Improved bacterial SOS promoter::lux fusions for genotoxicity detection

Yaakov Davidov a, Rachel Rozen a, Dana R. Smulski b, Tina K. Van Dyk b, Amy C. Vollmer b,c, David A. Elsemore b,1, Robert A. LaRossa b, Shimshon Belkin a,*

a Division of Environmental Sciences, the Fredy and Nadine Herrmann Graduate School of Applied Science, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
b DuPont Central Life Sciences, P.O. Box 80173, Wilmington, DE 19880-0173, USA
c Swarthmore College, Swarthmore, PA 19081-1397, USA

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Abstract

Escherichia coli strains containing plasmid-borne fusions of Vibrio fischeri lux to the recA promoter–operator region were previously shown to be potentially useful for detecting genotoxicants. In an attempt to improve past performance, the present study examines several modifications and variations of this design, singly or in various combinations: (1) modifying the host cell’s toxicant efflux capacity via a tolC mutation; (2) incorporating the lux fusion onto the bacterial chromosome, rather then on a plasmid; (3) changing the reporter element to a different lux system (Photorhabdus luminescens), with a broader temperature range; (4) using Salmonella typhimurium instead of an E. coli host. A broad spectrum of responses to pure chemicals as well as to industrial wastewater samples was observed. Generally, fastest responses were exhibited by Sal94, a S. typhimurium strain harboring a plasmid-borne fusion of V. fischeri lux to the E. coli recA promoter. Highest sensitivity, however, was demonstrated by DPD3063, an E. coli strain in which the same fusion was integrated into the bacterial chromosome, and by DPD2797, a plasmid-bearing tolC mutant. Overall, the two latter strains appeared to perform better and seemed preferable over the others. The sensor strains retained their sensitivity following a 2-month incubation after alginate-embedding, but at the cost of a significantly delayed response. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Ames test; Boluminescence; Escherichia coli; Genotoxicity testing; lux; recA; SOS response

1. Introduction

The obvious need for sensitive monitoring of environmental genotoxicants, to warn and protect against mutagenic, carcinogenic and teratogenic effects of pollutants, has led to the development of
numerous methodologies for genotoxicity detection. Assays relevant to human risk assessment often involve mammals, either using direct animal exposure or tissue culture tests. Simpler microbial bioassays, justified by the correlation between genotoxicity of chemicals in bacterial and animal systems [1,2], allow faster molecular insights and are gaining increasing usage. Of these, the most widely accepted and practiced is the Salmonella mutagenicity assay (the “Ames test”) [3], which quantifies reverse, prototrophic mutations in histidine-requiring strains of Salmonella typhimurium.

A different approach for the detection of environmental mutagens is based on the activation of the bacterial SOS system. Included in this category are the SOS Chromotest [4], the Rec–lac test [5] and the umu test [6]. In all of these cases, SOS induction is followed using lacZ as a reporter gene, and chromogenic b-galactosidase activity at a single time point serves as a measure of genotoxicity. Several recent reports have suggested detecting SOS activation by the use of bacterial bioluminescence (lux) as a reporter [7–12], an approach which allows the monitoring of the bacterial response in real-time by simple luminometry. Belkin et al. [7,8] and Vollmer et al. [12] described the use, in Escherichia coli, of either recA or uvrA promoters with Vibrio fischeri’s luxCDABE. In the Vitotox® assay [10,11], the same plasmid-borne reporter system was fused to the recN promoter of E. coli and introduced into S. typhimurium. Ptitsyn et al. [9] have used, in E. coli, the fusion of the cda promoter with the luxCDABE of Photobacterium leiognathi. Elasri and Miller [13] reported, in Pseudomonas aeruginosa, the fusion of its recA promoter to V. fischeri luxCDABE as a means for monitoring UV radiation, as previously shown by Vollmer et al. [12] in E. coli.

The systems reported in these studies share several disadvantages, among them the plasmid location of the promoter–lux fusion, and — at least for the E. coli and S. typhimurium constructs — the need to operate at a temperature range dictated by the luminescence enzymes rather than that of the host organism. In a continuation of our previous studies [7,8,12], we describe in this communication several of the avenues pursued to overcome these difficulties and thus improve genotoxicant detection capacities of bacterial strains carrying recA::lux fusions.

2. Materials and methods

2.1. Bacterial strains and plasmids

The different bacterial strains used in this study are listed in Table 1. Construction of the plasmid containing the recA::luxCDABE (pRecALux3) fusion in E. coli strain DPD2794 was previously reported by Vollmer et al. [12]. Strain DPD2797 contained the same plasmid in E. coli strain DE112 [14], a tolC derivative of strain RFM443 [15]. S. typhimurium strain Sal94 was obtained by transforming strain WG49 [16] with plasmid pRecALux3 [12].

Table 1
Bio luminescent bacterial strains used in the present study

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>SOS promoter</th>
<th>Location of lux fusiona</th>
<th>lux originb</th>
<th>tolCc</th>
<th>Host straind</th>
<th>Assay temperature (°C)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPD2794</td>
<td>recA</td>
<td>mcp</td>
<td>Vf</td>
<td>+</td>
<td>Ec RFM443 [15,41]</td>
<td>26</td>
<td>lexA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPD2797</td>
<td>recA</td>
<td>mcp</td>
<td>Vf</td>
<td>−</td>
<td>Ec DE112 [14]</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>DPD1718</td>
<td>recA</td>
<td>ci</td>
<td>Pl</td>
<td>+</td>
<td>Ec DPD1692</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>DPD1714</td>
<td>recA</td>
<td>ci</td>
<td>Pl</td>
<td>+</td>
<td>Ec DM800 [24]</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>DPD1709</td>
<td>recA</td>
<td>ci</td>
<td>Pl</td>
<td>+</td>
<td>Ec DM803 [24]</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>DPD3063</td>
<td>recA</td>
<td>ci</td>
<td>Vf</td>
<td>+</td>
<td>Ec W3110 [25]</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Sal94</td>
<td>recA</td>
<td>mcp</td>
<td>Vf</td>
<td>+</td>
<td>St WG49 [16]</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> mcp: multi-copy plasmid; ci: chromosomal integration.

<sup>b</sup> Vf: V. fischeri; Pl: P. luminescens.

<sup>c</sup> (+) Wild type; (−) mutant.

<sup>d</sup> Ec: E. coli; St: S. typhimurium.
E. coli strain DPD1707 contains a chromosomally integrated fusion of the E. coli recA promoter region to the Photorhabdus luminescens luxCDABE reporter in host strain JC7623 [17]. This strain was constructed by a previously described method [18] using plasmid pDEW14. Plasmid pDEW14 was constructed by ligating a PstI–EcoRI fragment containing a recA–lux fusion from plasmid pDEW65 into plasmid pBRINT.Cm [19]. Previously, plasmid pDEW65 had been made by ligating a fragment containing the recA operator-promoter region into BamHI- and SalI-digested pJT205 [20]. This recA operator-promoter region had been obtained by PCR amplification using pRecALux3 [12] as the template. Strain DPD1707 was used as the donor in the P1-mediated generalized transductions [21] with selection for chloramphenicol resistance to construct E. coli strains DPD1718, DPD1714, and DPD1709.

E. coli strain DPD1718 was constructed by using strain DPD1692 (Lac- Kanamycin-resistant; DuPont Microbial Genetics laboratory collection) as the recipient for the transduction with P1 via phage grown on strain DPD1707. The recA–lux fusion of strain DPD1718 is integrated into the lacZ locus of E. coli and oriented such that the direction of transcription is the same as that of that of the lacZYA operon. Addition of 30 μg/ml nalidixic acid, a DNA gyrase inhibitor that results in potent induction of the SOS response [22], to an exponentially growing culture of E. coli resulted in activation of transcription from the lac promoter, because the magnitude of induction observed with the tested chemicals exceeds that obtained with the lac operon inducer, IPTG.

E. coli strains DPD1714 and DPD1709 were constructed by using strains, DM800 (F-, metA28, lacY1 or Z4, thi-1, xyl-5 or -7, galK2, tss-6) [24] and DM803 (lexA<sup>ind</sup> of DM800) [24], respectively, as the recipients in transductions, with P1<sub>vir</sub> phage grown on strain DPD1707. lexA<sup>ind</sup> encodes a non-cleavable form of the LexA, the repressor of SOS-induced gene expression [22].

E. coli strain DPD3063 contains a chromosomal insertion of the recA promoter region fused to the V. fischeri luxCDABE reporter at the lacZ locus. It was constructed using P1<sub>vir</sub> phage grown on strain DPD3062 as the donor and E. coli strain W3110 (F<sup>+</sup>, prototroph) [25] as the recipient with selection for Kanamycin resistance. Prior to this, E. coli strain DPD3062 had been made by a previously described method [18] using plasmid pDEW100 that had been constructed by ligation of a 8.6-kbp BamHI–PstI fragment of pRecALux3 [12] into plasmid pBRINT.Km [19].

2.2. Experimental conditions, luminescence measurement and data analysis

Prior to the assay, the bacterial tester strains were grown overnight in LB broth [21], with shaking at 26°C or at 37°C, according to the tested strain (Table 1). The cells were then diluted to approximately 10<sup>7</sup> cells/ml and regrown under the same conditions until a cell density of ca. 2 × 10<sup>8</sup> was reached. Kanamycin (25 mg/l) was included in the overnight growth medium, to ensure maintenance of the plasmid, but omitted from the regrowth medium due to an inhibitory effect on luminescence (S. Belkin, unpublished data). For the plasmid-bearing strains, repeated controls demonstrated no loss of kanamycin resistance, and hence, no loss of plasmid, during the short assay period.

All samples tested were at pH 7.0. A twofold dilution series in LB was prepared in opaque white microtiter plates (Dynatech, Chantilly, VA, USA), to a final volume of 50 μl in each of the wells. LB broth served as a control. To all wells, 50 μl of the early exponential cell suspension was added, and the plates were incubated in a temperature-controlled (26°C or 37°C, see Table 1) microtiter plate luminometer (Lucy 1, Anthos Labtech, Salzburg, Austria).

Luminescence values are presented as arbitrary relative light units (RLU), or as the ratio of the luminescence of the induced sample to that of the uninduced control (response ratio) as described pre-
Maximal response ratios are the maximal ratios obtained during the course of an experiment. EC_{200} is the effective sample concentration causing a twofold increase in the maximal response ratio. As previously described [8,29,30], EC_{200} values were determined by plotting a value known as gamma \( (\Gamma) \) as a function of sample concentration. \( \Gamma \) is defined as \( (I_s - I_o)/I_o \), where \( I_s \) is the maximal luminescence obtained for the given sample concentration \( s \), and \( I_o \) is the luminescence of the control at the same time point. When \( \Gamma = 1 \), sample concentration is equal to EC_{200}.

All experiments were run in duplicate, and were repeated at least twice (three times in most cases) at different dates, using different batches of cells. Standard deviation of duplicates was smaller than 5%.

2.3. Alginate immobilization

Exponentially growing culture aliquots (5 ml) were gently mixed with an equal volume of Na-alginate (Sigma), and added dropwise (35–40 \( \mu l \) drop) into a continuously stirred solution of 0.2 M CaCl\(_2\). The Ca-alginate beads which were immediately formed were rinsed for several hours in a large volume of fresh CaCl\(_2\) (0.2 M) at 4\(^\circ\)C, and routinely stored under the same conditions.

2.4. Industrial wastewaters

Wastewater samples were neutralized to pH 7, filter-sterilized (0.22-\( \mu m \) pore size) and kept frozen until tested. Testing was carried out in microtiter plates as in Section 2.2 above, following a twofold dilution series in LB. Toxicity of the samples was determined by the Microtox\textsuperscript{\textregistered} bioassay [31,32], using lyophilized \textit{V. fischeri} preparations purchased from Azur Environmental (Carlsbad, CA, USA). The Microtox\textsuperscript{\textregistered} procedure was adapted to microtiter plate format in a manner similar to that published by Blaise et al. [33].

2.5. Chemicals

All chemicals used were analytical grade. Hydrogen peroxide (H\(_2\)O\(_2\)), mitomycin C (MC), and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) were obtained from Merck, Sigma and Fluca, respectively.

3. Results

3.1. Kinetics of light production and data analysis

Three compounds served for the initial comparison of the various \textit{E. coli} constructs examined in the course of this study: MC, H\(_2\)O\(_2\), and MNNG. To exemplify characteristics of light production by SOS-responsive promoter \(<\textit{lux}\>\textit{bearing}\ E. \textit{coli}\) in the presence of genotoxic hazards, the response of strain DPD3063 to the three model inducers is displayed in Fig. 1. The kinetics of luminescence development at different MC concentrations is presented in Fig. 1A, and the response ratios as a function of concentration for all three compounds are shown in Fig. 1B. A very similar pattern to that emerging from Fig. 1B could be viewed if maximal luminescence values were used rather than response ratios (not shown). In Fig. 1C, the calculation of EC_{200} values from the data in Fig. 1B is presented. These values represent the sample concentration causing a twofold increase [8,29].

![Fig. 1. Genotoxicity determination using the recA\textsuperscript{\textregistered}::lux-harboring strain DPD3063. (A) Kinetics of light development in the presence of increasing MC concentrations. (B) Maximal response ratios as a function of MC, H\(_2\)O\(_2\), and MNNG concentrations. (C) Calculation of EC\(_{200}\) values using the data in panel B. Gamma values are calculated as described under Section 2.](image-url)
increase in luminescence, and are characteristic of each tester strain/genotoxicant couple. When comparing the performance of different strains exposed to the same inducer, lower EC\textsubscript{200} values reflect greater sensitivity and a lower detection threshold of the tested strain; when comparing the response of a single strain to different compounds, lower EC\textsubscript{200} values imply greater genotoxicities of the tested chemicals.

3.2. LexA control of the luminescence response

To confirm that the luminescence response measured in the presence of known DNA-damaging agents is indeed SOS-regulated, we have tested chromosomal integrations of the recA:\textless lux fusion in an isogenic pair of E. coli hosts, DPD1714 (lexA\textsuperscript{+}) and DPD1709 (lexA\textsuperscript{ind}). lexA\textsuperscript{ind} encodes a non-cleavable form of the LexA, the repressor of SOS-induced gene expression. SOS induction requires cleavage of LexA by an activated RecA protein [22]. Both strains were luminescent, but background light emission of the mutant was at least 10-fold lower than that of the wild type. As shown in Fig. 2, which describes the reaction of both constructs to different H\textsubscript{2}O\textsubscript{2} peroxide concentrations, luminescence in the lexA\textsuperscript{ind} mutant was inhibited while that of the lexA\textsuperscript{+} strain was induced. Similar results were obtained for MC and other inducers (not shown), indicating that the luminescent response to genotoxicants of the integrated recA:\textless lux fusion in our constructs is, indeed, mediated by the RecA/LexA SOS system of E. coli.

3.3. Inhibiting efflux capacity by a tolC mutation

One potential difficulty in assessing the toxic potential of either defined chemicals or unknown samples lies in the permeability barriers posed by the bacterial membrane, hindering access of the toxin to the target macromolecule. It has been demonstrated [14] that the use of an E. coli host strain with a tolC\textsuperscript{+} mutation lowered the detection threshold of a sensor strain harboring a grpE:\textless lux fusion. The same approach was utilized in this study; of the isogenic E. coli strains, DPD2794 (tolC\textsuperscript{+}) and DPD2797 (tolC), the latter’s ability to pump out undesired molecules is limited [34]. Fig. 3 presents the response of both constructs to H\textsubscript{2}O\textsubscript{2} and MC. For the latter compound, but not for the former, the tolC mutation had a substantial effect on the response threshold, and thus on detection sensitivity.

3.4. Replicon for promoter:\textless lux fusion: multi-copy plasmid or a chromosomal integration

The original bioluminescent genotoxicity sensor strains [12] harbored the lux fusion on a multi-copy plasmid, thus enhancing the intensity of the observed response but introducing a potential instability into the maintenance of the extrachromosomal genetic element. Another possible disadvantage of a multi-copy plasmid-based fusion is a loss of responsiveness to the regulatory element due to a titration effect of hundreds of fusions containing operator-promoters on a fixed level of repressor. Strain DPD3063 (Fig. 1) chromosomally integrates a single...
copy of the same recA::lux segment found in the plasmid-borne multi-copy fusion strain DPD2794. The single-copy chromosomal integration generally led to a somewhat longer lag period, to lower luminescence levels (in both non-induced and induced states), to increased response ratios and to enhanced sensitivities. The two latter points — both direct consequences of the decreased background luminescence — are demonstrated in Fig. 4, which compares the response ratios of strain DPD3063 (chromosomal integration) to that of DPD2794 (multi-copy plasmid) in the presence of different MC concentrations.

3.5. Expanding temperature range by the use of a different reporter: P. luminescens instead of V. fischeri lux

One of the problems often accompanying the use of luminescent E. coli is the necessary compromise in working temperature: the optimal expression temperature of the V. fischeri luminescence enzymes is much lower than the 37°C required for "normal" E. coli functions. A solution may be provided by the lux system of P. luminescens [35], which readily operates at this temperature. As described under Section 2, P. luminescens luxCDABE was therefore fused to the E. coli recA promoter and chromosomally integrated to yield strain DPD1718. When the 37°C response of DPD1718 to the three model inducers was compared to that of DPD3063 at 26°C, an advantage in response time was clearly apparent. This is demonstrated in Fig. 5 for MC. When both strains were compared at 26°C, DPD1718 still exhibited faster responses than DPD3063, although the differences were less pronounced (not shown). At both temperatures, DPD1718 displayed a higher background luminescence (not shown), leading to lower response ratios and thus to a higher detection threshold. The advantage in response times was thus offset by a reduced sensitivity.

3.6. Changing the microbial host from E. coli to S. typhimurium

While E. coli is a very convenient test organism, the use of S. typhimurium as a sensor strain seemed attractive due to its general acceptance as a mutagenicity assay organism in the histidine reversion test [3], as well as to its current use in the Vitotox® assay [10,11]. S. typhimurium strain Sal94 was therefore constructed, containing the same recA::lux-bearing plasmid as E. coli DPD2794. Regardless of the inducer, Sal94 reproducibly displayed a much faster response (Fig. 6); it also exhibited an increased sensitivity to H2O2, but a lowered one to MC and MNNG (not shown).

3.7. Amenability to polymer immobilization

Several options can be envisioned for on-line application of microbial sensor cells to real-time...
genotoxicity monitoring; one of these includes immobilization in a polymer matrix. In a preliminary attempt to determine if these genotoxicant-responsive strains are amenable to such treatment, tester cultures were encapsulated in alginate and subjected to several maintenance regimes. When stored in a CaCl₂ solution at 4°C, the cells remained active for prolonged periods, but at the cost of an increasingly delayed response and reduced luminescence intensity. Fig. 7 summarizes the results of one such experiment carried out with strain DPD2797 (tolC, V. fischeri lux fusion on a multi-copy plasmid). Over the first month, maximal luminescence values and response ratios decreased only marginally, while the lag period doubled from 1 to 2 h. EC₂₀₀ values were not significantly affected during this initial storage time (not shown). During the second month, all three parameters deteriorated, and after 60 days, the response ratio elicited by 100 μg/l MC decreased to approximately 1.8. After the third month, although the cells were still luminescent, the reaction to MC was very weak and delayed by over 5 h.

3.8. Detection threshold for other chemicals

Table 2 displays EC₂₀₀ data for an expanded list of chemicals, most of them known or suspected genotoxicants [36,37], obtained by the use of selected strains from among those described in Table 1. In addition to the three model compounds used in the examples presented above, Table 2 contains representatives of two groups of environmentally significant chemicals: phenol and some derivatives, and halomethanes. At least for the compounds in Table 2, two strains stand out with the lowest detection levels: DPD3063 and DPD2797. While these strains exhibited positive responses to several Ames-negative compounds, except for one case, all of these chemicals were previously recorded as potential genotoxicants by at least one other established bioassay. For the one exception in Table 2, the Ames-negative 2,3 dichlorophenol, no conclusive data could be found to prove or disprove its genotoxic potential. There were no false-negatives among the tested chemicals; furthermore, numerous other Ames-negative compounds, with no recorded genotoxic activity, did not induce any of the recA<sup>·</sup>:lux tester strains. The list included various inorganic Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> salts, organic acids (acetic, salicylic), solvents (methanol, hexane, decane, p-xylene, 1,1 dichloromethane), urea, chlorinated benzenes, sugars (glucose, sucrose) amino acids (glycine, alanine) and more.

3.9. Chemical industrial wastewaters

Even ideal responses to solutions of defined chemicals, as sensitive as they may be, do not guarantee satisfactory performance with the complex samples often encountered in the course of environmental monitoring. Selected tester strains were therefore tested using complex samples from a large number of industrial wastewaters sources, and an example of the data obtained is presented in Fig. 8. The tested set included 20 discrete samples collected...
### Table 2
EC\textsubscript{200} values for selected chemicals

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS number</th>
<th>Tester strain\textsuperscript{a}</th>
<th>EC\textsubscript{200} (mg/l)</th>
<th>Ames test\textsuperscript{c}</th>
<th>Other genotoxicity tests\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>50-07-7</td>
<td>DPD3063</td>
<td>$10^{-1} \pm 8 \times 10^{-3}$</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>MNNG</td>
<td>70-25-7</td>
<td>DPD2797</td>
<td>0.05 $\pm$ 0.025</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>7722-84-1</td>
<td>DPD3063</td>
<td>0.8 $\pm$ 0.5</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Phenol</td>
<td>108-95-2</td>
<td>Sal94</td>
<td>16</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>2,4,6 Trichlorophenol</td>
<td>88-06-2</td>
<td>DPD2822</td>
<td>$10 \pm 3.5$</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>100-02-7</td>
<td>Sal94</td>
<td>0.25</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>87-86-5</td>
<td>DPD3063</td>
<td>0.008</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>Chlorodibromomethane</td>
<td>124-48-1</td>
<td>DPD3063/DPD2797</td>
<td>$20 \pm 8$</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>Bromodichloromethane</td>
<td>75-24-4</td>
<td>DPD2797</td>
<td>100</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chloroform</td>
<td>67-66-3</td>
<td>DPD3063</td>
<td>300</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Strain for which lowest EC\textsubscript{200} values were obtained.

\textsuperscript{b}EC\textsubscript{200} values for the strain in the previous column. Data represent average $\pm$ standard error (for three or more independent experiments) or average only (only two independent experiments). Each independent experiment was carried out in duplicate.

\textsuperscript{c}Data from Zeiger [37]. (+) Positive response; (−) negative response.

\textsuperscript{d}Data from Zeiger [37]. (+) Positive response in at least one of the following assays: cultured Chinese hamster in vitro chromosome aberration, in vitro Chinese hamster sister chromatid exchange, mouse lymphoma cell mutation, or Drosophila sex-linked recessive lethality; ND — no data.

In 10 consecutive days of February and March 1994, from both the influent and the effluent of a biological (activated sludge) treatment plant of the wastewaters of a chemical manufacturing facility. The influents were characterized by an average dissolved organic load of 640 mg C/l, a total dissolved solids content of 1050 mg/l, and a relatively high Microtox\textsuperscript{b} [31,32] toxicity (EC\textsubscript{50}: 1.5%). The effluents, in contrast, did not display any Microtox\textsuperscript{b} toxicity, and while their mineral contact remained unchanged, the dissolved organic carbon was reduced, on the average, to 18 mg/l. Fig. 8 presents the induction ratios of strain DPD2794 in response to this sample series (all at 10% concentration). All crude wastewater samples displayed a variable but clear SOS inductive effect, thus indicating a potential genotoxicity; this activity, however, was completely eliminated by the biological activity in the treatment plant. At least by our assay, the effluents did not contain the genotoxic hazards present in the raw influents, which were either degraded or neutralized during the treatment process.

#### 4. Discussion

While the need for sensitive, rapid, and cost-effective means for eco-genotoxicology monitoring is obvious, it is also clear that no single test can provide a definite and comprehensive picture of the genotoxic hazards inherent in a sample. As in acute toxicity bioassays, therefore, a “battery approach” may be needed for a complete coverage of all potential risks, although this may be hampered by cost considerations. Out of presently available methodologies, those based on reporter gene fusions to SOS promoters appear, in principle, to be highly cost-effective, at least for screening purposes. Though SOS
activation in itself does not imply mutagenicity, the two activities are certainly correlated [4]. The SOS Chromotest [4] and _umu_ test [6] both utilize this phenomenon: following an incubation period of a fixed length, a colorimetric assay is performed to quantify the β-galactosidase activity driven by SOS induction.

This communication describes developments in the use of a different reporter for SOS activation: microbial bioluminescence (_lux_ genes). Past reports [9–12] have described different pathways in the choice of the three major system components: SOS operator-promoter, _lux_ reporter and host organism. The end result, however, was similar in all cases: plasmid-bearing bacterial strains capable of sensitive bioluminescent reporting on the presence of DNA-damaging agents. US and European patents pertaining to the use of _lux_ reporter technology in this manner have recently been granted [38,39], and one of the tests described — Vitotox® — has recently become commercially available.

Our original report [12] described the use, in _E. coli_, of either _recA_ or _uvaA_ promoters with _V. fischeri_’s _luxCDABE_. Strain DPD2794, bearing the _recA::lux_ fusion on a multi-copy plasmid, has recently been shown [40] to distinctly differentiate between direct and indirect damaging agents. In an attempt to improve SOS responsive operator-promoter::_luxCDABE_ fusions, several modifications were investigated.

### 4.1. Change of host organism to an _E. coli_ _tolC_ mutant

This mutation [14,34] apparently affects the efflux capacity of _E. coli_ cells, thereby allowing higher-than-normal internal concentrations of toxic compounds which would otherwise be excluded from the cytoplasm. In such mutants, exposure to compounds with permeability limitations, due to, e.g., hydrophobicity, was expected to be facilitated. Indeed, as shown in Fig. 3, this was the case for MC, the detection threshold for which was lowered at least 10-fold to below 1 μg/l. As was to be expected, there was no effect on the response to _H_2O_2_, signifying that the improvement in sensitivity is limited to certain classes of chemicals. Different types of permeability mutations, singly or in combination, may be used in the future to endow the tester strains with a broader spectrum of enhanced sensitivity.

### 4.2. Change of reporter genes from _V. fischeri_ to _P. luminescens lux_

The main purpose of this step was to expand the temperature range of the reporter proteins into that optimal for _E. coli_. The higher temperature caused a faster induction of the SOS system and thus of the _lux_ operon, leading to a faster luminescence response; this advantage, however, was offset by much higher background luminescence, which significantly affected the sensitivity of the system.

### 4.3. Integration of the promoter::_lux_ fusion, previously on a multi-copy plasmid, into the bacterial chromosome

While a longer lag period preceded the induction of the single-copy chromosomally integrated fusion, response ratios were increased and sensitivities were enhanced — mostly due to a greatly reduced background luminescence. In addition to providing an inherently more stable system, therefore, this modification led to a generally more satisfactory performance.

### 4.4. Using a different bacterium as host

The attraction of this modification was in the use of _S. typhimurium_, the Ames test organism, instead of _E. coli_. While the faster response is clearly attractive, the observed variable effect on detection threshold makes this advantage questionable in our system.

Some of the modifications, therefore, led to distinct improvements while the effect of others was variable and genotoxicant-specific. The chromosomal integration, the _tolC_ mutation and, to some degree also, the _P. luminescens lux_ operon were clearly advantageous. Strains containing combinations of these characteristics were therefore selected for further studies, which included a more expanded list of chemicals, some of them known genotoxicants. Of the different variations we have constructed and tested, two _E. coli_ constructs stand out as of highest potential applicability: DPD3063, containing...
a single chromosomal integration of the recA::lux fusion, and DPD2797, a tolC mutant of the multicopy plasmid-borne fusion. The detection thresholds presented for these strains in Table 2 compare very favorably with those obtained, with greater cost and effort, using accepted methodologies. Strain DPD2794, when tested against a series of industrial wastewater samples, proved the potential usefulness for practically real-time monitoring of aquatic samples of environmental significance.

In general, our results clearly indicate the high applicability of the concept to the monitoring of genotoxicity. The assays are very simple, the results are rapidly available and the sensitivities are at least in the same order of magnitude as those of more traditional methodologies. Our preliminary immobilization experiments also point at one of several potential formats by which the cells can be used in a future on-line system. Nevertheless, extensive studies need to be carried out before such strains can be proposed for general use, and further modifications may be required in all system components.

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