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Genetically Engineered *pfabA pfabR* Bacteria: an Electrochemical Whole Cell Biosensor for Detection of Water Toxicity

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We describe here a bacterial sensor for electrochemical detection of toxic chemicals. The sensor constitutes recombinant bacteria harboring plasmids encoding the *fabA* and *fabR* genes and has high-resolution amperometric response to membrane-damaging chemicals. For example, it can detect phenol at concentrations ranging between 1.6 and 16 ppm within 20 min. The high sensitivity is achieved by using the *fabA* promoter fused to a reporter gene-encoded β -galactosidase on a low copy number plasmid, under the control of the FabR repressor. The use of electrochemical whole cell sensors enables sensitive, fast, easy to operate, and cost-effective detection of water toxicity threats.

Pollution of water resources is an increasing problem worldwide. Along with the growing industrial and domestic pollution there is the threat of terror-related poisoning. The pollutants constitute a wide variety of toxicants, including chemicals such as phenol and its derivatives, heavy metals, pesticides, and carcinogens. Therefore, detection of specific pollutants is unrealistic, and a general warning of acute water toxicity is essential.

The methods used for monitoring of water toxicants are either analytical or biological. Analytical chemistry provides accurate identification and quantification of specific compounds, but it is costly and time-consuming when the nature of the toxicant is unknown. In addition, analytical methods are not suitable for monitoring bioavailability, toxicity, and genotoxicity.

Biological assays provide a cost-effective determination of general toxicity and genotoxicity of the bioavailable toxicants. Standard toxicity bioassays use organisms such as daphnia or fish. The detection time is in the range of days (24–48 h).^{1–3} Microorganisms, in particular bacteria, have recently been used

extensively for determining toxicity^{4,5} due to their rapid growth, fast response time, low cost, and ease of genetic modifications. There are two main approaches of using bacteria as a whole cell biosensor, known as “turn off” or “turn on” mechanisms.⁶ In the “turn off” mechanism, a measurable signal decreases by toxicity, whereas in the “turn on” mechanism, a signal appears following exposure to a toxic compound. The commercial bioassay Microtox (Azur Environmental, Carlsbad, CA) is an example of the toxicity bioassay recognized generically as the *Vibrio fischeri* bioluminescence inhibition test.

Many recombinant bacteria are based on “turn on” mechanisms and are constructed by fusion of reporter genes to various stress-response promoters (see reviews by Kohler et al.⁷ and Daunert et al.)⁸ Stress-induced promoters may be activated by toxic or hazardous chemicals and can be fused to reporter genes to monitor the presence of such chemicals. Among the promoters used are heat shock genes, such as *grpE* and *dnaK*, which are sensitive to protein damage; oxidative sensitive genes, such as *oxyR*; genes sensitive to DNA damage, such as *recA*; and membrane-damage-responsive elements, such as the *fabA* gene.^{9–11} The proteins mostly used as reporters are β -galactosidase, measured by a colorimetric reaction and *luxCDABE* measured by light emission. Recently, the green fluorescence protein (GFP) from jellyfish has also been extensively used as a reporter.^{12,13}

Electrochemical monitoring is of special interest for in situ measurements, since it can be performed using simple, compact, and mobile equipment and is easily adaptable for on-line measurements. The potential application of electrochemical biosensors in

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real-time and on-line monitoring of hydrocarbons and heavy metals has been previously demonstrated.^{14–17} In addition, electrochemical sensors are especially suitable for designing high-throughput devices, such as the highly sensitive miniaturized electrochemical nanobiochip for rapid monitoring of water pollution.¹⁸

In this work, an electrochemical bacterial sensor for toxicity measurements based on genetically engineered *Escherichia coli* was constructed. The detection of toxicants is mediated by the *fabA* promoter controlled by the *fabR* gene. The *fabA* gene codes for hydroxydecanoyl-ACP dehydrase, a key enzyme in the synthesis of unsaturated fatty acids, and is induced when fatty acid biosynthesis pathways are interrupted. The use of the *fabA* promoter as the sensing element for membrane damage was previously described by Bechor et al.,¹⁹ who fused the *fabA* promoter to the *V. fischeri luxCDABE* reporter gene and obtained a biosensor sensitive to a broad concentration range of phenol and phenol derivatives. The detection limit was 7.3 ppm after 2 h of exposure to phenol.

We constructed a *fabA* promoter fused to the reporter gene *lacZ* that could be used in electrochemical measurement. High sensitivity was achieved by manipulating two factors: low copy number plasmid that contains the *fabA* promoter fused to the reporter gene *lacZ* and a second plasmid that contains the intact gene encoding for the *fabR* repressor gene, known also as *YijC* gene.^{20–25} The expression from the *fabA* promoter was tightly controlled by the presence of the gene coding for the FabR repressor. These manipulations resulted in a low background, a high signal-to-noise ratio (SNR), and increased sensitivity in detecting membrane-damaging reagents.

MATERIALS AND METHODS

Chemicals, Oligonucleotides and Enzymes. Antibiotics and the optical β -galactosidase substrate, *p*-nitro- β -D-galactopyranoside (PNPG) were from Sigma Chemicals, Ltd; *p*-amino- β -D-galactopyranoside was synthesized by Prof. Carmeli at the Department of Organic Chemistry, Tel-Aviv University, Israel. DNA primers were purchased from Sigma Chemicals (Israel) Plasmids, and restriction enzymes were from New England BioLab.

Plasmids and Bacteria. Plasmids pBR322 and pACYC184 (New England BioLab), *E. coli* TG1 (Bethesda Research Laboratories, U.S.A.) was used for transformation and plasmid manipula-

tion. *E. coli* MC1061 (Bethesda Research Laboratories, U.S.A.) carrying a deletion of the chromosomal *lac* operon was used to construct the biosensor strain.

Construction of Plasmids and Biosensor. The *fabA* promoter was obtained by PCR of 230 bp from genomic DNA of *E. coli* MG1655 as the template using 5'-GGTCTAGAGTTCTCTG-TAAGCCTTATTT-3' and 5'-GGGCATGCGGTGAAAGCGGTA-GAAGA-3' as primers with *Xba*I and *Sph*I restriction sites, respectively. Following digestion with *Xba*I and *Sph*I, the PCR products ligated into *Xba*I- and *Sph*I-digested pBR322, containing ampicillin resistance gene for selection. Plasmid *pfabA* was purified using a Qiagen kit and transformed into *E. coli* MC1061.

The *fabR* gene was obtained by PCR of 1200 bp from genomic DNA of *E. coli* (MG1655). The primers used were 5'-AACGGC-CGTATCGACGATACTCTCCG-3' and 5'-AATCCGGAATCGATA-AGCCAGCAATC-3', containing the *Eag*I and *Bsp*E1 restriction sites, respectively. Following digestion with *Eag*I and *Bsp*E1, the PCR products were ligated to *Eag*I- and *Bsp*E1-digested pACYC184 plasmid containing tetracycline resistance. The engineered plasmid was purified using a Qiagen kit and transformed into *E. coli* TG1 strain. After purification, the *pfabR* plasmid was transformed into competent *E. coli* containing *pfabA*. The transformed bacteria were grown on tetracycline and ampicillin to maintain the two plasmids.

The sequence, size, and orientation of the constructed plasmids were verified by DNA-agarose gel, restriction endonuclease analysis, and sequencing. DNA sequencing was performed by the dideoxynucleotide chain termination method using an ABI model 377 sequencer (ABI, U.S.A.).

Culturing Procedures. Cultures were grown with aeration at 30 or 37 °C in LB broth or agar with appropriate antibiotics (100 μ g/mL of ampicillin and 12.5 μ g/mL of tetracycline) when required. For biosensing experiments, *E. coli* harboring both plasmids was grown overnight at 37 °C on LB agar plates with the two antibiotics. A single colony was then picked and grown overnight on 2 mL of the same, but liquid, medium. The overnight culture was diluted 1:100 in LB containing the two antibiotics and grown at 30 °C with aeration by shaking at 250 rpm.

Electrochemical System. The electrochemical system consisted of an eight-channeled multipotentiostat built by Prof. Yarnitzky from the Technion, Israel Institute of Technology. The potentiostat was interfaced to the PC via an A/D converter, controlled by LabVIEW software. The electrochemical cells contain screen-printed electrodes (SPE) (Gwent, U.K.) and were attached to polystyrene 0.3-mL wells. The SPEs include three electrodes: carbon ink working electrode (3.14 mm²), carbon ink counter electrode, and Ag/AgCl reference electrode printed on a ceramic platform. A specific lab-made apparatus was used for the SPE electrical contacts, combined with a suction-expulsion stirring mechanism manufactured in our laboratory, which was controlled by a resonance regulator operated at a frequency of 8 Hz.

Amperometric Toxicity Measurements. Recombinant bacteria were exposed to the tested chemicals in 300- μ L electrochemical cells. The induced β -galactosidase activity was determined amperometrically using the substrate *p*-amino- β -D-galactopyranoside at a final concentration of 0.8 mg/mL. The product of the enzymatic reaction, *p*-aminophenol (PAP) is oxidized at the

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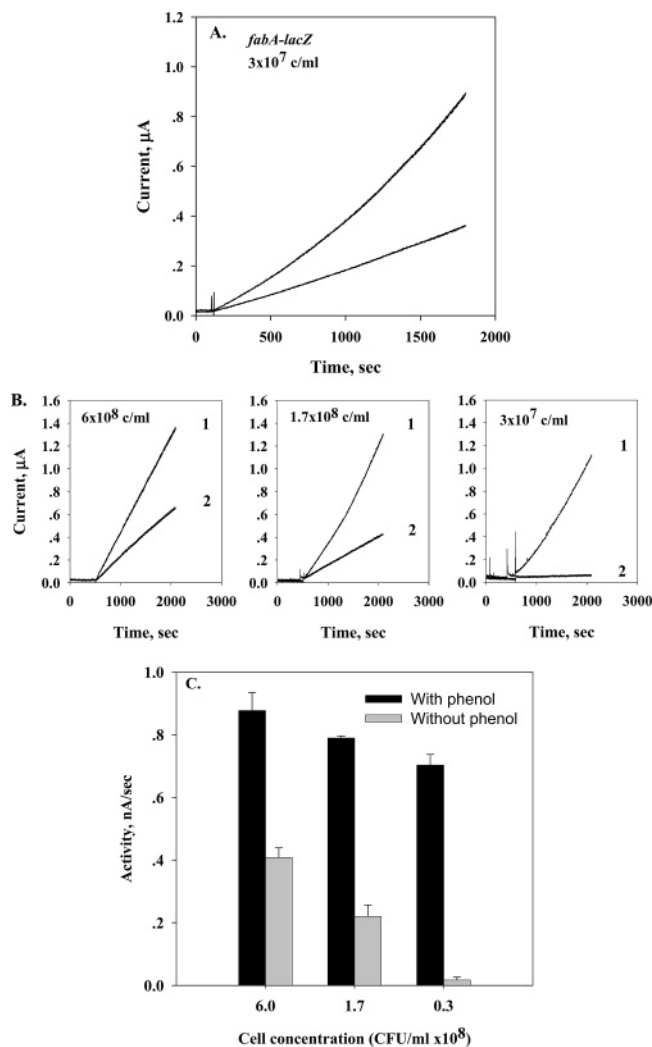


Figure 1. Signal-to-noise measurements. (A) Amperometric signals of β -galactosidase activity obtained with bacteria containing *fabA-lacZ* plasmid in response to 6 ppm phenol (1) and without phenol (2). (B) Amperometric signals of β -galactosidase activity obtained by different cell concentrations of the bacterial sensor, containing both *fabA-lacZ* and *pfabR* plasmids in response to 6 ppm phenol (1) and without phenol (2). (C) Comparison of the β -galactosidase activity induction in the absence or presence of phenol. Activity is presented by $\Delta A/\Delta t$. Amperometric measurements were performed at 220 mV.

carbon working electrode (220 mV working potential). The electrochemical measurements were performed on-line.

RESULTS AND DISCUSSION

Sensing of Phenol. To construct a biosensor for membrane-damaging agents, such as phenol, we used the *fabA* gene. This gene codes for hydroxydecanoyl-ACP dehydrase, a key enzyme in the synthesis of unsaturated fatty acids, and is induced when fatty acid biosynthesis pathways are interrupted. The induction process is controlled by the FabR repressor and by the FadR activator. The expression of the *fabA* gene is kept low by the repressor, which is removed from the promoter in the presence of membrane-damaging agents. The promoter of the *fabA* gene was fused upstream to a promoterless *lacZ* gene, coding for the enzyme β -galactosidase. Addition of phenol to bacteria containing the *fabA-lacZ* fusion results in induction of β -galactosidase activity (Figure 1A). However, the signal-to-noise ratio was small due to

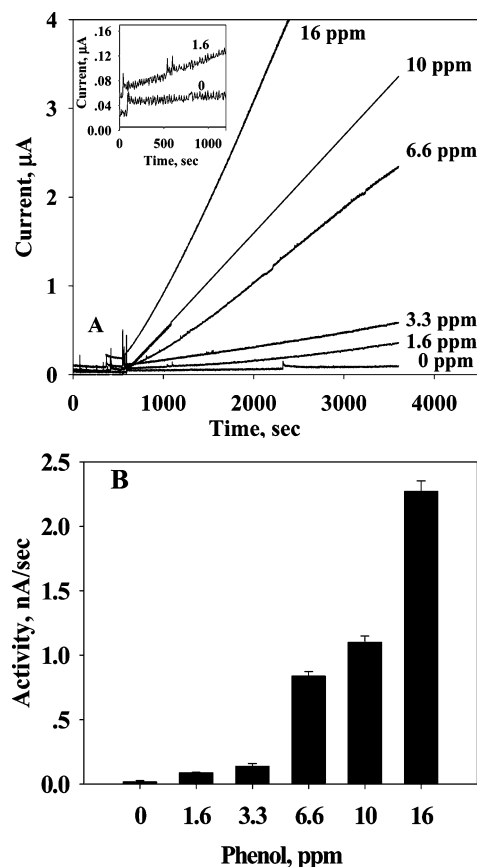
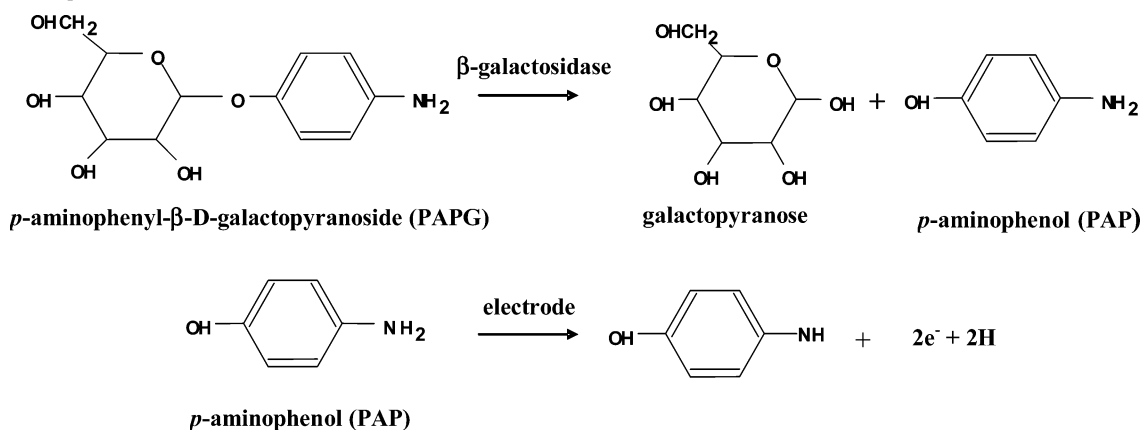


Figure 2. Response of the bacterial *pfabA pfabR* sensor to phenol. (A) Electrochemical signal obtained with *E. coli* cultures, *pfabA pfabR*, exposed to different phenol concentrations. Inset: Signals obtained after 20-min exposure to the lowest concentration of 1.66 ppm phenol. (B) β -Galactosidase activity induction in the presence of phenol. Bacterial concentration used for the experiment was 3×10^7 cell/mL. Activity is presented by $\Delta A/\Delta t$. Each result represents the mean of three measurements.

a relatively high background (Figure 1A). To reduce the background resulting from the incomplete shut-off of the promoter, we added a plasmid that carries the *fabR* gene, coding for the repressor of the *fabA* gene.²² The presence of multiple copies of the repressor gene resulted in a reduction of the background level and in a significantly higher signal-to-noise ratio (Figure 1B). In bacteria carrying the cloned repressor gene, induction of the *fabA* gene was obtained by 6 ppm of phenol, with a fast response time of less than 15 min. Several bacterial concentrations were examined, and a response was obtained in all of them. The optimal bacterial cell concentration was 3×10^7 cells/mL, which yielded the highest induction activity relative to background (Figure 1C). High *E. coli* concentrations of 6×10^8 and 1.7×10^8 cells/mL resulted in very high background activity, above which the induced activity showed an increase of less than 50% measured as the $\Delta \text{current}/\Delta \text{time}$ ($\Delta A/\Delta t$). The enzyme activity is presented as nanoamperes per second; 1 nA corresponds to 20 pmol of PAP.

Response of the Bacterial-Based Sensor to Phenol. The response of recombinant bacteria containing *pfabA* and *pfabR* to increasing phenol concentrations is shown in Figure 2. We could detect as low as 1.6 ppm phenol in less than 20 min, a fast response in comparison to other studies reported.^{19,26} The curve exhibits a direct correlation between the phenol concentration and the

Scheme 1. Enzymatic Reaction Catalyzed by β -Galactosidase and the Final Products Galactopyranose and *p*-Aminophenol^a



^a PAP is oxidized at the working electrode at $V = 220$ mV.

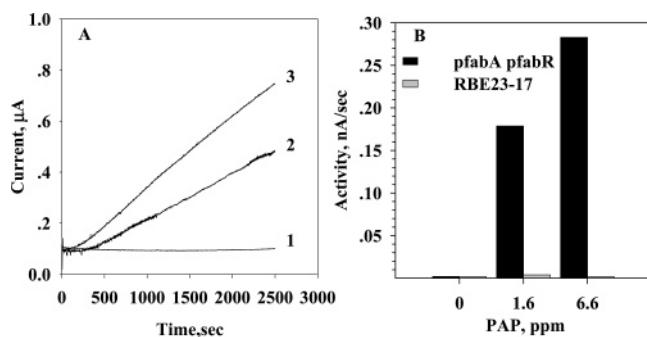
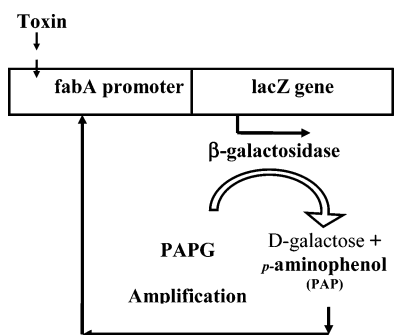


Figure 3. Electrochemical signal amplification. (A) Exposure of the *E. coli* sensor bacteria *pfabA pfabR* to the enzymatic product, PAP, and the resultant *fabA* promoter activation: (1) 0, (2) 1.66, and (3) 6.6 ppm phenol. (B) Comparison between *pfabA pfabR* and control bacteria sensitive to heavy metals, RBE23-17 exposed to PAP, and the calculated enzymatic activity measured as $\Delta A/\Delta t$. Bacterial concentration used for all the experiments was 3×10^7 cell/mL.

Scheme 2. Amplification Mechanism by PAP, the Enzymatic Product, and Activation of the *fabA* Promoter by Further Induction of β -Galactosidase Activity



induced current (Figure 2B), with a clear signal difference in the range of 1.6–16 ppm. These results constitute a potential for developing a quantitative biosensor for membrane-damaging agents.

The low background signal leads to a high SNR, and therefore, this biosensor is highly sensitive and accurate. To compare, the reported bioluminescence-based sensors (constructed *E. coli*

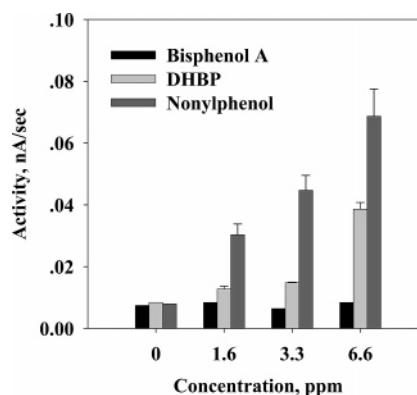


Figure 4. *E. coli* sensor *pfabA pfabR* response and induction by the phenol derivatives bisphenol A, DHBP, and nonylphenol at different toxicant concentrations. Reporter β -galactosidase activity is presented by $\Delta A/\Delta t$.

containing a plasmid with the *fabA* promoter fused to the *V. fischeri luxCDABE*) have high background levels, which leads to reduced sensitivity and increased detection time.¹⁹ This reporter could detect 7.3 ppm of phenol after more than 2 h of exposure.

Signal Amplification. An additional factor responsible for the high SNR is the amplification of the reaction by the product of the enzymatic activity, which is by itself a phenol derivative. This product, *p*-aminophenol, obtained by β -galactosidase cleavage of *p*-aminophenylgalactoside (Scheme 1), further activates the *fabA* promoter, thus amplifying the reaction. This assumption was supported by the experiments summarized in Figure 3, indicating that PAP induces the activity of *lacZ* when under the control of *fabA* promoter. The amplification described here is specific to the *fabA* promoter, because exposure to the same concentrations of PAP did not affect the activity of β -galactosidase when the *lacZ* gene was fused to other promoters, such as a heavy-metal-sensitive promoter²⁷ (Figure 3B). The PAP induction of the *fabA-lacZ* gene, which resulted in -0.1787 and 0.2830 nA/s activities following exposure to 1.6 ppm and 6.6 PAP, respectively, was 2–3 orders of magnitude higher than the induction of RBE23-17 bacteria sensitive to heavy metals (3.8734×10^{-3} and 9.5812×10^{-4} nA/sec, respectively). The amplification is illustrated in Scheme 2.

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Table 1. Summary of Chemicals Tested with *pfabA* *pfabR* Sensor Bacteria

chemical	concn ^a ppm or %	β -galactosidase activity nA/s	induction factor ^b
phenol	0	0.017	1.00
	1.6	0.089	5.20
	3.3	0.139	8.11
	6.6	0.839	49.00
	10	1.101	64.28
	16	2.273	132.72
DHBP	0	0.008	1.00
	1.6	0.013	1.52
	3.3	0.015	1.76
	6.6	0.039	4.59
	0	0.008	1.00
nonylphenol	1.6	0.030	3.79
	3.3	0.045	5.59
	6.6	0.069	8.58
	0.00	0.007	1.00
bisphenol A	1.6	0.008	no induction
	3.3	0.006	no induction
	6.6	0.008	no induction
	0.00	0.017	1.00
hydrazine	1.6	0.018	1.05
	6.6	0.033	1.94
	0.00	0.035	1.00
DDVP	20	0.035	no induction
	0.00	0.042	1.00
toluene	46	0.140	3.35
	0.00	0.043	1.00
ethanol	1%	0.065	1.54
	0.00	0.097	1.00
methanol	1%	0.114	1.17

^a The concentration is described in [vol/vol] percentage (%).

^b Calculated as the slope ratio between the generated current (shown as nA/s) in response to the inducer and the generated current without the inducer. The slope values represent the average of at least three experiments with relative standard deviation of less than 5% ($n = 3$).

Exposure to Other Phenol Derivatives and Nonphenolic Compounds. To characterize the toxicity detection potential of the recombinant bacteria containing the two plasmids *pfabA* and *pfabR*, we examined the influence of other phenol derivatives on the expression of β -galactosidase. We tested nonylphenol, bisphenol A, and 2,4-dihydroxybenzophenone (DHBP), and the results are exhibited in Figure 4.

There was a dose–response behavior to increasing concentrations of nonylphenol and DHBP, but not to bisphenol A. Non-

ylphenol is used in the production and processing of rubber, plastics, and phenolic resins.²⁸ It is very toxic, LD₅₀ of 1.3 g/kg, due to decreased weight gain, liver damage, and hemorrhages.²⁹ Bacteria exposed to nonylphenol displayed a higher induction level at all concentrations in comparison to DHBP. The latter is a member of the benzophenones chemicals and is considered as a possible water pollutant and an estrogen disrupter.³⁰ Although DHBP is considered nontoxic, addition led to induction of the *fabA* promoter, in particular, at the 6.6 ppm concentration, probably due to interference with a membrane-associated biochemical processes. The results with bisphenol A, which showed no influence on the *fabA* promoter, are in accordance with those reported by Gu et al.,^{31,32} who showed no effect of bisphenol A in a luminescence-based biosensor.

Detection of Other Types of Toxic Chemicals. Because the *fabA* promoter should sense chemicals that induce membrane damage, we tested the effect of additional relevant compounds: hydrazine (caustic reducing agent frequently found in industrial wastewater), the organophosphate DDVP (2,2-dichlorovinyl dimethyl phosphate), toluene (aromatic hydrocarbon), ethanol, and methanol (Table 1). The sensor bacteria responded to hydrazine, toluene, and ethanol, but there was a low response to methanol and no response to DDVP. This specificity of the response may be useful to determine the nature of the toxicant and to characterize its involvement in membrane damage.

CONCLUSIONS

Rapid and sensitive toxicity monitoring provides valuable information on the level of toxicity of wastewater and the quality of drinking water. Therefore, development of biosensors, which offers a high signal-to-noise ratio, is highly important. Here, we describe the development of an improved membrane-damage biosensor that produces very low background signal. Moreover, enhanced sensitivity was achieved by the enzymatic product PAP, which further activated the *fabA* promoter. This biosensor contains two plasmids: *pfabA* encoding β -galactosidase under the control of the *fabA* promoter; and *pfabR*, which encodes the repressor of this promoter. This sensor is highly sensitive, rapid, and responds to very low concentrations of phenol (1.6 ppm) within 20 min. The recombinant bacteria respond to nonylphenol, DHBP, toluene, hydrazine, and ethanol while remaining insensitive to bisphenol A and the organophosphate DDVP. The use of whole cell sensors with amperometric measurements enables the fast, easy to operate, and economical sensing of water toxicity.

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