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An arsenic-specific biosensor with genetically engineered *Shewanella oneidensis* in a bioelectrochemical system

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ABSTRACT

Genetically engineered microbial biosensors have yet to realize commercial success in environmental applications due, in part, to difficulties associated with transducing and transmitting traditional bioluminescent information. Bioelectrochemical systems (BESs) output a direct electric signal that can be incorporated into devices for remote environmental monitoring. Here, we describe a BES-based biosensor with genetically encoded specificity for a toxic metal. By placing an essential component of the metal reduction (Mtr) pathway of *Shewanella oneidensis* under the control of an arsenic-sensitive promoter, we have genetically engineered a strain that produces increased current in response to arsenic when inoculated into a BES. Our BES-based biosensor has a detection limit of ~40 μM arsenite with a linear range up to 100 μM arsenite. Because our transcriptional circuit relies on the activation of a single promoter, similar sensing systems may be developed to detect other analytes by the swap of a single genetic part.

1. Introduction

Microbes are a promising platform for biosensing since they are: i) simple to cultivate; ii) able to thrive under adverse conditions; iii) sensitive to analytes in a highly specific manner; and iv) amenable to recombinant DNA technologies (D'Souza, 2001). Microbial biosensors have been principally developed for environmental applications, since microbes have the tools to sense both bioavailability and toxicity of chemical species (Belkin, 2003). Many of these systems have relied on the analyte-induced expression of reporter genes that produce a bioluminescent (Ramanathan et al., 1997), fluorescent (Sagi et al., 2003; Wang et al., 2013a), or colorimetric signal (Joshi et al., 2009). However, genetically engineered microbial biosensors have yet to see commercial success in their target environments (Shin, 2011).

One possible explanation for the slow path towards commercialization is the impracticality of remotely detecting the expression of common reporter genes such as luciferase, green fluorescent protein, or β-galactosidase (Lei et al., 2006). Because remote monitoring does not require on-site technicians or transportation of samples to laboratories, the ability of microbial biosensors to transmit information across distances will be essential to their penetration of the environmental monitoring market. Consequently, there has been recent interest in the development of deployable bioluminescent biosensors for remote monitoring (Nivens et al., 2004). However, this technology has not gained traction because it is difficult to create a biosensor that both: i) transduces and transmits bioluminescent information; and ii) provides extended maintenance of engineered microbes (Bjerketorp et al., 2006).

We developed a novel genetically encoded bioelectrochemical system that overcomes this limitation, utilizing microbes to reduce an anode in an electrochemical cell, thereby producing an electric signal as a direct output (Friedman et al., 2012b). Microbial fuel cells are common BESs, which are designed to harvest electric power from chemical energy stored in organic waste. Alternatively, the direct electronic output of BESs is ideal for many biosensing purposes, since signal transduction is not required (TerAvest et al., 2011). Not surprisingly, BES-based biosensors have been designed to detect broad environmental signals such as biological oxygen demand (Kim et al., 2003), carbon source availability (Kim et al., 1999), and general toxicity (Wang et al., 2013b). Only recently, a BES-based sensor was developed to detect a specific chemical signal (Golitsch et al., 2013). While the foundational work of Golitsch et al. was pertinent to show the

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promise of genetically engineered BES-based biosensors with a specific signal, its target analyte (arabinose) is not of environmental concern. Here, we move beyond this proof-of-principle and describe a genetically encoded biosensor for arsenic that employs a *Shewanella oneidensis* strain growing in a BES. The sensing strain is a genomic Δ*mtrB* mutant, and is complemented by a plasmid-encoded copy of *mtrB* that is driven by an arsenic-inducible promoter. Because MtrB is essential for electrode reduction (Coursolle et al., 2010), current production increases in response to arsenic when this strain is inoculated into a BES.

2. Materials and methods

2.1. Strains and growth conditions

S. oneidensis is an environmental isolate from Lake Oneida, New York (Myers and Nealson, 1988). We used an *S. oneidensis mtrB* deletion mutant (Δ*mtrB*) as a basis for the construction of our arsenic-sensing strain (Coursolle and Gralnick, 2012). During experiments, we employed minimal M4 medium (Supplemental materials; Rosenbaum et al., 2010). When appropriate, sodium L-lactate and Fe(III) citrate were added to minimal M4 media at final concentrations of 20 and 10 mM, respectively, to provide a carbon source and electron acceptor.

Escherichia coli DH5α and WM3064—a diaminopimelic acid (DAP) auxotroph derived from strain β2155 (Dehio and Meyer, 1997)—were used for routine cloning and conjugation into *Shewanella* strains, respectively. For growth of *E. coli* WM3064, we supplemented all media with 300 μM DAP. All *Escherichia* strains were grown at 37 °C unless otherwise noted; all *Shewanella* strains were grown at either room temperature or 30 °C. All strains were maintained on solid lysogeny broth (LB) medium containing 1.5% agar. Overnight cultures were grown in 5 mL volumes of LB from either single colonies on agar plates or from frozen stocks maintained in glycerol. When appropriate, kanamycin was added to growth media at a final concentration of 50 μg/mL.

2.2. Strain construction

We used standard molecular biology techniques to construct a plasmid containing an arsenic-responsive genetic circuit. We obtained BBa_K098994 and BBa_J33201—derived from the genomes of *S. oneidensis* and *E. coli* JM109 (Diorio et al., 1995), respectively—from the Registry of Standard Biological Parts (http://partsregistry.org). BBa_J33201 contains both an arsenic-inducible promoter (P_{ars}) and its downstream transcriptional regulator (*arsR*). This fragment was amplified *via* PCR using a forward primer (ArsR_1) upstream of an EcoRI/XbaI cloning site, and a reverse primer (ArsR_2) with a 5′ extension introducing AscI, SpeI, and PstI cutsites (Table S1 in Supplemental materials).

Upon digesting this PCR product with EcoRI and SpeI, the coding region of *mtrB*, along with its native upstream region, was directly digested out of BBa_K098994 with XbaI (upstream) and PstI (downstream). Because the SpeI and XbaI enzymes produce compatible sticky ends, the final arsenic sensing plasmid (pArsR/MtrB) was constructed *via* simultaneous ligation of each fragment into the EcoRI/PstI cloning site of pBBRBB (Addgene plasmid 32549; Vick et al., 2011). This is a broad host range mobile vector with a kanamycin resistance cassette, which was derived from pBBR1MCS-2 (Kovach et al., 1995).

Plasmid pArsR/MtrB and an empty vector control were electroporated into electrocompetent *E. coli* WM3064 stocks—prepared as described by Wu et al. (2010)—in 1 mm cuvettes at a voltage of 1.8 kV, a capacitance of 25 μF, and a resistance of 200 Ω. After selecting for transformants, the resulting *E. coli* strains were grown overnight and plated on LB+DAP in a suspension with *S. oneidensis* after being washed of residual kanamycin. Specifically, *S. oneidensis* Δ*mtrB* was employed in the construction of the arsenic-sensing strain and a negative control, while wild-type *S. oneidensis* was used as a positive control. Following a protocol based on Coursolle and Gralnick (2012), each conjugation proceeded for 8 h at 30 °C after which a new LB+kanamycin plate, without DAP, was streaked for single colonies of *S. oneidensis*. Successfully conjugated *S. oneidensis* colonies were confirmed *via* colony PCR and Sanger sequencing.

2.3. Iron(III) citrate reduction

For initial characterization of our arsenic sensing strain, we employed ferrozine reagent (Stookey, 1970) to quantify its capacity to reduce Fe(III) to Fe(II) at concentrations of arsenite ranging from 0 to 100 μM. While the pathways responsible for electrode reduction and soluble iron reduction may not fully overlap, MtrB plays a crucial role in both pathways, making the quantification of iron reduction (by measuring Fe(II)) ideal for our purposes (Rosenbaum et al., 2010; Coursolle and Gralnick, 2012). In preparation for the assay, all strains were cultured aerobically overnight in LB at the arsenite concentration that would be employed in the subsequent assay. After growing to an OD_{600} of 1, cultures were washed and resuspended to a final OD_{600} of 0.015 in minimal M4 medium with 20 mM sodium lactate and 10 mM Fe(III) citrate. Inside an anaerobic chamber, 30 μL of each cell suspension was added to 270 μL of minimal M4 medium (and arsenite, where appropriate) in the wells of sterile 96-well plates. After incubating for 12 h, 5 μL from each well was transferred into clean wells of a new 96-well plate, along with 45 μL of 0.5 M HCl and 300 μL of ferrozine reagent (Coursolle et al., 2010; Stookey, 1970). For quantification, the absorbance of the resulting mixture was measured spectrophotometrically at 562 nm. Defined Fe(II) standards at an appropriate range of concentrations were used to calibrate the assay.

2.4. BES construction and electrochemical techniques

We employed single-chambered glass electrochemical reactors with a working volume of 100 mL during the characterization of the sensing strain. We operated the BESs as electrochemical half-cells by employing either a commercial potentiostat (VSP, Biologic USA, Knoxville, TN) or an open-source, microcontroller-based potentiostat (placed in a Faraday cage for electronic shielding; Friedman et al., 2012a) to control and monitor electrochemical conditions in a three-electrode system. We controlled the potential of a graphite rod serving as a working electrode (Poco Graphite, Inc., Decatur, TX) at +0.497 V_{SHE} using an Ag/AgCl/sat'd KCl reference electrode, while employing another graphite counter electrode as a current drain. In this arrangement, *S. oneidensis* biofilm formation and electron transfer occurs at the working electrode, because it is poised at a potential that is energetically favorable for electrode reduction. The headspace of the reactor was connected to the atmosphere through a 0.2 μm filter (Pall Corporation, Cortland, NY) to maintain a micro-aerobic environment. This environment provided the sensing strains with oxygen as an alternative to the electrode as an electron acceptor, so that electron acceptors would not be limiting when arsenic was not present (*i.e.*, in conditions where the Mtr pathway was incomplete). Previous work has indicated that *S. oneidensis* exposed to low levels of oxygen continue to express the Mtr pathway at functional levels and do not lose electrode respiration capabilities (TerAvest et al., 2014).

Investigations of the electrode reduction capacity of the *S. oneidensis* strain carrying pArsR/MtrB, alongside Δ*mtrB* and wild-type *S. oneidensis* empty vector controls, were performed in

batch operation at arsenite concentrations ranging from 0 to 100 μM. *S. oneidensis* strains were grown overnight in LB+kanamycin (without arsenite) to an OD_{600} just under 1.0 before being resuspended in minimal M4 (with kanamycin) to an OD_{600} of 0.015. Suspensions were inoculated into 100 mL of minimal M4 medium with kanamycin and arsenite (when appropriate) after at least 5 h of abiotic background current measurement. While current was recorded over the entire length of batch experiments, peak current was of particular interest as a response variable to arsenite concentration, and was defined as the absolute maximum current observed after processing raw data by subtracting background noise and performing a 30 min moving average. A schematic illustration representing our BES-based sensing system can be found in Supplementary materials (Fig. S1).

3. Results and discussion

3.1. Design of an arsenic-sensing genetic circuit

We relied on an *mtrB* complementation strategy to construct an arsenic-responsive genetic circuit in plasmid pArsR/MtrB, which would cause increased current production by *S. oneidensis* in response to arsenic. MtrB plays an essential role in the metal reduction (Mtr) pathway of *Shewanella* spp., stabilizing a complex formed between MtrA and MtrC (Coursolle et al., 2010; Hartshorne et al., 2009). Because this complex is required for electrode reduction, strains lacking the *mtrB* gene are unable to produce significant levels of current when inoculated into BESs. However, when *mtrB* is re-introduced into a knockout strain, the electrode reduction phenotype is restored—*i.e.*, when inoculated into a BES, current production will increase in response to increasing levels of MtrB. We exploited this by placing the *mtrB* coding sequence under the control of an arsenic-inducible promoter region.

The arsenic-inducible promoter (P_{ars}) is negatively regulated by ArsR (Fig. 1). When arsenic is excluded from a cell with these genetic components, the binding kinetics between ArsR and its associated operator region within P_{ars} are relatively strong, blocking the transcription of downstream genes (*i.e., arsR* and *mtrB*). In this arrangement, ArsR limits its own expression to a low, basal level. However, the circuit dynamic changes when arsenic compounds enter the cell. In this condition, arsenic associates with ArsR, inducing a conformational change that leads to its dissociation from the P_{ars} operator. While ArsR associates only with arsenite forms, arsenates may also be detected following intracellular conversation to arsenite (Scott et al., 1997). Because the binding kinetics between ArsR and the P_{ars} operator become less favorable with increasing arsenic concentrations, MtrB levels increase with increasing arsenic abundance until the point of saturation, resulting in an increase in electric current.

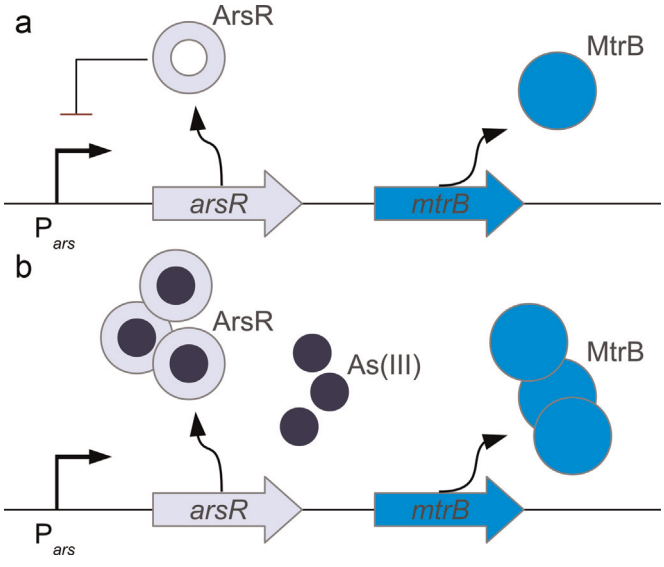

Fig. 1. Design of arsenite-responsive transcriptional circuit in in plasmid pArsR/MtrB. *arsR* is a negative auto-regulator, inhibiting the transcription of itself and downstream *mtrB*. (a) When relatively little arsenite is present, ArsR is able to bind to the operator of P_{ars}, which is the arsenite-sensitive promoter. (b) As arsenite enters the cell, ArsR dissociates from the operator, leading to an increase in *mtrB* expression.

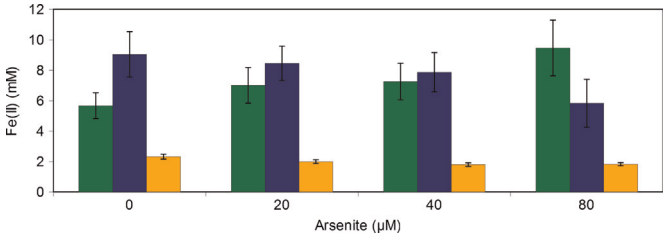

Fig. 2. Fe(II) concentration measured in growth media of *S. oneidensis* strains over 12 h at various concentrations of arsenite. A wild-type positive control (blue-middle bar) and a Δ*mtrB* negative control (orange-right bar) each show toxic responses to arsenic, as iron reduction capacity is diminished with increasing arsenite concentration; the *S. oneidensis* strain carrying pArsR/MtrB (green-left bar) shows an increasing capacity to reduce iron at increasing arsenite concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Effect of arsenic on iron reduction

The *S. oneidensis* strain carrying pArsR/MtrB had increasing capacity to reduce Fe(III) to Fe(II) at increasing arsenite concentrations (Fig. 2). A statistically significant linear correlation between arsenite concentration and Fe(III) reduction ($R^2=0.26$, $p=0.043$) was observed over the range of arsenite concentrations 0–80 μM. This suggests that our sensing strain could function as the biological component of an arsenic sensor with direct electronic output when inoculated into a BES, although the relatively high Fe (II) production when arsenite is not present indicated some background expression of *mtrB*.

It is interesting to note that increasing MtrB expression compensates for arsenic toxicity in our sensing strain. Conversely, both wild-type *S. oneidensis* and the Δ*mtrB* negative control showed a decreasing trend in iron reduction rates at increasing arsenite concentrations (Fig. 2), indicating that toxic effects of arsenite caused a growth defect in *S. oneidensis* strains at the range of concentrations under investigation. This trend was statistically significant for the Δ*mtrB* strain ($R^2=0.31$, $p=0.022$). Therefore, it is likely that increasing MtrB expression compensates for this defect in our sensing strains, possibly by enhancing energy generation by respiration of Fe(III), and thus, increasing energy available for export and detoxification. At arsenite concentrations above 100 μM, however, the *S. oneidensis* strain carrying plasmid pArsR/MtrB showed diminished capacity to reduce iron. This phenomenon may be explained by saturation of MtrB activity (disallowing compensation for arsenic toxicity) combined with the inherent toxic effects of overexpression of Mtr proteins, which was recently investigated by Goldbeck et al. (2013).

3.3. Effect of arsenic on BES current production

When inoculated into BESs, the *S. oneidensis* strain carrying plasmid pArsR/MtrB generated increasing peak current in response to increasing arsenite concentrations (Fig. 3). Peak current was consistently observed 1.5 day after inoculation, followed by a

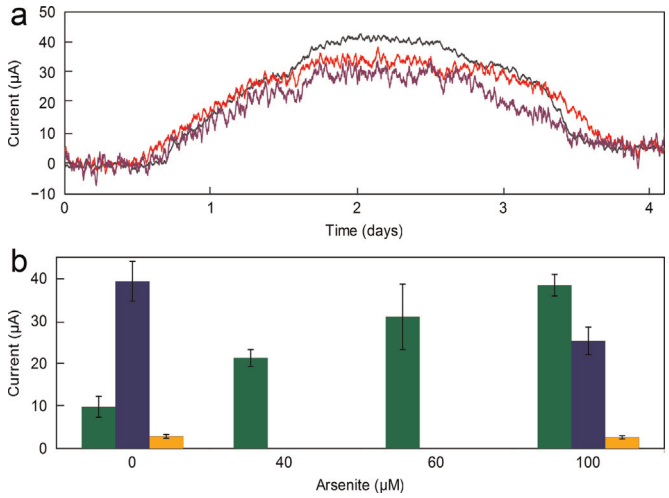

Fig. 3. Current production by *S. oneidensis* strains in triplicate batch BESs. (a) Current *versus* time, in triplicate, is shown for *S. oneidensis* strain carrying pArsR/MtrB at 100 μM arsenite. (b) A summary of peak current reveals the wild-type positive control (blue-middle bar) and a Δ*mtrB* negative control (orange-right bar) each show toxic responses to arsenic, as electrode reduction capacity is diminished with increasing arsenite concentration; the *S. oneidensis* strain carrying pArsR/MtrB (green-left bar; only this bar is shown for the 40 and 60 μM arsenite concentrations) shows increasing peak current production at increasing arsenite concentrations, with significantly different peak current at 40 μM arsenite ($p=0.02$, two-tailed unpaired *t*-test) in comparison to basal levels. Data was not collected for the positive and negative controls at intermediate concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

downturn in current caused by a depletion of carbon source—as seen, for example, in a plot of current *versus* time for triplicate experiments of *S. oneidensis* strain carrying pArsR/MtrB at 100 μM arsenite (Fig. 3a). A significant increase in peak current was observed for the arsenic-sensing strains at concentrations as low as 40 μM arsenite ($p=0.02$, two-tailed unpaired *t*-test). Over a range of 0–100 μM arsenite, a linear increase in mean peak current was observed ($y=0.29x+10.42$, $R^2=0.97$) (Fig. 3b). Just as with iron reduction, however, a decrease in mean peak current was observed at arsenite concentrations above 100 μM, which may be similarly explained by saturation of MtrB activity in combination with the toxic effects of overexpression of Mtr proteins (Goldbeck et al., 2013). However, it is interesting to note, again, that MtrB expression seems to compensate for arsenic toxicity, since the strain carrying pArsR/MtrB outperformed the positive control at arsenite concentrations at the upper limit of the linear range of the sensing strain (*i.e.*, 100 μM).

3.4. Considerations for deployment

Despite the capability of our strain to function as an arsenic sensor, it is important to note that the lower limit of 40 μM is orders of magnitude above the maximum chronic exposure limit of 10 μg/L (~133 nM) set by both the United States Environmental Protection Agency and the World Health Organization (McGuigan et al., 2010). However, in constructing and characterizing an alternate arsenic-inducible plasmid to that described herein, we observed that modification of the *mtrB* upstream sequence affects the limit of detection and linear range of arsenic sensitivity (Fig. S2; Supplementary materials), indicating that altering *mtrB* expression dynamics could improve performance of the strain. Several approaches may be used to optimize this strain in the future, including: i) chromosomal integration of the regulated *mtrB* construct to reduce background expression; ii) introducing constitutive expression of *arsR* to further decrease background expression; and iii) increasing flavin production to enhance electron transfer. These approaches, in combination with a parallel-directed evolution strategy to alter either the *mtrB* ribosomal binding site strength or the binding kinetics of the arsenic-inducible promoter, may be used to increase the sensitivity of the arsenic sensor.

We must emphasize that the successful deployment of a BES-based biosensor will likely be dependent on its ability to support continuous, remote monitoring of environmental contamination, which would confer a competitive advantage over current detection methods. These methods are expensive, time-consuming, and often require the transportation of samples from the field to a laboratory—disallowing adequate monitoring of regions of environmental risk due to inconsistent spatial and temporal sampling (Dowdeswell et al., 2010). As part of the 2012 International Genetically Engineered Machine (iGEM) Competition, Cornell iGEM addressed the challenge of supporting continuous, remote monitoring by building a prototype system employing an *S. oneidensis* sensing strain growing in continuous flow BES reactors. Briefly, this device is capable of supporting monitoring for up to six months and is capable of wirelessly transmitting water quality data by employing a modified version of a previously published open-source potentiostat (Friedman et al., 2012a). Unlike the bench top reactors discussed so far, this prototype includes two inlets: one taking a continuous sample; and one continuously feeding the reactor with growth media. With the help of a control reactor that contains *S. oneidensis* constitutively expressing *mtrB*, this prototype system compensates for environmental fluctuations.

Finally, we also investigated the specificity of the arsenic-sensitive promoter while active in our sensing strain by adding other toxic metals. Specifically, we investigated the iron reduction capacity of both our sensing strain (in the absence of arsenite) and an empty vector control in the presence of Co, Cu, Mn, Ni, or Zn ions at 80 μM levels using a ferrozine assay as described in Section 2.3. While it is known that ArsR associates with antimonite with similar kinetics as it does with arsenite (Ramanathan et al., 1997), we did not observe an increase in iron-reduction activity of the sensing strain with other toxic metals (Fig. S3; Supplementary materials). In regard to interference that may be caused by antimonite, we observe that many regions that suffer from arsenic contamination are not threatened by antinomy. For example, in groundwater samples taken by the British Geological Survey from Bangladesh, antimonite concentrations in 90% of arsenic-contaminated samples analyzed fell well below both the World Health Organization exposure limit and the measured concentration of arsenite (de Mora et al., 2011). Conversely, in regions affected not only by arsenic, but also by antimony, a sensor utilizing the sensing strain discussed herein may function as a multi-analyte sensor for more generalized toxicity. Perhaps more interestingly, however, the specificity of ArsR for either arsenic or antimony may be modulated by directed evolution approaches, as suggested above for enhancing arsenic sensitivity.

When investigating the specificity of the arsenic-sensitive promoter, we did observe the toxic nature of Cu ions causes a considerable decrease in the iron-reduction activity of both the sensing strain and the empty vector control (Fig. S3; Supplementary materials). Indeed, Cu toxicity has been shown to inhibit iron reduction by *Shewanella* spp. (Markwiese and Colberg, 2000). While more work is required to account for Cu interference over a range of Cu and As concentrations in real time, we believe the shared physiological response of our test and control strains serves as a foundation for our proposed method of control. Because the prototype device designed to house this control requires only biannual maintenance to acquire continuous data, it should reduce the cost per sample relative to chemical analysis. Further, while the day-long response time of this first-generation device is

considerably longer than current methods, a response time on the order of days is reasonable in our target application of first-pass monitoring: Because current environmental monitoring programs often do not support sampling on a daily basis, the prototype system provides an improvement over existing methods. Additionally, methods to improve the response time of first-generation device are readily attainable, as Golitsch et al. (2013) have recently demonstrated a chronopotentiometric technique that significantly reduces response time in BES-based biosensors such as our own, resulting in significant responses within 2 h. This work suggests that similar BES-based biosensors may be more than adequate for the task of first-pass monitoring of contamination events.

4. Conclusions

We have shown that it is possible to transduce chemical information of relevance to environmental quality into electronic form by employing a BES inoculated with genetically engineered *S. oneidensis*. By characterizing current responses of our engineered strains to arsenic, we have moved beyond the proof-of-principle, and have constructed a BES-based biosensor with direct environmental application. Because our biosensor produces an electric current directly, it may be more easily integrated into a deployable device for remote monitoring than traditional microbial biosensors. Further, Golitsch et al. (2013) have recently demonstrated that the modularity of the Mtr pathway of *S. oneidensis* may be exploited by placing multiple analyte-driven promoters upstream of various coding regions of the pathway. This modularity will facilitate widespread application of *S. oneidensis* based, genetically-encoded biosensors, since a multiple-promoter circuit controlling the Mtr pathway may be constructed to compute the presence of chemical analytes in either OR or AND logic. Further, because *S. oneidensis* strains can be modified to detect a multitude of analytes with the simple swap of a promoter, a number of customizable BES-based biosensors, tailored to target applications, may be constructed by exploiting the regulation of the Mtr pathway of *S. oneidensis*. Because of this, we believe *S. oneidensis* is an ideal host for development of biosensors not only for remote monitoring of environmental contaminants, but also for biocomputing challenges that may transfer information from the chemical environment directly to the logic of the digital world.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.07.003.

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