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MINI-REVIEW

Genetically engineered microbial biosensors for in situ monitoring of environmental pollution

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Abstract Microbial biosensors are compact, portable, cost effective, and simple to use, making them seem eminently suitable for the in situ monitoring of environmental pollution. One promising approach for such applications is the fusion of reporter genes with regulatory genes that are dose-dependently responsive to the target chemicals or physiological signals. Their biosensor capabilities, such as target range and sensitivity, could be improved by modification of regulatory genes. Recent uses of such genetically engineered microbial biosensors include the development of portable biosensor kits and high-throughput cell arrays on chips, optic fibers, or other platforms for on-site and on-line monitoring of environmental pollution. This mini-review discusses recent advances in microbial biosensors and their future prospects, with a focus on the development and application of genetically modified microbial biosensors for in situ environmental monitoring.

Keywords Microbial biosensor · Genetically engineered · In situ environmental monitoring

Introduction

Microbial biosensors are very useful for the detection of target chemicals and physiological signals in diverse fields. Considered to be appropriate first-step monitoring systems, they provide enough information for routine sample testing and screening and can complement analytical chemistry (Harms et al. 2006). Conventional chemical analysis can offer excellent accuracy with high sensitivity, but it is usually expensive and time-consuming and requires that the samples be transported to costly laboratory facilities that have the necessary instruments and trained personnel. In addition, some toxic pollutants (e.g., aromatic compounds) are volatile, and thus may be lost during transportation and the various steps of the analytic process, possibly leading to false negatives. In such cases, on-site monitoring by microbial biosensors could be a useful alternative to chemical analysis (Shin et al. 2005).

The use of microorganisms as the sensing elements of a biosensor has several advantages over the use of other sensing elements, such as enzymes, antibodies, or sub-cellular components. There are typically a variety of microorganisms suitable for a given purpose, and they may be easily prepared through simple cultivation in relatively inexpensive media. In addition, microorganisms are capable of detecting a wide range of chemicals, they are amenable to genetic modification, and they can often adapt to a broad range of reaction conditions (Lei et al. 2006; Yagi 2007).

More recently, recombinant DNA technology has been used to further tailor the utilized microorganisms, often by fusing natural regulatory genes (i.e., those encoding a transcriptional regulator plus a promoter/operator) with a reporter gene (Vollmer and Van Dyk 2004; Ron 2007). The most frequently used reporter genes are the *lux*/*luc* (encoding bacterial/firefly luciferase), *gfp* (encoding green fluorescent protein), and *lacZ* (encoding β-galactosidase) genes. In relatively nonspecific biosensors, a reporter gene is fused to a constitutive promoter, and the toxicity of the target compound is measured as a decrease in the activity of a reporter protein. In more specific biosensors, transcriptional regulators activated by specific target chemicals or classes of compounds and/or conditions that stress the cell

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interact with an inducible promoter to trigger the dose-dependent production of a measurable signal from a reporter gene (Harms et al. 2006; Sørensen et al. 2006; Ron 2007).

Several previous reviews have discussed the use of such constructs for environmental monitoring (D'Souza 2001; Hansen and Sørensen 2001; Keane et al. 2002; Belkin 2003). Although microbial biosensors were found to be effective tools for in situ monitoring, they traditionally yielded lower reproducibility and sensitivity than chemical analysis (van der Meer et al. 2004). More recently, however, there has been some progress in improving the efficacy of microbial biosensors. For example, their sensitivities and specificities have been enhanced through modification of the effector-binding sites of the regulatory proteins (Park et al. 2005a; Galvão and de Lorenzo 2006), as well as the use of surface-expressed proteins (Mulchandani et al. 2001; Lei et al. 2005) and periplasmic binding proteins (Medintz and Deschamps 2006).

Although significant progress has been made in the development of microbial biosensors, they have not yet been widely adopted in their target environments, and for the most part, they have not been commercially successful outside of academia. This may be because although many microbial biosensors perform properly under optimum conditions (i.e., in an aqueous phase and a buffered medium), such conditions often differ from those of the real environment, which may be harsh. In addition, the public may see the market implementation of genetically engineered microbial biosensors as carrying an unacceptable level of risk, decreasing the political priority of widespread biosensor adoption (Harms et al. 2006).

Despite these barriers, however, some systems have been successfully implemented and commercialized. Sayler and Ripp (2000) first demonstrated the field application of genetically modified microorganisms for bioremediation processes. Since then, a limited number of microbial biosensors have been applied for the in situ monitoring of environmental pollution (Table 1) and have been successfully commercialized (Table 2). Most of the commercially available biosensor systems are intended for the clinical, pharmaceutical, and food industries, where they are often used to detect contaminants and verify material conversion and product content (Patel 2006).

The previous reviews published on microbial biosensors for environmental monitoring addressed their potential field application based on the data obtained under optimum laboratory conditions. In contrast, this mini-review focuses on recent progress made in the use of genetically modified microbial biosensors especially for the in situ monitoring of real environmental pollution, discusses the issues in their environmental application and commercialization, and addresses some possible future directions for microbial biosensor development.

Regulatory genes

Genetically engineered microbial biosensors are constructed by fusing pollutant-responsive genes (encoding a transcriptional regulator plus a promoter/operator) to a promoterless reporter gene. These recombinant genes can then be transformed into microorganisms as a plasmid or integrated into the chromosome. When the target chemical or physiological condition (or a complex combination of conditions) is present, the regulator activates the promoter, which triggers expression of the reporter gene and its measurable signal. Therefore, the specificity and sensitivity of a biosensor are mainly determined by the responsiveness of the regulatory gene to its intended target(s). Although biosensors were previously described using several terms (Harms et al. 2006; Sørensen et al. 2006; Ron 2007), they are currently classified based on the properties of their target(s) and are designated as having either "chemical specificity" or "stress specificity". Chemical-specific biosensors respond to one specific or several structurally related chemicals whereas stress-specific biosensors respond to the presence of toxic chemicals or adverse physiological conditions that cause cellular stress responses (e.g., DNA or protein damage).

Regulatory proteins often intrinsically respond to structurally similar chemicals that all fit into the effector-binding sites of the regulatory proteins. For example, regulatory proteins that are capable of reacting with benzene usually also react with toluene, ethylbenzene, and xylene (Stiner and Halverson 2002; Xu et al. 2003; Kim et al. 2005; Dawson et al. 2008), while those that are capable of detecting cadmium typically also detect other heavy metals, such as mercury and zinc (Biran et al. 2000). Thus, it is noteworthy that even highly specific chemical biosensors might detect related substances. Some examples of chemical-specific biosensors include those used for the detection of polycyclic aromatic hydrocarbon (Park et al. 2003; Xu et al. 2003; Kim et al. 2005; Park et al. 2005a, b; Shin et al. 2005; Paton et al. 2009), heavy metals (Ivask et al. 2001; Stocker et al. 2003; Trang et al. 2005; Fujimoto et al. 2006), and pesticides/herbicides (Védrine et al. 2003; Lei et al. 2005).

A number of groups have sought to understand the molecular mechanisms through which regulatory proteins activate promoters in the presence of effectors, in the hopes of constructing genetically engineered microbial biosensors with modified specificities and sensitivities. Diaz and Prieto (2000) scrutinized the various bacterial regulator–promoter pairs capable of biodegrading aromatic pollutants and gained insight into the molecular mechanisms through which regulatory proteins sense a given signal and activate transcription from their cognate promoters. More recent studies have shown that mutagenesis of the effector-binding sites of regulators involved in the degradation of aromatic

Table 1 Genetically engineered microbial biosensors used for in situ detection

Microorganism	Analyte	Characteristic	Matrix	Reference
E. coli with *lacZ*	Cadmium	On-line 25 nM 5 μM	Water Sea water Soil	Biran et al. (2000)
E. coli with *lux*	Toxicity	RBL[a] 1.5–5 0.2~1.0	Water from treatment plant Stream water	Gu et al. (2001)
Ralstonia eutropha with *lux*	Ni^{2+} Co^{2+}	0.1 μM 9 μM	Soil	Tibazarwa et al. (2001)
E. coli with *luc*	Methyl Hg Phenyl Hg Dimethyl Hg	0.2 nM (50 ng/L) 1 nM (0.34 μg/L) 10 μM (2.3 mg/L)	Soil extract	Ivask et al. (2001)
Pseudomonas fluorescens with *luc*	Mercury Arsenite	0.003 μg/kg 0.7 μg/kg	Soil extract	Petänen et al. (2001)
E. coli with *lux*, *lacZ*, *gfp*	Arsenite	0~0.5 μM (*lux*) 0.1~0.6 μM (*gfp*) Above 8 μg/L (*lacZ*)	Potable water	Stocker et al. (2003)
E. coli with *luc*, *lux*	Heavy metals (Hg, As, Cu, Zn, Pb, Cd)	20 μg/L to 400 mg/L	River water	Hakkila et al. (2004)
E. coli with *lux*	Arsenite	10~100 μg/L	Groundwater	Trang et al. (2005)
E. coli with *lacZ*	Phenolics	0.1 μM to 10 mM With the naked eye	Soil extracts Wastewater	Shin et al. (2005)
Salmonella typhimurium TA1535 with *lux*, *gfp*	Genotoxicity Cytotoxicity	–	Groundwater Sediment	Baumstark-Khan et al. (2005)
Ralstonia eutropha JMP with *lux*	2,4-Dichlorophen-oxy acetic acid	0~25 mg/L 0~50 mg/kg	Soil	Toba and Hay (2005)
Burkholderia sp. RP007 with *gfp*	Phenanthrene	–	Soil	Tecon et al. (2006)
Pseudomonas putida TVA8 with *lux*	Benzene Toluene Ethylbenzene m-Xylene	0.195 mM 0.218 mM 0.036 mM 0.043 mM	Soil extracts	Dawson et al. (2008)
E. coli and *Pseudomonas putida* with *lux*	Hydrocarbon degradation	–	Soil	Diplock et al. (2009)
Pseudomonas fluorescens HK44pUTK21 (*lux*)	Naphthalene degradation	0~15 mg/L	Soil extracts	Paton et al. (2009)

[a] Relative bioluminescence values

compounds may increase their sensitivities and specificities to toxic aromatic compounds (Park et al. 2005a; Galvão and de Lorenzo 2006). Furthermore, Looger et al. (2003) reported that regulator specificity could be improved through computer-aided systematic design. Indeed, structure-based computational methods have radically changed the ability of researchers to manipulate the molecular recognition between chemicals and proteins, improving the development of

Table 2 Commercially available microbial biosensors

Product	Analyte	Strain	Company (country)
Microtox®	Water toxicity	*Vibrio fischeri*	Azure, Bucks (UK)
ToxAlert®	Toxicity	*Vibrio fischeri*	Merk, Darmstadt (Germany)
Cellsense®	Toxicity	*Escherichia coli*	Euroclon, Ltd. (UK)
SPR-CELLIA	Toxicity	Whole cells	Nippon Laser and Electronics Lab (Japan)
Micredox®	BOD toxicity	Microorganisms	Aotea Bio, Ltd. (New Zealand)
BOD_5 Biosensor	BOD	*Pseudomonas putida*	Nisshin Electric (Japan)
Remedios	Toxicity	Luminescent bacteria	Remedios (Scotland)

engineered regulators that can function as biosensors for a wide range of target compounds.

Because specific proteins may result in common intracellular reactions, some biosensors can detect classes of functionally related substances; these include classes of antibiotics (e.g., tetracyclines) (Bahl et al. 2004), endocrine disruptors (Hock et al. 2002), and quorum-sensing molecules (Andersen et al. 2001; Burmølle et al. 2003). Based on this, the regulatory networks involved in the bacterial recognition of attractants (e.g., food) or repellents (e.g., inhibitors or toxins) are considered promising targets for biosensor construction. If the promoter for a certain chemical-specific biosensor is not yet available, relevant candidate promoters may be cloned by the polymerase chain reaction (PCR) using proteomic technologies. Cells are exposed to the target chemicals, responsive gene expression is then identified from two-dimensional gel electrophoresis, and the relevant promoters are PCR amplified.

Similarly, stress-specific biosensors can be constructed by putting a reporter gene under the control of stress-regulated promoters that are known to respond to toxic compounds or stress-response-eliciting conditions (Vollmer and Van Dyk 2004). Such promoters include stress-response regulons such as the bacterial SOS system (e.g., ColD *cda*, *recA*, *umuDC*, *sulA*), which reacts to DNA damage (i.e., genotoxicity) (Mitchell and Gu 2004; Norman et al. 2005); the heat-shock promoters (e.g., *grpE*), which react to conditions that cause protein damage (Sagi et al. 2003); oxidative stress-responsive promoters (e.g., *katG*) (Mitchell and Gu 2004); and membrane damage-responsive promoters (e.g., *fabA*) (Bechor et al. 2002).

Notably, the stress-specific biosensors can provide direct information about the physiological symptoms caused by environmental pollutants. Given that a toxic chemical could include a variety of chemical species, stress-specific biosensors could be used for the initial and/or overall detection of cellular stress or toxicity in environmental samples of unknown composition. Other methods could then be used to identify the individual toxins. Indeed, Lee et al. (2005) successfully used several types of stress-responsive promoter/reporter gene fusions within 96-well plate- or chip-based screenings for high-throughput analysis that yielded specific information on toxicity.

Reporter genes

All microbial biosensors, both chemical- and stress-specific, give positive signals when the regulatory proteins are activated in the presence of target chemicals or environmental stresses, triggering expression of the reporter gene. The produced signal may be visible to the naked eye (e.g., a change in color, bioluminescence, or fluorescence), or it may require optical or electrochemical conversion into a measurable response (e.g., a change in light absorption, current, or potential).

Some reporter genes encode enzymes that catalyze easily monitored colorimetric reactions. For example, *lacZ* from *Escherichia coli* encodes the enzyme β-galactosidase, which splits the β-galactose bonds of substrates such as X-galactopyranoside. This reaction can be monitored by the naked eye or colorimetric methods, as the color intensity is proportional to the level of enzyme activity present in the reaction (within a certain range). Similarly useful reactions are governed by alkaline phosphatase (Durrieu and Tran-Minh 2002; Paitan et al. 2004) and horseradish peroxidase (Xu et al. 2003). Recently, novel photosynthetic bacterium-based biosensors were constructed using the *crtA* or *crtI* genes, which are responsible for carotenoid synthesis (Fujimoto et al. 2006; Yagi 2007; Yoshida et al. 2008); these biosensors do not require the addition of a substrate to manifest their color changes.

Other reporter genes encode luminescent enzymes. One of the most popular of such genes for biosensor-based environmental monitoring is that encoding luciferase, which has been used to examine environmental stress (Choi and Gu 2002; Kim and Gu 2003; Sagi et al. 2003; Vollmer and Van Dyk 2004; Lee et al. 2005), hydrocarbons (Park et al. 2003; Kim et al. 2005; Park et al. 2005a, b; Paton et al. 2009), and heavy metals (Ivask et al. 2001; Tibazarwa et al. 2001; Stocker et al. 2003; Trang et al. 2005). In these biosensors, the light emitted by the sensing bacteria is proportional to the concentration of the pollutants; unlike the long-lasting color produced by the β-galactosidase or alkaline phosphatase reactions, the emitted light has a short-life. The luciferase-encoding genes from the firefly (*luc*) and bacteria (*lux*) have been used in biosensor development. Firefly luciferase forms bioluminescent oxyluciferin in the presence of oxygen and adenosine triphosphate (ATP) by catalyzing the oxidation of benzothiazole-thiazole luciferin. It is more sensitive than bacterial luciferase, but requires the addition of a substrate and ATP for bioluminescence. Bacterial luciferase produces bioluminescence by catalyzing the oxidation of long-chain fatty aldehydes and reduced flavin mononucleotides to form fatty acid and flavin mononucleotides in the presence of oxygen. This reaction requires a set of two (*luxAB*) to five (*luxCDABE*) genes, and the produced protein is heat-labile, difficult to use in mammalian cells, and fairly ineffective under anaerobic conditions.

A biofluorescent molecule, the green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, produces fluorescence activity following irradiation at its excitation wavelength, without the addition of any exogenous substrate or cell extract (Stiner and Halverson 2002). Thus, GFP is useful for protein localization within microbial cells

and as a marker of individual microbial species in a mixed population, among other things (Andersen et al. 2001; Burmølle et al. 2003). The usefulness of GFP in bacterial biosensors has been somewhat limited by its high background signal and the relatively slow formation of the fully active fluorophore. However, various studies have demonstrated that the use of modified experimental and analytical techniques and the application of GFP variants that are ultrasensitive, emit brighter fluorescence, and/or respond more quickly could make *gfp* a viable alternative to the *luc* and *lux* reporter genes (Roberto et al. 2002; Wells et al. 2005; Tecon and van der Meer 2006). Mutant GFPs having increased stability, improved signal intensity, and altered spectral intensity have become commercially available in recent years and may prove useful in the future for biosensor applications.

Biosensors can use multiple reporter genes to sense and discriminate among environmental parameters, such as genotoxicity, oxidative damage, and cytotoxicity (Mitchell and Gu 2004; Baumstark-Khan et al. 2005). In terms of limitations, the sensitivity of bioluminescence-based systems can be decreased in turbid solution or in layers of immobilized cells due to light scattering. However, these systems can be remotely monitored using light-sensitive instruments. In the future, such work will benefit from the development of more compact and mobile detection devices geared toward on-line detection.

On-site monitoring

The need to perform field monitoring has driven the development of portable biosensors capable of providing cheap and simple (often on-site) analysis. At a contaminated site, microbial biosensors can provide fast and specific data on the chemicals and/or environmental stresses involved, as well as their biological effects (e.g., bioavailability and toxicity). Freeze-dried bacterial biosensors may be kept for months and transported to the necessary sites without losing their activity or viability, and the measurements are most often performed with little or no sample preparation needed. The genetically engineered microbial biosensors currently used for in situ monitoring of real environmental samples are listed in Table 1.

Biran et al. (2000) reported the novel use of a cadmium biosensor for on-site and on-line monitoring of water, sea water, and soil samples. They used a cadmium-responsive promoter from *E. coli* fused to a promoterless *lacZ* gene and monitored the results with an electrochemical assay of β-galactosidase activity. The substrate *p*-aminophenyl-β-D-galactopyranoside was converted to *p*-aminophenol, which was then oxidized at the electrode. This reaction was converted to a current-based signal using a chronoamperometric technique. The system was able to detect cadmium at concentrations as low as 25 nM in water and 5 μM in untreated soil samples under anaerobic conditions. Stocker et al. (2003) constructed arsenite biosensors with various reporter genes and compared their detection characteristics in the field. Among them, colorimetric qualitative paper strips using β-galactosidase as the reporter were found to produce a visible blue color at arsenite concentrations above 8 μg/L. Our own group reported a simple microbial biosensor that used naked eye detection of a color change for the on-site detection of phenolic compounds in soil and wastewater (Shin et al. 2005). For this purpose, we constructed a plasmid harboring the β-galactosidase gene fused with the phenolic-responsive CapR promoter (Park et al. 2003), inserted the plasmid into *E. coli* cells, and freeze-dried and stored the cells until use. Our results revealed that the relative color change of this system could be used to detect phenolic compounds in the range of 0.1 μM to 10 mM, even when used to analyze contaminated soil and wastewater (Table 3). The concentration levels measured by the biosensor were similar to those assessed by a typical chemical analysis; we hypothesized that our use of intact whole cells might have accounted for the system's excellent response at much higher concentrations of phenol (at 10 mM). Fujimoto et al. (2006) and Yoshida et al. (2008) constructed arsenite biosensors using the carotenoid biosynthesis gene, *crtA*, and the phytoene dehydrogenase gene, *crtI*,

Table 3 Comparison of the biosensor-based and chemical methods for measuring phenolic compounds in soil and wastewater

Soil or wastewater[a] source	Concentration (mM)	
	Biosensor[b]	Chemical analysis
Laboratory soil[c]	0.09±0.01	0.15±0.01
Dyeing industry complex	0.01±0.001	ND
Thermoelectric power plant	0.02±0.003	ND
Wastewater treatment plant		
A	ND	ND
B	ND	ND
C	ND	ND
Hospital waste		
A	8.06±0.10	10.96±0.43
B	9.93±0.12	4.44±0.15
C	1.07±0.02	2.07±0.01
D	3.08±0.05	1.38±0.05
E	10.61±0.23	6.14±0.13

ND not detected

[a] Wastewater samples were obtained from various sources in South Korea

[b] Concentrations were estimated using a regression curve or approximated in comparison to a color standard prepared with the same cells

[c] Laboratory soil was prepared as described elsewhere (Shin et al. 2005)

as reporters in the photosynthetic host bacteria, *Rhodovulum sulfidophilum* and *Rhodopseudomonas palustris*. In these systems, a color change could be detected with the naked eye in response to 5 or >10 μg/L arsenite, respectively, in liquid culture (artificial arsenic-contaminated water) without needing an additional substrate for color visualization. The time required for color development (12~24 h) was too long for these systems to be of practical on-site use (Fujimoto et al. 2006; Yoshida et al. 2008), but future developments may broaden their applicability. Regardless of this fact, the work to date has indicated that color changes mediated by *β-gal*, *crtA*, *crtI*, and other reporter genes can be appropriate for simple on-site detection.

In addition to the use of color development for detection, bioluminescence- and fluorescence-based microbial biosensors have been used for highly accurate and quantitative on-site monitoring of metals (Ivask et al. 2001; Petänen et al. 2001; Tibazarwa et al. 2001; Stocker et al. 2003; Hakkila et al. 2004; Trang et al. 2005), general toxicity (Gu et al. 2001; Baumstark-Khan et al. 2005), herbicides (Toba and Hay 2005), and hydrocarbon biodegradation (Dawson et al. 2008; Diplock et al. 2009; Paton et al. 2009). In the context of metals, organomercurials (Ivask et al. 2001), mercury and arsenite (Petänen et al. 2001), nickel (Tibazarwa et al. 2001), and arsenic (Stocker et al. 2003; Trang et al. 2005) have been monitored from real environmental samples using bioluminescent microbial biosensors with various detection limits (Table 1). Luminescent biosensors have also been successfully used to monitor changes in acute toxicity and the bioavailability of hydrocarbon compounds in soils during degradation (Tecon and van der Meer 2006; Dawson et al. 2008; Diplock et al. 2009; Paton et al. 2009). One example that has received enormous attention is the assessment of arsenic-contaminated groundwater in India and Vietnam. The application of luminescent arsenic-biosensing bacteria (Trang et al. 2005) to samples from nearly 200 groundwater wells in the Red River and Mekong River deltas of Vietnam resulted in more than 90% accurate measurements, suggesting that the utilized biosensor was quite reliable. The ease of use, low application cost, and urgent health care problems made this genetically modified biosensor a good choice for large screening campaigns in Asia. This is a vivid example of the successful application and large-scale environmental validation of genetically modified biosensors for monitoring of natural water resources under field conditions (Harms et al. 2006).

On-line monitoring

Another important aspect of biosensor use for environmental monitoring is the ability of the system to enable continuous on-line and real-time monitoring. The on-line monitoring of microbial biosensors can involve reporter enzymes whose activity can be monitored electrochemically (Biran et al. 2000; Paitan et al. 2003). Such systems can offer highly sensitive, reproducible, high-throughput measurement of multiple samples using a compact analyzer and disposable electrodes. Because these systems do not employ optical measurement, they can be used on crude or turbid solutions; this can be a critical benefit for monitoring pollutants in water systems and soil (Horsburgh et al. 2002).

On-line microbial biosensor systems can also be computerized and automated. For example, the toxicity of endocrine-disrupting chemicals and phenol in water was continuously monitored in the field over a period of 4 months by Gu et al. (2001), using a multichannel two-stage mini-bioreactor system and genetically engineered bioluminescent bacteria. Recombinant bioluminescent *E. coli* strains known to be responsive to various specific stresses (e.g., DNA, membrane and protein damage, or general cellular toxicity) were set up in parallel as two-stage mini-bioreactor systems. Samples (field or spiked) were filter-sterilized and pump-injected into the second stage of each mini-bioreactor. Bioluminescence was measured by a luminometer connected to the side port of each second-stage mini-bioreactor through an optic fiber probe and cable. DNA damage was detected in *E. coli* (*recA::luxCDABE*) exposed to as little as 2 ppm bisphenol A. Data from the luminometer could be received for on-line data acquisition via an IBM-PC based "Lumitrend" program, which was developed by this group. Differences in the bioluminescent signals from the second-stage mini-bioreactors before and after sample injection could be used to trigger an alarm from the on-line monitoring system (Gu et al. 2001). In another study, the on-line monitoring of tributyltin using a recombinant bioluminescent stain (*E. coli* :: *luxAB*) was optimized across a number of parameters known to influence microbial activity, including the bacterial physiology, medium, growth rate, dilution rate, and oxygen level. Real-time monitoring of bioluminescence was then performed with continuous addition of tributyltin up to 300 μM throughout the 7-day experimental period (Thouand et al. 2003). Recently, an on-line bioluminescence-based biosensor was developed to monitor the disinfection efficacy of chlorine dioxide gas under various treatment conditions in a pilot-scale gas treatment system (del Busto-Ramos et al. 2008). In this study, the recombinant bioluminescent reporter, *Pseudomonas fluorescens* 5RL (carrying the *Psal*::*luxCDABE* fusion), was used to emit intense luminescence in the presence of salicylate. The bioluminescence was detected on-line using a photomultiplier tube housed inside a light-tight chamber and connected to a computer. The results showed that increasing concentrations of chlorine dioxide gas corresponded to faster decreases in luminescence. The gas concentration capable of causing a tenfold

change in the log-reduction time was 3.4 mg/L of chlorine dioxide (del Busto-Ramos et al. 2008).

One of the limitations facing the on-line and real-time application of microbial biosensors is the need to maintain the activity of the microbial biosensors at a constant level over a long period of time. Various techniques for maintaining biosensor cells have been described, including continuous cultivation, immobilization in biocompatible organic/inorganic polymers, and freeze/vacuum drying (Bjerketorp et al. 2006). In the context of continuous culture, biosensor devices have been designed such that the supply of nutrients allows the recombinant inducible fluorescent or bioluminescent bacteria to continually grow and emit light, which is then measured using a fluorimeter or photodiode (Védrine et al. 2003; Pooley et al. 2004).

In the immobilization-based techniques, which is also frequently used to promote the stability and long-term use of microbial biosensors, the cells are immobilized on transducers or support matrices such as filters and membranes (Werlen et al. 2004; Toba and Hay 2005), optical fibers (Durrieu and Tran-Minh 2002; Védrine et al. 2003; Hakkila et al. 2004), and chip arrays (Van Dyk et al. 2001; Tani et al. 2004; Lee et al. 2005). The immobilization methods include both chemical and physical methods (Lei et al. 2006; Bjerketorp et al. 2006). Among the methods are physical adsorption to a solid surface, cross-linking between molecules, covalent binding to a surface, and entrapment within a membrane, surfactant matrix, polymer microcapsule, or sol-gel (Bjerketorp et al. 2006). One commonly used example of physical immobilization is that in nutrient agar. For example, agar-based immobilization in microtiter plates enabled the preservation of different toxicity-sensing *E. coli* strains for as long as 2 weeks (Kim and Gu 2003). Similarly, Lee et al. (2005) immobilized 20 recombinant sensor bacteria possessing different *lux*-fused promoters to construct a cell array biosensor. The cell–agar mixture was dispersed to a cell chip or a multiwell plate, and toxicity (taken as a bioluminescence change) was measured using a highly sensitive cooled charge-coupled device camera. Despite the advantages of this strategy, however, the various immobilization techniques may damage/stress the microorganisms, thereby activating the stress response and potentially inactivating the biosensor (Bjerketorp et al. 2006). In addition, immobilization may lead to poor long-term stability (and thus lower biosensor sensitivity and detection limits) due to desorption of the microbes or too slow diffusion of nutrients into the entrapment material (Lei et al. 2006). Thus, it remains challenging to store/maintain microbial biosensors at ambient temperatures for prolonged periods without re-growing the cells or losing cell viability/activity (Bjerketorp et al. 2006). Lei et al. (2005) reported that the service life of a microbial amperometric biosensor was 5 days when stored in operating buffer at 4°C. Kuang et al. (2004) reported that their biosensor (*E. coli* carrying a *recA::gfp* fusion), which was composed of a high-density living bacterial cell array, had an active sensing lifetime of more than 6 hours and a shelf life of 2 weeks. Mulchandani et al. (2001) reported a recombinant microorganism with surface-expressed organophosphorous hydrolase was stable up to 45 days, retaining 100% of the original activity when stored at 4°C. Therefore, considerable optimization is required for the fabrication of long-lived biosensors, and future work is warranted.

Limitations of microbial biosensors

Although many types of microbial biosensors have been developed, relatively few have been used for in situ monitoring (Table 1) or subjected to commercialization (Table 2). This is likely due at least in part to the inherent limitations of microbial biosensors. Almost no reported microbial biosensor can detect chemicals at concentrations below 0.1 μM (van der Meer et al. 2004), and biosensors often respond to groups of compounds rather than to a specific compound (Biran et al. 2000). Additionally, microbial biosensors can exhibit delayed responses due to the time required for reporter gene expression, which may be a drawback for real-time monitoring. Thus, researchers have sought to improve the sensitivities, specificities, and response times of microbial biosensors by exploiting more sensitive promoters (Kim et al. 2005; Norman et al. 2005), refining host strains (Bechor et al. 2002), designing strains that are capable of producing modifying enzymes (Oda et al. 2004), employing surface-expressed or periplasmic binding proteins (Mulchandani et al. 2001; Lei et al. 2005; Medintz and Deschamps 2006), and controlling bacterial physiology (Marqués et al. 2006). For example, surface-expressed or periplasmic proteins (i.e., those residing on the surface of microbial biosensors) can directly react with substrates without requiring them to enter the cell, thereby improving the speed and sensitivity of the microbial biosensors (Mulchandani et al. 2001; Lei et al. 2005; Medintz and Deschamps 2006). Alternatively, the engineering of effector-binding sites by site-directed mutagenesis can be used to change the range of specificity and sensitivity (Park et al. 2005a; see Galvão and de Lorenzo 2006 for review). Thus, in the coming years, we should seek to screen for natural variations that could improve microbial biosensors and to further extend their sensitivities and specificities.

Another limitation impacting the widespread use of microbial biosensors is the inherent difficulty of maintaining cell viability and activity in complex environments that may lack nutrients and/or contain inhibitory compounds. A

decrease in cell viability/activity could result in underestimation of toxicants. To overcome this issue, Baumstark-Khan et al. (2005) simultaneously assayed both cell viability and biosensor activity. In this case, the biosensor consisted of an SOS-inducible promoter fused to *luxCDABE* and a constitutively expressed promoter fused to *gfpuv*, and changes in relative luminescence were taken as reflecting biosensor activity. While viability/activity issues might have less of an effect on disposable microbial biosensors intended for shorter-term on-site monitoring, they could be a serious concern for continuous use on-line microbial biosensors. However, significant activity variations are seen among different species and strains, as well as within the same species/strain based on their physiological status during and after preservation (Bjerketorp et al. 2006). Therefore, microbial biosensor development could benefit from careful microorganism selection and the precise control of cell physiology. Ideally, a designed biosensor would survive and maintain its biosensor activities under the target field conditions, even in harsh environments such as those characterized by extreme temperatures, highly acidic or alkaline pH, and/or the presence of complex organic solvents (Lei et al. 2006). Once the limitations of the current cell preservation methods are overcome, microbial biosensors could potentially be exploited in automated on-line environmental monitoring devices, arrays of microchip-immobilized sensor cells, optic fibers, or other high-throughput platforms.

Finally, the use of microbial biosensors may be limited by public perception that the release of genetically modified microbial biosensors into nature could be risky. In reality, the risk of an adverse consequence is very low; most biosensor cells are immobilized on matrices or suspended in a compartment, and environmental samples are applied to the biosensor systems. In addition, the benefits can far outweigh the possible risks. The arsenic biosensor used for large screening campaigns is an excellent example, as this simple, sensitive, and cheap sensor kit helped prevent arsenite-associated diseases in Asia (Trang et al. 2005). In addition, the standardization and legislation of microbial biosensors could diminish some of the public misconceptions about genetically modified microbial biosensors.

Future trends in biosensor development

Along with efforts to resolve the limitations of the presently available biosensors, future tasks in biosensor research are likely to include studies aimed at developing the kinds of miniaturized, high-throughput, wireless/mobile, and automated devices that are currently being called for. These efforts are likely to involve emerging techniques in nanotechnology, genetic engineering, microelectronics and others, as well as the development and optimization of many new instruments. Given that genetically engineered microbial biosensors are presently used for in situ monitoring, future studies might focus on the need for more easily regulated, cost-effective, and safe biosensors. The future could further see the development of more rapid, sensitive, cheap, and disposable on-site biosensors as portable field devices, as well as high-throughput, automated, wireless/mobile, and stable on-line biosensors for early warning systems.

Such in situ monitoring applications will need stable microbial biosensors that can survive and function in harsh environments, contain varied microorganism populations (e.g., thermophiles, alkalophiles, metallophiles, osmophiles, etc.), and offer users the ability to control their physiologies (D'Souza 2001). For example, all of the metal resistance determinants in *Ralstonia* sp. CH34 are inducible, and thus their regulatory elements can be used to develop biosensors capable of measuring heavy metals in harsh environments contaminated with toxic metal ions (Niles 2000). In addition, biosensors with higher sensitivities and faster responses will be in constant demand, and genetic and metabolic engineering may be used to control (on/off) or create the desired metabolic pathway(s) for a given purpose. New surface-expressed enzymes or proteins (Mulchandani et al. 2001; Lei et al. 2005; Medintz and Deschamps 2006) as well as stronger promoters and new reporter genes could also become available in the future as we gain a better understanding of microbial genetics and develop new and improved recombinant DNA technologies.

In terms of technological innovations, another obvious development would be the generation of microbial biosensors capable of simultaneously monitoring multiple environmental parameters. These could include genetically engineered microbial arrays affixed to chips, optic fibers, or other platforms (Tani et al. 2004; Lee et al. 2005; Matsui et al. 2006). Bolton et al. (2002) reported high-throughput microarray analysis of general and specific bioreporters based on the integrated complementary metal oxide semiconductor photodetector-based detection of luminescence from nearly 5,000 induced biosensor cells. In the future, more advanced versions of such arrays that use less expensive and more user-friendly equipment could facilitate the in situ monitoring of both general toxicity and the individual components in a sample.

Single-cell biosensors for microscale detection (Tecon and van der Meer 2006) could conceivably monitor microenvironments that cannot be easily accessed by chemical analysis (Harms et al. 2006). Single-cell biosensors consist of live cell arrays fabricated by inserting recombinant bacteria (e.g., *E. coli* containing *zntA-lacZ* or *recA::gfp*) into a microwell array formed on one end of an imaging fiber bundle. Each microwell is occupied by only one cell

and each fiber has its own light pathway, enabling individual cell responses to be monitored simultaneously (Biran et al. 2003; Kuang et al. 2004). This type of array was found to respond to as little as 100 nM mercury or 1 ng/mL mitomycin C (a genotoxin). Thus, optical imaging fiber-based single-cell arrays could be a flexible and sensitive biosensor platform for monitoring the "quantum behavior" of various sensing microbes (Tecon and van der Meer 2006). However, this type of system requires sophisticated instrumentation, precise quantification, and accurate interpretation of the reporter signals obtained from individual heterogeneous cells.

As a basis for the future optimization of similar biochips, Ben-Yoav et al. (2009) studied the distribution and propagation of bioluminescence in microbial whole-cell biochips; the authors simulated and optimized the optical emission/detection with respect to fundamental system parameters using an optical ray tracing method. Their results should assist in the design of more efficient light-collecting biochips in the future. Overall, in the near future, the use of miniaturized and high-throughput biosensors with new instrumentation will allow the development of fully automated in situ monitoring systems. This can be expected to benefit both the environment and the economy, as it will reduce the required time and the volume of reagents, samples, and generated waste.

Outlook and perspectives

The microbial biosensors described herein have numerous advantages that would seem to make them ideal for in situ monitoring of environmental parameters. However, several limitations that must be resolved before they can hope to succeed in environmental applications and commercialization, including the need for sustainable cell viability and activity even under harsh environmental conditions, decreased response times, increased sensitivity, and improved selectivity. These critical advances might allow the growing biosensor industry to begin meeting the sizeable market demand for cheap, sensitive, selective, and fast biosensors. At the same time, we need to reassure the public that it is safe to use genetically modified microbial biosensors. In the future, the characteristics of reliable performance, low cost, and high sample throughput capability, as well as validation or acceptance by regulatory authorities, could make the microbial biosensor market a huge success. Microbial biosensors should also rapidly evolve into more compact, mobile, automated, and wireless devices for ubiquitous multi-functional, in situ and real-time monitoring of a large number of environmental variables. This is why genetically engineered microbial biosensors (especially portable microbial biosensor kits) are currently considered to have great promise for wider implementation in the near future.

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