CHAPTER TWO

# Engineering Rugged Field Assays to Detect Hazardous Chemicals Using Spore-Based Bacterial Biosensors

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## Contents



*Methods in Enzymology*, Volume 589
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2017.02.005

## Abstract

Bacterial whole cell-based biosensors have been genetically engineered to achieve selective and reliable detection of a wide range of hazardous chemicals. Although whole-cell biosensors demonstrate many advantages for field-based detection of target analytes, there are still some challenges that need to be addressed. Most notably, their often modest shelf life and need for special handling and storage make them challenging to use in situations where access to reagents, instrumentation, and expertise are limited. These problems can be circumvented by developing biosensors in *Bacillus* spores, which can be engineered to address all of these concerns. In its sporulated state, a whole cell-based biosensor has a remarkably long life span and is exceptionally resistant to environmental insult. When these spores are germinated for use in analytical techniques, they show no loss in performance, even after long periods of storage under harsh conditions. In this chapter, we will discuss the development and use of whole cell-based sensors, their adaptation to spore-based biosensors, their current applications, and future directions in the field.

# 1. INTRODUCTION

## 1.1 The Need for Portable, Field-Amenable Technology for Monitoring Environmental Pollutants

The spread and persistence of many different hazardous materials represents a significant threat to both human populations and the environment. These materials range from consumer products and the by-products of industry to naturally occurring elements. Given the toxic and persistent nature of many environmental pollutants there is a need to develop highly selective, inexpensive technologies to quickly, easily, and reliably monitor contamination levels in situ. This is especially important due to the likelihood of biological accumulation, a phenomenon where species high on the food chain become contaminated through consumption of their food (Perkins, 1986). There is also a significant risk of contamination occurring in water sources. Indeed, without quick and sensitive assays, contamination will have an increasingly detrimental effect as the long-term consequences of low-concentration chronic exposure to pollutants are often not as apparent as the symptoms of high-concentration acute exposure, despite remaining quite deadly (Aksoy, 1985; Buchancova et al., 1998; Dallaire et al., 2014; Ihrig, Shalat, & Baynes, 1998; Ludewig & Robertson, 2013; Tsuji, 2015; Wahlang et al., 2014). Additionally, many of the communities affected by pollution are in rural, underdeveloped areas that do not have access to laboratories equipped to perform environmental analysis. These communities will benefit tremendously from the use of portable technologies to monitor their local environment.

## 1.2 Current Methods of Detection

The range of physical and chemical characteristics found in hazardous materials presents a challenge for researchers since the variance in chemical and physical properties necessitates a number of analytical techniques in order to accurately assess biological and environmental samples for contamination. Historically, one of the most popular techniques was atomic absorption spectrometry, especially for the detection of heavy metals (Soodan, Pakade, Nagpal, & Katnoria, 2014). Despite its initial popularity, the method has largely been replaced by more recent innovations (Soodan et al., 2014). Some of these newer methods involve the use of mass spectrometry (MS). For example, methods designed to assay for organic molecules like polychlorinated biphenyls (PCBs), hydroxylated PCBs (OH-PCBs), benzene, toluene, ethylbenzene, and xylene contamination use gas chromatography-coupled MS (Grosser, 2006). Heavy metal detection is often accomplished using inductively coupled plasma MS (ICP-MS) (Ortega, 2002). In fact, ICP-MS is the "gold standard" for heavy metal analysis since it permits multielemental trace detection (Beauchemin, 2008).

These MS-based techniques are favored because of their excellent sensitivity, selectivity, and their established, reliable methodology. Despite these significant advantages, the nature of environmental pollution demands additional attributes from analytical methods for timely and efficient monitoring of contamination. The instrumentation in combination with the need for highly trained personnel makes the use of traditional methods expensive, often prohibitively so in rural or developing countries. Additionally, MS-based techniques can only be performed in a laboratory setting. Another concern that needs to be considered involves the analysis of complex matrices. This is particularly important in field-based assays designed to analyze biological and environmental samples, since it is difficult to incorporate sample preparation steps without increasing the need for additional materials and instruments. Other techniques, like atomic fluorescence spectrometry, ICP-optical emission spectroscopy, ICP-atomic emission spectrometry, and X-ray fluorescence spectrometry, are widely used and afford very low detection limits, but, unfortunately, also possess some of the same disadvantages as MS (Soodan et al., 2014).

The shortcomings of the current methodologies in field applications have led scientists to explore alternative approaches that employ portable instrument platforms for detection of compounds in the environment. As a result, a host of nanoparticle-based sensors have been developed for a wide range of compounds (Ibrahim, Hayyan, AlSaadi, Hayyan, & Ibrahim, 2016).

Many of these nanoparticles take advantage of surface-enhanced Raman spectroscopy (SERS) (Li, Zhai, Li, & Long, 2014). This technique has been used to develop SERS substrates to detect a wide variety of analytes, including arsenate (Han, Hao, Xu, & Meng, 2011), PCBs (Bantz & Haynes, 2009), pesticides (Pang, Yang, & He, 2016), trinitrotoluene (Mahmoud & Zourob, 2013), and uranium (Ruan, Luo, Wang, & Gu, 2007). Assays using SERS fingerprints as a mode of detection require specialized instrumentation. These tools have been miniaturized and implemented in microfluidic and analytical platforms configured for field studies (Qu et al., 2012), but their use still requires specialized training and access to this technology may be limited by its price. It is worth noting that the nanomaterials themselves may be quite expensive as many of these materials incorporate gold and silver. Although these methods generally have excellent sensitivity and can be performed rapidly, they often have difficulty discriminating between analytes, especially metals (Li et al., 2014). An ideal scenario for field studies would include use of analytical technology that can be adapted to address the need to assay samples containing multiple contaminants. It would also be inexpensive and require minimal training and expertise for its use. Indeed, in some cases, these technologies should be easy to use so that they may be employed by the general public to ensure that contamination in homes and communities is monitored.

## 2. WHOLE CELL-BASED BIOSENSORS

### 2.1 Adapting Biology to Serve as Technology

Whole cell-based biosensors (WCBs) have emerged as an alternative to traditional instrumentation in that they are inexpensive to produce, require no special equipment or training beyond basic laboratory facilities and techniques, and exhibit high selectivity and sensitivity of detection. Furthermore, the design and execution of these assays makes them very easy to miniaturize and facilitates their use in a field-based platform. The data produced by these assays can be particularly useful since the living organisms that are employed provide valuable information about the toxicity and bioavailability of the analyte being measured (Belkin, 2003). Many different designs and methods have been developed for WCBs; however, herein we focus on WCBs that have been genetically engineered to exploit the regulatory mechanisms of species of bacteria that have developed resistance to toxic materials.

Many species of bacteria have evolved to become resistant to toxic substances through a variety of mechanisms. For example, in *Escherichia coli*, the plasmid R773 confers resistance to arsenic species by encoding for a number of genes that form an ATP-driven efflux pump that removes arsenic from the cytosol (Carlin, Shi, Dey, & Rosen, 1995). Similar mechanisms have developed in *Synechococcus* to remove cadmium, lead, and zinc from the cell (Erbe, Taylor, & Hall, 1995). Other organisms have developed resistance to toxins through degradation pathways. *Pseudomonas azelaica* has developed a resistance to hydroxybiphenyls by evolving an operon consisting of enzymes that degrade these compounds into carbon and energy sources (Kohler, Kohler-Staub, & Focht, 1988). Similarly, *Pseudomonas putida* has established degradation pathways for volatile organic compounds that ultimately produce Krebs cycle intermediates (Marques & Ramos, 1993).

These resistance mechanisms provide excellent candidates for exploitation in biosensor development, not because of the enzymes that confer resistance but because of the machinery involved in the regulation of the transcription of these enzymes. Two core components form this machinery: a promoter which controls the transcription of the resistance genes, and a constitutively expressed regulatory protein, typically a binding protein. There are two general paradigms in which this occurs as the regulatory protein may be a transcriptional repressor or a transcriptional activator (Snyder & Champness, 1997). If the regulatory gene codes for a transcriptional repressor, the corresponding protein will bind to the promoter and prevent transcription in the absence of the ligand. In the presence of the compound, the repressor will bind its ligand and allow for transcription of the resistance genes; thus, the repressor negatively regulates expression of the resistance genes. Conversely, if the regulatory gene encodes for a transcriptional activator, the corresponding regulatory protein will only bind to the promoter in the presence of ligand, possibly due to a conformational shift upon binding the ligand or oligomerization following binding that allows for DNA binding. This allows for the initiation of transcription of the resistance genes. Gene expression may also be regulated through other mechanisms, but these will not be discussed because they are not pertinent to the development of WCBs. Regulation schemes using transcriptional repressors as sensing elements form the foundation of many WCBs because this particular design is easy to engineer, use, and produces results that are easy to interpret.

WCBs have been developed for a wide range of analytes with diverse chemical and physical properties. Metals like antimony, arsenic, cadmium, chromium, cobalt, copper, lead, mercury, nickel, and zinc have all been

detected with WCBs (Collard et al., 1994; Feliciano, Liu, & Daunert, 2006; Klein, Altenbuchner, & Mattes, 1997; Ramanathan, Shi, Rosen, & Daunert, 1997; Shetty, Deo, Liu, & Daunert, 2004; Shetty et al., 2003; Stocker et al., 2003; Tauriainen, Karp, Chang, & Virta, 1998). WCBs have also been engineered to respond to a variety of organic compounds that range from volatile organics to stable, persistent organic pollutants, including benzene, chlorocatechols, ethylbenzene, toluene, middle chain alkanes, naphthalene, organophosphates, phenols, xylene, PCBs, and OH-PCBs (Behzadian, Barjeste, Hosseinkhani, & Zarei, 2011; Feliciano, Xu, et al., 2006; Guan et al., 2000; Kobatake, Niimi, Haruyama, Ikariyama, & Aizawa, 1995; Liu, Germaine, Ryan, & Dowling, 2010; Mulchandani, Mulchandani, Kaneva, & Chen, 1998; Shin, Park, & Lim, 2005; Sticher et al., 1997; Turner et al., 2007). These sensors have found applications in environmental analysis since many of these compounds are dangerous and common environmental contaminants. Indeed, arsenic, lead, mercury, cadmium, benzene, and PCBs are among the most concerning compounds on the Agency for Toxic Substances and Disease Registry (ATSDR) biannual priority list (ATSDR, 2015). Biosensors are also useful for the detection of pesticides since many popular insecticides contain chlorocatechols and organophosphates. Devices have also been developed to monitor water sources by engineering a chip that uses a charge-coupled device to monitor the bioluminescent signal of WCBs (Tsai et al., 2015). In this device, water flows through microfluidic channels and pollutant detection in the water was possible after only 15 min. Although the environmental applications of WCBs have received a great deal of attention, the technology has proven to be useful in many other instances. Biomedical applications have been seen in the detection of amino acids, sugars, and viruses, like tuberculosis and human respiratory syncytial virus (Jacobs et al., 1993; Jaeger, Lindow, Miller, Clark, & Firestone, 1999; Olivo, Collins, Peeples, & Schlesinger, 1998; Shetty, Ramanathan, Badr, Wolford, & Daunert, 1999). The technology has been further exploited to be used as a tool to evaluate clinical samples for bacterial quorum-sensing molecules, and as laboratory tools to demonstrate bacterial proliferation in the gut of humans and animals such as mice (Kumari, Pasini, & Daunert, 2008; Kumari et al., 2006; O'Connor et al., 2015; Raut, Joel, Pasini, & Daunert, 2015; Raut, Pasini, & Daunert, 2013; Struss, Pasini, Ensor, Raut, & Daunert, 2010). WCBs have even been developed to detect undetonated explosive ordnance by engineering sensors responsive to chemicals found in the vapor signature of a landmine (Garmendia, de las Heras, Galvao, & de Lorenzo, 2008; Yagur-Kroll

et al., 2014). Kabessa et al. developed a system using WCBs for the remote detection of landmines based on fluorescence (Kabessa et al., 2016). Bacteria are quickly evolving resistance to many different compounds and WCBs take full advantage of this rapidity, as new biosensors can be created as soon as the genes responsible for the resistance to the compounds and the genes that regulate the expression of these enzymes are identified and mapped.

## 3. DESIGN, CREATION, AND USE OF WCBs

### 3.1 WCB Design

The core component of these biosensors is a plasmid engineered to contain a sensing element and a reporting element (Figs. 2G and H and 3I). The reporting element is composed of a reporter gene whose transcription is linked to a promoter that allows the reporter gene's expression to be controlled by a regulatory protein that has also been placed in the same plasmid in a manner that allows it to be constitutively expressed (Fig. 1A). This regulatory protein functions as the sensing element (also called a recognition element) in tandem with the promoter. These regulatory genes are specifically chosen for their ability to interact with the intended target analyte of the biosensor and influence the expression of genes linked to a specific promoter. The reporting element, usually a gene whose corresponding protein emits a detectable signal or an enzyme that can be used to produce a signal, is placed under the control of the sensing element's promoter. This means that the addition of the target analyte will induce expression of the reporter (Fig. 1B), which allows for the detection of the target. Given the simplicity of this scheme, it should be no surprise that these sensors can be created using only the most basic molecular cloning techniques.

Engineering a biosensing plasmid requires several steps shared with vectors for insertion and expression of other genes. Selection of the plasmid vector that carries the promoter and genes for the recognition and reporter proteins will primarily depend on the availability of restriction sites and other necessary expression-related genes that the vector contains. Vectors often contain multiple genes in addition to an origin of replication and promoter. Most vectors include a gene conferring antibiotic resistance to allow for selection. Some will already contain a reporter gene. The ideal vector for developing a WCB plasmid would have the following: desirable restriction sites, a resistance gene, the appropriate reporter, and (if required) a promoter that will allow the regulatory gene to be constitutively expressed. Restriction enzymes that produce overhangs are generally preferred for cloning

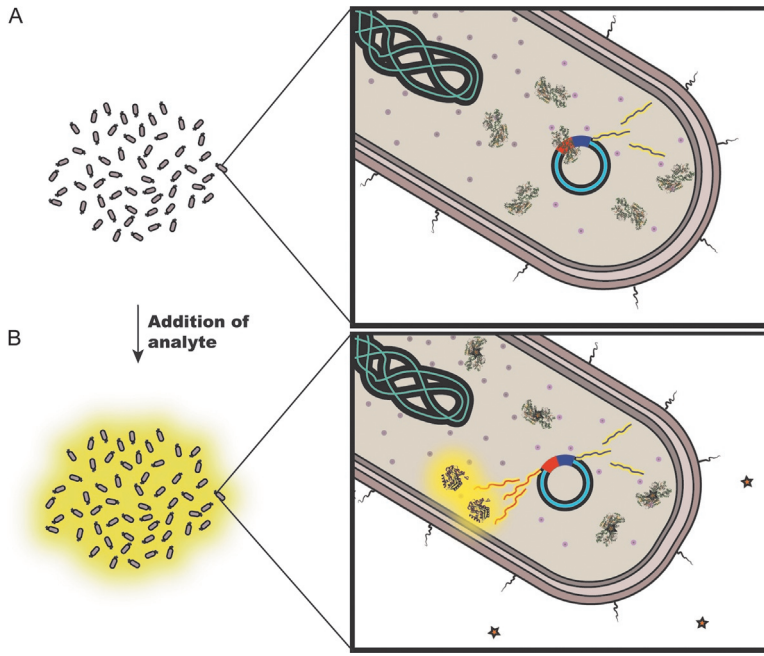


**Fig. 1** The whole-cell biosensor and its constitutive parts. (A) In the absence of the target analyte, the transcriptional repressor will bind to the inducible promoter and prevent transcription of the reporter gene. (B) When the analyte (depicted as a *star*) is introduced to the cellular environment, the transcriptional repressor will begin to bind its ligand and allow for the transcription of the reporter from the inducible promoter. The reporter will begin to emit a signal and detection can be accomplished.

applications since they allow for the directional annealing of the insert and the vector (Fig. 2B). Since most inserts produced via restriction digestions will be inserted into the appropriate vector using a DNA ligase (Fig. 2E) to catalyze the formation of the phosphodiester bond, restriction enzymes that produce blunt ends should be avoided if possible. The ligation reaction depends on the association of the insert, vector, and ligase. This is much easier to achieve when the insert and vector have overhangs since the annealing of the two DNA pieces will increase the time of their association and will maintain the reading frame. Blunt-ended ligations are still possible, and even common, but overhangs enhance the chances of a successful ligation and often save the user time in the creation of the desired plasmid. Several considerations need to be taken into account when designing a plasmid for biosensing applications, specifically when there is a need for using restriction endonucleases to isolate the gene of interest. First, enzymes must be chosen

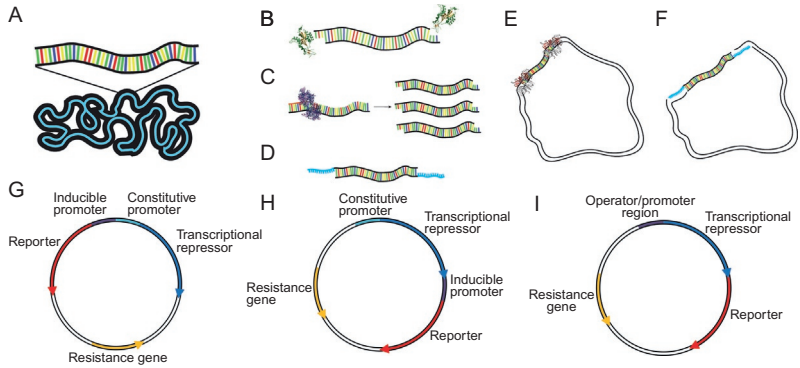


**Fig. 2** Creation and design of plasmids engineered for WCBs. (A) Regulatory proteins and inducible promoters are identified and mapped in their native organisms. (B) The target sequence may by isolated from the template using restriction endonucleases. (C) Alternatively, the target may be amplified from the template using PCR. (D) Long 5′ and 3′ overhangs may be incorporated into the insert using PCR to allow for the use of ligation-independent cloning. (E) The insert may be ligated into the desired vector if it contains the appropriate restriction sites. (F) If overhangs have been created for ligation-independent cloning, the insert can be annealed to the vector and transformed into the host organism following treatment with T4 DNA polymerase. (G) In some cases, the transcriptional repressor and inducible promoter may be isolated in a way that incorporates a constitutive promoter to drive expression of the repressor. (H) In other cases, the gene will need to be inserted into a vector that contains a promoter that will allow for constitutive expression of the repressor. (I) Some WCBs have been engineered using a promoter that allows for the autoregulation of the transcriptional repressor.

according to the sequence of the vector. If there is no corresponding restriction site, the enzyme may not be used to cut the vector. Although the vector's sequence may be mutated to allow for the use of a particular enzyme, other methods (such as PCR) may be considerably easier. It is also important to consider the sequence of the insert. If the enzyme also cuts the insert, it is not optimal since it will not be possible to generate overhangs through a restriction digestion. Reaction conditions are also an important consideration, especially when using multiple enzymes. Ensuring that the enzymes function well at the same temperatures and in the same buffers leads to easier protocols, as do other enzyme characteristics like heat inactivation and short reaction times. It should be noted that plasmids can eventually be lost after several generations from a given cell. To overcome this drawback of plasmid-based whole-cell sensors, vectors can be used that recombine into the host's genome to reduce genetic instability and ensure that the genetic

material and the properties it endows can be permanently inserted in a given cell.

Generally, the genes of interest are isolated in one of two ways: through the use of restriction endonucleases or a polymerase chain reaction (PCR).

Since the genes are often isolated from the genome of the organism from which they originate, it can be difficult to use restriction enzymes as there may not be restriction sites that allow for the isolation of the target gene in frame and without unnecessary nucleotides on either side of the gene. PCR is often the better choice for isolation (Fig. 2C). It allows genes to be selected regardless of the availability of restriction sites and will increase the flexibility in choosing different vectors. It is also easier to isolate a large amount of the gene since it will be amplified many times over from the original copy. Users can insert restriction sites at regions 5′ and 3′ to the gene to allow for easier insertion into the vector via ligation. It is also possible to use PCR to create inserts that can be inserted into a vector without ligase, perhaps the easiest way to go about cloning the insert into the vector. To do this, PCR primers are designed to create large 5′ and 3′ regions that are complementary to the regions of the vector that are directly up- and downstream of the intended insertion site (Fig. 2D). Both the vector and insert are then treated with T4 DNA polymerase. In the absence of dNTPs, T4 polymerase exhibits 3′ exonucleolytic activity. This can be used to create vary large overhangs (>20 bp) that cause the vector and insert to anneal when mixed (Fig. 2F). The resulting mixture can be transformed into the appropriate host after the addition of dNTPs to halt the nucleolytic activity of T4 polymerase. The machinery of the cell will complete the insertion of the gene with its own ligases. This can be much easier (and less frustrating) than more traditional techniques.

When planning experiments for the isolation of the regulatory gene and the corresponding promoter, it is important to consider the orientation that will be required when inserted into the vector. It is imperative that the regulatory gene is constitutively expressed. As such it must be inserted into the vector in a manner that puts the gene downstream of a promoter that will accommodate this need. Sometimes, the regulatory gene can be isolated with both the promoter it controls (the inducible promoter) and another promoter which will allow it to be constitutively expressed (Fig. 2G) (Turner et al., 2007). Other times, the regulatory gene may autoregulate (Fig. 2I) (Ramanathan et al., 1997). If such a design is not possible, care must be taken to select a vector with a promoter appropriate for this task and insert the gene accordingly (Fig. 2H). Care must also be taken

to ensure that the inducible promoter controls the expression of the reporter gene. If using a vector that already contains the reporter gene, the promoter is simply inserted upstream. If using a vector without the desired reporter, it is often easier to perform an overlap extension PCR to splice the regulatory gene, promoter, and reporter gene together. This reduces both the number of restriction sites required and overall effort by requiring only one reaction.

## 3.2 Selection and Use of Reporter Genes

Selection of the reporter gene depends upon the intended application of the system, its platform, and the equipment available, with each option possessing its own distinct advantages and disadvantages. Many different reporter genes have been employed in WCBs that employ a variety of modes of detection, including electrochemistry, colorimetric, absorbance, fluorescence, and bioluminescence (Close, Ripp, & Sayler, 2009; Daunert et al., 2000). Electrochemical detection of analytes has been accomplished using the *lacZ* gene, which codes for the hydrolase β-galactosidase. Though this method is highly sensitive, it requires both specialized equipment and substrate for β-galactosidase (Biran, Klimentiy, Hengge-Aronis, Ron, & Rishpon, 1999). Colorimetric assays have also been developed using *lacZ* for use in a qualitative assay without any instrumentation or as a quantitative reporter using absorbance. However, this method also requires the addition of a substrate and usually lacks the sensitivity of other methods (Naylor, 1999). Although it may not be entirely necessary, employing host strains where endogenous *lacZ* has been removed will reduce the background signal produced by native expression of the enzyme. Fluorescent reporters, like the well-known green-fluorescent protein, are sensitive and reliable but require instrumentation and are often encumbered by high background signal due to autofluorescence of the cells and other molecules in cell culture media. Therefore, special care must be taken when performing fluorescence-based assays and may require the use of specially prepared buffers and media. Bioluminescence is also a popular mode of detection. Many bioluminescent reporter genes are available for use, including firefly luciferase, *rluc*, *gluc*, *luxAB*, the full lux cassette, and aequorin (Roda et al., 2016). Because of the lack of endogenous reporter expression in most strains of bacteria, reporting elements using bioluminescence are often more sensitive than other methods. In order to produce a viable assay for use in the field, it is important to select a bioluminescent reporter that suits this aim, ideally

one with no required exogenous substrate, although stable and easily stored substrates can be used in these assays with tremendous success. Electrochemical, colorimetric, fluorescent, and bioluminescent reporters have been among the most popular methods of detection; however, others have been used. Chloramphenicol acetyltransferase (CAT) has been employed in many cell-based assays and was one of the first reporter genes to be widely used (Lewis, Feltus, Ensor, Ramanathan, & Daunert, 1998). Reporter production is often monitored using radiolabeled chloramphenicol, but the limited linear range and the requirement for radioactive isotopes make CAT an unpopular choice for WCBs (Daunert et al., 2000). Many of the reporters mentioned earlier can be very useful for laboratory-based assays, but their dependence on equipment (fluorimeters, spectrophotometers, luminometers, etc.) and reagents makes them less useful in field-based assays. Consequently, this has traditionally limited the usefulness of fluorescent, bioluminescent, and electrochemical reporters since the assay will neither be as cheap nor as field amenable as is required. Nevertheless, recent advances in adapting these detection approaches using cell phone-based and other technologies show promise for their use in portable field-applicable devices. The use of a colorimetric assay using β-galactosidase is an attractive option as a reporter gene for a truly field-amenable system, despite the potential loss of sensitivity and requirement of substrate addition.

## 3.3 Reporter Induction Assays With WCBs

The protocol for the assay used to induce reporter expression in WCBs is quite simple, as is illustrated in Fig. 1. Cells harboring the biosensing plasmid are grown to an optimized growth phase, generally measured using optical density, in one of a number of different cell growth media. LB Miller broth, Terrific broth, and minimal media are among the most common media chosen for this task. The analyte of interest is then added at known concentrations and incubated for an optimized amount of time at a specified temperature, with or without shaking. In some cases, the cells may be harvested via centrifugation and suspended in new media or a specially prepared buffer prior to the addition of analyte. The relative volumes of cells, buffer, and analyte will vary from sensor to sensor and are optimized to ensure the greatest analytical performance. Following incubation with the analyte, the reporter expression is evaluated. The method used depends on the assay and the reporter gene. For a luminescent reporter, the mixture of cells and analyte is read on a luminometer following substrate addition, if

needed. Fluorescent reporters generally require a centrifugation step to harvest the cells prior to resuspension in a buffer chosen to reduce the matrix effect produced by the autofluorescence of the cells. The signal is then read on a fluorimeter. Colorimetric assays are very similar; however, absorbance is read at a required wavelength on a spectrophotometer or a qualitative assessment of color intensity could be employed depending upon the substrate used. Electrochemical assays are also performed in an analogous manner, but require quantification of reporter production using a specially designed electrode. It is worth noting that some host organisms, especially Gram-positive strains, require lysis before evaluation of reporter expression.

Because of the disparity of hosts and gene constructs, it is difficult to make general statements regarding the analytical characteristics of WCBs. The signal produced can disappear, can appear as new, can shift (for example, if optical, shift to a different wavelength), can increase from a steady-state level, or decrease. For increased sensitivity of detection in the measurement, ideally, it is best to have a biosensor with a reporter system that will produce a dose-dependent response as a result of increasing concentrations of the analyte. The sensitivity of WCBs is generally in the micromolar range, but some WBCs have been produced with limits of detection (LODs) in the nanomolar range (Park, Tsai, & Chen, 2013) and even attomole detection has been reported with bioluminescence detection (Ramanathan et al., 1997). WCBs usually offer exquisite selectivity (He, Yuan, Zhong, Siddikee, & Dai, 2016). WCBs are considerably more rugged than many other methods, especially methods employing enzymes and other proteins due to the robustness of bacterial cells compared with most proteins alone. Changes in buffer composition, temperature, and pH will have little effect, but the addition of some solvents may disrupt analytical performance due to their toxicity.

## 3.4 Use in Portable Platforms

Assays are most often performed in microtiter plates since they allow for the convenient use of fluorescence-, luminescence-, or absorbance-based detection methods. Although this is acceptable for assays performed in a laboratory, other platforms have been developed to be of more use in the field. Microfluidic platforms constructed of polydimethylsiloxane (PDMS) are among the most popular and have been used with great success for the detection of arsenic and other analytes using WCBs (Date, Pasini, & Daunert, 2010; Rothert et al., 2005). Rothert et al. designed and imparted a pattern

into a PDMS compact disc (CD) that caused sample to flow through channels into wells containing biosensing cells with the application of centrifugal force with rotation. Despite the advantages of PDMS, it can be very expensive and cumbersome to use as the basis for microfluidic devices. Another cheaper, easier method involves the use of filter paper. Biosensing cells were lyophilized onto filter paper and used to assay water and saliva samples for the presence of acyl homoserine lactones (Struss et al., 2010). There are a few different methods to produce these portable analytical devices, but the basic scheme remains the same (shown in Fig. 3): cells containing biosensing plasmids are grown to an optimized optical density (Fig. 3A), dried or immobilized on filter paper (Fig. 3B and C), assays are performed under optimized conditions (Fig. 3D), and a signal is detected as a result of reporter gene production (Fig. 3F).

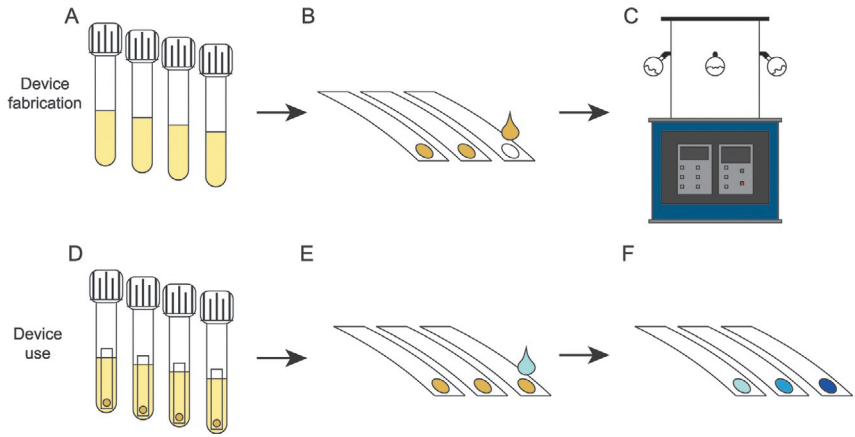


**Fig. 3** The development and use of paper strips and microfluidic platforms with WCBs. (A) A WCB strain is grown to an optimized growth phase. (B) After harvesting cells via centrifugation, the cells are resuspended in a drying protectant, cryoprotectant, or immobilizing matrix and applied to the devices. (C) The devices are allowed to dry. If a drying protectant or cryoprotectant was used, they are dried under vacuum or lyophilized. Otherwise, the devices may be left at room temperature to dry or under whatever conditions are required for the polymerization of immobilization matrices. (D) The analytical devices are incubated in media containing either known concentrations of the analyte or samples collected from the field for an optimized period of time. (E) The devices are removed from the media and substrate is applied if required. (F) The signal produced by the reporter is measured. The method used to evaluate reporter expression will depend upon the reporter, with the most popular options being fluorescence, luminescence, or colorimetric signals. An incubation period may be required to allow for full color development when using colorimetric reporters.

In some cases the filter paper is modified with wax printing, where wax designs are melted onto filter paper to create hydrophobic surfaces to allow for laminar flow. This technique can also be used to sequester cells and the applied sample from the rest of the device. Although this increases the cost of device production, wax printing can greatly increase the usefulness of the device because it allows for multiplexing and the application of sample to channels that feed into multiple wells. Since these assays are designed for quick and easy field use, it is advantageous to be able to assay for multiple analytes at once, especially if the device is designed for use by the general public to monitor the safety of the environment and water sources in local communities.

Cell immobilization can be accomplished using many methods but has most commonly been done through lyophilization within a drying protectant, or via an agar immobilization. *lacZ* has been used in WCBs as a reporter gene in microfluidic and paper-based devices in order to create a colorimetric assay that requires no additional instrumentation using the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, better known as X-Gal (Struss et al., 2010). The use of colorimetric detection may prevent these assays from being employed as quantitative methods in the field; however, software may be used to quantify the intensity of color produced relative to blanks to produce a semi-quantitative method. These software techniques are easy and reliable, but the requirement of specialized software may place some limitations on this technology's use in the field. Strategies to overcome these limitations are discussed later in this chapter.

## 3.5 Addressing the Concerns of Life Span and Robustness

Although they have many advantages, WCBs have limitations that can make them challenging to use, especially in the context of a field assay. Since these assays employ living cells, all the limitations and weaknesses of the cells are conferred on the assays. The cells require very specific conditions for storage and handling, most likely requiring refrigeration or freezing. Additionally, if the cells are not kept frozen or refrigerated, they may have a limited shelf life, ranging from days to months. They are also vulnerable to a wide variety of factors that can either kill the cells or disrupt their function as biosensors, including excursions in temperature or pH, irradiation, and organic solvents, to name a few. Researchers have developed many strategies to address these limitations with varying degrees of success. Most of these strategies fall into two broad categories: drying methods and immobilization.

Perhaps the most common method uses lyophilization to freeze dry cells in a cryoprotectant solution. Typically, cells are grown to an optimized growth stage, collected, resuspended in the drying protectant, frozen, and dried under vacuum. This method requires careful optimization of both the growth phase chosen to begin lyophilization and the components of the cryoprotectant solution. The growth phase can not only impact the viability of the cells upon rehydration (Costa, Usall, Teixido, Garcia, & Vinas, 2000), it can also interfere with analytical performance. Therefore, it is important for the proper growth phase to be identified as it will vary from species to species and even strain to strain. The composition of the cryoprotectant will vary from strain to strain as well since it has been shown that varying concentrations of sucrose, glucose, fructose, mannose, salts, glycerol, glycol, and DMSO can greatly impact the survivability and analytical capability of the sensing cells (Shin et al., 2005). A cheaper, easier alternative to lyophilization is the use of vacuum drying. Although this method is very similar to lyophilization, it tends to be harsher on the cells and can reduce the survival rate (Manzanera et al., 2002; Manzanera, Vilchez, & Tunnacliffe, 2004; Stocker et al., 2003). It is also worth noting that moisture can reduce the viability and analytical performance of WCBs when drying methods are employed so that, although refrigeration may be unnecessary for short-term storage, care needs to be taken in storing lyophilized cells, especially in humid climates.

Immobilization methods involve encapsulating the sensing cells in solid matrices composed of organic or inorganic polymers in order to sequester the cells and enhance their survivability. Some of the most common matrices are nutrient agar (Kim & Gu, 2003; Lee, Mitchell, Kim, Cullen, & Gu, 2005; Yagur-Kroll et al., 2014), alginate (Corbisier et al., 1999; Elasri & Miller, 1999; Heitzer et al., 1994; Peter, Hutter, Stöllnberger, & Hampel, 1996; Polyak, Geresh, & Marks, 2004), polyvinyl alcohol-based cryogels (Busto, Meza, Ortega, & Perez-Mateos, 2007; López-Fouz et al., 2007; Philp et al., 2003), and inorganic sol-gels (Amoura, Nassif, Roux, Livage, & Coradin, 2007; Lan, Dunn, & Zink, 2005; Nassif et al., 2002; Premkumar et al., 2002; Yu et al., 2005). Generally, this can extend the life span of the cells from weeks to months. Generally, immobilization can extend the shelf life of bacterial sensors to several months when stored at room temperature (Durand, Thouand, Dancheva-Ivanova, Vachon, & DuBow, 2003; Gu, Choi, & Kim, 2001; Shin et al., 2005). Immobilization-based strategies can protect WCBs to some degree, but the cells are still vulnerable to many environmental insults and proper care

still needs to be undertaken during storage to ensure full analytical performance, especially with biodegradable polymers and polymers where cross-linking may increase over time. Despite the advances in the methodology used to preserve bacterial biosensors, it is clear that these methods still lack the robustness and life span of ideal field equipment. In order to more completely address these problems, researchers adopted a philosophy similar to those who initially developed WCBs: rather than develop new technologies to solve current problems, they repurposed phenomena common in nature to suit their needs, and turned their attention to natural bacterial spores (Cano & Borucki, 1995). By incorporating the biosensing gene constructs into bacteria that produce endospores, researchers were able to develop rugged biosensors resistant to most environmental factors with a functionally unlimited shelf life (Cano & Borucki, 1995; Date, Pasini, & Daunert, 2007).

## 4. THE DEVELOPMENT OF SPORE-BASED BACTERIAL BIOSENSORS

### 4.1 Bacterial Endospore Formation

Some species of bacteria, notably strains of *Bacillus* and *Clostridium*, undergo a stress response called sporulation when they encounter conditions that are unfavorable for growth. This occurs as a multistep process shown in Fig. 4.

Sporulation initiation occurs as a result of nutrient-deficient conditions and a quorum-sensing mechanism indicating high population density. There is no single nutrient that disproportionately influences sporulation, though there is evidence that GTP concentrations play a major role (Molle et al., 2003). Sporulation contains a number of regulatory pathways, many of which are connected and redundant. Sporulation begins with the activation of Spo0A (Burbulys, Trach, & Hoch, 1991), the master regulator of sporulation initiation (Fujita & Losick, 2003) and expression of the sigma factor $\sigma^H$ (Britton et al., 2002). Following initiation, the cell undergoes asymmetric cell division where a septum forms near the cell pole. Under normal circumstances, cell division is prevented in the polar regions of the cell (Harry, 2001). However, during sporulation, DivIVA recruits the machinery responsible for cell division to the poles (Thomaides, Freeman, Karoui, & Errington, 2001). Two proteins, Spo0J and Soj (Marston & Errington, 1999), are possibly involved in organizing and moving the chromosome toward the poles, where DivIVA will interact with

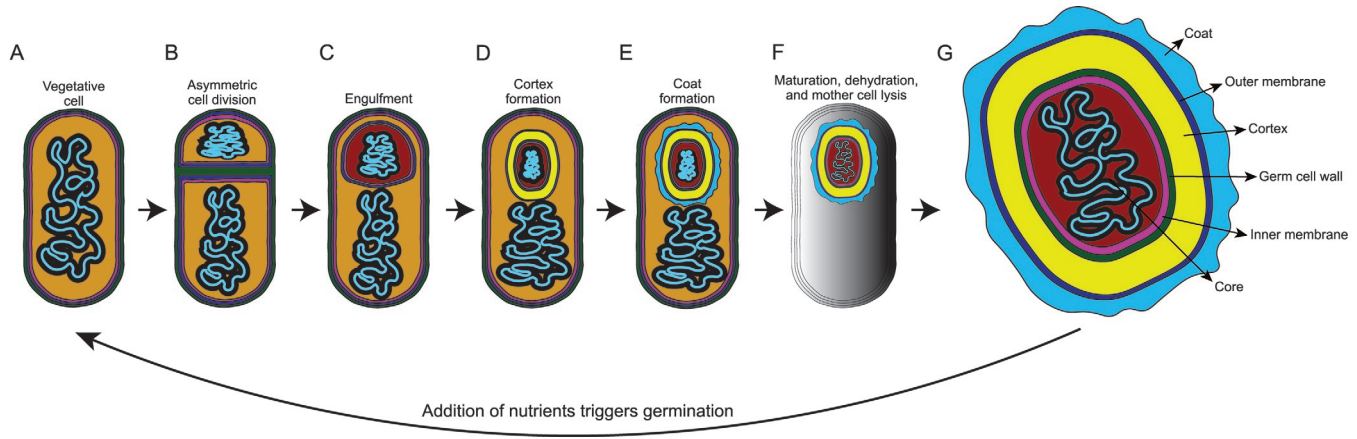


**Fig. 4** Depiction of the sporulation cycle in *B. subtilis*. (A) Nutrient deficiency causes the vegetative cell to initiate sporulation. (B) A septum forms toward the pole of the cell during asymmetric cell division. (C) The cell wall material of the septum is degraded and the prespore is engulfed by the plasma membranes of the mother cell. (D) The cortex forms on top of the germ cell wall surrounded the prespore. (E) The coat forms on the exterior of the outer membrane of the cortex. (F) The spore fully matures, dehydrates, and lytic enzymes are released to degrade the mother cell. (G) Enlarged depiction of the fully formed spore.

RacA that has bound the chromosome at positions surrounding *oriC* (Wu & Errington, 2003).

Septum formation between the prespore and the mother cell initiates alterations in gene expression that continue sporulation via the activation of the sigma factors $\sigma^E$ and $\sigma^F$, located in the prespore and mother cell, respectively, ensuring that specific sets of genes are transcribed in each cellular compartment. Following the completion of asymmetric cell division, the cell wall material in the septum is degraded. Enzymes precede the septal membranes as they migrate around the prespore cytosol in a process known as engulfment where the hydrolysis of linkages in the cell wall likely allows the membranes to move around the prespore (Abanes-De Mello, Sun, Aung, & Pogliano, 2002). The membranes then meet and fuse, causing the prespore to be released as a protoplast, which initiates the expression of two more sigma factors, $\sigma^G$ and $\sigma^K$, that facilitate the formation of the spore cortex and the later stages of sporulation (Kunkel, Losick, & Stragier, 1990; Sun, Stragier, & Setlow, 1989). The cortex is comprised of layers of peptidoglycan that make the spores robust and also help to achieve and maintain the dehydration of the spore core. The germ cell wall forms first and has a high degree of cross-linking between peptide side chains. The cortex then forms on top of the cell wall with a much lower level of cross-linking. Once the cortex is formed, the spore coat begins to assemble. The spore coat consists of two layers, composed of between 25 and 35 proteins ranging in size from 8 to 65 kDa. The inner layer has a thin, lamellar-like structure relative to the thick, electron-dense outer coat. Bonds between these coat proteins may have major roles in the hardiness of the spore. SpoVID and CotE will then assemble on top of a SpoIVA layer and form a ring with a small gap between the ring and the prespore (Driks, Roels, Beall, Moran, & Losick, 1994; Price & Losick, 1999). Subsequently, other coat proteins assemble on top of this ring and form the spore coat. Mature spores can then be released from the mother cell following complete dehydration and the release of autolysins to degrade the mother cell.

## 4.2 The Exceptional Resilience of the Spore

The resistance of spores to radiation, temperature, pH, and chemical insult is conferred by morphological features of the spore. Spore resistance to irradiation is not completely understood, especially resistance to ionizing radiation, but much investigation has been done to elucidate the mechanisms of resistance to UV light at 254 nm. In the late stages of sporulation a number

of α/β-type small acid-soluble proteins (SASPs) are expressed in the prespore that saturate the core and bind to DNA (Setlow, 1988). The complexation of SASPs and DNA significantly alters the photochemistry of the DNA in a manner that makes it more resilient. Rather than forming cyclobutane dimers and (6-4)-photoproducts when exposed to 254-nm light, as would typically occur, DNA complexed with SASPs will form "spore photoproducts" (SPs) (Mehl & Begley, 1999), a less lethal form of DNA damage that is quickly repaired at the outset of germination. Consequently, DNA repair mechanisms play a large role in UV resistance. Typical repair mechanisms like nucleotide excision repair and recombination-mediated repair play roles, but SP lyase (Spl), an enzyme unique to spore-forming bacteria, is responsible for the quick repair of SPs. Spl is expressed during sporulation but only becomes active during germination and can reverse thymine dimerization SPs into two thymine residues (Mehl & Begley, 1999). It is important to note that the roles played by α/β-type SASPs and DNA repair mechanisms in resistance to irradiation are also a major feature of spores' resistance to other environmental factors as they prevent accumulated DNA damage from impairing germination and the cellular mechanics of the vegetative cell. This likely explains why spores inactivated by wet heat rarely show significant DNA damage.

Most spores are resistant to temperatures of 95°C, approximately 40°C higher than vegetative cells. This resilience is likely due to the low relative water content of the spore core. Where the cortex and coat contain roughly 80% water (much like a vegetative cell), the core contains less than 50% and even as low as 25% (Gerhardt & Marquis, 1989). The inhibited movement of molecules in the core and reduced protein activity as a result of insufficient hydration are major contributors to the heat resistance of spores. The exact mechanism of core dehydration is unknown, but cortex formation, the addition of pyridine-2,6-dicarboxylic acid (better known as dipicolinic acid or DPA), and the mineralization of the core play significant roles. DPA is produced in the mother cell only during sporulation and is transported into the prespore where it is thought to contribute to core dehydration (Paidhungat, Setlow, Driks, & Setlow, 2000). DPA comprises over 10% of the dry weight of the spore (Setlow, 1994) and also may play a role in radiation resistance as it has been shown to be a photosensitizer (Paidhungat et al., 2000). The high divalent cation content of the spore core, especially the high concentration of $Ca^{2+}$ ions, also plays a role in the dehydration of the core (Gerhardt & Marquis, 1989). This is not fully understood but there may be a relationship between the mineralization of the core and the presence of DPA, which

appears to chelate the divalent cations. The spore's high resistance to chemical insult most likely comes from the coat and inner membrane. It is thought that the coat proteins may form a kind of protective barrier. Even more importantly, the low permeability of the inner membrane to both hydrophilic and hydrophobic small molecules prevents many chemicals from penetrating the core (Scherrer, Beaman, & Gerhardt, 1971). The cause of this low permeability is unknown, but it may be related to the compression of the inner membrane relative to normal cell membranes. It is also worth noting that the exceptional DNA repair mechanisms and the dehydration of the core also contribute to chemical resistance as many genotoxic chemicals will have limited effects even if they are able to enter the core. The spores also exhibit no metabolic activity and have an exceptional life span, with reports of spores estimated to be millions of years old (Cano & Borucki, 1995).

## 4.3 The Return of Endospores to Their Vegetative State

When the spores are introduced to a nutrient-dense environment, they undergo germination and once again become vegetative cells. This occurs via nutrient interaction with Ger receptors on the inner membrane of the cortex (Paidhungat & Setlow, 2000). The signal transduction that occurs during germination is not fully understood, but it is clear that germinant travels through the outer layers of the spore to reach the receptors on the cortex. This may happen via pores in the structure of the spore coat formed by the proteins composing the coat (Moir, Corfe, & Behravan, 2002). This process also involves ion transfers, membrane-associated changes, and ion movements that move water to the core to partially rehydrate it. Signals are then transmitted to outer layers of the spore to activate lytic enzymes to remove the cortex and fully rehydrate the core (Moir et al., 2002). Metabolic activity then resumes as the spores become vegetative cells. The addition of dipicolinic acid and calcium ions induces germination by directly activating cortex lytic enzyme, regardless of Ger receptor activation (Paidhungat & Setlow, 2000). These characteristics make sporulating bacteria an ideal choice of host organism for WCBs as they directly address the major disadvantages of whole-cell sensors in other organisms. The gene construct may be developed using traditional molecular cloning techniques and transformed into the host. Then, sporulation may be initiated using well-developed methodology to store the biosensors. When the WCBs are needed for use, they may then be germinated and used.

## 4.4 Bacterial Endospores as Analytical Tools

Although the WCB assays largely remain the same, alterations must be made to accommodate the need for germination. This is depicted in Fig. 5. In ideal conditions, germination can occur concurrent with the incubation of the cells and sample but, if this results in inefficient germination or poor reporter induction, a germination step where the spores are incubated with growth media must be undertaken prior to attempting a reporter induction assay. Typically, this takes between 1 and 3 h, but the addition of DPA and calcium chloride may reduce the time required by enhancing germination efficiency.

The ability of *Bacillus* spores to function as biosensors in an analytical assay has been thoroughly demonstrated. Date et al. designed plasmids incorporating operator/promoter regions and the corresponding regulatory proteins from organisms that possess resistance to heavy metals along with reporter proteins that produce a detectable signal (Date et al., 2007). One of these plasmids, pMUTin-23 (Fig. 6A), takes the O/P region and several genes from the *E. coli* resistance plasmid R773 that confers resistance to arsenic and antimony. The plasmid is organized such that the regulatory protein ArsR, a protein capable of binding As(III) and antimonite, regulates the transcription of *lacZ*. The gene encoding for the reductase ArsC was also included in order to reduce As(V) to As(III) and thereby increase the functionality of the assay. The plasmid pSD202 (Fig. 6B) was engineered to contain the gene *smtB*, which encodes for a transcriptional repressor that binds zinc and its corresponding promoter. This promoter was positioned upstream of *egfp*, which functions as the reporter. pMUTin-23 and

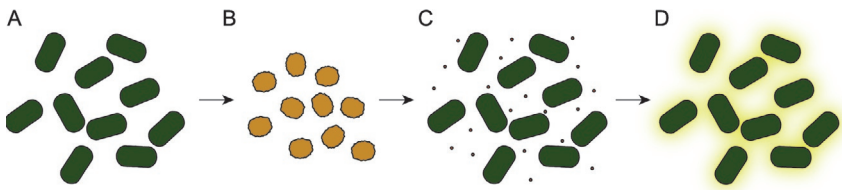


**Fig. 5** The preparation and use of sporulated WCBs. (A) Vegetative cells are cultured in specially prepared media for several days to induce and undergo sporulation. (B) The sporulated WCBs may be stored under nearly any conditions prior to use. (C) The spores are incubated in media to induce germination. They are then incubated with either known concentrations of the target analyte or samples collected from the field. In optimal circumstances, these two steps may be performed concurrently. (D) After an optimized incubation period, the signal produced by the reporter may be evaluated. Depending upon the reporter, the cells may need to be lysed to ensure the signal is efficiently read.

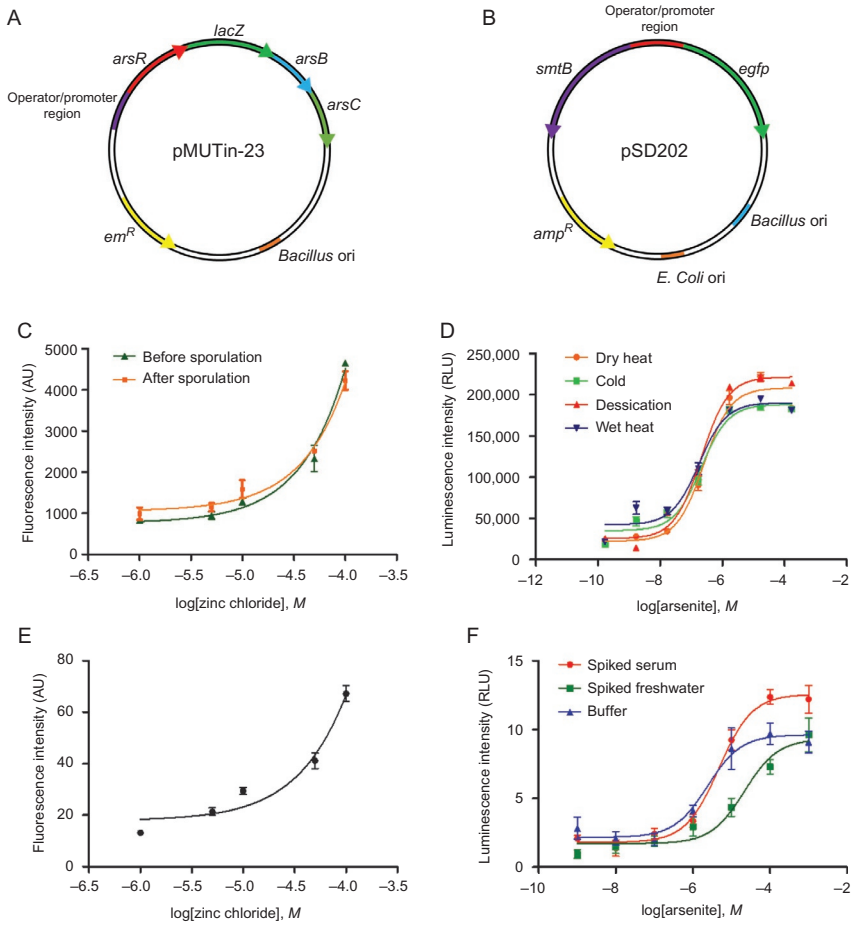


**Fig. 6** Detection of arsenite and zinc using sporulated WCBs. (A) Plasmid map of the vector pMUTin-23 used for arsenite detection. (B) Plasmid map of the vector pSD202 used for zinc detection. (C) Calibration curve showing pSD202-harboring cells' response to zinc before and after sporulation. (D) Calibration curves of pMUTin-23 spores stored for 12 months under various conditions. (E) Fluorescent response of pSD202 spores immobilized on a microfluidic CD and measured using an optical fiber. (F) Luminescent response of pMUTin-23 spores immobilized on a microfluidic CD where spiked samples have been prepared in human serum, collected freshwater, and buffer.

pSD202 were transformed into *Bacillus subtilis* and *Bacillus megaterium*, respectively, and reporter induction assays were performed. When compared, the vegetative biosensing *Bacillus* cells performed similarly to whole cell-sensing systems in *E. coli* with LODs ranging from $2.8 \times 10^{-8}$ to $1 \times 10^{-6}$ *M*. The authors then compared the analytical characteristics of

the sensing cells following sporulation and germination to vegetative cells that had not undergone sporulation, shown in Fig. 6C. As expected, the LODs and dynamic ranges remained consistent for both strains. In order to demonstrate the stability of the spores, the authors then subjected the cells to three sporulation/germination cycles. Again, the sensors remained very consistent, with LODs and dynamic ranges remaining comparable after every cycle. Even after 6 months of storage, the analytical characteristics of the assay remained unchanged. *Bacillus* cells harboring the pMUTin-23-biosensing plasmids have also been shown to be able to survive long-term storage in extreme conditions. In order to demonstrate this, Sangal, Pasini, and Daunert (2011) created a variety of simulated climates designed to replicate some of the more extreme situations under which these biosensors may be employed across the world. The four chosen conditions were dry heat (an oven set to 37°C), wet heat (a water bath set to 28°C with a relative humidity of above 90%), cold (a −20°C freezer), and desiccation (a desiccator at room temperature). Spores were left in these conditions for 12 months. Aliquots were taken at monthly intervals and the analytical performance was evaluated. Unsurprisingly, the LODs and dose–response curves remained consistent across all conditions and time points (Fig. 6D).

Although these experiments prove that sporulated *Bacillus* cells can effectively preserve the biosensing capabilities of these strains, it still needed to be shown that these sensors could function in a context meaningful to researchers and users in the field. Since the matrices seen when analyzing biological and environmental samples are a great deal more complex than the spiked deionized water used in previous experiments, it was very important that the spores be shown to germinate efficiently and retain the same analytical performance when the sample is composed of freshwater or human blood serum. This was demonstrated by using the biosensing strains in simulated freshwater, freshwater collected from various water sources in Indiana and Kentucky, commercially available serum, and serum drawn from healthy volunteers, shown in Fig. 6F. Though some matrix effects were seen when using serum that caused high background signals, LODs remained below $1 \times 10^{-6}$ *M*, meaning they were still comparable to the results seen with spiked deionized water and in the *E. coli*-based whole-cell sensors.

These strains have also been used on a centrifugal CD microfluidic device that can easily function as a portable system for sample analysis (Fig. 6E) (Date et al., 2010). Samples are applied to specific reservoirs and the disk is spun at a particular angular frequency to create the flow, which

pushes the samples into wells containing immobilized biosensing spores. The signal could then be read using a spectrophotometer with a fiber optic cable. In the case of pSD202, readings were taken at 20-min intervals in order to optimize the incubation time, whereas pMUTin-23-harboring cells were only measured once since the assay requires the addition of substrate. This was done using spiked deionized water, serum, and freshwater. As expected, the cells' analytical performance was comparable to *E. coli*-based systems, vegetative *Bacillus* cells, and assays performed in microtiter plates. Fantino et al. developed what they termed "sposensors" in *B. subtilis* using recognition elements based on the gene *czcD* to detect zinc and *bceA* to detect the antibiotic bacitracin (Fantino, Barras, & Denizot, 2009). Both systems employed *lacZ* as a reporter for colorimetric detection. In these assays, the germination and incubation steps were performed concurrently, making the assay very simple to perform. When spotted on filter paper, zinc and bacitracin detection occurred at $1 \times 10^{-6}$ and $3.5 \times 10^{-9}$ *M*, respectively. Given these results, it is clear that the strategy of using *Bacillus* cells as a host for biosensing plasmids and exploiting the robustness and longevity of spores in order to facilitate storage is viable for field-based assays analyzing a variety of matrices.

## 5. CHALLENGES, INNOVATIONS, AND FUTURE DIRECTIONS

### 5.1 Achieving Incredible Sensitivity Through Directed Evolution

Since chronic exposure to environmental pollutants at low levels is still a major concern due to possible carcinogenicity and birth defects, among other concerns, it is imperative that WCBs be sensitive enough to detect contamination at very low concentrations. This is also a necessity for the biomedical applications of WCBs, as being able to diagnose and treat diseases at their outset improves the efficacy of many treatments. Although some WCBs may have passed this threshold, many have not. Some researchers have employed directed evolution to address this issue. Researchers were able to increase the sensitivity of a WCB designed to detect dinitrotoluene by performing multiple rounds of random mutagenesis and selecting for the mutations which produced the most sensitive assay with the strongest signal (Yagur-Kroll et al., 2014). The same techniques have been used to push the native selectivity of a binding protein from its native ligand, D-xylose, to dinitrotoluene for use in the detection of landmines (de Las Heras &

de Lorenzo, 2011). This directed evolution strategy using random mutagenesis has promise, since, after several rounds of random mutagenesis have been performed and the characteristics of the regulatory protein and the promoter have been further elucidated, researchers can begin to make rational designs for mutations based upon the data obtained by the directed evolution of the original regulatory protein.

## 5.2 Employing Cell Phones to Enhance the Utility of Cell-Based Assays

Recent work in the field holds great promise for WCBs, especially spore-based sensors. These advances in methodology and the platforms used make for a bright future for bacterial biosensors because these innovations address the major limitations of WCBs or spore-based sensors and make previously impossible techniques feasible in the field. The chief innovation is the use of cell phone technology in analytical applications. Since smartphones are nearly ubiquitous anywhere in the globe, including remote and difficult to reach locations, they are an outstanding tool for field-based methods because they are relatively inexpensive and, thus, available to almost everyone even in resource-poor countries as well as incorporating powerful light sensing, computational, navigational, and communications capabilities. Recently, researchers have used smartphones to quantify luminescent signals using a smartphone camera (Roda, Guardigli, et al., 2014; Roda, Michelini, et al., 2014). This was initially done using enzyme-based assays, but it has since been shown that the toxicity of a sample may be evaluated using human kidney cells and the bioluminescent signal they produce, quantified using a smartphone (Cevenini, Calabretta, Tarantino, Michelini, & Roda, 2016). It has also been demonstrated that electrochemical and colorimetric signals may be interpreted by smartphones (Aronoff-Spencer et al., 2016; Vashist, Marion Schneider, Zengerle, von Stetten, & Luong, 2015). This is very promising for WCBs as this would provide a cheap and easy to use portable luminometer to be used in conjunction with a paper or microfluidic platform. Previously, the use of these biosensors for field applications has been limited to employing β-galactosidase as a reporter because qualitative colorimetric assays require no instrumentation. This is a practical method and works well in point-of-care, on-site, and field applications, but it generally lacks the sensitivity needed for many important analytes. Also, the fact that it is a semi-quantitative test is a disadvantage when there

is a possibility of high background signal due to the "leakiness" of the promoter. This can be a major problem since it is difficult to perform a baseline correction with the naked eye.

With a smartphone luminometer, many different luminescent and electrochemical reporters are now an option outside of the laboratory. Not only would this allow for a more sensitive assay, but it would also make quantitation much more feasible as it would even be possible to estimate the concentration of contaminating materials from samples using a standard curve. Special device cases have been designed and produced using 3D printing to accommodate the analytical devices for use with any type of smartphone (Roda, Guardigli, et al., 2014). Also, software may be developed to interpret results and provide users with information concerning the safety of samples, including correcting the data relative to a blank sample (Cevenini et al., 2016). This creates a platform that is affordable and ideal for use by the general public as it requires materials for its fabrication that are easily attainable and relatively inexpensive. Performance of the assay and interpretation of the results would also require no special training or knowledge while, at the same time, producing a more sensitive assay with greater utility.

## 6. CONCLUSIONS

Though challenges still remain, WCBs in sporulating host organisms provide an excellent option for use in field-based analytical assays because of their ruggedness, low cost, ease of use, and adaptability to field applications. The ease of creation and use of WCBs together provide many advantages compared to traditional methodologies, including robustness, reliability, easily interpretable results, field amenability, and low cost of manufacture. Moreover, the reagents needed for biosensor detection are not expensive, and thus, the typical cost of the assays is affordable even in developing and resource-poor countries. Many of these advantages are directly related to the simplicity of the system, which allows them to be easily adapted to portable platforms amenable to use in a variety of environmental conditions, including extreme climates and polluted environments. The versatility and ease of use of these analytical technologies make WCBs ideal candidates for further investigations and inspiration in the design of novel technologies that can be used by the general public to ensure the health and safety of people, communities, wildlife, and the environment all around the globe.

## ACKNOWLEDGMENTS

The authors would like to thank the National Science Foundation (grant CHE-1506740) and the Government of Israel MOD (P.O. 4440563897) for funding this work. S.D. would also like to thank the Miller School of Medicine of the University of Miami for the Lucille P. Markey Chair in Biochemistry and Molecular Biology.

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