, and the resulting chemiluminescence was measured. The results showed that the response to ions with size, charge, and structure similar to that of antimonite/arsenite was negligible when compared to the response to the target analytes, thus confirming the selectivity of the sensing strain (data not shown). Overall, the analytical performance of the *Bacillus*-based sensing system (Table 1) proved to be consistent with that of other sensing systems developed for arsenic using the same reporter gene and nonsporulating bacteria, such as *Escherichia coli*, as the host microorganism.[11]

In order to develop a bacterial sensing system for zinc, plasmid pSD202 was designed to incorporate the gene for the reporter protein eGFP under transcriptional control of the promoter and the regulatory protein SmtB of the *smt* operon (Figure 1). While the β-galactosidase-based reporter strain for arsenic described above served as proof of concept, a fluorescent protein was chosen as a reporter herein because it presents several advantages over enzymatic reporters for on-site applications. These include no need for adding an exogenous substrate and for lysing the sensing cells to detect the reporter protein activity, along with the possibility of monitoring the reporter protein formation and accumulation in real time. Specifically, the *smtB* gene is placed upstream while the *egfp* gene is located downstream of the O/P region of the *smt* operon. In the absence of zinc ions, the SmtB protein binds to the O/P region of the *smt* operon and inhibits the transcription of the eGFP protein. Alternatively, the presence of zinc ions, which bind to SmtB, causes a conformational change in the SmtB protein that leads to transcription of the *egfp* gene located downstream of the O/P region. After transformation of the reporter plasmid pSD202 into *B. megaterium*, the bacterial cells were incubated with varying concentrations of zinc chloride, and the signal produced by the eGFP expressed by the bacteria was measured to evaluate their response. The fluorescence increased until the analyte level reached 1×10^{-4} M. At higher concentrations, a decrease in signal was observed due to cell death resulting from the analyte toxicity. At concentrations lower than 1×10^{-6} M, there was no noticeable change in the fluorescence. A dose-dependent response was observed in a range of concentrations over 2 orders of magnitude, with a detection limit for zinc of 1×10^{-6} M (see Figure 5). Reproducibility studies exhibited intra- and interassay coefficient of variation values below 14%. Selectivity studies were also performed with *B. megaterium* WH130 cells harboring plasmid pSD202. Cells were treated with solutions at different concentrations of Cd^{2+}, Ni^{2+}, and Co^{2+} ions, respectively, and the resulting fluorescence intensity was measured. It was

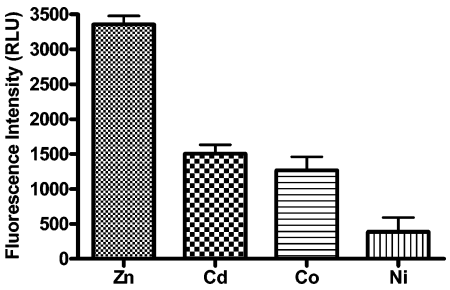

Figure 3. Selectivity study performed with *B. megaterium* cells harboring plasmid pSD202. Cells were incubated for 1 h at 37 °C with a 1×10^{-4} M solution of each ion, and the resulting fluorescence intensity was measured with a λ_{ex} of 480 nm and a λ_{em} of 510 nm. The signals have been corrected with respect to the blank. Data are the average ±1 SD ($n = 3$).

observed that the biosensor also responded to these ions, although the response was less than that to Zn^{2+} ions. The maximum response to each of the tested metals was observed at the concentration of 1×10^{-4} M. These results are reported in Figure 3. The SmtB protein is also known to be responsive to Cd^{2+} and Co^{2+} because of its sequence similarity to other metalloregulatory proteins, such as CadC.[29] The sensor's ability of responding to Cd^{2+} should not interfere with Zn^{2+} sensing in biological fluids because cadmium is a toxic heavy metal not expected to be found in humans at significant levels, unless accidental exposure occurred. On the other hand, cobalt is an essential element present in the human body as a constituent of vitamin B_{12}. However, cobalt blood levels are reported to be in the order of nanomolar concentrations, which are at least 3 orders of magnitude lower than optimal zinc blood levels (1.2×10^{-5} to 3.8×10^{-5} M).[30]

After evaluating the analytical performance of the developed whole-cell sensing systems, cells of *B. subtilis* containing *arsR-lac-Z* genes and of *B. megaterium* containing *smtB-egfp* genes were sporulated following standard sporulation protocols as described in the Experimental Section. A scanning electron micrograph of the spores thus obtained, at a magnification of 4500×, is shown in Figure 4. The size of spores was determined to be approximately 1 μm by 0.5 μm. A spore is composed of a set of protective structures arranged in series of concentric shells. The DNA is stored in a desiccated form within the core of the structure. When provided with the essential germinating agents and nutrients, spores can germinate and regain complete cellular activities. Upon these considerations, spores of each of the two biosensing systems were exposed to germination conditions and the obtained vegetative cells were then evaluated for their ability to respond to the respective analytes, by using the protocols described above. The results for both sensing systems indicated that, not only were the germinated cells responsive to the analytes in a dose-dependent fashion, but they also maintained their analytical performance in terms of detection limit, dynamic range, and reproducibility. A representative example for the zinc biosensor is shown in Figure 5.

The sustainability of spores as a means for preserving, storing, and transporting whole-cell sensing systems was further evaluated by exposing the sensing bacteria to three sporulation−germination

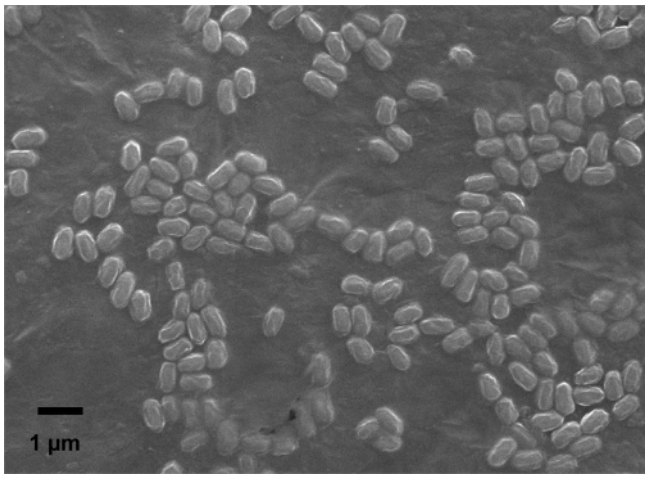

Figure 4. Scanning electron micrograph of spores of *B. subtilis*. Magnification 4500×. Scale bar represents 1 μm.

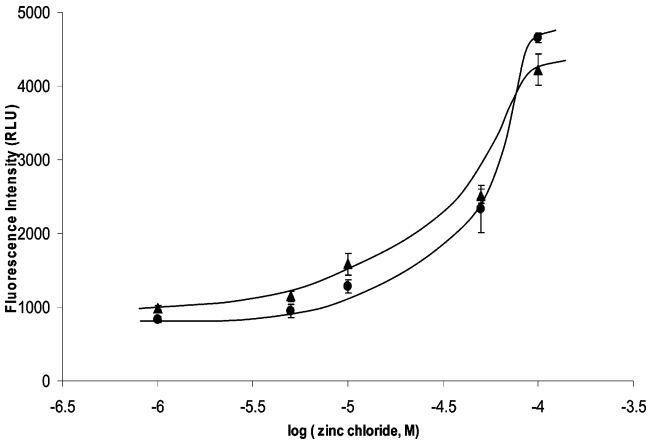

Figure 5. Dose−response curves of the *B. megaterium* sensing cells harboring the pSD202 plasmid, generated before (●) and after (▲) sporulation. Cells were incubated with various concentrations of zinc chloride for 1 h at 37 °C. Fluorescence intensity was measured with a λ_{ex} of 480 nm and λ_{em} of 510 nm. The signals have been corrected with respect to the blank. Data are the average ±1 SD ($n = 3$).

cycles at intervals of 1 month. The detection limits and dynamic ranges for the analytes, as well as the reproducibility of the spore-based biosensing systems, were determined after germination in each cycle. A schematic of the experimental setup is presented in Figure 6. The results obtained in these sets of experiments for the antimonite/arsenate/arsenite biosensor are summarized in Table 1. For the zinc biosensor, the detection limit of 1×10^{-6} M and dynamic range of 1×10^{-4} to 1×10^{-6} M, achieved before sporulation, were also obtained after each cycle, as well as after 8 months storage. It is evident that both limits of detection and dynamic ranges did not change significantly after each sporulation−germination cycle, with comparison to presporulation. Importantly, both of the spore-based sensing systems retained their analytical performance also after being stored at room temperature for at least 6 and 8 months, respectively, thus supporting the long-term storage of the sensing bacterial cells in the form of spores. The coefficients of variation were also consistent with those calculated before sporulation (data not shown), thus confirming the reproducibility of the system over time and after sporulation−germination cycles. It is noteworthy that, along with the perfor-

(30) Zapsalis, C.; Beck, R. A. *Food Chemistry and Nutritional Biochemistry*; Wiley: New York, 1985.

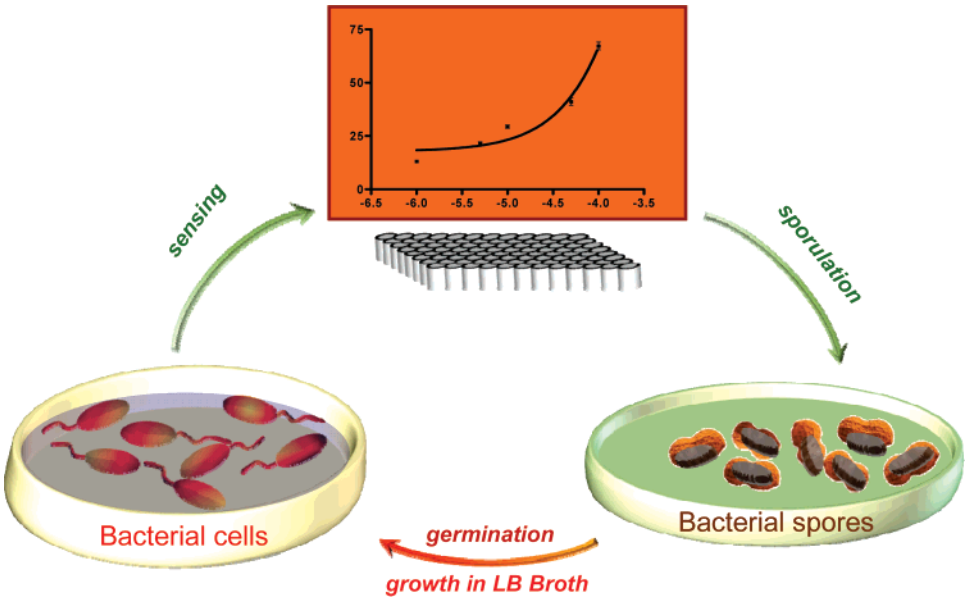

Figure 6. Schematic representation of the experimental setup including sensing−sporulation−germination cycles.

mance being virtually identical before and after sporulation, the cell's response in terms of signal intensity is also virtually identical (Figure 5). These results show that the reported spore-based approach allows addressing one of the most relevant drawbacks of whole-cell sensing systems, that is, their limited stability.

CONCLUSIONS

We have demonstrated a new method of preservation, storage, and transport of whole-cell biosensing systems based on the formation of spores that will allow for their broad use in on-site applications, thus circumventing some of the current drawbacks of cell-based biosensors. Furthermore, the possibility of long-term storage of the whole-cell sensing systems should enable their incorporation into various analytical devices such as micro-total-analytical systems (μ-TAS) by packaging them with all the needed reagents in a dry form. Living whole-cell biosensing systems can be integrated into portable analytical devices, such as microcentrifugal microfluidic platforms.[31] These devices allow for rapid responses in the order of minutes, with simple and shortened procedure as compared to conventional benchtop protocol. In particular, the use of fluorescent and bioluminescent photoproteins as reporters is expected to facilitate the integration of whole-cell biosensing systems into such miniaturized analytical devices.[32] This would further enhance environmental on-field applications, as well as biomedical applications at the patient bedside and the physician's office. The use of miniaturized analytical systems would also allow incorporating multiple biosensing systems in one small device, thus providing a portable solution to environmental and clinical multiplexed, on-site analysis. Moreover, the well-documented resistance of spores to extreme conditions such as dry and wet heat, freezing temperatures, and desiccation, among others, could prompt the use of whole-cell sensing systems in harsh environments, upon evaluation of the stability of the spore-based whole-cell sensing systems in those environments. This would be of particular importance in developing countries, which often experience difficult environmental conditions and have poor storage and transportation facilities, or in the challenging environments of a battlefield.

(31) Rothert, A.; Deo, S. K.; Millner, L.; Puckett, L. G.; Madou, M. J.; Daunert, S. *Anal. Biochem.* **2005**, *342*, 11−19.
(32) Dikici, E.; Rowe, L.; Moschou, E. A.; Rothert, A.; Deo, S.; Daunert, S. Luminescent Proteins: Applications in Microfluidics and Miniaturized Analytical Systems. In *Photoproteins in Bioanalysis*; Daunert, S., Deo, S. K., Eds.; Wiley VCH: Weinheim, Germany, 2006; pp 179−198.

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