Genetically Engineered Whole-Cell Sensing Systems: Coupling Biological Recognition with Reporter Genes

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I. Introduction

A biosensor is a measurement device or system that is composed of a biological sensing component, which recognizes a chemical or physical change, coupled to a transducing element that produces a measurable signal in response to the environmental change. Biosensing systems can be classified into three basic types based on the sensing component: molecular, cellular, and tissue.[1,2] Molecular-based biosensing systems employ subcellular components or macromolecules as the sensing element. These sensing elements include antibodies, nucleic acids, enzymes, ion channels, and lipid bilayers. Cell- and tissue-based biosensors are derived from isolated whole cells or intact tissue, respectively, from a wide range of plants and animals.

The advantages of molecular-based biosensors are their high specificity, selectivity, and rapid reaction times. However, these systems do not provide functional information such as analyte bioavailability. Furthermore, the expense of macromolecule isolation and extraction combined with the relatively short shelf life of the molecules can impose undesirable restrictions on their applicability for biosensing systems. Through advancements in genetic engineering, cell-based systems can be designed to afford high specificity and selectivity for the analyte and are stable in various environmental settings including fluctuations of temperature and pH. Furthermore, these systems can also be designed to exhibit nonspecific or effect selective properties which have been used to identify chemical toxins and genotoxins. The greatest advantage of cell-based systems is their ability to provide physiologically relevant data in response to the analyte and to measure the bioavailability of the analyte. Although the use of the complex metabolic pathways in whole cells can afford several benefits, it can also present problems associated with loss of specificity due to interference from molecules within the cell and nutrients in the media. Tissue-based biosensing systems are comprised of several cell types and, like cell-based systems, provide functional data; however, these types of sensors are generally less stable, and therefore, their applicability as biosensors is limited.

Cell-based biosensing systems can be classified according to the response of their sensing element, e.g., changes in cellular metabolism, pH, altered gene expression in genetically modified organisms, etc. Genetically engineered cell-based sensing systems can elicit a response in the presence of an analyte by coupling the sensing element to a reporter gene through gene fusion, which upon expression produces a readily measurable signal. The sensing element is

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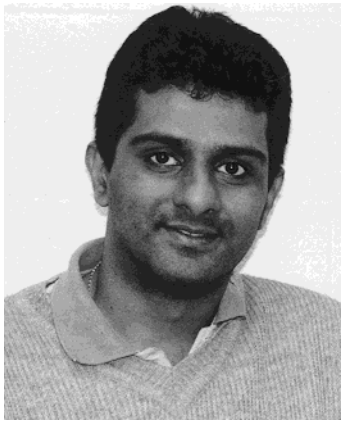
Sylvia Daunert is an Associate Professor of Chemistry and Pharmaceutical Sciences at the University of Kentucky and an Associate member of the Center of Membrane Sciences. She received her Pharm.D. degree from the University of Barcelona, her M.S. degree in Medicinal Chemistry from the University of Michigan in 1985, and her Ph.D. degree in Bioanalytical Chemistry from the University of Barcelona in 1991. She has been a Fullbright Scholar and has received among others the Juan Abelló Pascual Award in Biochemistry from the Spanish Royal Academy of Doctors, the Van Slyke Research Award from the American Association for Clinical Chemistry, a National Science Foundation-CAREER Award, a Cottrell-Scholars Award, and a Lilly Analytical Faculty Award. Her research interests lie in the area of bioanalytical chemistry, at the interface between analytical chemistry and molecular biology. More specifically, her group employs recombinant DNA technology to design new assays and biosensors based on genetically engineered proteins and cells. An additional research focus of her group is in the design of sensing arrays for the detection of molecules in small volumes and microfluidic platforms. (Photographed by Lee P. Thomas.)

Gary Barrett was born in Liverpool, England, in 1968. His undergraduate studies for a Zoology (B.Sc. Honors) degree included research in superparasitic behavior supervised by Dr. I. Harvey. This was followed by a postgraduate course in Environmental Diagnostics at Cranfield University England. He remained at Cranfield for his Ph.D. studies under Dr. D. Cullen. His Ph.D. research was in the development of optical biosensors for bacterial contamination in water systems. In 1999 he joined Dr. Daunert's research group at the University of Kentucky working on the centrifuge-based microfluidic platform.

Jessika S. Feliciano was born in Ponce, Puerto Rico, in 1975. She received her B.S. degree in Chemistry (Magna Cum Laude, 1997) from the Pontifical Catholic University of Puerto Rico, where she worked with Professor Gabriel Infante in radiochemistry and cancer research. During her undergraduate years she went to the Harvard School of Public Health to work with Dr. Bruce Demple in cellular and molecular toxicology and to the University of Kentucky to work with Professor Sylvia Daunert on immunoassays that employ reporter genes. After graduation, she interned at E. I. DuPont where she worked with Dr. Tina Van Dyk and Dr. Robert LaRossa on using microbial genetic technologies to discover sites and modes of action for crop protection chemicals. In 1998, Jessika was awarded a National Science Foundation Graduate Fellowship and then enrolled in the graduate program at the University of Kentucky, where she is currently pursuing her Ph. D. degree in Bioanalytical Chemistry. She later joined the research group of Professor Sylvia Daunert to perform her graduate work. Jessika was also awarded the National Science Foundation-IGERT Fellowship in 1999, the Ford Foundation Pre-Doctoral Fellowship in 1998, and the National Consortium for Graduate Degrees for Minorities in Engineering & Science (GEM) Fellowship in 1998, among others. Her current research focuses on the use of reporter gene technology in the development of novel bioanalytical sensing systems, particularly developing bacterial sensors for the detection of environmental pollutants.



Ranjit S. Shetty was born in Mangalore, India, in 1972. He grew up in Bombay, India, and attended S. I. E. S. College where he received his B.S. degree in Chemistry in 1992. Later he received his M.S. degree in Organic Chemistry from the University of Bombay in 1994. He joined the Ph.D. program in Chemistry at the University of Kentucky in 1995, where he is currently working under the tutelage of Dr. Sylvia Daunert. His research focuses at the interface of biology and chemistry coupling genetic engineering and analytical chemistry for the development of biosensors. In particular, he is interested in designing and developing bacteria-based biosensors for the determination of sugars, organic, and heavy metal pollutants.

often composed of regulatory proteins and promoter sequences of chromosomal or plasmid DNA.[3] The specificity of these elements for the analyte confers selectivity to the system, while the reporter protein determines the system's sensitivity and detection limits.[3,4] A schematic showing the cellular events that result in the generation of a measurable signal is depicted in Figure 1. A bioavailable analyte can pass through the cell membrane via absorption, active or passive transport and binds to a regulatory protein, thus activating transcription of the reporter gene. Subsequent translation of the reporter mRNA produces a protein that generates a signal in the

Suresh Shrestha is currently a Ph.D. candidate in Bioanalytical Chemistry working under the supervision of Professor Sylvia Daunert at University of Kentucky. He received his B.S. degree in Chemistry from Eastern Kentucky University in 1996. His doctoral research focuses on development of fluorescence-based sensing systems utilizing bacterial periplasmic binding proteins and genetically engineered cells.

Wendy Smith-Spencer was born in Florida in 1970. She received her B.S. degree in Chemistry from Clemson University in 1993, where she worked with Professor James Fanning studying the removal of Cs^+ ions from nuclear waste by precipitation with the anion Co-dicarbollide. She earned her Ph.D. degree in Toxicology at the University of Kentucky (1999), under Professor Ramesh C. Gupta. Her doctoral research was sponsored in part by a National Institute of Environmental Health Sciences training grant and was concerned with the effect of potential cancer preventive agents on DNA damage induced by polycyclic aromatic hydrocarbons. Wendy began her postdoctoral work in the laboratory of Professor Sylvia Daunert at the University of Kentucky in 1999. Her current research interests concern the development of cell-based biosensing assays employing recombinant DNA technology.

presence of a substrate or an external stimulus (e.g., light). An overview of the analytes or target responses, promoters, and regulatory proteins that mediate reporter gene expression in cell-based biosensing systems is depicted in Table 1.

It is our aim, in this review, to discuss the design, development, and analytical applications of genetically engineered cell-based biosensing systems employing reporter genes. In the first section we have provided an overview of the chemical reactions and structural properties of the reporter genes currently used in cell-based biosensing assays. The advantages and disadvantages of these genes as reporters are also discussed. In the following sections we have detailed the design, specificity, and sensitivity of cell-based biosensing systems for nonspecific (effect selective) and specific, namely, metal-resistant and metabolism-based, analyte detection. Finally, with regard to the future development of these systems, their application in high-throughput screening techniques and multianalyte detection strategies is discussed.

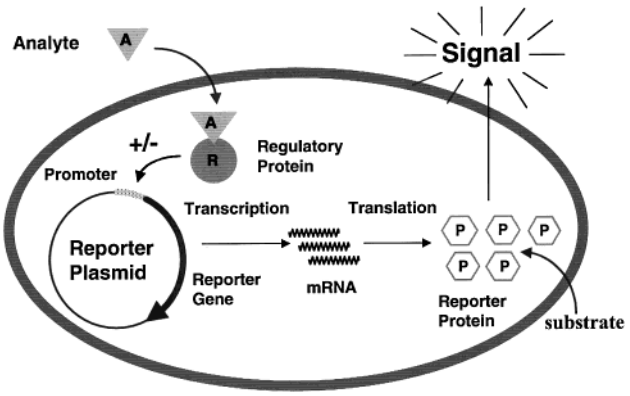


Figure 1. Schematic representation of the cellular events that result in the expression of a reporter protein. A bioavailable analyte passes through the cell membrane and binds to a regulatory protein, thus activating transcription and translation of the reporter gene. Upon addition of an external substrate, a readily measurable signal results.

II. Reporters for Cell-Based Biosensing

Expression of a reporter gene produces a measurable signal, which can be readily distinguished over the background of endogenous proteins. For analytical uses, reporter genes or their corresponding proteins are often coupled to a sensing element which recognizes an analyte and thus confers selectivity to the system while the reporter protein produces a detectable signal, thus determining the system's sensitivity.[4] The sensing or recognition component can be an enzyme, a receptor, or an antibody. Several characteristics are required for a gene to be useful as a reporter gene. First, quantification of reporter gene expression or activity must be conducted using a simple assay; second, the amount or activity of the reporter protein is reflective of the analyte being studied; and finally, similar endogenous proteins or enzyme activity are absent or minimal in the target cells.[5]

Reporter genes have a wide variety of applications in modern science. Since recent reviews[6,7] have discussed these applications in detail, only a brief summary will be presented here. One of the earliest and most common uses of reporter genes is to study *cis*-acting genetic elements, such as promoters and enhancers, in the upstream regions of genes.[6,7] Cell culture as well as recent studies with transgenic animals and plants have employed reporter genes to identify transcription elements responsible for basal and tissue-specific gene expression and *cis*-acting elements involved in human diseases. Reporter genes have also been employed to study gene expression and gene transfer. The identification of reporter genes (e.g., firefly luciferase and green fluorescent protein), which can be used as noninvasive markers of gene expression, and recent technological advancements in detection strategies employing charge-coupled devices (CCD) imaging cameras and fluorescence microscopy have provided temporal as well as

Table 1. Promoters and Regulatory Genes That Mediate Reporter Gene Expression Elicited by an Analyte or Target Response in Whole-cell Sensing Systems

target response/analyte	promoter	regulatory gene
protein damage	*grpE, dnaK, lon, ibp, clpB, P3,* Hsp104	*rpoH*
oxidative damage (hydrogen peroxide)	*katG*	*oxyR*
oxidative damage (superoxide)	*micF*	*soxRS*
growth limitation	*uspA*	Unknown
amino acid starvation	*his*	*relA, spoT*
stationary phase	*xthA*	*rpoS*
osmotic pressure	*osmY*	*rpoS*
DNA alkylation	*alkA*	*ada*
phosphate starvation	*phoA*	*phoB, phoM, phoR, phoU*
nitrogen starvation	*glnA*	*glnB, glnD, glnG, glnL*
carbon starvation	*lac*	*cya, crp*
membrane synthesis	*fabA*	*fadR*
DNA damage	*recA, uvrA, umuC, sfiA, rec*N, *sulA, RAD54,* ACOX	*lexA, recA*
antimonite/arsenite	*ars*	*arsR*
copper	*cup1*	*Ace1*
cadmium	*cad*	*cadC*
lead	*pbr*	*pbrR*
mercury	*mer*	*merR*
linear alkanes	P_{alkB}	*alkS*
toluene and its derivatives	P_u	*xylR*
isopropylbenzene	*ipbo/p*	*ipbR*
naphthalene, salicylate	P_{nah}	*nahR*
chlorocatechols	P_{clc}	*clcR*
L-arabinose	P_{BAD}	*araC*
lactose	P_{lac}	*lacI*

spatial information on gene expression at the single-cell level and have been previously reviewed by Welsh and Kay,[8] and Naylor.[7] A rapidly growing application of reporter genes is to study transcription factors and cell signaling mechanisms. This use of reporter genes not only provides intrinsic information regarding the function of these intracellular proteins at the basal level and crosstalk between these various pathways, but also has led to the discovery of new therapies and novel targets of human diseases, including cancer, viral, inflammatory, and cardiovascular diseases.[7] In fact, kits (for example, PathDetect and Stratagene) that can determine the involvement of various proteins in signal transduction pathways are commercially available.[7] Finally, reporter genes have also been used to study extracellular signaling mechanisms and their effects on gene regulation.[6,7] This application of reporter genes has been incorporated in the development of biological screens for drug discovery and, the focus of this review, genetically engineered biosensors, specifically those employing whole cells to detect various analytes.[7]

Cell-based biosensors employing reporter genes have been developed to study a wide variety of structurally diverse endogenous and exogenous analytes. These include metals such as antimony, arsenic, mercury, cadmium, lead, cobalt, nickel, chromium, copper, and zinc;[3,9−12] organic toxins such as chlorocatechols, benzene, toluene, ethylbenzene, xylene, organophosphates, middle-chain alkanes, and naphthalene;[13−19] genotoxins;[20−22] viruses such as tuberculosis and human respiratory syncytial virus;[23,24] antigens;[25] and various endogenous substances including sugars and amino acids.[26,27] Seven unique reporter proteins have been employed in cell-based biosensing systems, namely, chloramphenicol acetyltransferase, β-galactosidase, bacterial luciferase, firefly luciferase, aequorin, green fluorescent protein, and uroporphyrinogen III methyltransferase. Each of these reporters has advantages and disadvantages based on the assay conditions and the detection method employed (Table 2). The choice of reporter is dependent upon the background endogenous activity of the cell line used, gene expression and transfection efficiency, and the detection method[7,28] as well as the analytical application of the system.[4]

A. Chloramphenicol Acetyltransferase (CAT)

CAT, derived from *Escherichia coli*, was one of the first proteins used as a reporter and has been traditionally used to monitor gene transfer. Although still used in this fashion, CAT's use has been extended as a reporter protein for cell-based biosensing systems. The CAT-Tox (L) assay has been developed to identify and measure the molecular mechanisms of toxicity.[29] This assay couples the *cat* reporter gene with the promoter regions of 14 stress-related genes and stably transforms human liver cells, HepG2, with these fusion constructs. The stress response elicited by a wide variety of agents, including genotoxins, heavy metals, and planar aromatic hydrocarbons, can be assessed with this assay. Additionally, in a recent study CAT has been employed in a cell-based biosensing assay for the nongenotoxic carcinogens, peroxisome proliferators, in which a fusion construct of CAT with the promoter for rat acyl-CoA oxidase, the rate-limiting enzyme in the peroxisomal β-oxidation pathway, was transfected into a rat liver cell line.[30]

CAT was first identified in the 1960s following an epidemic spread of resistance to a broad-spectrum of antibiotics of which chloramphenicol was the first.[31,32] The mechanism of chloramphenicol resistance resulted from CAT-mediated inactivation of chloramphenicol by *O*-acetylation of the C-3 hydroxyl position yielding 3-*O*-acetyl chloramphenicol (Table 3).[33,34]

Table 2. Advantages and Disadvantages of Reporter Proteins Used in Cell-Based Biosensing Systems

reporter protein	advantages	disadvantages
chloramphenicol acetyltransferase	No endogenous activity.	Often employs radioisotopes. Requires addition of a substrate. Requires separation of substrate and product. Narrow linear range.
β-galactosidase	Sensitive and stable. Moderate linear range. Applicable in anaerobic environment.	Endogenous activity. Requires addition of a substrate.
bacterial luciferase	High sensitivity. Does not require addition of a substrate. No endogenous activity in mammalian cells.	Heat labile therefore limited use in mammalian cells. Narrow linear range.
firefly luciferase	High sensitivity. Broad linear range. No endogenous activity in mammalian cells.	Requires addition of a substrate. Requires an aerobic environment and ATP.
aequorin	High sensitivity. No endogenous activity in mammalian cells.	Requires addition of a substrate and the presence of Ca^{2+}.
green fluorescent protein	Autofluorescent, therefore, does not require addition of a substrate or cofactors. Spectral variants. No endogenous homologues in most systems. Stable at biological pH.	Moderate sensitivity. Requires posttranslational modification. Background fluorescence from biological systems may interfere. Potential cytotoxicity in some cell types.
uroporphyrinogen III methyltransferase	Autofluorescent, therefore, does not require addition of a substrate or cofactors. May have better signal-to-noise ratio than green fluorescent protein. Does not require costly reagents or special host strains.	Endogenous activity.

Table 3. Reporter Proteins, Chemical Reactions, and Detection Methods[a]

reporter protein	catalyzed reaction	detection method
chloramphenicol (CM) acetyltransferase	CM + acetyl CoA → 3-acetyl CM + CoA 3-acetyl CM ⇌ 1-acetyl CM 1-acetyl CM + acetyl CoA → 1,3-diacetyl CM + CoA	RI, FL
β-galactosidase	hydrolysis of β-galactosides	CR, HC, FL, EC, CL
bacterial luciferase	$FMNH_2 + R{-}CHO + O_2 \rightarrow FMN + H_2O + RCOOH + h\nu$ (490 nm)	BL
firefly luciferase	firefly luciferin + O_2 + ATP → oxyluciferin + AMP + P_i + $h\nu$ (550 − 575 nm)	BL
aequorin	coelenterazine + O_2 + Ca^{2+} → coelenteramide + CO_2 + $h\nu$ (469 nm)	BL
green fluorescent protein	postranslational formation of an internal chromophore	FL
uroporphyrinogen III methyltransferase	urogen III + SAM → precorrin-2 precorrin-2 → sirohydrochlorin precorrin-2 + SAM → trimethylpyrrocorphin	FL

[a] RI, radioisotope; FL, fluorescence; CR, colorimetric; HC, histochemical; EC, electrochemical; CL, chemiluminescence; BL, bioluminescence; SAM, *S*-adenosyl-L-methionine.

Since its discovery, the acetylation by CAT was found to be specific to chemical species similar to chloramphenicol and only CoA thioesters were found to be applicable cofactors for acyl transfer.

Numerous variants of CAT have been identified of which the type III enzyme is the most active (k_{cat} = 600 s^{-1}).[32] CAT, in its endogenous form, is a trimer with each of the three subunits containing a six-stranded mixed parallel and antiparallel β-pleated sheet, which forms the backbone, and five α-helices along a single side. A small 3-stranded β sheet is also formed by the N-terminus. The β-pleated sheet of each subunit extends across the subunit interface of the trimer forming eight hydrogen bonds between the three subunits, contributing to the stability of this protein.[32] The interface between the substrates forms a deep pocket which binds the substrate, chloramphenicol, via both hydrophobic and polar interactions. Interestingly, although the catalytic efficiency is highly variable between CAT variants, they all show similar affinities for chloramphenicol.[35] Studies by Kleanthous et al.[36] identified His 195, located within the substrate binding pocket, as playing a primary role in CAT catalysis by acting as a base to deprotonate the C-3 hydroxyl group of chloramphenicol, thus facilitating the attack of the hydroxyl oxygen by the carbonyl of acyl CoA.

In analytical bioassays, the product of the CAT-mediated acetylation reaction is predominantly measured using radiolabeled chloramphenicol or acetyl-CoA.[6] This method has been used to detect levels of CAT as low as 2 pg (www.euro.promega.com). However, assays for CAT generally have a narrow linear range (up to 3 orders of magnitude of enzyme activity) and, therefore, require testing of several sample dilutions to verify that the values obtained are within this range. Moreover, the health risks associated with the use of radioisotopes and the increasing restrictions on waste disposal of these agents are further disadvantages of this assay.[6]

Fluorescent assays have also been developed to measure CAT activity. These assays employ fluorescent chloramphenicol substrates, which can be quantified following their acetylation by CAT (Table 3).[37] The detection limits of the fluorescence-based assay are comparable to those using the radioisotope assay, 10^{-5}−10^{-6} units of activity in 1 h, while the linear range has been reported to be greater than 3 orders of magnitude. A primary disadvantage of both the radioisotope and fluorescent assays for measuring CAT activity is the requirement for separation of the substrates and products.[4] Despite the limitations of these assays, CAT is still widely used as a reporter protein due to its stability and lack of endogenous expression in mammalian cells.[4,7]

B. β-Galactosidase (β-Gal)

E. coli β-galactosidase (β-Gal), encoded by the *lacZ* gene, catalyzes the hydrolysis of β-galactosides. Like CAT, β-Gal is a widely used reporter protein that has historically been used to study transcriptional and translational gene regulation based on the pioneering work of Beckwith, Silhavy, and colleagues.[38,39] Although some β-Gal activity may be found endogenously, this reporter protein continues to be used in biosensing assays. Development of cell-based biosensors employing an inducible promoter and β-Gal as a reporter have been used to identify a wide variety of analytes including heavy metals,[11] toxic salts,[3] chlorocatechols,[13] and viruses in controlled as well as natural environments.[24] Early electron micrograph studies[40] and subsequent X-ray crystallography analysis[41] have identified β-Gal as a tetramer composed of 40% β-sheet, 35% α-helix, 13% β-turn, and 12% random coil.[42,43] Several amino acids have been identified that contribute to the enzymatic activity of β-Gal.[43] Notably, Tyr-253, Tyr-503, and Tyr-588 are believed to be important based on amino acid substitution, iodination, and proteolysis studies. Site-directed inhibition and site-specific replacement studies have further determined that Glu-461, a strictly conserved residue, may be involved in electrostatic stabilization of a galactosyl cation transition state and is probably a Mg^{2+} ligand. A second Glu residue, Glu-537, has been shown to covalently bind to the galactose substrate, namely, 2-deoxy-2-fluoro-β-D-galactopyranoside, and is thus important for catalysis. Finally, both monovalent (Na^+ and K^+) and divalent (Mg^{2+} and Mn^{2+}) cations mediate β-Gal activity by acting as cofactors, although the exact mechanism of action has not been determined.

Several detection methods for β-Gal are available including colorimetric, histochemical, fluorescent, luminescent, and electrochemical. These detection strategies are dependent on the substrates used. The most common substrates employed for β-Gal are *o*-nitrophenyl β-D-galactopyranoside (ONPG) for colorimetric detection, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) for histochemical detection, 4-methylumbelliferyl-β-D-galactopyranoside (MUG) for fluorometry, 1,2-dioxetane substrates for luminescence, and *p*-aminophenyl-β-D-galactopyranoside (PAPG) for electrochemical analysis. The advantages of colorimetric assays lie in their simplicity and rapidity; however, their low sensitivity (100 pg)[44] and narrow dynamic range have led to their replacement by other methods of detection.[7] Histochemical measurements are similar to the colorimetric ones in terms of sensitivity. While these assays are typically time-consuming, they have the advantage of tissue specificity as well as an overall measure of gene expression.[45] In the late 1970s and early 1980s, sensitive (2 pg)[44] fluorometric assays were developed to measure β-Gal activity.[46−48] Since then, these assays have been further refined to detect ultralow levels of β-Gal in pL volumes. Using fluorescein-di-β-D-galactopyranoside as the substrate and capillary electrophoresis laser-induced fluorescence detection strategies, Craig et al.[49] were able to obtain detection limits as low as 6.5×10^{-14} M β-Gal in as little as 40 pL of the enzymatic mixture (2.6×10^{-24} mol). Luminescence-based assays employing 1,2-dioxetane substrates are typically 3 orders of magnitude more sensitive than fluorescent-mediated assays having detection limits as low as 2 fg.[5,44] Moreover, chemiluminescent detection methods of β-Gal are not only rapid but extend over a dynamic range of more than 5−6 orders of magnitude. One last and promising method for detection of β-Gal activity is electrochemistry. Although this method requires the addition of an external substrate, it does not require lysis or permeabilization of the cells and thus is ideal for online continuous measurement of enzymatic activity.[50] Moreover, this detection strategy can be performed in turbid solutions and under anaerobic conditions. In summary, the wide variety of highly sensitive detection strategies, especially chemiluminescent and electrochemical methods, coupled with their simplicity make β-Gal a valuable reporter in cell-based biosensing.

C. Bacterial Luciferase (Lux)

Luciferase is a generic name for any enzyme that catalyzes a light-emitting reaction.[51] The measurable release of visible light by organisms containing luciferase is termed bioluminescence. Bioluminescent organisms, including bacteria, algae, dinoflagellates, fungi, jellyfish, clams, fish, insects, shrimp, and squid, are ubiquitous and can be found in aquatic as well as terrestrial environments with the vast majority occurring in the marine communities.[52] Among them, bacteria are the most abundant luminescent organisms and have been classified into three genera *Vibrio*, *Photobacterium*, and *Xenorhabdus*.[52] The light-emitting proteins (Lux) of several of these bacterial species have been purified, and the ability to transfer the cDNA coding for Lux proteins into prokaryotic and eukaryotic organisms has resulted in their common use as reporters of gene expression.

Bacterial luciferase catalyzes the oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde to FMN and the corresponding fatty acid in the presence of molecular oxygen. This reaction results in the emission of a blue-green light with a maximum intensity at 490 nm and quantum efficiency between 0.05 and 0.15 (Table 4).[53] Differences in the color of the emitted light occur and are believed to be the result of other proteins found in the organisms, which can induce shifts in the emitted light. Tetradecanal is synthesized by the bacteria and is believed to be the natural fatty aldehyde substrate for the bacterial luciferase. However, fatty aldehydes of chains ranging from 7 to 16 carbons in length are also effective substrates for the reaction.[53] In fact, shorter chain aldehydes, including decanal, have been found to induce a higher luminescent response by bacterial luciferase than tetradecanal.[4,10]

All bacterial luciferases are heterodimeric proteins composed of two subunits, α (40 kDa) and β (37 kDa), whose amino acid sequence can differ by up to 45% and 55% between bacterial species, respectively.[53,54] The crystal structure of luciferase from only one species, *Vibrio harveyi*, has been determined in the absence of substrates and has been extensively reviewed by Baldwin et al.,[51] and therefore, it will

Table 4. Excitation and Emission Maxima of Reporter Proteins and Their Most Commonly Used Substrates

reporter protein	excitation λ	emission λ	quantum yield
chloramphenicol acetyltransferase	504−545 nm	510−570 nm	
BODIPY[a] 1-deoxychloramphenicols			
β-galactosidase			
methylumbelliferyl β-D-galactopyrosanides	350 nm	450 nm	0.2−0.4
fluorescein-di-β-D-galactopyrosanides	488 nm	530 nm	0.91
resorufin β-D-galactopyrosanides	550 nm	600 nm	0.2−0.4
1,2-dioxetanes (no enhacer)		462 nm	
saphire enhancer		463 nm	
emerald enhancer		542 nm	
ruby enhancer		620 nm	
bacterial luciferase		490 nm	0.10
firefly luciferase		550−575 nm	0.90
aequorin		469 nm	0.15
green fluorescent protein	395 nm	509 nm	0.80
blue fluorescent protein	380 nm	440 nm	0.18
cyan fluorescent protein	433 nm	475 nm	0.40
yellow fluorescent protein	513 nm	527 nm	0.61
red fluorescent protein	558 nm	583 nm	0.23
uroporphyrinogen III methyltransferase	378 nm	608 nm	

[a] BODIPY stands for borondipyrromethene difluoride.

not be discussed in detail here. The catalytic site of the enzyme is postulated to lie in a deep pocket on the α-subunit.[55−57] Although the role of the β-subunit has not yet been determined, its presence is essential for a high quantum yield reaction.[51]

The structural subunits, α and β, of bacterial luciferase are encoded by the *luxA* and *luxB* genes, respectively, which are located adjacently in the *lux* operon. Three additional genes in the *lux* operon, *C*, *D*, and *E*, code for proteins that associate to form the fatty acid reductase required for synthesis and recycling of the fatty aldehyde.[54,57] These five common genes are conserved in all bacterial species identified to date.[54] The expression of *luxA* and *luxB* in the host organism is sufficient for signal bioluminescence; however, expression of all five genes has the advantage of not requiring the addition of a substrate. Several additional *lux* genes, whose function may or may not be known, have been identified in different bacterial species.[54] These have been reviewed by Meighen.[52]

One of the earliest uses of bacterial luciferase as a reporter was the development of the Microtox assay, which involved the exposure of naturally luminescent bacteria, *Photobacterium phosphoreum*, to potentially toxic samples.[54] Decreases in luminescence of the exposed bacteria, upon comparison to control groups, suggested potential toxicity of the sample. Since these early studies, cDNA cloning and expression of the *lux* genes has been used to develop novel biosensors for numerous analytes, including those based on whole-cell systems. By fusing the *lux* operon to pertinent promoters and subsequent expression in host cells, bacterial luciferase has been used as a marker of exposure to heavy metals,[9] toxic organics,[18] and nitrate[58] in whole-cell bioassays. Although bacterial luciferases are useful for sensitive detection and measurement of prokaryotic gene transcription, their applicability in mammalian systems is limited since these enzymes are heat labile (>30 °C).[7,53] Moreover, the linear range of these assays is somewhat low (3 orders of magnitude) compared to that of other bioluminescent reporters.[7,59,60]

D. Firefly Luciferase (Luc)

Firefly luciferase catalyzes the oxidation of its substrate, a benzothiazolyl−thiazole luciferin, to oxyluciferin, in the presence of ATP, O_2, and Mg^{2+}, producing CO_2 and visible light.[4] The maximum emissions of different firefly species occur between 550 and 575 nm and are attributed to different amino acid substitutions in the luciferase.[7,61] Studies employing site-directed mutagenesis resulting in single amino acid substitutions yielded emissions of light ranging from the green to the red spectrum.[57,62] Therefore, expression of these mutants in host organisms under the control of independent promoters can result in the generation of distinct signals, a property that can be exploited for multianalyte detection. The light emitted by the luciferase reaction is characterized by a transient flash peaking at 300 ms. This emission time can be significantly extended (several minutes) by the addition of coenzyme A, which promotes the dissociation of the oxidized substrate from the enzyme, thus preventing its decay.[7]

In contrast to bacterial luciferase, firefly luciferase is a 62 kDa monomer that has 40−50% amino acid sequence homology between luciferases in a single family of beetles.[57] Two domains, namely, a large N-terminal domain linked by a four-residue flexible loop to a small C-terminal domain, have been identified by X-ray crystallography.[57,63] The active site of the enzyme is postulated to lie facing a cleft located between the two domains and along the connecting loop, where the most conserved residues of beetle luciferases occur.

Like bacterial luciferase, there is no endogenous activity of firefly luciferase in mammalian cells. Its high sensitivity (subattomole level), broad dynamic range (7−8 orders of magnitude), and simplicity are the primary advantages of this bioreporter.[7] Moreover, the sensitivity (femtogram levels) of firefly luciferase has the potential to be greater than that of bacterial luciferase since its quantum efficiency (≅ 0.9) is approximately 10-fold higher.[54] Firefly luciferase, under the control of various promoters, has

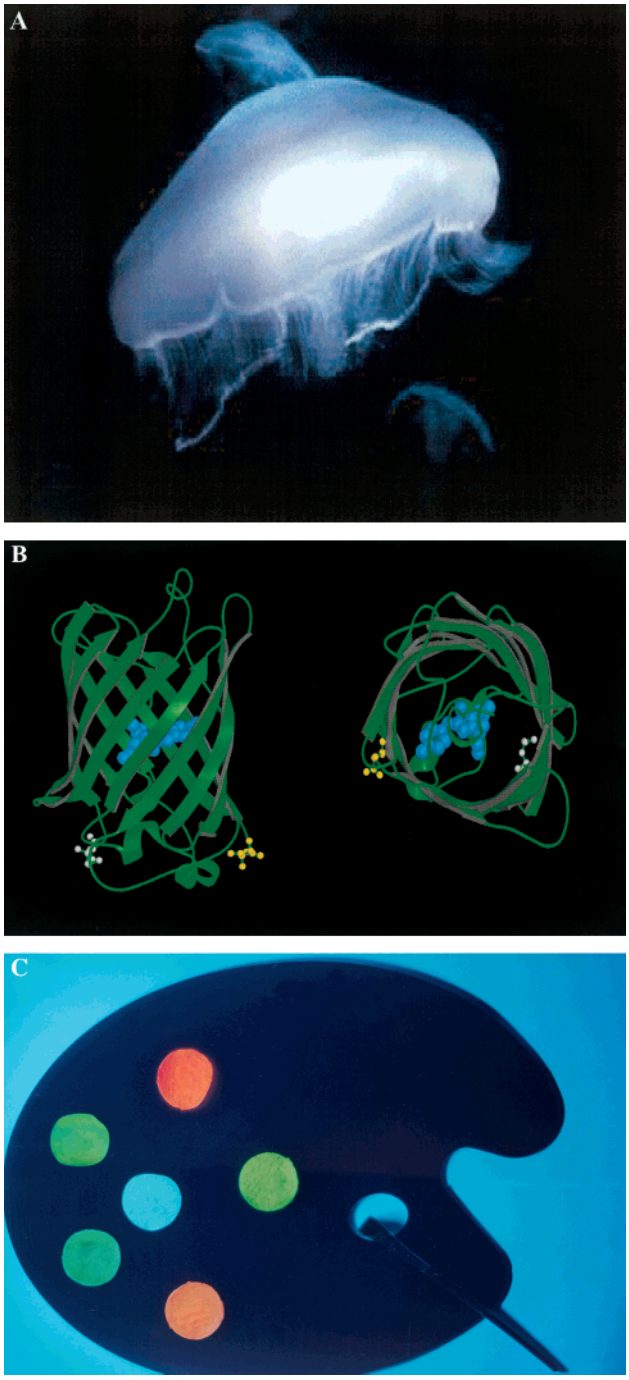


Figure 2. (A) Photograph of the bioluminescent jellyfish *Aequorea victoria*. (B) X-ray crystal structure of green fluorescent protein (GFP) pictured from the side and the top, respectively. The internal chromophore lies within the β-barrel network of the protein and is represented by a ball-and-stick model. (C) Expression of fluorescent reporter proteins in *E. coli*. Clockwise from the top: CobA; GFP; RFP; GFP mutants, cyan, yellow, and blue (center).

been employed in whole-cell biosensors for the detection of heavy metals, such as cadmium and lead,[12] and aromatic organics.[19]

E. Aequorin

Aequorin is a Ca^{2+}-binding photoprotein that has been isolated and cloned from the bioluminescent jellyfish *Aequorea victoria* (Figure 2A). Its substrate or luciferin, coelenterazine, is noncovalently bound to the apoprotein along with molecular oxygen. Following the addition of Ca^{2+}, the coelenterazine is oxidized to coelenteramide and blue light is emitted in the range of 460−470 nm. CO_2 is also produced as a byproduct of the reaction. The emitted light, like in firefly luciferase, occurs as a brief flash lasting less than 3 s with a quantum yield of 0.15.[4] Aequorin, like other Ca^{2+}-binding photoproteins, can be regenerated by removal of Ca^{2+} followed by the addition of fresh coelenterazine in a reducing environment.[64]

Aequorin is formed by two components, an imidazopyrazine coelenterazine and apoaequorin, a 22 kDa single polypeptide chain. The protein contains three Ca^{2+} binding sites that upon occupation produce a conformational change resulting in oxidation of the luciferin. Each of these Ca^{2+} binding sites is comprised of two α-helices separated by a β-pleated sheet that form an EF-hand motif. The oxygen binding site within the photoprotein occurs most likely via His-169.[65] Site-directed mutagenesis studies have been employed to provide information regarding the function of specific residues within its primary structure. These data have recently been reviewed by Lewis and Daunert.[66] Briefly, a single substitution of each of three cysteine residues with serine resulted in a substantial loss (95%) of enzymatic activity while unexpectedly, subsequent replacement of all three cysteine residues with serine yielded a slight increase (16%) in enzymatic activity.[66,67] The C-terminal region of aequorin is also believed to be important for its native protein activity since deletion or addition of amino acid residues in this region have resulted in considerable loss of protein (>95%) activity.[68] Additionally, four of the six tryptophan residues of apoaequorin occupying positions 12, 108, 129, and 173 have also been found to contribute to aequorin's activity, and an observed loss (>95%) in native protein activity occurs upon residue substitution.[69] However, recent studies from our laboratory do not confirm these findings.[70]

Aequorin has been extensively used as an indicator of intracellular calcium as well as in several additional applications including immunoassays, nucleic acid probe assays, and biosensing systems.[4] However, to our knowledge only one cell-based assay has used aequorin as a reporter of analyte exposure. The Cellular Analysis and Notification of Antigen Risks and Yields (CANARY) assay, reported by Rider et al., is currently being applied to the rapid (<1 min) detection of pathogenic bacteria and viruses.[25] Cultured B cells containing surface antibodies, specific to antigens of different pathogens, are genetically engineered to produce aequorin. Upon exposure to the antigen, the B cell containing aequorin emits light as a result of activation of an intracellular signaling cascade that releases calcium ions inside the cell. Despite its limited use to date in cell-based biosensing assays, aequorin's high sensitivity (detection limits in the attomole to subattomole levels), stability, and lack of endogenous expression make this protein an excellent choice as a bioreporter.

F. Green Fluorescent Protein (GFP)

The green fluorescent protein (GFP), like aequorin, is a photoprotein which has been isolated and cloned from the jellyfish *Aequorea victoria*. Variants of GFP have also been cloned from the sea pansy, *Renilla reniformis*, but are not strongly homologous to *Aequorea* GFP, although their chromophores are identical.[57] The primary advantage of GFP as a reporter protein is its autofluorescence, and therefore, its use does not require the addition of cofactors or exogenous substrates to produce light.[7] The autofluorescent nature of GFP is a result of its internal covalently bound imidazolinone chromophore. The formation of this chromophore occurs by a posttranslational modification resulting from cyclization and subsequent oxidation of three amino acid residues of the protein and is the rate-limiting factor thus determining the reporting speed of gene expression by GFP.[71] GFP acts as an accessory protein to aequorin or luciferase, the primary proteins, by shifting the emission spectrum from blue bioluminescence to green fluorescence.[72] This process occurs by a radiationless energy transfer from the primary protein to GFP. The excitation spectrum of GFP has a maxima at 395 nm with a minor peak at 475 nm, while the emission maxima occurs at 509 nm with a small shoulder at 540 nm.[8] Although, the fluorescence quantum yield of GFP (0.72-0.85) is comparable to that of the well-known fluorescent dye, fluorescein (0.91), its molar absorptivity is somewhat lower, causing GFP to have approximately 1 order of magnitude lower intensity than fluorescein.[66]

The recent identification of the three-dimensional structure of GFP indicates that this protein has a unique folding pattern, which has been termed the β-can.[72,73] GFP is a single polypeptide chain that forms an 11-stranded β-barrel (diameter 30 Å and length 40 Å) and is "capped" at the ends by small α-helical sections (Figure 2B). The cyclic chromophore resides inside of the cylinder forming an irregular α-helical segment. The barrel structure contributes to the stability of the protein, as well as in protecting the fluorophore from quenching agents such as molecular oxygen.[72] Three posttranslationally modified amino acids contribute to the formation of the fluorophore, namely, Ser65-Tyr66-Gly67. It has been postulated that cyclization of these residues in GFP, and not other proteins, occurs as a result of acid−base chemistry, which is a catalyst for the cyclization and requires the close proximity of the backbone atoms of Ser65 and Gly67. In fact, it is known that the glycine residue at position 67 is required for fluorescence to occur.[72]

GFP has numerous qualities that make it an ideal reporter protein. One particularly useful aspect of GFP as a reporter is the ability to alter its stability and spectral properties through structural alterations of the native protein, especially within the fluorophore region.[8,64] Although the majority of modifications of GFP result in decreases or losses of its activity, some notable mutants have been identified that show improved fluorescence intensity, thermostability, and chromophore folding. For example, the mutation S65T was found to render a GFP with these properties. Importantly, several mutants have been developed with altered excitation and emission spectra of GFP. Substitution of Tyr66 yields a family of variants with excitation and emission spectra in the blue range, while other structural alterations yield cyan-, red- and yellow-shifted variants (Figure 2C). It has been proposed by us and others that these spectral variants can be used for multianalyte detection purposes.[66] Additional advantages of GFP include high stability at biological pH, assay simplicity, and lack of endogenous homologues in most target organisms.[6,7] However, the sensitivity of GFP may be compromised by the prevalence of other fluorescent molecules found in certain biological systems.[6] Additionally, a recent trasfection study has found, in contrast to other reports dealing with prokaryotes, yeast, and plant cells,[71,74,75] that GFP is toxic to some mammalian cell types including NIH/3T3, BHK-21, HepG2, Hep3B, Huh-7, T24, and TSGH-8301.[76] Further, this observed cytotoxicity is suspected to be linked to apoptosis.

Despite these disadvantages, the unique characteristics of GFP have led to its successful application as a reporter protein. GFP has been used to measure gene expression, identify transformed cells, study cell-trafficking mechanisms, and as a reporter for various analytes. A recent study from our laboratory has used GFP as a reporter in a fiber-optic whole-cell biosensor to detect L-arabinose.[26] In this study, expression of GFP was under the control of the regulatory protein AraC of the *araBAD* operon and the promoter region P_{BAD}. In the presence of L-arabinose, GFP was expressed and its relative amount was determined via fluorescence detection. This system was determined to be highly selective for L-arabinose with the ability to discriminate this sugar from its stereoisomer, D-arabinose, and a wide variety of pentoses and hexoses.

G. Uroporphyrinogen (Urogen) III Methyltransferase

Uroporphyrinogen (urogen) III methyltransferase (UMT), important for the biosynthetic pathways of vitamin B_{12} and siroheme,[77] catalyzes the *S*-adenosyl-L-methionine (SAM)-dependent addition of two methyl groups to the substrate, urogen III, producing dihydrosirohydrochlorin (precorrin-2). Precorrin-2 can be oxidized to a fluorescent product, sirohydrochlorin, or accept the addition of a third methyl group from SAM through further action of UMT yielding a second fluorescent product, trimethylpyrrocorphin. Both products emit a red to red-orange (590−770 nm) fluorescence when illuminated with UV light at 300 nm.[78] Moreover, the substrate, urogen III, is ubiquitous and can be found in all organisms;[78] thus, UMT can function as a reporter gene without requiring the addition of a substrate or other cofactors.

UMT has been identified and purified from several different organisms and exists in two forms.[77] The first form is required for vitamin B_{12} synthesis and is encoded by the *cobA* genes in *Bacillus megaterium*,[79] *Methanobacterium ivanovii*,[80] *Propionibacterium freudenreichii*,[77] and *Pseudomonas denitrificans*.[81] The second form of UMT is encoded by the

cysG gene in *E. coli*[82] and *S. typhimurium*[83] and is required for siroheme, cysteine, and vitamin B_{12} synthesis. This latter form, but not CobA, has, in addition to methylase activity, NAD^+-dependent precorrin-2 oxidase and ferrochelatase activity, which are attributed to siroheme production in *E. coli*. CysG is a 458 amino acid protein that has a molecular mass of 52 kDa. CobA, however, is somewhat smaller than CysG and is comprised of only 280 amino acids with a molecular mass of 30 kDa.[77] In fact, CobA is homologous only to the C-terminal region of CysG.[77] On the basis of these characteristics, the methylation activity of CysG is attributed to the C-terminal region of the protein while its NAD^+-dependent precorrin-2 oxidase and ferrochelatase activities are attributed to the N-terminal region.

UMT has been used as a reporter protein for the selection of recombinant plasmids[78] and recently as a marker for gene transcription in bacterial, yeast, and mammalian cells.[84] Additionally, studies currently being conducted in our laboratory have employed UMT as a reporter protein in a whole-cell biosensor for the detection of the toxic salts arsenite and antimonite.[85] In these studies, a recombinant plasmid is constructed by placing a regulatory region of the *ars* operon, coding for the protein ArsR, upstream of the *cobA* gene. The recombinant plasmid is then incorporated into *E. coli* and the genetically modified bacteria are used to detect antimonite and arsenite. The fluorescent signal of sirohydrochlorin, produced by the enzymatic activity of UMT, has been reported to have a similar intensity to that of GFP.[84] However, the red fluorescent properties of sirohydrochlorin are believed to yield a greater signal-to-noise ratio compared to the green fluorescent signal of GFP since autofluorescence and light scattering of endogenous materials are lower in the red wavelengths.[84] For these reasons, UMT may prove to be a valuable reporter protein for a number of applications including whole-cell biosensor development.

A variety of reporter genes are available for use in cell-based biosensing systems. The unique characteristics, advantages, and disadvantages of these reporter genes have been described above. It is our opinion that the luminescent reporters offer distinct advantages as a result of their high sensitivity and broad linear range. The GFP class of reporters offer additional advantages because of their multiple spectral properties which make them ideal for noninvasive, multianalyte, and high-throughput screening assays.

III. Nonspecific Biosensing Systems (Effect Selective)

The ability of intact cells to recognize a group of substances has been exploited in a wide range of biosensing systems using complex variables. Many nonspecific microbial tests have been developed for the detection of toxic substances in the environment. These test systems are nonspecific in the sense that they are based on the response mechanism that they activate; therefore, they can be referred to as "effect selective" sensing systems. These nonspecific whole-cell sensing systems employ toxin-sensitive cells that express a reporter gene upon interaction with toxic substances. Critical amounts of toxins affect the cells in two different ways: (i) they can decrease the metabolic activity of the cells or (ii) they can cause cell death. An example of this type of nonspecific sensing systems for toxin detection is the commercially available Microtox assay.[86] This technique relies on the changes in light production in living luminescent bacteria (*Photobacterium phosphoreum*) as a result of chemical inhibition. The rate and amount of light produced can be altered either by chemical inhibition of any of the enzymes involved in these reactions or by toxins interfering with the formation of NADPH. Moreover, the relative level of toxicity can be assessed with this assay.

A second type of nonspecific sensing system involves the use of genetically engineered cells to express a reporter protein in the presence of an analyte. In these whole-cell sensing systems, different strains are engineered through molecular biology to contain the desired genes. Toxin-sensitive nonspecific biosensing systems employ a promoterless reporter protein (e.g., luciferase, GFP, β-galactosidase) fused downstream of the gene corresponding to the stress promoter of interest. These whole-cell sensing systems can be visualized as an environmental switch which is turned on when critical amounts of the toxins are present. Although these types of biosensing systems are used to detect any compound that is toxic to the cells, they do not provide any information on the type of toxin present.

A. Heat-Shock and Other Stress Related Proteins

Nonspecific biosensing systems for toxin detection are mainly based on stress response, such as the heat-shock response. This kind of system employs a transcriptional promoter, involved in the stress response that regulates protein expression and production. Heat-shock gene expression is a general stress response caused by elevation in temperature, the presence of abnormal proteins, and exposure to a variety of chemicals, including organics, heavy metals, oxidative agents, and antibiotics.[87] Exposure of *E. coli* cells to these stressors results in the induction of a specific set of proteins, termed the heat-shock proteins. The resulting induction is mediated primarily at the transcriptional level; however, some translational regulation also occurs.[88] The cellular mechanism of the heat-shock expression system is activated to minimize or avoid protein denaturation; thus, by inducing heat-shock protein synthesis, the cell is able to survive the stress condition. These proteins are involved in essential events that affect cell viability including folding, assembly, transport and degradation of proteins, DNA replication, protein RNA synthesis, and cell division.

The major heat-shock proteins have been classified into five families corresponding to their molecular size: 100−110, 90, 68−75 (hsp70), 60 (hsp60), and 16−28 kDa.[89] Further, on the basis of their role in metabolism, heat-shock proteins can be divided into three categories: They can serve (i) as chaperones, assisting in protein folding processes; (ii) as proteases, taking part in native and foreign protein

denaturation; and (iii) as effector proteins, activating the heat-shock protein synthesis. Although the heat shock or stress response has been observed in a wide variety of organisms, including humans, the best studied stress response is that of *E. coli*.[88] In *E. coli*, the *rpoH* gene encodes a 32 kDa sigma factor, σ^{32}, that drives transcription of approximately 20 genes corresponding to the heat-shock proteins. The *rpoH* gene directs RNA polymerase (E) to heat-shock promoters which are recognized only by Eσ^{32}. In addition, two other alternative sigma factors control different heat-shock regulons in *E. coli*. The σ^{E} (σ^{24}) factor belongs to a class of sigma factors that respond to extracytoplasmic stimuli and is regulated my a more complex mechanism.[90] The second alternative sigma factor, σ^{54}, controls the expression of the phage-shock operon.[91]

The σ^{32} regulon includes the DnaK-DnaJ-GrpE chaperone team and the GroEL-GroES chaperone team. Members of both chaperone teams are key proteins involved in the regulation of heat-shock gene expression in *E. coli*. Heat-shock protein production has been linked to an increase in temperature. Overexpression of GroEL-GroES chaperone team permits growth up to 40 °C. In addition, simultaneous overexpression of both chaperone teams supports growth up to 42 °C. However, some of these heat-shock proteins are required at all temperatures, since they play a role in regular events in the cell.

DnaK is a member of a well-conserved group of heat-shock proteins (the hsp70 family) and plays a protective role in supporting growth of the cell at temperatures above the normal physiological range of *E. coli*. DnaJ is a member of the hsp40 family and along with DnaK affects replication of bacteriophage λ. GrpE is an accessory protein that interacts with the products of the DnaK and DnaJ proteins. Together, they form a chaperone team that binds and releases target proteins in cellular folding reactions.

As the DnaK chaperone team, the GroEL-GroES chaperone team is involved in bacteriophage growth, cellular folding reactions, and general proteolysis. However, they play distinct roles in protein folding. In addition, the GroEL-GroES chaperone team mediates cellular mutagenesis. Members of this chaperone team include the serine protease *lon* and *clpB*, whose role as a protease or as a chaperone is unclear.

The stability and activity of σ^{32} regulate the expression of the heat-shock genes. The activation of the σ^{32} regulon is sensitive to both an increase and a decrease in temperature. Consequently, the rate of transcription increases at high temperatures, and decreases at low temperatures. However, the signal that regulates the expression of the σ^{32} regulon is not known yet. Several different mechanisms have been proposed, and all of them indicate that the DnaK chaperone team plays a regulatory role by detecting the signals produced by the inducers. From all of these proposals, there are several versions of the homeostatic mechanism. In the homeostatic mechanism, the DnaK chaperone team is the central element (Figure 3). Folded and unfolded proteins compete with σ^{32} for free DnaK, DnaJ, and GrpE. Upon inducing signals, such as the ones produced by

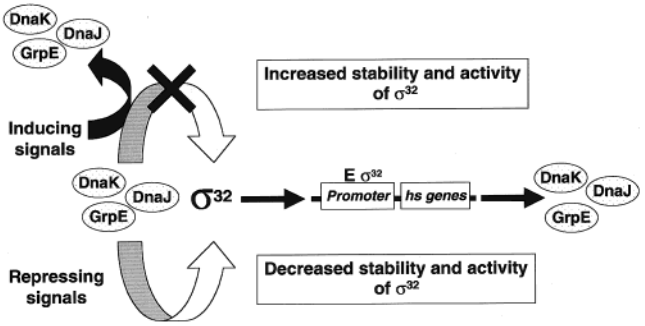


Figure 3. Speculative model of the regulation of σ^{32} in *E. coli*. A homeostatic mechanism by which free chaperones, DnaJ, DnaK, and GrpE, regulate the activity and stability of the σ^{32} regulon. Inducing signals (e.g., temperature upshift, ethanol, unfolded proteins) titrate these heat-shock proteins (DnaK, DnaJ, and GrpE) away (indicated in the figure as a black arrow), relieving their negative regulatory effects, thus increasing the stability and activity of σ^{32}. Conversely, upon repressing signals such as temperature downshift, an increase in the levels of these heat-shock proteins will inactivate σ^{32}.

ethanol, temperature upshift, and misfolded proteins, an increase in the substrates for these chaperones will suppress the negative regulatory effects of the chaperones. In contrast, repressing signals such as temperature downshift will decrease the substrates for these chaperones, releasing them, and therefore inactivating σ^{32}. It should be noted that in other species of bacteria the heat-shock response is much more complex and involves alternative regulatory mechanisms other than σ^{32}, for example, the σ^{B}, CIRCE/HrcA,[92] and CtsR elements in *Bacillus subtilis*, the HAIR/HspR and OrfY elements in *Streptomyces albus*, and the ROSE element in *Bradurhizobium japonicum*.[93−95]

E. coli as well as other organisms undergo different cellular stress responses besides the heat-shock response. Bacteria have developed several complex additional mechanisms that allow them to survive under stress conditions. These cellular stresses can result not only from heat shock but also from cold shock (*cspA*), cytoplasmic stress (*ibp*), nutrient starvation (universal stress protein *uspA*), oxidative stress (*oxyR, soxRS*),[96] growth limitation, osmotic stress (*osmY*),[97] DNA damage (SOS),[98] amino acid starvation, and stationary phase[99] among others. All of these conditions have been exploited in developing nonspecific whole-cell biosensing systems (Table 5).

GFP, β-Gal, CAT, and luciferase have been employed as reporter proteins in whole-cell sensing systems based on stress induction. Cellular stresses caused by heat shock, osmotic pressure, ethanol, phenol, isopropyl β-D-thiogalactopyranoside (IPTG), and serine hydroxamate have been monitored using the GFP.[100] This system employs three heat-shock promoter elements isolated from *E. coli*'s transcriptional factor σ^{32}, the protease subunit ClpB, and the chaperone DnaK. Fusion of these stress promoters upstream of the *gfpuv* gene, a mutant form of the *gfp* reporter gene,[101] resulted in a plasmid that detects the stress response. Upon exposure to the different chemical and physical inducers, the corresponding heat-shock proteins were activated and a green fluorescent signal was released as the protein

Table 5. Stress-Responsive Mechanisms in Whole-Cell Sensing Systems

stress inducer	regulatory gene	stress response	reporter genes	host organisms	references
protein damage	*rpoH*	heat shock	*gfp, lacZ, cat, lux*	*E. coli* *S. cerevisiae* human liver cells (HepG2)	29,100,102−108, 112,114−118, 120−122
oxidative damage	*oxyR*	hydrogen peroxide	*lux*	*E. coli*	109−111,114−118, 120−122
oxidative damage	*soxRS*	superoxide	*lux*	*E. coli*	109,111,114−118, 120−122
growth limitation	unknown	universal stress	*lux*	*E. coli*	108,114,116,117, 120,121
amino acid starvation	*relA, spoT*	stringent	*lux*	*E. coli*	114
stationary phase	*rpoS*	resting state	*lux*	*E. coli*	114
osmotic pressure	*rpoS*	hyperosmotic	*lacZ, lux*	*E. coli*	50,119
DNA alkylation	*ada*	adaptive	*lux*	*E. coli*	109,114,138
phosphate starvation	*phoB, phoM, phoR, phoU*	P utilization	*lux*	*E. coli*	114
nitrogen starvation	*glnB, glnD, glnG, glnL*	N utilization	*lux*	*E. coli*	114
carbon starvation	*cya, crp*	catabolite activation	*lux*	*E. coli*	114,120,121
membrane synthesis	*fadR*	fatty acid synthesis	*lux*	*E. coli*	114,115,120,121
DNA damage	*lexA, recA*	SOS	*lacZ, lux, gfp, cat, luc*	*E. coli, S. cerevisiae, P. aeruginosa, S. typhimurium*, Rat liver cells (H-4-II-E)	20−22,30,109, 113−118,122, 130−138,140−146

was produced. An example of a compound analyzed with this system is ethanol, which was detected down to a concentration of 1% with a linear response ranging from 2% to 4% concentration. Measuring the fluorescence produced during the resting cell state, however, resulted in a relatively slow method.

Measurement of the β-Gal activity of stress-responsive strains has been reported using *Saccharomyces cerevisiae* yeast cells. Like other organisms, *S. cerevisiae* undergo a typical stress response when exposed to elevated temperatures. In this study, Fujita and co-workers developed a practical method for evaluating the potential toxic risk assessment of disinfectants in pesticides.[102] They employed the *hsp104* gene of *S. cerevisiae* to determine the toxicity of pesticides such as Captan, tetrachloroisophthalonitrile (TPN), and bis(quinolin-8-olato-*O,N*) copper (Oxine-copper) based on β-galactosidase expression.

Recently, Bianchi and Baneyx took advantage of new advances in the understanding of stress responses to develop *lacZ* gene fusions for the detection and characterization of new antibacterial agents.[103] *E. coli* strains harboring three unique promoters induced by cold shock (*cspA*), cytoplasmic stress (*ibp*), or protein misfolding in the cell envelope (P3*rpoH*) form the basis of a minimal assay that can be used to detect and categorize the modes of action of antibacterial agents by monitoring the enzymatic activity of the β-galactosidase produced. Model antibiotics such as chloramphenicol (cold shock induction), streptomycin (cytoplasmic stress), and polymicin B (protein misfolding in the cell envelope) were detected by the system. However, antibiotics that selectively affect the ribosomes or cause DNA damage cannot be detected. Importantly, detection of antibacterial compounds in natural extracts from *Streptomyces venezuelae* shows potential for the discovery of new antibacterial agents from nature.

Biran et al. developed a whole-cell biosensing system for on-line monitoring of gene expression.[50] The system employs an *E. coli* strain harboring the *osmY::lacZ* gene fusion and is based on the amperometric determination of β-Gal activity using *p*-aminophenyl-β-D-galactopyranoside (PAPG) as the substrate. The product of the *osmY* is a periplasmic protein whose function is not known.[97] Transcription of *E. coli*'s *osmY* is regulated and induced by hyperosmolarity nutrient starvation and possibly by other regulatory circuits that ultimately lead to the stationary phase. A disposable three-electrode cell that employs screen-printed electrodes forms the basis of the detection system that can either be performed directly in the electrochemical cells, requiring 300 μL of culture, or be adapted inside the culture flask.

Todd et al. developed a CAT whole-cell sensing system to measure stress responses in human liver cells (Hep G2).[29] This system, the CAT-Tox (L) assay, consists of a panel of 14 strains, each one employing a unique stress promoter. The relevance of this technique over other toxicity assays is that the CAT-Tox (L) assay provides a stress profile that could serve as a chemical toxicological fingerprint, since it gives information on the mode of action of the chemical on human cells. This panel of stress-responsive strains bears the genes corresponding to enzymes involved in the transcription of a variety of cellular and molecular mechanisms that can result in liver cell injury as well as in the transcription of the CAT gene. Through this CAT-based bioassay, both toxic and nontoxic stresses, such as xenobiotic response (CYP IA1, GST Ya, XRE), antioxidant response (ARE, GST Ya), DNA damage (GADD 45 and 153 gene, p53), heavy metals (MT IIA), heat-shock response (hsp70), and protein damage (GRP78 and HSP70), can be measured. The sensitivity achieved for polycyclic aromatic hydrocarbons was 1 nM (0.32 ppb), while a detection limit in the pM range was recorded for dioxin. This study demonstrated that the CAT-Tox (L) assay can be used to distinguish between closely related compounds as well as to indicate the molecular mechanisms of cellular damage. Because of the ability to provide

molecular information, the CAT-Tox (L) assay has found applications in the evaluation of chemical mixtures from environmental sites.

Toxicity assays based on bioluminescence are often referred to as "lights on" or "lights off" assays. A "lights on" signal results when the cell is stressed by various factors resulting in activation of the heat-shock or other stress related proteins and expression of the light emitting genes. In contrast, the "lights off" signal is indicative of the inhibition of the luciferase enzyme or some toxic effect on cellular metabolism. Therefore, toxins can be detected by two ways: by induction of the stress response at low concentrations (e.g., "lights on") or by inhibition of bioluminescence at higher concentrations (e.g., "lights off"). Bioluminescence-based whole-cell biosensing systems have been extensively used to monitor cellular stress responses. Fusing the *luxCDABE* of *V. fischeri* and *P. phosphoreum* to various promoter genes that control several regulatory networks has been the approach undertaken by LaRossa and co-workers. Promoter genes of the heat-shock response [hsp70 (*dnaK*), hsp60 (*groEL*), *grpE*, and *lon*],[104−108] SOS regulatory system,[109] oxidative damage,[109−111] and the universal stress protein (*uspA*)[108] were activated in these circuits resulting in the development of bioluminescence upon exposure to metals, solvents, and pesticides. By introducing a mutation in the *tolC* locus, a minor outer membrane protein in *E. coli* that extrudes hydrophobic compounds from the cell, an increase in sensitivity to pentachlorophenol (detection limit of 37 ppb) was achieved.[107] Further, comparison studies between *lux* fusion strains harboring the heat-shock protein *grpE* and the universal stress protein *uspA* revealed that although the relative level of inducing stress is similar, the *grpE::lux* fusion strain has stronger induction responses (assessed with equal or lower concentrations), better detection limits, and detects a wider range of stresses.[108]

A notable application of stress-responsive bacterial strains is in the development of a minibioreactor for wastewater biotreatment plants (WWBP).[112] The system showed some limitation due to the overloading of highly toxic chemicals and usage of daughter cells of bacteria previously exposed to toxic chemicals. However, in further studies, they improved the sensing device by coupling a second vessel to the system.[113] This new bioreactor employs two minibioreactors in series, the first vessel is used to grow the cells, while in the second vessel toxic chemicals are pumped and bioluminescence is measured. By employing two vessels, healthy and fresh sensing cells were delivered into the second vessel continuously. Due to the ease of operation and miniaturization achieved as the system was optimized, it shows greater promise in the implementation in a WWBP. Importantly, this bioreactor can serve as an early warning system of wastewater toxicity as well as to monitor accidental spills, discharges, or failures in plant operation.

Detection of toxins using bioluminescent bacterial strains has been broadened as the panel of stress-responsive promoters has emerged.[114,115] This panel of microbial toxicity sensing systems employs different *E. coli* strains, each one carrying a unique stress promoter that activates a different regulatory circuit, including the bacterial heat-shock response to monitor protein damage, the SOS regulatory network (see below) involved in protection against DNA damage, and the *oxyR* and *soxRS* regulons for oxidative stress. The most significant advantage of this kind of technology is that the bioluminescence response will not only indicate the presence of the stress-inducing agent, but also give some information on its character, namely, its mechanism of action. Results, obtained in 1−2.5 h, were similar or lower in detection limits than those obtained with the Microtox test. Moreover, the use of a microtiter plate makes it simple, allowing the screening of a large number of samples over a short period of time. Applications of this "panel of stress-responsive proteins" include characterization of stress-inducing agents,[116−119] identification and characterization of toxic chemicals,[120,121] and monitoring toxicity of industrial wastewater treatment facilities.[122]

B. Detection of Carcinogens

A carcinogen is a compound capable of inducing malignant neoplasms. On the basis of their chemical or biological properties, carcinogens can be classified into two main categories, DNA-reactive (e.g., genotoxic) and epigenetic. The first group, genotoxic agents, comprises those carcinogens that chemically interact with DNA. In this category the majority of carcinogens are those that function as electrophilic reactants. The second category, epigenetic carcinogens, is composed of those agents whose carcinogenicity is the result of some biological effect other than direct DNA damage. Epigenetic carcinogens include cytotoxic agents and peroxisome proliferators.

Several microbial biosensing systems have been developed for the specific detection of chemical carcinogens. The most widely used assay for genotoxic detection is the Ames test.[123−126] This test employs a set of strains of *Salmonella typhimurium* to measure the rate of reversion of His^- mutants to His^+ mutants that have the ability to grow on histidine-deficient medium. Each mutant strain reacts specifically to either frameshift-inducing mutagens or chemicals that cause base-pair substitutions. However, the Ames test cannot be used to detect chemicals that produce epigenetic or homologous recombination (recombination between homologous DNA sequences). Moreover, the use of several bacterial strains to detect different kinds of mutagens is its major drawback. Different genotoxicity assays have evolved from the Ames test to overcome the cost and time limitations required by the assay. These tests are based on the expression of genes using easily detectable methods such as the production of light.

On the basis of this concept, Ulitzur and co-workers developed the bioluminescence test (BLT) to monitor DNA synthesis inhibitors.[127] In this test, a dark variant (SD-18) of the luminous bacteria *Photobacterium leioghnati* BE8 is employed to determine the

Table 6. Comparison of the Sensitivity of Mutagenecity or Genotoxicity Tests to Some Genotoxins[a]

genotoxin (unit of measurement)	lower limit of detection[b]			
	Ames test	SOS chromotest	*umu* test	SOS *lux* test
MMC (M)	2.3×10^{-10}	1.7×10^{-8}	1.5×10^{-7}	5.0×10^{-9}
MNNG (M)	3.6×10^{-9}	5.0×10^{-7}	2.0×10^{-6}	7.1×10^{-7}
DMS (M)	1.6×10^{-6}	6.7×10^{-6}	3.0×10^{-4}	7.5×10^{-6}
γ Rays (Gy)	3−4	<5	ND	2.56

[a] MMC, mitomycin C; MNNG, *N*-methyl-*N*′-nitro-nitrosoguanidine; DMS, dimethyl sulfate. ND, not determined. [b] Dose of the genotoxin or mutagen which increases the response by a factor of 2 over background levels. (Adapted with permission from ref 133. Copyright 1997 American Society for Microbiology.)

ability of the test compounds to restore luminescence. In contrast to the Ames test, the bioluminescence test can use the same dark mutant to detect both base-pair and frameshift mutagens. Using the BLT, Ulitzur and co-workers tested three base substitution agents [*N*-methyl-*N*′-nitrosoguanidine (NTG), hydroxylamine (HA), and ethyl methanesulfonate (EMS)], three frameshift agents (20-methylcholanthrene, 2-aminoanthracene, and 9,10-dimethyl-1,2-benzanthracene), and four acridine agents (acriflavine sulfate, 9-aminoacridine, and acridine orange). Among the most important advantages of this system over the Ames test are (i) applications in food and biological fluids analysis, since it is not affected by the presence of amino acids or other nutrients; (ii) ability to assay volatile and gaseous samples; (iii) ability to perform both toxicity and genotoxicity studies; and (iv) detection of all the chemicals active in the Ames test and a large number of carcinogens that are not active.[128] Further, the BLT has also been employed for the detection of hydrazine derivatives and anticancer drugs (DNA synthesis inhibitors or DNA-damaging agents) in patients' urine to determine the pharmokinetics of the drug.[129] This system was the first genotoxin detection assay able to monitor the kinetics of genetic events in a continuous and nondestructive manner.

Another genotoxicity test based on natural occurring luminescent bacteria is the commercially available Mutatox genotoxicity test system (AZUR Environmental). The Mutatox test was developed to detect the presence of genotoxins using dark mutants of the luminescent *V. fischeri*, strain M169. This system has been used to detect organic chemicals, *N*-methyl-*N*′-nitro-nitrosoguanidine (MNNG), and proflavine in pure and complex mixtures. Detection of genotoxic agents that cause base-pair substitutions or frameshifts, SOS inducing agents, or DNA intercalating agents can be achieved by both the Mutatox assay and the BLT. The LUMIStox assay is another bioassay that employs *V. fischeri* strains to detect genotoxins based on decreased luminescence.

Exposure to genotoxins can have different consequences in cellular biochemistry and physiology: (i) if the damage remains, it can lead to cell death; (ii) damage may be repaired with no further consequences to the cell; or (iii) damage activates the SOS repair network. Several genotoxin detection systems have been developed based on the SOS response. Such whole-cell sensing systems that employ genes involved in DNA repair and recombination rely on β-Gal, GFP, CAT, and bacterial and firefly luciferase to monitor mutagenicity. Among them are the SOS chromotest[130] and the *umu* test,[131] which employ the β-galactosidase reporter protein, and the VITOTOX test[132] and the SOS-*lux* test,[22,133,134] which utilizes the luciferase system. A comparison of the sensitivity to some genotoxins of the SOS chromotest, the *umu* test, and the SOS-*lux* test to that of the Ames test is shown in Table 6.

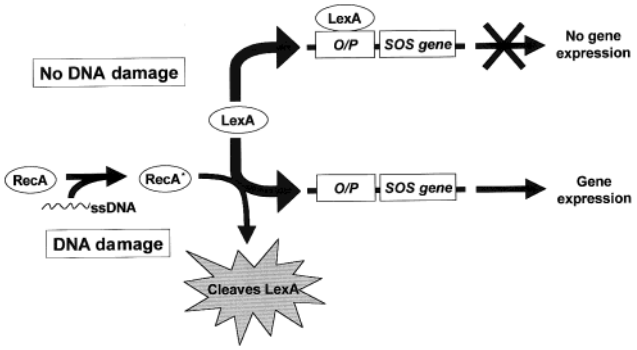


Figure 4. Model of the SOS regulatory network. In the absence of DNA damage, the LexA protein binds to the operator sequences, inhibiting transcription. In contrast, when DNA damage is present, an inducing signal consisting of ssDNA regions will be generated. As RecA binds to these ssDNA regions, it is converted to the activated form. The activated RecA exhibits a coprotease activity that results in the cleavage of LexA and further SOS gene expression.

The SOS system is a regulatory network that is activated to repair DNA damage.[98] Activation of this repair system, caused by DNA damaging agents (e.g., mutagens, genotoxic agents), results in the SOS response. In *E. coli*, a circuitry involving the RecA and LexA proteins controls the expression of over 20 genes that are responsible for the SOS response (Figure 4). This response results in the activation of synthesis of a series of proteins, including RecA and UmuC/D, the proteins related to mutagenesis.

When the SOS network is activated, the resulting gene products directly repair DNA or allow the cell to tolerate the DNA lesion until repair occurs. The SOS response is under the control of two proteins, LexA and RecA. The LexA protein is the common repressor of the SOS genes, including the *recA* gene. The RecA protein is a DNA recombinase which, in the presence of DNA damage, can act as a specific protease, inducing a signal. The characteristics of this signal are still unknown, although evidence suggests that it consists of single-stranded DNA regions produced as a consequence of the inhibition of DNA replication or by some other circumstances. RecA is activated upon binding to these single-stranded DNA regions. Once activated, the RecA protease can cleave

or promote self-cleavage of the LexA repressor, leading to the activation of the SOS genes. After the DNA has been repaired, LexA levels are restored, repressing the SOS genes again.

The SOS chromotest is a bacteria-based assay that activates a repair (SOS) response using an enzyme-linked reporter system (i.e., β-galactosidase).[130] This system employs a plasmid with the *lacZ* fusion located downstream of *sfiA*, a gene involved in the inhibition of cell division during the SOS response. The ability of genotoxic agents to induce the expression of the *sfiA::lacZ* fusion is used to classify compounds according to the SOS inducing potency (SOSIP). This bioassay requires only a single strain harboring the designed plasmid whose response to the genotoxic agent can be observed within a few hours. Since the system does not require survival of the strain, detection of toxic compounds can be assessed through this biosensing system. The SOS chromotest is a simple, direct, colorimetric assay with results closely related to those obtained with the Ames test.

Another quantitative assay for the detection of genotoxic agents is the *umu* test, which like the SOS chromotest uses a β-galactosidase colorimetric assay.[131] The *umu* operon, coding for UmuD and UmuC, is expressed late in the SOS response and is the only operon whose induction, caused by DNA-damaging agents, is required for SOS mutagenesis. On the basis of this principle, a plasmid was designed with the *lacZ* gene downstream from the *umuC′* gene, which is then regulated by the *recA* and *lexA* genes. In another study, upon the introduction of the rat glutathione *S*-transferase (GST) gene into this plasmid in *Salmonella typhimurium* NM 5004, detection of 10 known genotoxic agents, previously not achieved by the *umu* test and whose activity is mediated by GST, was assessed.[135] Unlike the *umu* test, which employs the *lacZ* gene, Justus et al. constructed a plasmid for the detection of mutagenic DNA repair by fusing the DNA damage-inducible *umuC* gene to the *luxAB* genes from *Vibrio harveyi*.[136] Using the UmuC′-LuxAB fusion protein, lower detection limits (defined as the minimum dose at which luminescence doubles the background level) were achieved. MNNG was detected down to 0.033 μg/mL and MMS down to 4.3 μg/mL, while the limits of detection achieved through the *umu* test were 0.6 and 27 μg/mL, respectively. The importance of these systems relies on the use of a well-characterized gene, *umu*, specifically involved in the late stages of SOS mutagenesis.

The SOS-*lux* assay is a bacteria-based sensing system for the detection of environmental genotoxins.[22,133,134] On the basis of the SOS induction, the pPLS-1 plasmid was designed fusing the *lux* operon of the marine photobacteria *Photobacterium leioghnathi* downstream of a SOS-controlled promoter, *cda*. Chemicals or radiation produce damage to the DNA molecules inside the cells, resulting in an increase in bioluminescence in a dose-dependent manner. Detection limits in the millimolar range for mitomycin C (MMC), in the micromolar for MNNG, nalidixic acid (NA), and dimethyl sulfate (DMS), and in the millimolar range for H_2O_2 and CH_2O were achieved. The advantages of the SOS-*lux* assay over other bioassays include (i) fast results available within 1−2 h; (ii) ability to perform in vivo analysis without disruption of the cells; (iii) increase in precision of number of data obtainable since it can be measured repeatedly within a few seconds; (iv) ability to perform kinetic studies of the SOS induction from the same culture; (v) detection of genotoxins with different DNA-damage mechanisms using the same test strain; (vi) sensitivity of the test can be increased when different host strains for the pPLS-1 plasmid are employed; and (vii) discrimination between genotoxins and cytotoxic effects due to simultaneous measurement of cell concentration and light emission.

As previously mentioned, a number of tests to determine mutagenic activity have been developed employing reporter proteins. On the basis of β-galactosidase expression, Shirakawa et al. constructed an *E. coli* strain harboring the *recA::cro::lacZ* fusion.[137] Under normal growth conditions, a limited amount of the RecA protein is produced since the *lexA* gene represses the *recA* gene. In the presence of DNA-damaging agents (e.g., genotoxins, mutagens), the SOS regulatory system is induced and an increase in RecA expression is observed. Upon treatment with NA, the β-galactosidase activity increased linearly with a maximum induction of 14-fold higher than the background level observed 8 h after the induction.

Genotoxicity biosensing systems using GFP as the reporter protein have been reported by Billinton and co-workers.[20] In their work, they fused the Rad54 promoter, involved in yeast cells' DNA repair mechanisms, to the *Aequorea victoria* green fluorescent protein *yGFP* using the yeast *S. cerevisiae* as the host organism. Although they exploited the potentials of the *Rad54* gene in a GFP-based biosensing system, some problems emerged during the assay. For instance, fluorescent compounds present in the media, including tyrosine, tryptophan, phenylalanine, reduced nicotinamides, and oxidized flavins, produced background fluorescence and, therefore, caused some interference in the resulting signal. Thus, many washing steps to remove traces of media were performed prior to the measurements. As a result, further experiments by Knight and colleagues were required to optimize this biosensing system.[21] The use of media with low fluorescent properties allowed in situ measurements of the GFP fluorescence without separating the protein from the media. A distinction between cytotoxicity and genotoxicity can be assessed through this biosensing system, since the system simultaneously measures yeast cell growth rate and GFP production. When the system was tested with the well-known genotoxin MMS, an inducing signal was recorded in less than 4 h, as compared to 24 h needed with the Ames test. However, the system has not yet been validated with other known mutagens previously identified by the Ames test. Further, upon optimization of this GFP-based sensing system, a continuous flow-through detection system was developed for genotoxity assessment.

A CAT-based whole-cell sensing system was reported by Lee et al.[30] A screening test for peroxisome proliferators was developed upon transfection of the rat *Acyl-CoA oxidase (ACOX) promoter::CAT* fusion into the rat liver cell line H-4-II-E. Peroxisomes are organelles that oxidize fatty acids via the β-oxidation. The fatty acyl-CoA-oxidase, the enoyl-CoA hydratase/3 hydroxyacyl-CoA dehydrogenase bifunctional enzyme, and the 3-ketoacyl-CoA thiolase comprise the peroxisomal β-oxidation system. Among the different peroxisomal fatty acyl-CoA oxidases, is the inducible form palmitoyl-CoA oxidase (ACOX), the one that plays a role in oxidative DNA damage and hepatocarcinogenesis. Upon fusion of the rat ACOX promoter upstream to the *CAT* gene, a plasmid was developed for the detection of peroxisome proliferators. In this bioassay, the level of induction does not correspond to the amount of compound present in the sample but to the ability of the compound to activate the peroxisome proliferator-activated receptor.

The use of the bacterial luciferase *luxCDABE* operon in genotoxicity assays has been extensively reported. Vollmer and colleagues reported the construction of a panel of stress-responsive bacterial strains harboring three *E. coli* stress genes.[138,139] These stress genes, coding for the *E. coli* stress promoters *recA*, *uvrA*, and *alkA*, have been fused to the *luxCDABE* operon of the promoterless *V. fischeri*. An increase in the production of light in these DNA repair promoter::*luxCDABE* fusions can be monitored in the presence of genotoxins. Further, the *uvrA* and *recA* strains were modified by employing the *Phorhabdus luminescens luxCDABE* operon and *S. typhymurium* as the host organism. The higher working temperatures, conferred by the *P. luminescens*, resulted in a faster response.[140] These bacterial strains are part of a larger panel of whole-cell biosensing systems that, under less-specific stress responses, detect heat shock, protein damage, and oxidative stress.[114,115]

An *E. coli* strain harboring the *recA::luxCDABE* fusion has been reported by Min et al.[141] This bioassay is used to distinguish genotoxic agents that cause direct DNA damage (DDD) such as UV, X-ray, benzo[*a*]pyrene, MMC, and MNNG from indirect DNA- damaging agents (IDD). Indirect DNA damage is caused by an increase in the intracellular level of reactive oxygen species (ROS) and leads to oxidative damage, strand breaks, and base modifications. IDD agents include cadmium chloride, H_2O_2, and some pesticides. Upon fusing the *recA* gene downstream to the *V. fischeri luxCDABE* operon, continuous monitoring of gene expression was assessed. Distinction between DDD and IDD agents was possible by understanding their detection limits, response ratios, and dose-dependent response patterns. For instance, a higher concentration of IDD agents was needed for DNA damage to occur. Consequently, the detection limit for DDD agents was 1−5 orders lower, depending on the mutagens group, than that for IDD agents. For example, benzo[*a*]pyrene was detected as low as 5.2×10^{-11} M (equivalent to 0.013 ppb).

Other organisms, besides *E. coli*, have been used in whole-cell sensing systems. Elasri and Miller explored the potentials of *Pseudomonas aeruginosa* in a biosensing system.[142] Since *P. aeruginosa* is a member of aquatic and soil microbial communities, environmental applications can be performed as it can be incorporated into microbial habits in a noninvasive fashion. Upon fusing the *recA* promoter upstream of the promoterless *V. fischeri lux* operon, the plasmid RM4440 was developed for the detection of ultraviolet radiation. Taking advantage of the nondestructive and substrate-free characteristics of the system, they incorporated this sensing system into a biofilm to study stress in microbial communities.[143] The cells were immobilized into an alginate matrix to simulate the natural organization of this microorganism in freshwater (e.g., biofilm). Thus, RM4440 can be implemented in a biosensor for environmental stress detection in microbial communities.

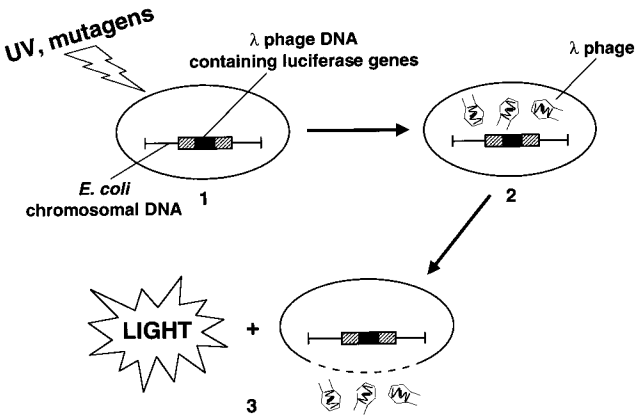


Figure 5. Proposed principle of bioluminescence by phage induction on the *E. coli* lysogenic strain. (1) Genes encoding firefly luciferase (indicated by the solid black rectangle) are cloned into phage λ and then infected into *E. coli* chromosomal DNA. (2) UV light or other mutagens induce the growth of λ phage inside the cell. (3) Induction of prophage mediates the expression of the luciferase and results in bacteriolysis, thus releasing luciferase. (Adapted with permission from ref 145. Copyright 1992 American Chemical Society.)

A different approach for the detection of mutagens is by prophage induction. DNA-damaging agents trigger the SOS system, activating the RecA protein, which in turn cleaves the prophage λ repressor thus inducing the lytic cycle of λ phage. One of such systems is the Inductest, a microbial-based sensing method that detects carcinogens by their ability to induce prophage λ from lysogenic *E. coli* cells.[144] A similar system was developed by Lee et al. based on bioluminescence.[145] The genes encoding firefly luciferase were cloned into phage λ and then infected into *E. coli* (Figure 5). The recombinant phage was integrated in the chromosomal DNA of *E. coli*. The induction of the prophage by the mutagen MMC results in cell lyses. The induction of prophage also mediates the transcription and expression of the luciferase gene. The bioluminescence emitted by luciferase was used in the determination of the levels of mutagen present. More recently, Maillard et al. reported a test that employs bacterial luciferase for the detection of mutagens that induce prophage λ.[146] A wide range of mutagens can be detected with this test, including alkylating agents, base analogues,

DNA cross-linking agents, and oxidizing agents. Whole-cell sensing systems based on prophage induction show promise for the rapid and inexpensive screening of a variety of mutagens.

IV. Specific Biosensing Systems Employing Metal Resistance

Metal ions have been involved in all phases of microbial existence since primordial times and play critical roles in cell growth and metabolic functions. Some metals are essential for microbial life while others are toxic. Metals considered essential for growth are s-block elements, such as Na, K, Mg, and Ca, which are present in high concentrations, and transition metals, such as Mn, Zn, Fe, Co, Ni, and Cu. These metals have a plethora of functions ranging from stabilization of structure to being cofactors in cellular reactions, such as electron transfer, redox reactions, and hydrolysis.[147] For example, ions such as Na^+, K^+, and Mg^{2+} are essential for the maintenance of chemical gradients across the plasma membrane; transition metal ions, such as Zn^{2+}, are required as cofactors for DNA-binding proteins.[148,149] Other metals, such as cadmium, arsenic, tin, aluminum, silver, and mercury, are toxic toward microorganisms; the extent of the toxic effects depend on the chemical form of the metal species. These metals have no known biological functions and often compete with or replace a functional metal resulting in toxicity. For example, Al^{3+} replaces Mg^{2+} in biological systems because of its higher affinity for ligands. Paradoxically, even essential metals, like copper and zinc, become toxic for the organisms when present at high concentration levels in the cell.[148] In the case of certain heavy metal ions, the toxicity arises due to the ability of these metals to form complexes within the cells and cause physiological damage. Certain metals are capable of interfering or blocking essential functional groups of proteins, modifying the active forms of important biomolecules, thereby preventing essential metals from performing their biological roles.[147] For example, Cd^{2+} ions bind nucleotides in DNA leading to strand breaks.

Typically, metal ions can enter the cell in two different ways,[150,151] one of which is a nonspecific uptake mechanism by which several metals quickly gain entry into the cytoplasm. This occurs mainly due to a chemiosmotic gradient developed across the cell membrane. Metals can also enter the cells via a slower mechanism that is highly specific and energy dependent. This metal ion uptake generally utilizes energy released from ATP hydrolysis within the cells.

Due to the toxic properties and ability of metals to accumulate inside the cells, microorganisms have evolved resistance machinery in order to survive in an environment containing metals. There are several reviews that deal extensively with metal toxicity and how microorganisms counteract these toxic metals.[151−155] Microorganisms can use one or more of the following resistance mechanisms to render them tolerant to metals:[156−162] (i) blocking the entry of metal ions into the cell by changing the uptake pathway; (ii) intracellular sequestering of metal ions by binding of the metal ions to specific proteins, such as metallothioneins, to avoid interference with the active components of the cells; (iii) extracellular sequestering of metal ions by binding of certain proteins to the metal ion on the outside of the cell wall, thus preventing its entry into the cytoplasm; (iv) chemically modifying the metal ions to less toxic forms in the cytoplasm; there are several oxidases, reductases, alkylases, and other structural enzymes present in the organisms that serve this purpose; and (v) active transport of the metals from the cytoplasm to the cell wall to prevent them from accumulating in the cell. The latter is accomplished by metal ion-specific efflux pumps that transport these toxic metals out of the cytoplasm.

The genetic determinants required for the resistance machinery are organized in operons. Typically, genes in these operons code for proteins and enzymes that carry out functions required to make the cells tolerant to the metal ions. The operons can be plasmid-, transposon- or chromosome-borne and in many cases can confer resistance to more than one metal ion. Usually the expression of these genes in the operon is tightly regulated by the presence or absence of the specific metal ion in the cell. The presence of the metal in the cell triggers the expression of the resistance genes to recognize the intruding ions and assists the microorganism in its survival. By exploiting the specific regulation and induction properties of operons with respect to specific metal ions, biosensing systems for detecting these metal ions have been developed. Further, by coupling the genetic information from the operon (part of it or entirely) with reporter proteins, such as β-galactosidase and luciferase, a number of cell-based sensing systems have been developed for the detection of metals such as mercury,[163−165] chromium,[166,167] cadmium,[12,168] copper,[167,169] aluminum,[170] lead,[12,167] arsenic,[3,171−175] and antimony.[3,171−175] Table 7 summarizes various metal-specific bacterial strains that have been engaged in designing bacterial sensing systems for metal ions. This overview will emphasize the resistance mechanisms present in microorganisms for certain metals and how they have been adapted in the development of sensing systems using reporter genes.

A. Arsenic/Antimony

Toxic metalloid arsenic exists in a +5 oxidation state as arsenate AsO_4^{3-} and in a +3 oxidation state as arsenite AsO_2^-. The structural similarity with phosphate makes arsenate toxic to the organisms as it has the ability to mimic phosphate, thus interfering in different metabolic processes. Antimony is a silvery-white metal that has numerous applications in lead storage batteries, solder, sheet and pipe metal, bearings, castings, paints, ceramics, and fireworks and as enamels for plastics, metal, and glass. Its oxoanion form, antimonite (SbO_2^-), is isoelectronic to arsenite. Exposure to antimony at high levels can result in a variety of health effects, such as eye irritation, heart and lung problems, and gastric disorders.

Not surprisingly, microorganisms have developed suitable resistance mechanisms to counter the ac-

Table 7. Examples of Specific Bioluminescence-Based Bacterial Strains for Sensing Metal Ions[a]

bacterial strain	plasmid	regulation unit	inducing metal ions
E. coli			
MC1061	pTOO31	*ars*	arsenite, antimonite, arsenate
CM1166	pC200	*ars*	arsenite, antimonite, arsenate
CM1569	pC202	*ars*	arsenite, antimonite, arsenate
MC1061	pTOO11	*mer*	mercury
CM781	pLD41	*czc*	cobalt
CM685	pLD13	*czc*	zinc
S. aureus			
RN4220	pTOO24	*cad*	cadmium, lead
SA3	pC200	*ars*	arsenite, antimonite, arsenate
SA9	pC300	*cad*	cadmium
R. eutrophus			
AE1696	pC202	*ars*	arsenite, antimonite, arsenate
AE714	pMOL284	*chr*	chromate, dichromate, chromium
AE1298	pMOL50::MiniTn*5-luxIAB*	*chr*	chromate, dichromate, chromium
AE859	pSSD50	*chr*	chromate, dichromate, chromium
AE1239	pMOL90::Tn*4431*	*cup*	copper
AE891	pMOL55::Tn*4431*	*cnr*	nickel
AE1433	pMOL28	*czc*	cadmium, cobalt, lead, zinc
AE1101	(pMOL30)(pSS50)	*tll*	tellurium

[a] Adapted with permission from refs 174 and 209. Copyright John Wiley & Sons Ltd.

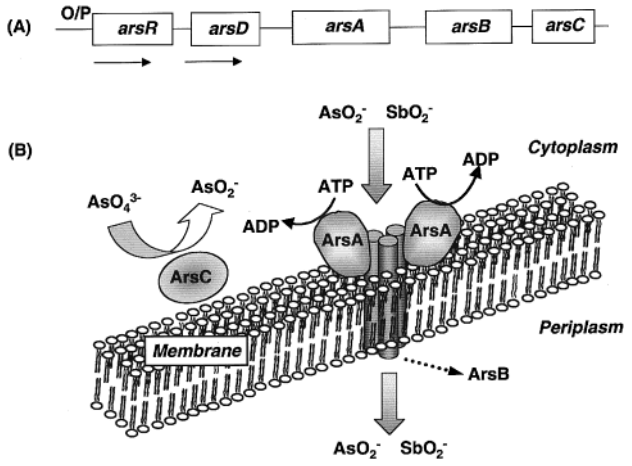


Figure 6. (A) Organization of the *ars* operon in *E. coli*. Genes are represented by the boxes. The *arrows* indicate the direction of mRNA transcripts. (B) Machinery of the arsenite pump in Gram-negative bacteria. (Adapted with permission from ref 181. Copyright 1993 Blackwell Science Ltd.)

tions of these toxic metalloids.[176,177] The resistance machinery used by bacteria against arsenate, arsenite, and antimonite is encoded by the *ars* operon found in plasmids and chromosomes.[162,178,179] It consists of a set of structural genes (*arsA*, *arsB*, and *arsC*) and two regulatory genes (*arsR* and *arsD*) (Figure 6A).[180] The structural genes encode for proteins that form the efflux pump (Figure 6B) capable of extruding antimonite and arsenite out of the cell.[181] ArsA, encoded by the *arsA* gene, is a membrane-linked ATPase protein stimulated by arsenite and antimonite. It is responsible for driving the protein pump by providing energy via ATP hydrolysis. ArsB is a membrane transport protein that forms the channel through which antimonite and arsenite are effluxed from the cells. ArsB combines with ArsA to form an ArsAB complex that can make the efflux pump either ATPase or chemiosmotic type.[182] However, this pump cannot transport arsenate out of the cell. To alleviate this problem, an arsenate reductase ArsC, encoded by *arsC* gene acts on the arsenate when it enters the cell, reducing it to the more toxic arsenite, which then can be removed by the protein pump.[183]

ArsR is a *trans*-acting regulatory protein responsible for the basal expression of the *ars* operon.[184,185] In the absence of the toxic metalloids, ArsR binds to the operator/promoter (O/P) region of the *ars* operon preventing expression of the other genes. When the oxoanions enter the cell, they bind to ArsR forcing some conformational change that leads to the dissociation of ArsR from the O/P region and subsequent expression of the genes needed for the resistance mechanism. Thus, ArsR is a repressor of the *ars* operon in the absence of the oxoanions. ArsD is another regulatory protein that is present in some *ars* operons and is instrumental in controlling the overexpression of the *ars* operon.[186]

Using the specificity of the *ars* operon for antimonite and arsenite, whole-cell sensing systems have been developed for these oxoanions.[3,168,171−173] Ramanathan et al. engineered a recombinant plasmid pRLUX to contain the *ars* promoter and the *arsR* gene upstream of a promoterless bacterial luciferase (*luxAB*) gene.[171] By transforming an *E. coli* strain with this plasmid, they developed a bacterial system that could sense antimonite and arsenite (Figure 7) at subattomolar concentrations. The detection limits obtained using this system were 3 orders of magnitudes higher than those obtained using conventional techniques, such as neutron activation analysis, gas chromatography/photoionization, anodic stripping voltammetry, and atomic absorption spectroscopy. In addition, the sensing system was highly specific for antimonite and arsenite as the luminescence signal obtained for other metal ions such as Bi^{3+}, Cd^{2+}, and Co^{2+} as well as for other oxoanions such as phosphate, sulfate, and nitrate was insignificant with respect to the blank.

In separate studies performed in our laboratory, bacterial sensing systems for antimonite/arsenite employing β-galactosidase as the reporter were de-

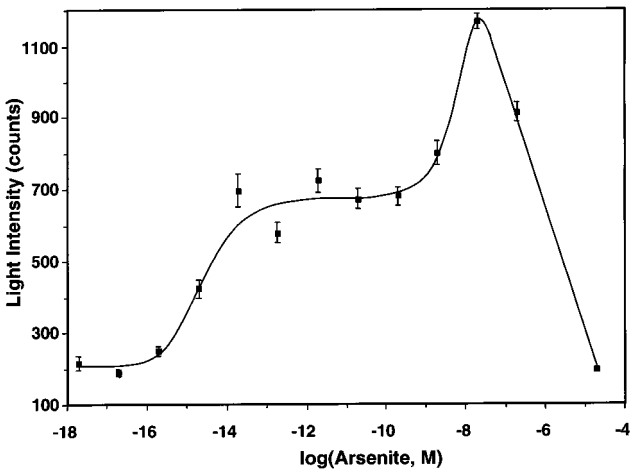


Figure 7. Calibration plot for arsenite performed after *E. coli* strain, JM109, harboring plasmid pRLUX were incubated with sodium arsenite solutions for 3 h. The bioluminescence signal has been corrected with respect to the blank. Data are the average ± standard deviation ($n = 3$). (Reprinted with permission from ref 171. Copyright 1997 American Chemical Society.)

veloped. A reporter plasmid pBGD23 that contained the *ars* O/P region, the regulatory *arsR* gene, and a fusion *arsD′::lacZ gene* was used in designing these sensing systems. By employing *arsD′::lacZ gene* fusion, the expressed fusion protein maintains the β-galactosidase activity although it lacks the function of ArsD protein. In *E. coli* cells harboring plasmid pBGD23, the ArsR protein regulates the expression of plasmid-borne β-galactosidase depending on the bioavailability of antimonite/arsenite. Activity of the expressed β-galactosidase, which is related to the amount of antimonite/arsenite in the sample, can be determined by electrochemical or chemiluminescent methods. Scott et al. monitored β-galactosidase activity with the substrate, *p*-aminophenyl-β-D-galactopyranoside (PAPG), whose reaction product *p*-aminophenol was detected electrochemically.[172] The *E. coli* strain JM109, transformed with pBGD23, was used in this study. A higher sensitivity was achieved by increasing the induction times. For instance, the detection limits for antimonite were 10 and 0.1 μM at 30 min and 17 h induction times, respectively. Similar detection limits were obtained for arsenite. The bacterial sensing system was specific to arsenite/antimonite as the response obtained with other oxoanions, such as phosphate, sulfate, carbonate, and nitrate, was not significantly different from that obtained with the blank. This was the first time that electrochemical detection has been coupled to reporter gene technology for the development of sensing systems for metal ions. In another study, the activity of expressed β-galactosidase was assayed using the chemiluminescent substrate Galacto-Light Plus by Ramanathan et al.[3] Figure 8 demonstrates a stepwise relationship between the chemiluminescence signal and the concentration of antimonite in the sample and detection of antimonite at levels as low as 10^{-15} M. This stepwise behavior observed in the calibration plots is attributed to the presence of a chromosomal *ars* operon present in *E. coli*. To substantiate this hypothesis, pBGD23 was introduced into a different strain of *E. coli* (strain AW10) in which the chromosomal *ars* operon was deleted and the response for arsenite and antimonite was evaluated. It was observed that the step-response was absent in the plot as shown in Figures 8 and 9. The response observed at higher concentrations of antimonite was similar in both strains of *E. coli* and could be attributed to the regulatory action of plasmid-borne ArsR, which is present in both systems. The detection limit for antimonite with this system was in the nanomolar range using a 30 min induction time. By using the JM109 cells in which the chromosomal *ars* operon is present, a more sensitive system is obtained in comparison to the one that employs the AW10 strain. However, the response with the JM109 strain is biphasic, and so it is appropriate to use the genetically engineered AW10 cells for measuring samples in the nanomolar levels. For more sensitive sensing, the JM109 cells can be used with some modification to the assay protocol. In this case, prior to inducing

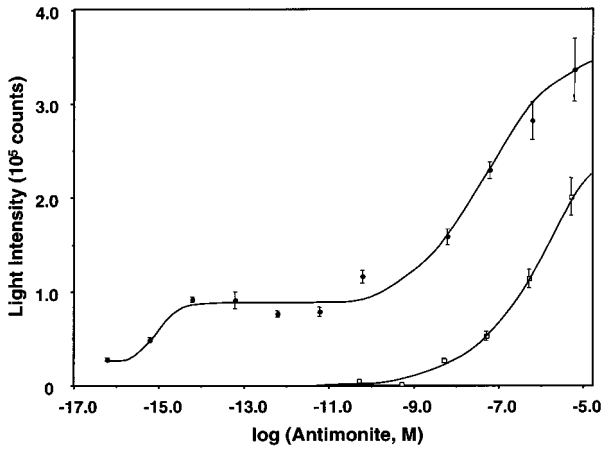


Figure 8. Calibration plots for antimonite performed after *E. coli* strains, JM109 (●) and AW10 (□), harboring plasmid pBGD23 were incubated with potassium antimonyl tartrate solutions for 30 min. The chemiluminescent signal has been corrected with respect to the blank. Data are the average ± standard deviation ($n = 3$). (Reprinted with permission from ref 3. Copyright 1998 Elsevier Science B.V.)

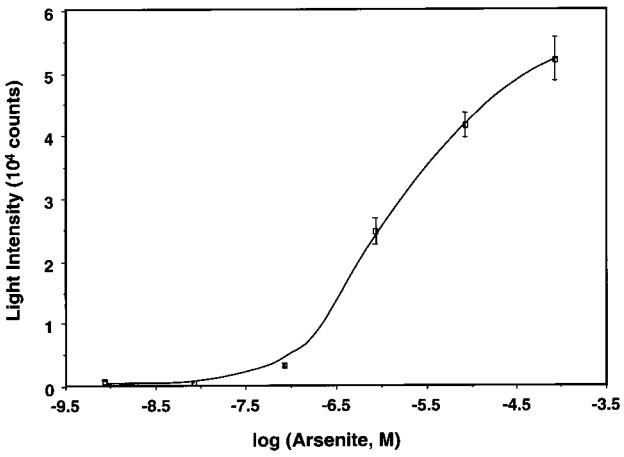


Figure 9. Calibration plot for arsenite performed after the bacteria (*E. coli* AW10) harboring plasmid pBGD23 were incubated with sodium arsenite solutions for 10 min. The chemiluminescent signal has been corrected with respect to the blank. Data are the average ± standard deviation ($n = 3$). (Reprinted with permission from ref 3. Copyright 1998 Elsevier Science B.V.)

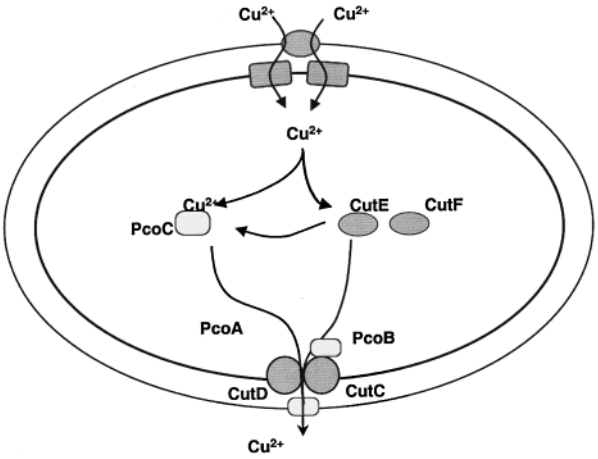


Figure 10. Diagram depicting plasmid- and chromosome-encoded proteins in *E. coli* that are responsible for rendering resistance to copper. (Adapted with permission from ref 194. Copyright 1994 Marcel Dekker.)

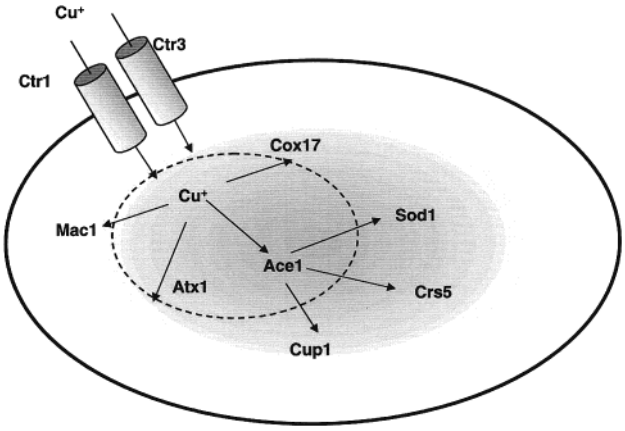


Figure 11. Schematic of the copper chaperone proteins involved in sequestration of copper ions in *S. cerevisiae*. (Adapted with permission from ref 200. Copyright 1998 Annual Reviews http://www.AnnualReviews.org.)

the bacterial system, the samples can be either spiked with known concentrations of the metal ions (antimonite/arsenite) or diluted.

B. Copper

Copper is an essential nutrient that plays a vital role as a catalytic cofactor for several enzymes including cytochrome *c* oxidase, superoxide dismutase, and other multi-copper oxidases.[187] However, its radical-forming characteristics, especially with molecular oxygen, make it toxic as it can cause damage to various biomolecules such as proteins and nucleic acids.[188] Moreover, irregularities in copper levels in the cell can lead to diseases such as Menkes' syndrome and Wilson's disease.[189,190] Menkes' syndrome is a lethal hereditary disease caused by the inability of cells to absorb and transport copper, leading to arterial changes and severe brain damage.

Microorganisms have evolved genetic determinants to ensure that cells are provided with nutritional amounts of copper and also to prevent aggregation of copper above toxic levels.[158,191] For this purpose, copper chaperones and efflux proteins are involved in keeping the copper levels in control by supplying copper to various copper-requiring enzymes and removing excess copper from the cell. Several microorganisms maintain copper homeostasis by maintaining equilibrium between the uptake and efflux systems in the cells. P-type ATPases are the most common copper-efflux pumps, which typically have cuprous ion as the substrate. In *E. coli*, plasmid- and chromosome-borne genes are responsible for copper efflux and lower cellular accumulation of copper (Figure 10).[192−194] The plasmid-borne *pco* determinant encodes for two proteins PcoA and PcoB that play a role in removal of copper ions from the cell and a third protein, PcoC, that binds copper within the cytoplasm. PcoR and PcoS are regulatory units of the *pco* operon. The chromosomal *cut* operon encodes protein molecules that are involved in transport of copper (CutA and CutB), intracellular binding of copper (CutE and CutF), and copper-efflux from the cells.[195] Under normal conditions, chromosomal *cut* genes encode proteins that mediate copper homeostasis. CutA and CutB are influx proteins that transport copper ions to the cytoplasm where copper-binding proteins (CutE and CutF) and efflux proteins (CutC and CutD) control the levels of copper ions within the cells. However, under adverse conditions where the copper levels are higher in the cells, the plasmid-encoded *pco* genes are responsible for copper homeostasis.

In the Gram-positive bacterium *Enterococcus hirae*, the *copA* and *copB* genes encode for two P-type ATPases involved in the regulation of copper ions within the cell.[196−199] Two regulatory genes *copY* and *copZ* encode for small metal-binding proteins CopY and CopZ that control expression of the structural genes of the *copYZAB* operon depending on accessibility of copper ions to the cells. CopA helps in copper uptake under copper-starved conditions, whereas CopB is an ATP-driven copper-efflux pump that plays a role when higher levels of intracellular copper ions are present. Plasmid-encoded *copABCD* genes found in *Ralstonia eutrophus* (formerly known as *Alcaligene eutrophus*) that are similar to the *pcoABCD* genes of *E. coli* and *copABCD* genes of *Pseudomonas syringae* confer resistance to copper ions.

Microorganisms such as *S. cerevisiae* employ copper homeostasis machinery that includes copper-binding cytoplasmic proteins called copper chaperones involved in nutritional and detoxification processes.[191] In *S. cerevisiae*, copper ions, after reduction by ferric/cupric reductases to cuprous ions, are transported to the cell by separate transport proteins Ctr1 and Ctr3 present in plasma membrane. Once the ions are inside the cell, the copper chaperones bind and transport them to specific proteins within the cell using specific pathways (Figure 11). For example, copper chaperones, such as Cox17, Atx1, and Lys7, bind copper ions and distribute them specifically to cytochrome *c* oxidase, CCC2 P-type ATPase, and superoxide dismutase, respectively.[200] The copper transport *ctr1* and *ctr2* genes are induced in copper-starved cells, and a highly copper-specific transcriptional activator Mac1 protein is involved in the regulation of these transport genes.[201,202] When copper ions are in excess, another transcriptional activa-

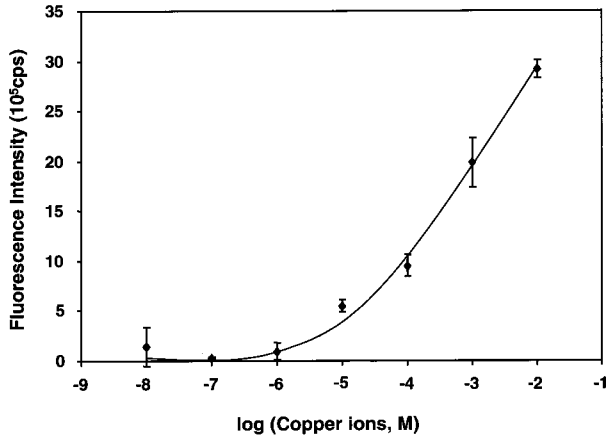


Figure 12. Calibration plots for copper ions performed after *S. cerevisiae* harboring plasmid pCuGFP were incubated with copper sulfate solutions for 3 h. The fluorescence signal has been corrected with respect to the blank. Data are the average ± standard deviation ($n = 3$).

tor, the Ace1 protein, regulates the *sod1* gene and detoxification genes *cup1* and *crs5*.[203−207] This copper-responsive Ace1 protein binds to the cuprous ions forming a metal complex, which subsequently binds to the promoter region of the *cup1*, *crs1*, and *sod1* genes. The resulting Cup1 and Crs5 proteins are metallothioneins involved in sequestering and possibly delivering the copper ions to a vacuolar copper transporter.

In recent studies from our laboratory, we have developed a fluorescence-based sensing system for detection of copper ions by employing the copper-specific properties of the Ace1 protein present in yeast.[208] Specifically, *S. cerevisiae* cells were genetically modified to harbor the reporter plasmid pCuGFP, which contains the *cup1* promoter and a promoterless *GFPuv* gene. In these genetically engineered cells, the expression of *GFPuv* gene is regulated by the *cup1* promoter and the Ace1 protein depending on the bioavailability of the copper ions. This sensing system is capable of sensing copper ions at micromolar levels in a specific manner (Figure 12). The fluorescence intensities exhibited by the cells following exposure to other metal ions, such as zinc, cobalt, iron, nickel, and cadmium ions, were negligible compared to the signal obtained from copper ions.

A cell-based biosensor for copper ions employing the reporter *luxCDABE* gene was developed by Corbisier et al.[167] A recombinant strain of *R. eutrophus* (*A. eutrophus*), AE1239,[169] obtained by transposon-mutagenesis (pMOL90::Tn*4431*) was immobilized in alginate or agarose matrixes. Freeze-dried AE1239 cells were also previously used for sensing copper ions present in incinerator fly ashes and contaminated soils.[209] The immobilized AE1239 cells were characterized with varying concentrations of copper ions. In both cases, the detection limits obtained for copper were in the 20−40 μM range for 90 min induction times, which were subsequently improved to 1 μM when used in an optimized reaction media.

C. Cadmium/Lead

To date, cadmium has not been found to mediate cellular metabolism; in fact, it is toxic to the cells. It usually enters the cell via the chromosomal manganese transport system in Gram-positive bacteria, by the magnesium uptake system in *S. cerevisiae*, and by the calcium uptake mechanism in plants.[154] There are several resistance mechanisms that have evolved to avoid cadmium toxicity in various organisms, though not all these mechanisms are well understood.

In Gram-positive bacteria, resistance is conferred by a P-type ATPase efflux pump, whereas in Gram-negative bacteria, a three-protein non-ATPase efflux is instrumental in removing cadmium ions out of the cells.[210,211] In *Staphylococcus aureus*, the *cad* operon is responsible for making the bacterium cadmium tolerant.[212−214] The genetic determinants of the plasmid-borne *cad* operon are the *cadA* and *cadC* genes. CadA is expressed when the cells are exposed to cadmium ions (and zinc), and it forms the protein pump required for transporting cadmium ions out of the cells. It is also believed to be involved in binding Cd^{2+} and in the initial transport of metal ions from the cytosol to the cell membrane. CadC is required for regulation of the cadmium pump. This P-type ATPase is also responsible for conferring resistance to lead in *S. aureus*. In some microorganisms, metallothioneins are responsible for protecting the cellular components from cadmium ions. In cyanobacteria, metallothioneins encoded by the genes present in the *smt* operon confer resistance to cadmium and several other metal ions.[215] In eukaryotes such as *S. cerevisiae*, there are multiple resistance mechanisms to make the cells cadmium resistant, one of which is by binding cadmium to glutathiones, forming metal complexes that can be subsequently transported out of the cell.[216] In some Gram-negative bacteria, such as *Ralstonia eutrophus*, the *czc* operon is involved in detoxification as it extrudes cadmium and other metal ions out of the cells via a cation antiporter efflux system.[217,218]

Tauriainen et al. developed sensing systems for cadmium and lead by transforming *S. aureus* RN4220 and *Bacillus subtilis* BR151 strains with plasmid pTOO24 that contained the reporter *luc* gene under transcriptional control of CadC and *cad* promoter of the *cad* operon (from *S. aureus* plasmid pI258).[12] These two recombinant strains were tested with different metal ions and found to respond to cadmium and lead ions. The detection limits obtained with the *S. aureus* strain for cadmium and lead ions were 10 and 33 nM, respectively and those obtained with the *B. subtilis* strain for cadmium and lead ions were 3.3 and 33 nM, respectively. At higher concentrations of the metal ions there is a notable decrease in the luminescence intensity, which can be attributed to the cytotoxicity of the metal ions. This effect can lead to false "negatives" yielding similar results with samples containing high levels of metal ions as well as with samples containing no metal ions. This problem can be alleviated by spiking the sample with a known metal ion concentration, which will either decrease or increase the luminescence intensity accordingly. These recombinant strains also show some response to other metal ions such as antimony and zinc. The cells were also tested for metal induction

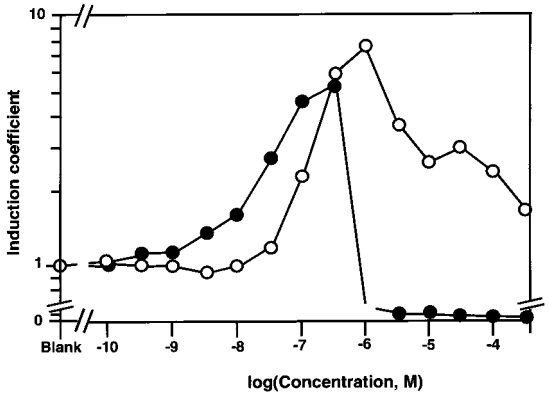


Figure 13. Calibration plot of freeze-dried RN4220 cells harboring plasmid pTOO24 after induction with cadmium (●) and lead (○). Luminescence measurements were carried out in 96-well microtitration wells. (Reprinted with permission from ref 12. Copyright 1998 Elsevier Science S.A.)

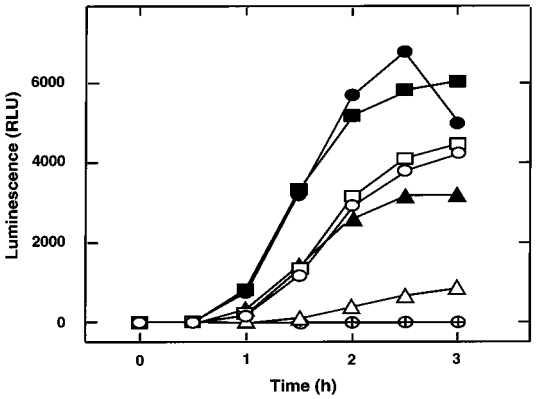


Figure 14. AE104(pEBZ141) as a chromate biosensing system. Induction plots of AE104(pEBZ141) obtained with 1 (○) and 10 μM (●) chromate were compared with induction plots with 1 (□) and 10 μM (■) dichromate, 1 (△) and 10 μM (▲) chromium ions, and cells in the absence of chromium salts (⊕). (Reprinted with permission from ref 166. Copyright 1998 American Society for Microbiology.)

after freeze-drying (Figure 13). It was observed that, after reconstitution, there was a reduction in induction efficiency and sensitivity of *S. aureus* cells whereas the *B. subtilis* cells did not show marked difference in the induction curves nor in sensitivity.

Corbisier et al. designed a luminescence-based bacterial system for sensing lead by employing the lead-responsive regulatory properties of the plasmid-borne *pbr* operon from *R. eutrophus*.[167] The *pbr* operon consists of two genes, *pbrR* and *pbrA*, that are responsible for regulation and conferring resistance to lead, respectively. The *pbrR* gene and part of the *pbrA* gene was coupled to the *luxCDABE* gene for designing the reporter plasmid. *R. eutrophus* harboring the reporter plasmid was specifically induced by lead ions and showed insignificant response to other metal ions, such as copper, cadmium, zinc, mercury, bismuth, tellurium, and gold.

D. Chromium

The transition metal chromium is usually found in its +3 oxidation state as metallic Cr^{3+} and in its +6 oxidation state as the divalent anions chromate CrO_4^{2-} and dichromate $Cr_2O_7^{2-}$. Chromium has been used extensively in manufacturing various metal alloys, in metallic dyes, and in the leather industries. It is also an essential nutrient required by humans for metabolism of fats and sugars.[219,220] It has been clinically demonstrated that patients with sugar-related disorders such as glucose intolerance respond favorably to chromium. On the other hand, chromates and dichromates are highly toxic and are established human carcinogens.[221,222] In addition, there is no evidence regarding the beneficial role of chromium in microorganisms. Chromates and dichromates can easily penetrate the cell membranes of eukaryotic and prokaryotic organisms. In several microorganisms, these oxoanions are transported into the cells by the sulfate transport pathway.[223] In the bacterium *R. eutrophus* CH34, chromate resistance is attributed to a plasmid-borne *chr* determinant.[224] Though there is not sufficient evidence indicating chromate efflux from the cells, the resistance to chromate can be attributed to a combination of reduction of the oxyanions to its less toxic form and its subsequent removal from the cell membrane.[166] The *chr* determinant in *R. eutrophus* CH34 encodes three open reading frames, namely, *chrA*, *chrB*, and *ORF3*. ChrA, a membrane-bound protein, is probably involved in the metal efflux from the cell. Although its role is not clear, ChrB is believed to be involved in rendering the cells resistant to chromium. Further, studies have indicated that the absence of the *chrA* and *chrB* genes lead to chromium sensitivity and hyperaccumulation of chromium in the cells, respectively.[225]

Taking advantage of the fact that chromate resistance in *R. eutrophus* CH34 was triggered by the presence of chromate, a bacterial sensing system for this metal ion was developed.[166] A mutant form of *R. eutrophus*, AE104, was used in designing the bacteria-based sensing system. Unlike the strain CH34, AE104 does not harbor the plasmids that contain the genes required to provide resistance to various toxic metals. This metal-sensitive strain was genetically engineered to harbor plasmid pEBZ141 that contained a fusion between the *chrBA′* and the *luxCDABE* genes (*chrBA′::luxCDABE*). The fusion protein is expressed in the presence of chromates and dichromates in this bacterium. By monitoring the enzymatic activity of the luciferase produced, the response of the bacterial system to various metals was evaluated. This system was found to be highly specific for chromates. The luminescence obtained by the bacterial system for other metals, such as molybdate, vanadate, and manganese salts, was insignificant. For example, the signal obtained by inducing the bacterial cells with 0.1 mM vanadate was 0.2% of that obtained with 0.1 μM chromate. This system responded favorably to Cr^{3+} with the best signals obtained with chromates and dichromates (Figure 14). The signal obtained for Cr^{3+} was 10 times less compared to that of chromate ions. This system was also used to understand the possible interactions between the plasmid-borne *chr* resistance system and the sulfate transport pathway in the bacterium. Similarly, a luminescence-based sensing system for chromium employing part of the *chr* determinant

from *R. eutrophus* CH34 was developed by Corbisier et al.[167]

E. Mercury

Mercury is a reactive and a highly toxic metal that exists in various forms in the environment. It has been used for a variety of applications ranging from amalgams in dental fillings to extracting gold in Brazilian mining fields.[226] Mercury is released into the environment from waste disposals and natural deposits and is also found in igneous rocks formed during volcanic activities. The burning of fossil fuels and the exhaust from manufacturing plants release inorganic mercury into the earth's atmosphere. In addition, some microorganisms are capable of producing small organic mercuric compounds, such as methyl mercury, that can pollute aquatic environments and eventually bioaccumulate.

Exposure to high levels of mercury can affect vital organs, such as the kidneys and brain. It can also cause damage to important biomolecules, namely, proteins and DNA, which can lead to neurological disorders. Furthermore, its high toxicity and ubiquitous nature makes it a major environmental problem. Due to widespread distribution of mercury and its compounds in the environment, different microorganisms have developed resistance mechanisms to help them survive in these polluted atmospheres. There are at least five mechanisms reported in the literature which can account for the resistance to mercury and its compounds by different microorganisms.[226−230] A few examples are outlined below.

(i) Some strains of bacteria *Enterobacter aerogenes* became resistant to mercuric compounds by reducing permeability of the cells to mercuric ions. This reduced permeability is due to two plasmid-encoded proteins present in the cells.

(ii) Some microorganisms chemically modify the more toxic forms of organic mercury to less toxic and insoluble forms. *Clostridium cochlearium T-2P* contains two plasmid-encoded enzymes that allegedly catalyze the demethylation of methylmercury and generates hydrogen sulfide in the cytosol. The demethylated moiety is subsequently converted to insoluble mercuric sulfide by reacting with the hydrogen sulfide previously produced by the second enzyme.

(iii) In certain strains of the bacterium *Desulfovibrio desulfuricans*, methylmercury levels within the cells are maintained at nontoxic levels by an enzyme-catalyzed reaction that produces hydrogen sulfide, which is required for the conversion of methylmercury its less toxic insoluble sulfide forms.

(iv) Certain soil and water bacteria are capable of mercury methylation via series of enzyme-mediated reactions. Although methylmercury is among the more toxic forms of mercury, some plasmid- or chromosome-borne enzymes are involved in the methylation reaction to form methylmercury, which is then effluxed from the cell by volatilization or is sequestered into some other insoluble forms.

(v) In the vast majority of Gram-positive and Gram-negative bacteria found throughout the world, the most commonly encountered mercury resistance mechanism involves the enzymatic reduction of the mercuric ion to its elemental form and its subsequent volatilization from the cell. The enzymes involved in this mechanism are encoded by genes of the *mer* operon.[160,230]

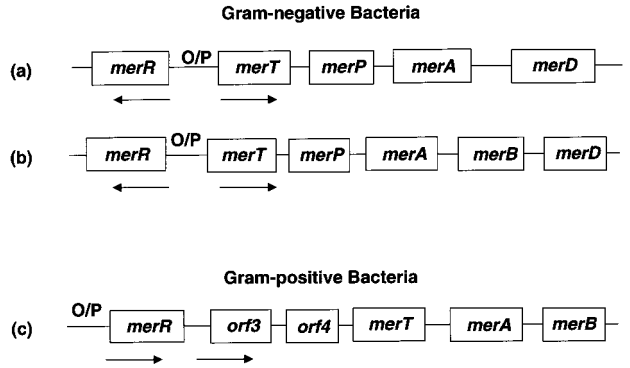


Figure 15. Organization of the *mer* operons of Gram-negative and Gram-positive bacteria. Parts a, b, and c represent the *mer* operons of Tn*501*, pDU1358, and pI258, respectively. Genes are represented by the boxes. The arrows indicate the direction of mRNA transcripts. (Adapted with permission from ref 231. Copyright 1992 American Society for Microbiology.)

Of the previously mentioned resistance mechanisms for mercury and mercury-containing compounds, the latter one involving reduction of mercuric ions is the best understood. The *mer* operon is most commonly found in Hg-resistant organisms and can be either plasmid-, chromosome-, or transposon-borne. In some cases, more than one of these operons is present. The genes from the *mer* operon code for protein molecules that carry out the regulation, transport, and other enzymatic functions to detect and eliminate mercuric compounds from the cells. The *mer* operon consists of the *merR* gene that encodes for the regulatory protein MerR, and it is typically located at one of the ends of the operon. In many Gram-negative bacteria, the *merR* gene is transcribed in a divergent fashion with respect to the other genes of the operon (Figure 15).[231] The *merP* and the *merT* genes encode for transport proteins responsible for the sequestration and transfer of the mercuric ions to reducing enzymes within the cells.[232,233] Some microorganisms also contain a *merC* gene that assists in the mercuric ion transport across the membrane.[234] The structural *merA* gene is usually located downstream of the transport genes of the operon. It encodes for the enzyme MerA, a mercuric reductase, which reduces inorganic mercuric ions to their elemental form.[235] This type of reductive resistance mechanism is termed "narrow spectrum resistance". In some bacteria, an additional gene *merB* is present, which encodes for the enzyme mercuric lysase. The presence of this enzyme confers resistance to organic mercurial compounds in the bacterium as it can break the carbon-mercury bond to form an organic moiety and inorganic mercury.[236] This type of resistance is termed "broad-spectrum resistance" due to their capability to confer resistance to a wider range of mercuric compounds.

MerR and MerD are the regulatory proteins of the *mer* operon.[237−240] In the absence of mercuric ions, MerR binds to the O/P region of the operon forming a MerR-DNA complex that represses the expression

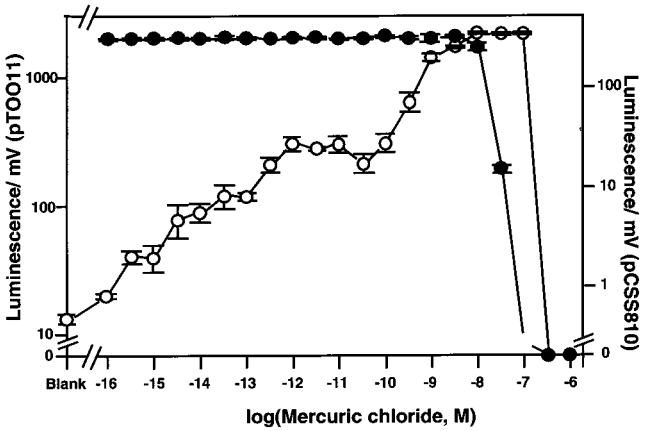


Figure 16. Bacterial sensing system for mercury based on *E. coli* MC1061 harboring plasmid pTOO11. Calibration plot for mercuric ions after *E. coli* MC1061 (pTOO11) were incubated with mercuric chloride. Luminescence measurements were done in triplicate. (Reprinted with permission from ref 165. Copyright 1995 American Chemical Society.)

of the genes of the operon. When mercuric ions enter the cell, they bind to the MerR protein inducing a change in conformation of the MerR-DNA complex, which in turn leads to transcription of the rest of the genes of the operon.[241] A pair of vicinal cysteine residues of the transport protein MerP binds mercuric ions initially in the periplasm and then passes them to another cysteine residue in MerT. Thus, the MerP and MerT proteins sequester the mercuric ions before sending them finally to the active sites of MerA. Here, the mercuric reductase enzyme, MerA, reduces the mercuric ions via an NADPH-dependent reaction. The reduced mercury is then volatilized out of the cell.

Virta et al.[165] developed a sensitive luminescence-based biosensing system for detection of mercuric ions by employing the regulatory protein MerR and the *mer* promoter sequence from the transposon Tn*21*[242] in conjunction with the reporter firefly luciferase (*luc*) gene. They constructed a recombinant plasmid pTOO11 containing the *mer* promoter, *merR gene*, and promoterless *luc* gene and transformed a strain of *E. coli* MC1061. In this genetically engineered bacterium, the *mer* promoter and MerR protein regulate expression of the reporter protein, firefly luciferase. The expression of the *luc* gene is repressed in the absence of mercuric ions in the cells. When mercuric ions are present, MerR activates the *mer* promoter and subsequently luciferase is expressed in the cells. This sensing system for detection of mercuric ions is more sensitive than the other systems previously developed. The detection limit obtained using this sensing system was in the femtomolar range (0.1 fM). A linear response was obtained with concentrations of mercuric chloride ranging from 0.1 fM to 100 nM. Any further increase in mercuric chloride resulted in a sharp decrease in the chemiluminescent signal, which can be attributed to the toxicity of mercuric ions at higher levels to the cells (Figure 16). This system was highly specific for mercuric ions as it responded negligibly to other metal ions, such as zinc, copper, manganese, and cobalt, at millimolar concentrations. Some induction with cadmium chloride was observed; however, 10^7 times higher concentrations of cadmium ions were required to generate the same response as mercuric ions.

V. Whole-Cell Sensing Systems Based on Cellular Metabolism

Metabolism is an essential process for the survival of organisms. It serves two fundamentally important functions in the cells: (i) generation of biological energy to drive vital functions and (ii) the formation of precursors, which is essential for the synthesis of biological molecules and cellular constituents. Prokaryotics have highly diverse metabolic pathways as they can be chemoheterotrophic, photoautotrophic, photoheterotrophic, or chemoautotrophic. Microorganisms with unusual metabolic capabilities have been discovered from the deep subsurface, ocean floor, and extreme environments such as volcanic springs.[243] In general, microorganisms exhibit enormous flexibility as to the substrates they can metabolize to derive energy. These substrates are diverse as sugars, organics, and toxic metals. Multiple and interrelated pathways for the metabolism of nutrients are known to exist in bacteria. These pathways are controlled by numerous regulatory mechanisms.[244] This ability of microorganisms to metabolize a wide range of substances has been utilized by industries as well as by research facilities to produce desired products. For example, anaerobic breakdown of sugars by yeast to produce alcohol is used in fermentation industries. In recent years, bioremediation processes have been developed to metabolize pollutants using microorganisms. The efforts undertaken by researchers to understand the mechanisms of cellular metabolism have led to the development of sensing systems for various analytes.

One of the major problems faced by modern society is the contamination of soil and groundwater by pollutants. The capability of microorganisms to metabolize these pollutants is exploited in bioremediation processes, and it is currently explored as an option for the cleanup of contaminated sites as it is more economical and environmentally safer than most of the established procedures.[245] Bioremediation of soil polluted by chlorinated solvents and petroleum products has been well documented.[246,247] Sensing systems based on the metabolism of cells in response to factors, like toxicity, growth, and other cellular events, are currently being widely explored. These systems monitor metabolism by measuring cellular pH, oxygen consumption, CO_2 production, lactate production, or redox potential.[1,248−250]

Whole-cell sensing systems have been developed based on the ability of microorganisms to metabolize an analyte of interest.[4] Microorganisms take in and metabolize various types of chemicals available in the environment for their survival. The analyte or its metabolic product induces the expression of genes encoding enzymes that are necessary for its transport and/or metabolism. A strategy employed in the development of whole-cell sensing systems is based on the genetic fusion of reporter genes to the genes in a metabolic operon induced by its respective analyte. Sensing systems can, therefore, be developed

by relating induction of these genes to the concentration of analyte of interest used as the inducer.[251] Thus, when the induction takes place in the presence of the target analyte, the reporter gene is coexpressed along with the other genes of the operon. Consequently, the concentration of the inducer can be quantified by measuring the signal generated by the reporter protein. There have been studies performed with bacteria, algae, and yeast for various types of analytes employing this strategy.[6] In this section, we review whole-cell sensing systems based on metabolic pathways developed for various organic compounds and sugar analytes.

A. Organic Compounds

Organic compounds are one of the major contributors to environmental pollution and, consequently, have been one of the primary target analytes for sensor development.[252,253] Many different bacterial species have the capacity to use organic molecules present in their environment as a source of carbon.[254] These microorganisms have evolved to produce diverse enzymes that metabolize these organics. The mechanism of regulating the expression of these enzymes has been the focus of a number of studies and is well understood. Such bacteria are currently widely used to degrade petroleum-product contaminants as well as industrial wastes.[255] Some of the problems faced in the degradation of these pollutants include the determination of their bioavailability and their sensitive and selective determination. A whole-cell biosensor for these organic pollutants can be engineered by placing the expression of a reporter gene under the control of a particular transcriptional activator involved in their metabolic pathway. Reporter-based biosensing systems are rapid, nondestructive, and noninvasive and, consequently, are becoming a popular tool for monitoring the bioavailable concentration of pollutants, as most of the organic pollutants have low water solubility and the extent of biodegradation of these pollutants is dependent on the transport processes to the microorganism.[107] Some of these systems for organic pollutants are reviewed below.

1. Alkane Sensing

Diesel oil mainly consists of alkanes and aromatic compounds. Alkanes, being hydrophobic in nature, are not bioavailable for degradation by microorganisms in contaminated sites.[16] Thus, determination of the bioavailability of alkanes is an important issue. A whole-cell sensor for linear alkanes has been developed by Sticher et al. on the basis of induction of the *alk* regulon in the presence of alkanes.[16] The *alk* regulon consists of the *alkBAC* operon and the *alkR* locus.[256] The locus *alkR* contains the *alkS* and *alkT* genes. The *alkBAC* operon encodes the genes of the catabolic enzymes involved in the hydroxylation of alkanes and dehydrogenation of the resulting alkanols. The alkane induces expression of a protein, AlkS, which in turn induces the *alkB* gene that codes for the enzyme involved in the hydroxylation of alkanes. In this system, the extent of gene expression was measured by coupling a reporter gene, namely, bacterial luciferase, to the induced gene. Specifically, the plasmid, pJAMA7, was constructed which consists of a fusion between the *alkB* promoter of *Pseudomonas oleovorans* and the *luxAB* genes of *Vibrio harveyi*. The plasmid was then transformed into *E. coli*, strain DH5α. In this system, the *alkB* promoter is activated by the transcriptional activator protein AlkS (its gene was originally present on another plasmid, pGEc74) cloned into the host cell. The cells were induced by octane, and the light emitted from the reaction of expressed luciferase with decanal was measured. The amount of light observed correlated to the amount of octane used for induction (Figure 17). The linear range obtained extended from 24.5 to 790 nM octane, and from the data fitted to a hyperbolic function, it was reported that the system can detect octane concentration as low as 3 nM. Other linear alkanes from pentane to decane also induced the cells to some degree (Table 8).[16] Aromatic and cyclic hydrocarbons and branched alkanes did not interfere in this sensing system. This biosensor was employed to determine the concentration of alkanes in groundwater samples. However, the alkane concentration in these samples, as determined by this cell-based sensing system, was found to be lower than the actual concentration, presumably due to the presence of an unknown inhibitor in the sample.

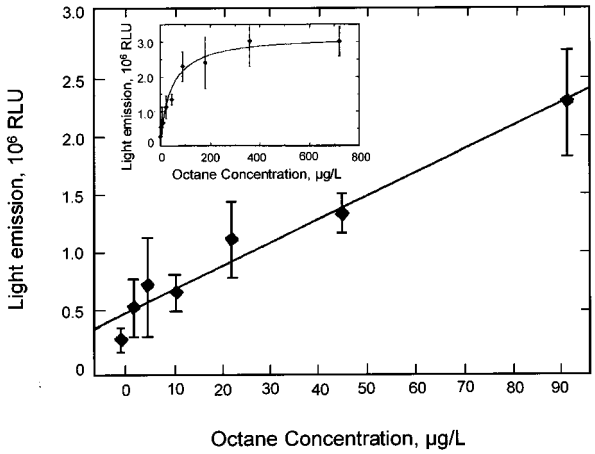


Figure 17. Light emission by *E. coli* DH5α (pGEc74, pJAMA7) after a 60-min induction period as a function of octane concentration. (Reprinted with permission from ref 16. Copyright 1997 American Society for Microbiology.)

2. Benzene and Benzene Derivatives Sensing

Monitoring chemical pollutants such as benzene-related compounds, halogenated compounds, and alkanes in drainages of chemical plants requires highly sensitive detection systems. To detect benzene-related compounds, reporter gene-based whole-cell sensors were developed by Ikariyama et al.[257] and de Lorenzo et al.[258] A bacterium, *Pseudomonas putida* mt-2, harbors the TOL plasmid which consists of the genes encoding for the proteins responsible for the degradation of xylene, toluene, and their derivatives to pyruvate and acetaldehyde. The regulation of the genes in the TOL plasmid is shown in Figure 18. The first operon in the TOL plasmid, *xylCAB*, is used to transform toluene derivatives into benzoate and toluate. The second operon, *xylDLEGF*, converts

Table 8. Relative Luciferase Activity in *E. coli* DH5α (pGEc74, pJAMA7) after Induction with Different Compounds

compound	relative induction[a] (%)
Linear Alkanes	
pentane	13
hexane	44
heptane	81
octane	100
nonane	100
decane	69
undecane	6
dodecane	11
hexadecane	11
branched alkanes	
heptamethylnonane	11
3-methylheptane	36
pristane	11
Cyclic Hydrocarbons	
cyclohexane	9
methylcyclohexane	11
dimethylcyclohexane	11
cycloheptane	11
Aromatic Hydrocarbons	
benzene	10
toluene	10
m-xylene	11
trichlorobenzene	9

[a] Induction was measured after 69 min. Light output (expressed in percent) was related to octane-induced light emission, arbitrarily set at 100%. (Adapted with permission from ref 16. Copyright 1997 American Society for Microbiology.)

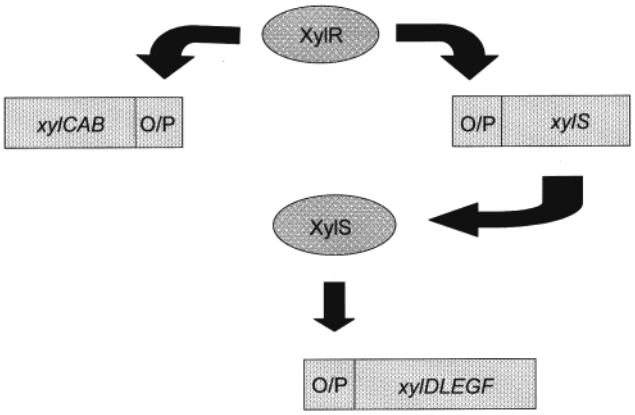


Figure 18. Regulation of the genes in the TOL plasmid. The product of the *xylR* gene, the protein XylR, regulates the expression of *xylCAB* and *xylS* in the presence of benzene derivatives. XylS activates the *xylDLEGF* operon involved in the complete degradation of benzene derivatives. (Adapted with permission from ref 257. Copyright 1997 American Chemical Society.)

these aromatic acids to pyruvate and acetaldehyde. These operons are under the control of two regulatory genes, *xylR* and *xylS*. The XylR protein, in the presence of benzene derivatives, induces the *xylCAB* and *xylS* genes. The product of *xylS*, the XylS protein, induces *xylDLEGF*. In the study performed by Ikariyama and co-workers, a sensing system was developed by constructing a plasmid, pTSN316, in which the firefly luciferase gene, *luc*, was fused to the promoter of *xylS*, which is activated by XylR. *E. coli* HB101 cells were transformed with this plasmid and immobilized at the tip of the fiber optic covered with a dialysis or a polycarbonate membrane. The light

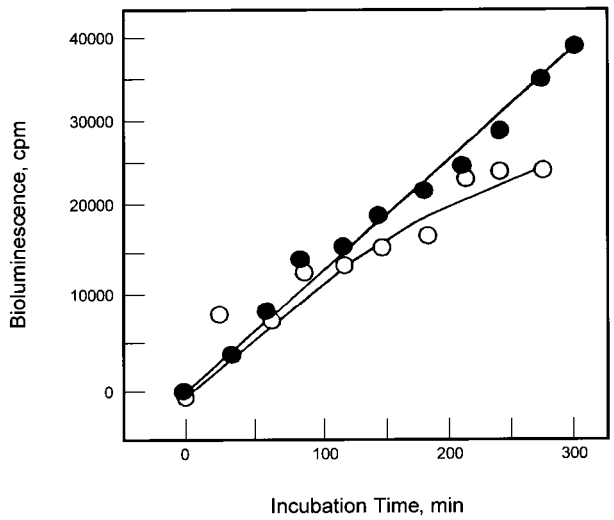


Figure 19. Luminescence behaviors of a cell slurry immobilized behind a dialysis membrane (●) and in a polycarbonate membrane (○). (Reprinted with permission from ref 257. Copyright 1997 American Chemical Society.)

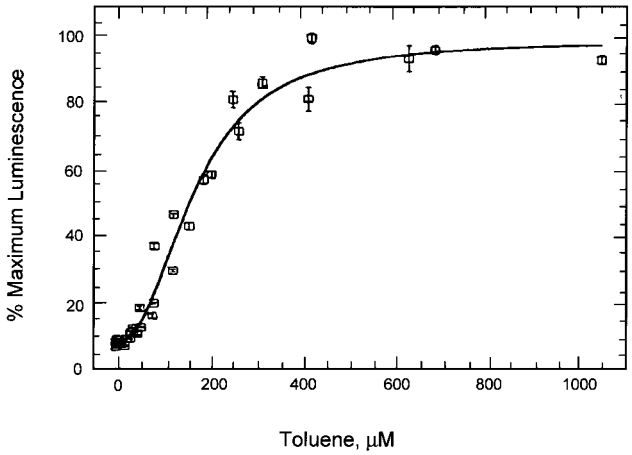


Figure 20. Toluene concentration dependence of luminescence emission from toluene sensing cells. Data from three separate experiments were normalized for maximal luminescence and combined. The error bars represent the standard deviations of three replicates of each sample within the same experiment. (Reprinted with permission from ref 18. Copyright 1998 American Society for Microbiology.)

intensity obtained correlated to the amount of benzene derivatives present in the sample as the inducer. Bioluminescence obtained from the cell slurry immobilized behind a dialysis membrane and behind the polycarbonate membrane induced with *m*-xylene is shown in Figure 19. This system was also reported to have a strong linear relationship with luminescence in the concentration range from 0.05 to 1 mM *m*-xylene. This whole-cell sensing system was shown to be sensitive to *m*-xylene, toluene, and other benzene derivatives.

In a similar manner, a biosensor for toluene was developed by Willardson et al.[18] In this study, a plasmid was constructed by fusing the reporter gene *luc* to the promoter P_u of the TOL operon. This plasmid consists of the transcriptional activator gene, *xylR*. In the presence of toluene, XylR binds to toluene and interacts with the promoter, P_u. This interaction initiates the transcription of genes under

Table 9. Effect of BTEX[a] and Phenol on Bioluminescence Emission of *P. putida* TVA8

treatment	specific bioluminescence[b] (nA)
buffer (control)	0.2 ± 0.1
benzene	242 ± 9
toluene	234 ± 7
ethylbenzene	1.0 ± 0.2
	47 ± 6[c]
o-xylene	0.5 ± 0.1
m-xylene	38 ± 3
p-xylene	24 ± 2
phenol	70 ± 2

[a] BTEX stands for benzene, toluene, ethylbenzene, and xylene. [b] Bioluminescence signal emitted by luciferase after a 2 h of induction. [c] Bioluminescence signal obtained after 4 h of induction with ethylbenzene. (Adapted with permission from ref 14. Copyright 1998 American Society for Microbiology.)

the control of P_u. The plasmid was transformed into *E. coli* cells. The sensing system developed was responsive to toluene and its derivatives. This system also demonstrated a strong correlation between toluene concentration and the emission of luminescence (Figure 20). The limit of toluene detection was reported to be between 10 and 20 μM.

Whole-cell sensing systems for benzene, toluene, ethylbenzene, and xylene (BTEX) were also developed using *Pseudomonas putida* F1 bacteria by Applegate et al.[14] In this case, unlike in the previous studies where the sensing system is plasmid-based, gene fusions were introduced into the bacterial chromosome employing a transposon delivery system. The *tod* operon, which is induced by BTEX compounds, was fused to the firefly luciferase genes, *luxCDABE*, and cloned into the transposon delivery vector pUTK211 to yield plasmid pUTK214. This plasmid was transformed into *P. putida* F1, and colonies carrying transposon insertions were selected for induction studies with BTEX compounds. A significant amount of bioluminescence was observed in the presence of benzene, toluene, *m*- and *p*-xylenes, and phenol (see Table 9). Since the complete *lux* cassette (*luxCDABE*) was fused to the *tod* operon, on-line monitoring of bioluminescence could be performed as it did not require any substrate for luciferase. Good correlation between the bioluminescence measured and the concentration of toluene in the sample was also reported with a detection of limit of 30 μg/L.

A whole-cell reporter gene-based sensing system for hydrophobic pollutants was developed by Selifonova et al. using the *ipb* operon involved in isopropylbenzene catabolism.[259] The *ipb* operon consists of the *ipbA*, *ipbB*, *ipbC*, and *ipbD* genes.[260] These genes code for the enzymes involved in the metabolism of isopropylbenzene. The gene *ipbA* encodes for isopropylbenzene dioxygenase, which converts isopropylbenzene to 2,3-dihydro-2,3-dihydroxyisopropylbenzene, the first step in the metabolism of isopropylbenzene. A plasmid, pOS25, containing a fusion between the *ipbA* and *luxCDABE* genes encoding luciferase from *Vibrio fischeri* was constructed. The regulatory gene *ipbR* and the *ipb* operator/promoter region were located upstream from the *ipbA* gene. The plasmid was then transformed into the HMS174 strain of *E. coli*. In the presence of isopropylbenzene, the gene *ipbA* is induced resulting in coexpression of luciferase. This system was also induced by many other hydrocarbons including toluene, substituted benzenes, alkanes, and cycloalkanes (see Table 10). Such a system may find potential uses as a bioindicator of hydrocarbon pollution in the environment.

3. Naphthalene and Salicylate Sensing

Sensors that utilize the *Pseudomonas* species as a sensing element have been developed for a wide range of organic compounds. Reports by Sayler and co-workers on the development of whole-cell sensing systems for naphthalene and salicylate use bacterial luciferase as the bioluminescent reporter gene.[17,261] In their study, a genetically engineered bioluminescent bacterium, *Pseudomonas fluorescens* HK44, was constructed by inserting the reporter plasmid pUTK21. This plasmid consists of two *nah* operons. The first operon consists of the *nahABCDEF* genes, and the second operon consists of the *nahGH* genes.[262−264] The first operon converts naphthalene to salicylate, and the second operon is involved in the oxidation of salicylate through the catechol *meta*-cleavage pathway. In pUTK21, the regulatory gene, *nahR*, is located upstream of *nahG* gene. The product of the *nahR* gene, the NahR protein, is constitutively expressed and exists in an inactive form. In the presence of an inducer, the NahR protein is activated and starts the transcription of the *nah* operon. The *luxCDABE* gene cassette from *Vibrio fischeri* was fused genetically to the *nahG* gene. A cell culture of *P. fluorescens* HK44 was immobilized on the surface of an optical liquid light guide by using strontium alginate. Upon exposure of this biosensor probe to naphthalene or salicylate, the increase in gene expression was observed through an increase in the

Table 10. Induction of Luciferase Expression in *E. coli* HMS174 (pOS25) by Organic Aromatic Compounds[a]

chemical	concentration (μM)	bioluminescence signal ratio after a given induction time[b]	
		100 min	250 min
benzene	1,000	66	4100
toluene	100	21	2300
ethylbenzene	100	14	4800
isopropylbenzene	100	5	4900
n-butylbenzene	500	6	25
n-amylbenzene	1,000	1.5	1.5
3-isopropylcatechol	100	3.5	11
2,3-dihydrozy-2,3-dihydroisopropylbenzene		NR	NR

[a] NR, no response. [b] Ratio of light produced by induced cells to light produced by uninduced cells. (Adapted with permission from ref 259. Copyright 1997 American Society for Microbiology.)

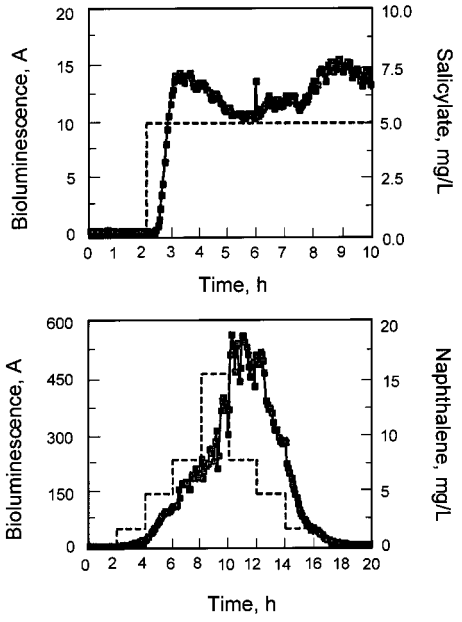


Figure 21. Comparative bioluminescence sensor response to salicylate and naphthalene waste streams. (A) Biosensor response to a step change in salicylate concentration: (□) time course of the bioluminescence response; (- - -) salicylate concentration (5 mg/L) in the biosensor cell. (B) Biosensor response to gradual changes in naphthalene concentration: (□) time course of the bioluminescence response; (- - -) naphthalene concentration in the biosensor cell. (Reprinted with permission from ref 17. Copyright 1994 American Society for Microbiology.)

bioluminescence emitted by the cells. Time course of the bioluminescent response from a step change in naphthalene and salicylate concentration is shown in Figure 21. The inducer concentrations of 0.5 mg/L of salicylate and 1.55 mg/L of naphthalene were reported to have a significantly longer response time compared to inducers at higher concentrations. Bacterial nutrients, such as glucose or other organic compounds, showed little or no interference in the sensing of naphthalene and salicylate.

4. Polychlorinated Biphenyl Sensing

Polychlorinated biphenyls (PCB's) are toxic environmental pollutants which bioaccumulate in the food chain. Therefore, there is increased interest in their detection and degradation using microorganisms.[265] A method, based on reporter genes, has been developed by Layton et al. for detecting biphenyls using bacterial luciferase.[266] A plasmid pUTK60 was constructed for this study. This plasmid consists of the *orf0-bphA1* gene from the biphenyl operon fused to the *luxCDABE* cassette. In pUTK60, the promoter region and a putative regulatory gene (*orf0*) are located upstream from the *bphA1* gene. The role of *orf0* in the regulation of the biphenyl operon, however, is unclear. Biphenyl dioxygenase is a multisubunit enzyme. The gene *bphA1* encodes for the largest subunit of this enzyme. The biphenyl operon converts biphenyl/chlorobiphenyl to benzoic acid/chlorobenzoic acid. In the presence of biphenyl, bioluminescence was generated in a concentration-dependent manner (Figure 22). Addition of benzoic acid repressed the bioluminescence. This result was consistent with previous observations that benzoic acid had an inhibiting effect on PCB degradation.[267]

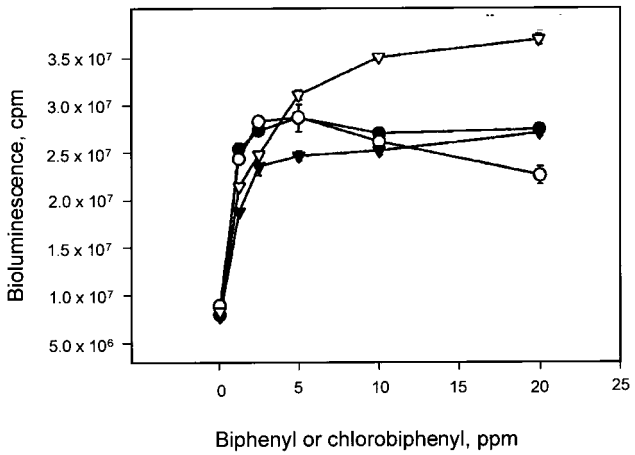


Figure 22. Bioluminescence emission from cells harboring plasmid pUTK60 after 2 h incubation with different concentrations of biphenyl and chlorinated biphenyl (CB): (●) biphenyl; (○) 2CB; (▼) 3CB; (▽) 4CB. (Reprinted with permission from ref 266. Copyright 1998 American Society for Microbiology.)

5. Chlorocatechol Sensing

Chlorocatechols are intermediates produced during the biodegradation of chlorinated compounds such as PCBs, which are major environmental pollutants.[268] Therefore, there is a need to develop sensitive methods for detecting chlorocatechols. A whole-cell sensing system for chlorocatechols using β-galactosidase as a reporter protein has been developed in our laboratory.[13] Chlorocatechols are metabolized by the enzymes encoded by the *clc* operon.[269] Three enzymes encoded by this operon, *clcA*, *clcB*, and *clcD*, metabolize catechol to intermediates that are shuttled into the Kreb's cycle. The regulatory protein, ClcR, regulates the expression of this operon. In this study, a plasmid, pSMM50R-B′, was used that consists of a reporter gene, *lacZ* encoding the β-galactosidase enzyme, fused to the *clcB* gene as shown in Figure 23. The regulatory gene, *clcR*, is located upstream of the *clcA* gene in this plasmid. The PRS4020 strain of *Pseudomonas putida* was transformed with this plasmid and employed as the sensing system for chlorocatechols. In the absence of chlorocatechol, the regulatory protein ClcR binds to the operator/promoter region of the plasmid and prevents the transcription of the genes. In the presence of chlorocatechol, ClcR binds to chlorocatechol releasing it from the O/P region, thus activating the transcription of the *clcA* and the *lacZ* fusion genes. The activity of the expressed β-galactosidase was monitored by chemiluminescence using Galacto-Light Plus (Tropix, Bedford, MA) as the substrate of the reaction. This sensing system showed detection limits of 6×10^{-10} M for 3-chlorocatechol and 2×10^{-9} M for 4-chlorocatechol. Distinction between these two isomers can be achieved by reducing the induction time. Selectivity studies with structurally similar organic compounds such as catechol, 2-chlorophenol, 4-chlorophenol, biphenyl, and 4-chlorobiphenyl showed no interference (Figure 23). Thus, a highly sensitive and

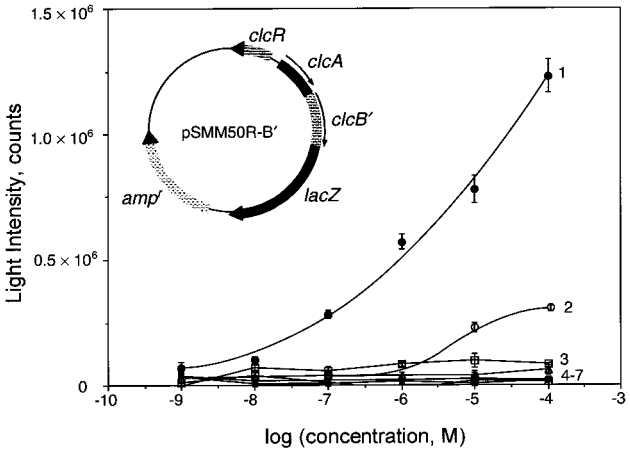


Figure 23. Selectivity of the bacterial sensing system based on *Pseudomonas putida* harboring the pSMM50R-B′ plasmid. The bacteria were incubated with a series of analytes for 30 min: (1) 3-chlorocatechol, (2) 4-chlorocatechol, (3) 4-chlorobiphenyl, (4) catechol, (5) biphenyl, (6) 2-chlorophenol, (7) 4-chlorophenol. The chemiluminescence signal was corrected with respect to a blank consisting of bacteria in deionized distilled water. Data are the average ± one standard deviation (n = 3). Insert: Plasmid pSMM50R-B′ showing the fusion between the *clcB* and the *lacZ* genes. (Reprinted with permission from ref 13. Copyright 2000 American Chemical Society.)

selective sensing system for 3-chlorocatechol and 4-chlorocatechol was developed.

B. Sugars

Sugars are another important set of analytes, besides organic compounds, for which development of sensors is desirable. There are different analytical methods available for the detection of sugars.[270,271] Detection of monosaccharides by conventional spectroscopic methods is difficult as these sugars lack a chromophoric group. Therefore, the sugars are derivatized with the chromophore for spectroscopic detection. Electrochemical methods developed to date lack specificity due to the structural similarity of sugars. Thus, separation by a chromatographic method is a necessary step before electrochemical detection. Whole-cell sensors utilizing reporter genes can be one of the best strategies for sugar detection as they are based on specific induction of a reporter gene in the presence of a specific sugar and thus provide higher selectivity.

1. Arabinose Sensing

A cell-based sensing system for L-arabinose has been developed in our laboratory by coupling the L-*ara* operon with GFP as a reporter.[26] The L-*ara* operon consists of three structural genes *araB*, *araA*, and *araD* under the control of a promoter, P_{BAD}. The gene *araA*, which encodes L-arabinose isomerase, converts L-arabinose into L-ribulose. L-Ribulose is phosphorylated by L-ribulose kinase, encoded by the *araB* gene. The product thus formed, L-ribulose-5-phosphate, is converted to D-xylulose-5-phosphate by L-ribulose-5-epimerase, encoded by the *araD* gene. The *araC* gene, which codes for AraC, the L-arabinose regulatory protein, is transcribed in the opposite direction from *araBAD* and is expressed independently like many other regulatory proteins. AraC has a binding site for the activator regions, $araI_1$ and $araI_2$, and the operator region $araO_2$ upstream from the promoter P_{BAD} as shown in Figure 24. The binding of the AraC dimer to $araO_2$ and $araI_1$ creates a DNA loop that inhibits the transcription of the *ara* genes. In the presence of L-arabinose, the AraC dimer dissociates from $araO_2$ and binds to the activator region $araI_2$ causing unlooping of the DNA. This event activates the transcription of the *ara* genes. The plasmid pBAD-GFPuv employed in this sensing system for L-arabinose consists of the *gfpuv* gene, fused in such a way that it is under the control of the P_{BAD} promoter and is regulated by the AraC protein. In the presence of L-arabinose, AraC causes the transcription of the *gfpuv* gene. The expression of GFP thus obtained is proportional to the amount of L-arabinose present. This is a reagentless sensing system, as the fluorescence of GFP is monitored by excitation of the bacterial cells at 397 nm. The sensing system is highly selective toward L-arabinose, and other hexose and pentose sugars showed very little or no interference, as shown in Table 11. This system can detect L-arabinose at concentrations as low as 5×10^{-7} M. To demonstrate the feasibility of using this system in remote sensing, the cells containing plasmid pBAD-GFPuv were entrapped behind a dialysis membrane at the tip of a fiber-optic

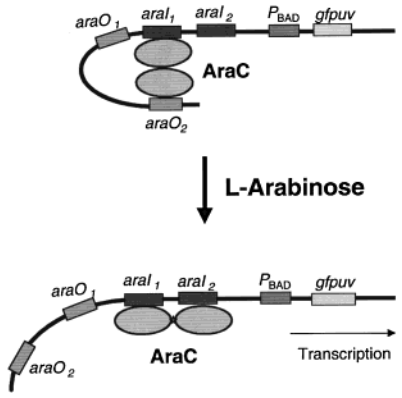


Figure 24. Schematic showing the regulation of GFP expression by AraC in the presence of L-arabinose. (Reprinted with permission from ref 26. Copyright 1999 American Chemical Society.)

Table 11. Selectivity Studies of the Bacterial Sensing System Using *E. coli* Harboring pBAD-GFPuv

sugar (1×10^{-3} M)	fluorescence[a] (%)	SD (%)[b]
L-arabinose	1178	3.88
D-arabinose	98	0.06
D-fructose	98	1.74
D-mannose	102	3.18
L-mannose	130	0.07
D-ribose	102	1.07
L-ribose	96	0.66
D-xylose	99	0.50
L-xylose	94	1.12
D-glucose	98	0.96
L-glucose	110	8.83

[a] Data are the average of three replicates. % Fluorescence = [fluorescence intensity (sample)/(fluorescence intensity (blank)] × 100. [b] SD = standard deviation. Reprinted with permission from ref 26. Copyright 1999 American Chemical Society.

bundle. The results obtained using this fiber-optic probe arrangement showed that the detection limit was 1 order of magnitude higher than that obtained with the solution-based sensing system. Although there is a slight decrease in sensitivity when using the fiber-optic system, the result proved its potential for the sensitive and selective on-line sensing of L-arabinose.

2. Lactose Sensing

The *lac* operon consists of three structural genes *lacZ*, *lacY*, *lacA* and the operator/promoter region. The *lacI*, which is expressed independently from the *lac* operon, produces a repressor protein that binds to operator in the O/P region of the operon.[272] As discussed above, the *lacZ* gene encodes for β-galactosidase, which is responsible for breaking down lactose. The gene *lacY* produces the transport protein, lactose permease, that transports lactose into the cytoplasm of the cell. The *lacA* gene encodes for the enzyme acetyltransferase. In the absence of lactose, the repressor protein binds to the O/P region and turns off the transcription of the *lac* genes. When lactose is present, lactose enters the cell utilizing a basal amount of permease protein and is subsequently degraded by β-galactosidase. Further, allolactose, which is one of the products of the metabolism of lactose, acts as an inducer of the *lac* operon by binding to the repressor protein. This binding event causes the repressor protein to change its conformation and be released from the O/P region, allowing for transcription to commence. In designing a sensing system for lactose, a reporter gene can be fused to the structural genes so that the corresponding reporter protein can be coexpressed in the presence of the inducer, lactose in this case. As the bacteria senses increasing amounts of lactose, an increased amount of the proteins involved in its metabolism are produced. Consequently, proportional amounts of reporter protein are being expressed. The signal observed from the reporter protein is directly proportional to the amount of lactose present in the sample. Currently, this strategy has been applied in our laboratory to develop a sensing system for lactose that employs the blue fluorescent protein (BFP), a variant of the GFP, as the reporter. Initial studies showed expression of BFP in the presence of lactose in a concentration-dependent manner. Optimization of this system is currently underway in our laboratory.[273]

This strategy has been taken a step further in the development of a dual-analyte whole-cell detection system using reporter genes. A dual-analyte detection system for L-arabinose and lactose is being developed using the above-mentioned systems by simultaneously detecting GFPuv and BFP in the presence of the respective sugars, namely, L-arabinose and lactose. The effect of the incubation time between the inducers, L-arabinose and lactose, and the cells on the emission of fluorescence by GFPuv and BFP is shown in Table 12. These results indicate that there is a promising future in the development of array detection systems employing whole cells coupled to reporter genes.

Table 12. Fluorescence Emission of Induction Time Studies of BFP and GFPuv in the Presence of 1×10^{-3} M L-Arabinose and Lactose

hours of induction (h)	% increase in fluorescence[a]	
	BFP[b]	GFPuv[c]
1	30.02	22.82
2	55.65	46.59
3	98.58	85.02
4	105.50	97.21
5	90.52	82.65
6	95.40	98.19

[a] % Increase in fluorescence = {[(fluorescence intensity of induced cells) − (fluorescence intensity of uninduced cells)]/(fluorescence intensity of uninduced cells)} × 100. [b] Emission collected at 443 nm. [c] Emission collected at 506 nm.

VI. Overview and Future

The use of genetically engineered whole cells containing reporter genes coupled to biological recognition components allows for the design of rapid, highly specific, and sensitive biosensing systems. This whole-cell approach avoids the need for extraction of enzymes or antibody development; thus, the effect of analytes on a "living" component provides information regarding cellular events not easily monitored by other cell-disrupting methods. The benefits of data derived from these cell-based systems are especially important in pharmacology and drug discovery whereby they can be used to screen for specific substances that act as agonists or antagonists in cellular processes; this is of paramount importance in high-throughput screening and functional genomics. A wide range of applications of this technology are currently under investigation in areas including biotechnology, pharmaceutical analysis, diagnostics, and environmental monitoring. To date cell-based biosensing systems employing reporter genes have been used to assess cellular toxicity, mutagenicity, and carcinogenicity and for the detection of endogenous and exogenous agents including metals, sugars, organics, and viruses.

Advancements in detection strategies and identification of novel regulatory elements and reporter genes will expand the applicability of these cell-based systems by permitting multianalyte detection, high-throughput screening, miniaturization, portability, and real-time detection. Recent studies from our laboratory have already focused on dual-analyte detection using GFP and its mutant, BFP.[273] Expansion of this application to the detection of several analytes using a single system requires the use of an equal number of reporters which can be detected and differentiated within that system. The identification of functional mutants of reporter proteins such as GFP with unique light emitting properties are currently underway. These studies coupled with the discovery of novel reporter genes, through advances in genetic research and the continued exploration of extreme environments, particularly the deep marine regions, will greatly advance multianalyte detection strategies. Moreover, the identification of reporter genes, which do not require addition of a substrate, such as GFP and UMT, will be particularly useful for on-line real-time detection.

One important parameter in the development of whole-cell biosensors is that of an efficient immobilization of the cells onto the surface of the sensor. The technique employed should maintain the cells' viability by employing immobilization matrixes that are nontoxic to cells and allow for permeation of nutrients that will ensure the cells' survival. In addition, the matrixes need to be permeable to the target analyte being sensed. This immobilization of cells should be homogeneous, reproducible, and render long lifetimes to the sensor. There are several immobilization techniques that are being currently used for this purpose. These include, for example, adsorption on membranes;[274−276] entrapment in agar/agarose,[277] in alginate,[17,143,167,278] in carrageenan,[279] in polyacrylamide,[280] and in sol-gels;[281] physical entrapment behind dialysis membranes;[257,282] and immobilization using porous glass beads.[283,284] Although these techniques are adequate and provide different kinds of immobilization strategies for different needs, advancements in new immobilization techniques should further aid in the development of improved cell-based biosensors with extended lifetimes.

One challenging but realistic goal of biosensor development is their miniaturization and portability. This technology is invaluable for applications such as point-of-care diagnostics, on-site environmental monitoring, and home testing systems. The limitation for these devices is often their sensitivity for use with small sample sizes. The higher sensitivity of cell-based reporter gene systems as compared to more traditional sensing techniques coupled with the additional information (e.g., bioavailability) and the selectivity provided by these systems has already indicated their potential for miniaturization. Further advances in electronics and computer science undoubtedly will improve detection strategies and, thus, will allow the monitoring of reporters with a greater degree of sensitivity than currently available at ultralow levels of analyte.

The selectivity, sensitivity, stability, and versatility of cell-based sensing systems employing reporter genes provide an ideal platform for developing diagnostic and biomonitoring strategies. Advancements in biotechnology and transduction mechanisms will ultimately provide the means for rapid and sensitive real-time analyte detection at ultralow levels.

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