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Cell damage detection using *Escherichia coli* reporter plasmids: fluorescent and colorimetric assays

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Abstract Bacterial reporter assays are powerful tools used to study the effect of different compounds that affect the physiology of cellular processes. Most bacterial reporters are luciferase based and can be monitored in real time. In the present study we designed and implemented two sets of *Escherichia coli* bacterial reporter assays, using a multicopy plasmid system. Each reporter strain was constructed using either green fluorescent protein or β-galactosidase (LacZ) proteins. The designed reporter strains are capable of responding in a specific manner to molecules that either oxidative stress, or membrane, protein, or DNA damage. In order to respond to the desired stimulus, promoter sequences from *E. coli* were used. These sequences correspond to the promoter of the major catalase (KatG) activated with cellular oxidative damage, the promoter of the β-hydroxydecanoyl-ACP dehydrase (FabA) which is activated with membrane perturbation, the promoter of DNA recombinase (RecA) which is activated by DNA lesions. For protein misfolding, the promoter of the heat-shock responsive chaperon (DnaK) was used. Our constructs displayed activation to damage from specific stimuli, and low response to nonspecific stimuli was detected. Our results suggest that these types of bacterial reporter strains can be used in semiquantitative (fluorometric) and qualitative (β-galactosidase activity) studies of different xenobiotic substances and pollutants.

Keywords Cellular damage · DNA damage · *Escherichia coli* · β-Galactosidase · Green fluorescent protein · Membrane damage · Oxidative damage · Protein damage

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Introduction

Since the emergence of recombinant DNA technology, scientists have being able to achieve the control of transcriptional regulatory circuits and gene expression, modifying organisms to respond to environmental changes. Whole-cell reporter assays have become powerful tools to address important toxicological, environmental and physiological questions (Raut et al. 2012). They can shed light on the primary action mechanism of antibiotics, anti-microbial compounds and xenobiotics. Likewise, whole-cell reporter assays can help evaluate the toxicity of several chemicals and the fraction of these pollutants that interact with living organisms.

Bacterial reporter strains are simple to generate, fast and reliable tools that use expression levels of induced genes that are activated with certain stimulus and are useful to understand the behavior of bacteria in contaminated soil or water (Robbens et al. 2010). Bacterial reporter strains offer the possibility of measuring in real time the effect of toxic compounds and may be used to assess the cell targets of novel molecules as well as in automated systems (Jones et al. 2013).

In the less complex bacterial reporter design, a sensor module, capable of activation upon interaction with the desired stimulus, is fused to an output module, usually a

reporter protein such as β-galactosidase, luciferase or fluorescent proteins. A sensor module usually comprises a well-characterized promoter sequence and its regulatory elements (Raut et al. 2012; Gu et al. 2004). This system has been applied to many organisms, both prokaryotic and eukaryotic to detect different compounds (for review Daunert et al. 2000). The best output module genes are easy to detect and, using the data collected as input, qualitative and semiquantitative data can be generated and can even be coupled to a portable device (Truffer et al. 2014).

Escherichia coli is a Gram-negative bacterium that represents a landmark in genetic manipulation. The profound knowledge that has been gained about the metabolism and genetics of this bacterium make it the ideal host for plasmid-based reporter constructs (Robbens et al. 2010; Vangnai et al. 2012). Previously, the promoters of *katG*, *fabA*, *recA* and *dnaK* genes have been used as sensors to monitor oxidative stress generation, membrane damage (fatty acid metabolism), DNA damage and protein damage, respectively (Van Dyk et al. 1994; Belkin et al. 1996; Vollmer et al. 1997; Bechor et al. 2002; Lu et al. 2005). By fusing these promoter sequences to the *lux* reporter operon, it has been shown that the activation of these sensing system sequences is stimulus specific (Van Dyk et al. 1994; Belkin et al. 1996; Vollmer et al. 1997; Bechor et al. 2002). Most of the studies that used reporter constructs were only tested with the stimulus that is specific for the reporter strain generated or exhibited similar effects, although cross-reactivity is rarely evaluated. Using these promoters in bacterial reporter constructs can allow researchers and environmental monitoring agencies to determine the primary cell damage effect of xenobiotics, pollutants and other molecules that represent potential hazards to living organisms.

Plasmids harboring the *lux* operon can be integrated as single-copy reporter constructs or as multicopy plasmids. However, the length of the construct may render problems such as transformation efficiency or plasmid stability. Luminescent reactions can be detected easily, but the enzyme is temperature sensitive, linear range is narrow, and if the signal is poor, detection may become difficult (Roelant et al. 1996; Daunert et al. 2000; Kohlmeier et al. 2007). If luciferase alone is used, detection requires complex reaction mixtures, exogenous substrate addition, and thus, the assay becomes time-consuming and destructive for the cell sample (Daunert et al. 2000). Therefore, individual data points must be obtained in order to achieve continuous expression patterns.

Green fluorescent protein (GFP) is an alternative to such problems since it does not require any added substrate. It is a stable protein and can monitor single cell activity, although background fluorescence and cell toxicity can be observed. The constructs can be easily obtained and are stable. The detection and semiquantitative measurements can be obtained with a standard fluorometer.

In the present work we developed and characterized a set of GFP- and LacZ-based plasmid reporter strains capable of sensing oxidative, membrane, protein and DNA damage in a specific manner. The designed reporter strains are easy to use, stable and can be obtained in a short period of time. These reporter strains can be useful in gaining knowledge of the primary mechanism of action of cell-damaging compounds and materials.

Materials and methods

Cell growth

Transformant *E. coli* XL1-Blue cells (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacI^{q}ZΔM15 Tn10 (Tet^r)], Stratagene) were grown in Luria–Bertani medium containing 100 μg ampicillin/ml. For fluorometric assays, cells were resuspended in M9 minimal medium and supplemented with 100 μg ampicillin/ml (Affymetrix).

Plasmid constructs

Promoter and reporter gene fusions were constructed using pGlow and pBlue plasmids (Invitrogen), following the manufacturer's instructions. pGlow plasmid fuses reporter promoters to cycle3 mutant version of GFP (Crameri et al. 1996). Promoters were PCR amplified using the primers listed in Table 1. They were designed based on previously reported sequences (Van Dyk et al. 1994; Belkin et al. 1996; Vollmer et al. 1997; Bechor et al. 2002) except for the 3′ modifications which were required to maintain the reading frame of either GFP or LacZ in the pGlow and pBlue plasmids, respectively. PCR conditions were as follows: Reactions were carried using 0.5 μM of each primer, 20 ng of *E. coli* total DNA and 1X GoTaq Green master mix (Promega), in a total reaction volume of 20 μl; PCR program: 94 °C for 3 min, 30 cycles of 94 °C for 1 min, alignment for KatG 62.5 °C, DnaK 40.0 °C, FabA 51.0 °C and RecA 53.0 °C for 30 s, and 72 °C for 30 s; finally, 1 cycle of 5 min at 72 and 4 °C to end the program. Plasmid DNA for sequencing was purified using Wizard plus SV Miniprep DNA purification kit following the manufacturer's instructions (Promega). All plasmids were sequenced using T7 primer. Transformants were kept as glycerol stocks at −70 °C. All molecular biology techniques used were as previously described (Sambrook and Russell 2001).

Table 1 Primers used in the present study

Promoter	Primer name	Sequence (5′ → 3′)	Tm (°C)	Product size* (bp)
KatG (catalase/oxidative stress)	KatGFw	TAACTGCAGCGAAATGAGGGCGGGAAA	64.3	264
	KatGpGlow	GCATCAATGTGCTCCCCTCT	62.5	
DnaK (chaperon/protein damage)	DnaKFw	AGCCTGCAGAAAAGCACAAAAAAT	40.2	183
	DnaKpGlow	CCATCTAAACGTCTCCAC	51.0	
FabA (β-hydroxydecanoyl-ACP dehydrase/membrane damage)	FabAFw	TAACTGCAGGCCATTACGTTGGCTGAA	58.2	109
	FabApGlow	CCATGTTCTCTGTAAGCC	51.2	
RecA (recombinase/DNA damage)	RecAFw	TAACTGCAGAGAGAAGCCTGTCGGCAC	59.1	243
	RecApGlow	CCATTTTTACTCCTGTCATG	53.1	

* *katG* promoter spans from −261 to +3. *dnaK* promoter spans from −179 to +4. *fabA* promoter spans from −105 to +4. *recA* promoter spans from −239 to +4. Only in the case of *katG* an extra G was added at the 3′ end to preserve the reading frame in pGlow and pBlue plasmids

Fluorometric assays for GFP

Fluorescence was determined using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific). Briefly, bacterial cells were grown to an OD_{600} of ~0.5 to avoid differences due to cell growth. Cells (1 ml) were transferred to a 1.5-ml micro centrifuge tube and collected for 5 min at 3000 rpm. Pelleted cells were resuspended in 1 ml of M9 minimal medium supplemented with 100 μg/ml ampicillin; the cell suspension was transferred into a 96-well black plate (Thermo Scientific). M9 minimal medium was used since LB lowered the fluorescence intensity (data not shown). Hundred microliter of M9 minimal medium was used as blank reading and was subtracted for total fluorescence intensity determination. GFP fluorescence was recorded using 395 nm as primary excitation and emission at 507 nm. Under the detection conditions used, we verified that the stimuli used did not exhibited fluorescent signal (data not shown). Untransformed *E. coli* cells were also tested and did not show significant fluorescence signal (data not shown). Induction of the reporters was achieved by adding different concentrations of a specific stimulus to each reporter strain and measured for different time intervals. The concentrations used were under the same range as previously reported (Van Dyk et al. 1994; Vollmer et al. 1997; Bechor et al. 2002; Lu et al. 2005), and SDS was used in accordance to the sub lethal concentrations used in (aWakayama et al. 1984). Figures 1 and 2 show a representative example of four independent experiments with no more than 5 % error between replicates. Data collection was determined on the time where the maximal activation was observed for each reporter, except for the cross-reaction experiments, where data points were collected for the same time interval. Total fluorescence intensity minus blank signal is shown as Fluorescence Units and plotted using Excel software (Microsoft). All samples were grown to the same cell density so that the activation profile was not due to differences in cell growth.

Viability assay

To determine qualitatively the amount of viable cells remaining after each treatment with stimulus-specific chemicals in each fluorometric assay, we conducted a spot test. It consisted of plating serial dilutions of cell suspensions previously exposed either to control stimulus (water) or to the specific stimulus. Three microliter of these cell suspensions was spotted on LB agar plates containing 100 μg ampicillin/ml.

β-Galactosidase enzymatic assay

β-Galactosidase activity was measured qualitatively. Biodisk activity assays were done as follows: 200 μl of cells grown in LB to an OD_{600} of ~0.5 were overlay plated using 5 ml LB agar (0.6 % w/v) on an LB plate containing 40 μg X-gal/ml and 100 μg ampicillin/ml plate. On top of the soft agar, five filter paper disks were layered at even distances; the maximal concentrations of stimulus used in the fluorometric assays were added (10 μl); and water was used as control. Plates were incubated at 37 °C. Blue halos were visualized 24 h after exposure and recorded using an Image Station 2000R (KODAK).

Results and discussion

Reporter characterization

Bacterial reporters can elicit different responses according to the concentration and the chemical nature of the stimulus. Therefore, it is crucial to design reporter plasmids that are easy to construct, sensitive, specific and with reduced cross-reactivity with other molecules. Most bacterial reporter strains are thoroughly tested to characterize the specific response to which they were designed for. Nevertheless, less effort is conducted to

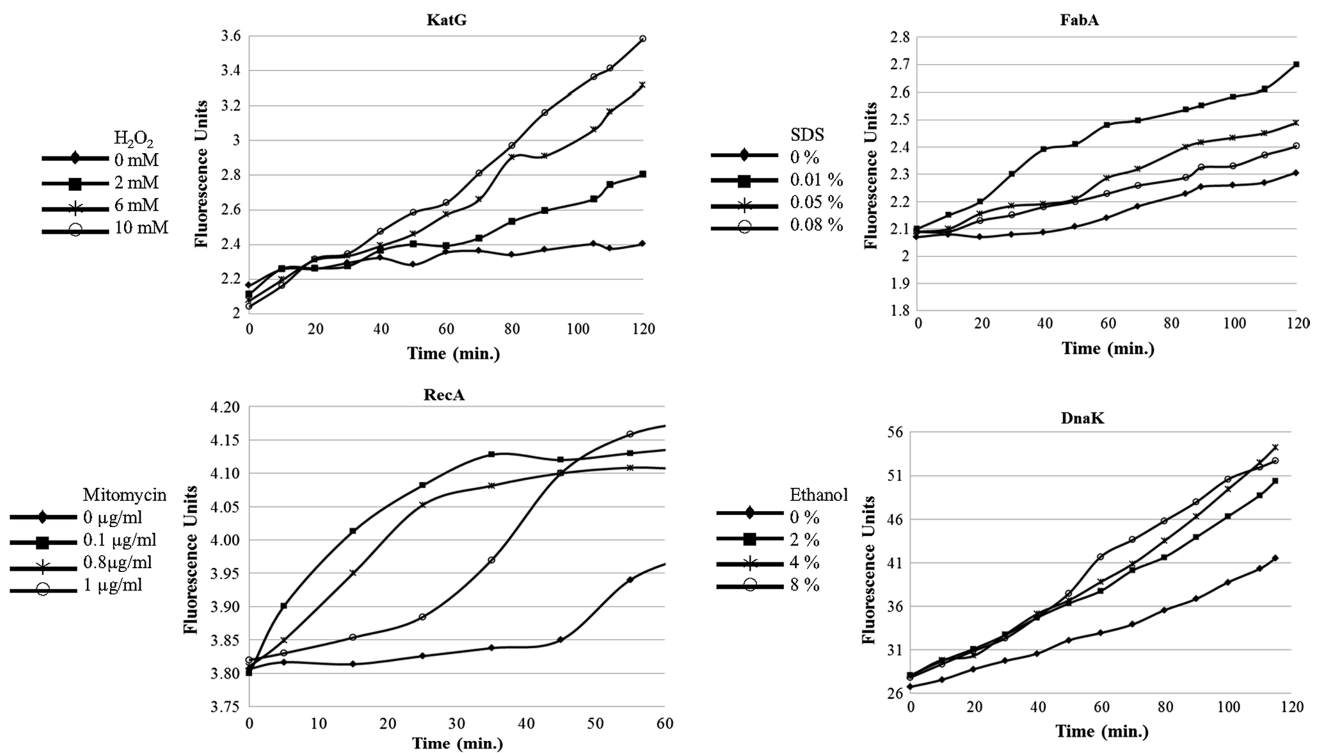

Fig. 1 Fluorometric analyses of the generated reporter strains. A representative experiment for each condition is shown. All experiments were done in quadruplicates. Fluorometric analyses with specific stimuli for KatG, FabA, RecA and DnaK reporter strains

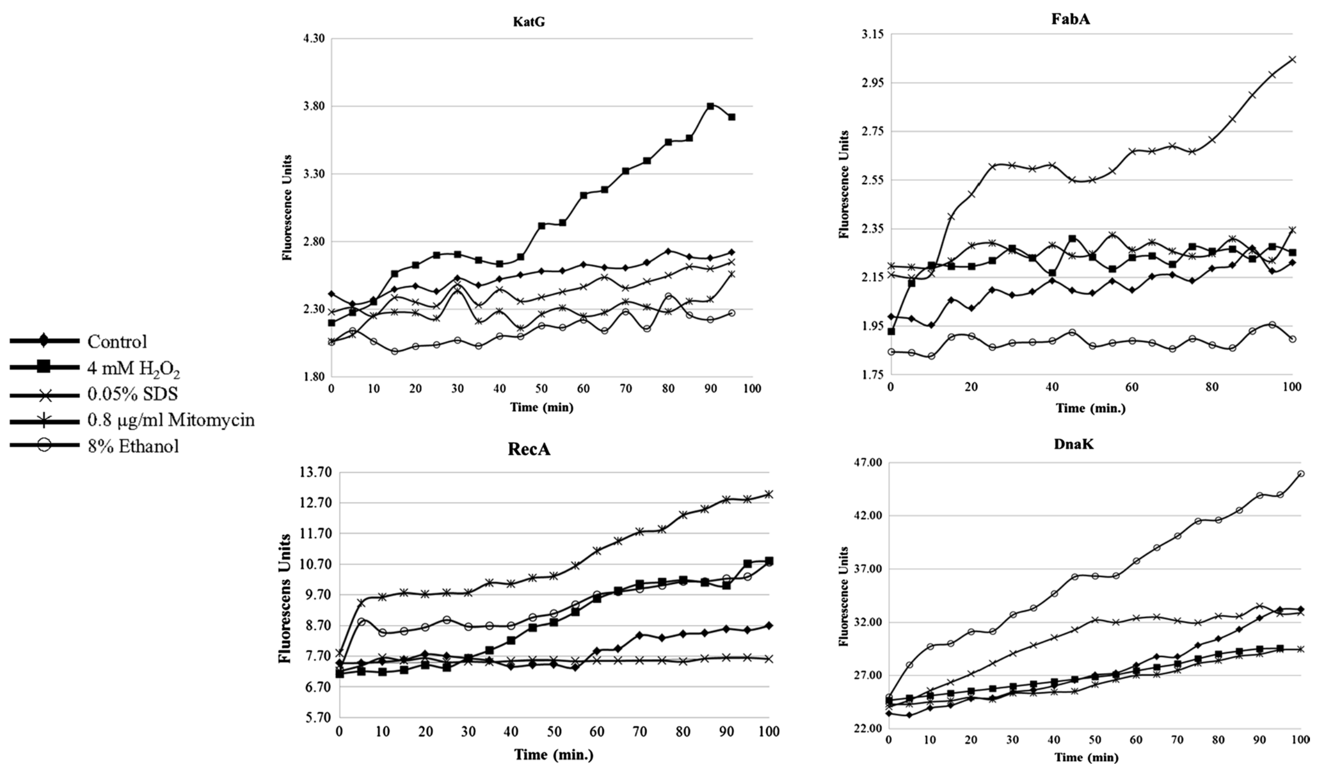

Fig. 2 Fluorometric analyses testing cross-reactivity of *KatG*, *FabA*, *RecA and DnaK* reporter strains. *Legends* indicate the stimuli and concentrations used. A representative experiment for each condition is shown. All experiments were done in quadruplicates

determine which other molecules they are capable of activating response in the designed strain. Hence, we conducted an analysis regarding the expression threshold of each reporter strain, using the specific stimulus toward which it was initially designed against. Also, the capacity of each reporter strain to react to different stimuli was assayed. XL1-blue strain was used since it carries a *rec*A mutation that prevents recombination, thus impeding plasmid loss. RecA expression is under the control of LexA; therefore, we did not expect any expression problems using this strain with the reporter plasmids, and similar results were obtained using DH5α and MG1655 (data not shown). Strains carrying the same mutation have been used previously (Lu et al. 2005).

We tested the reporter strains ability to react with specific cell-damaging molecules. Results shown in Fig. 1 indicate that the reporter strains exhibited specific response to known stimulus affecting cellular components that activated the promoter sequences used at similar concentrations previously tested (Van Dyk et al. 1994; Belkin et al. 1996; Vollmer et al. 1997; Bechor et al. 2002). The reporter strains responded to the agents that activate the specific response against which they were designed for, and in longer time courses no decay of signal was observed (data not shown). Stimuli used H_2O_2, which induces oxidative stress (KatG); ethanol, which induces protein misfolding in a similar way heat shock does (DnaK); SDS, which induces membrane disruption (FabA) and mitomycin, which induces DNA alkylation induced damage (RecA). Reporter strains showed moderate increasing fluorescence in the control conditions during each assay. We hypothesized that this may be due to the cellular requirement of these proteins due to cell-growth-related stress; upon addition of stressing agents transcription is enhanced. The effect observed with SDS at the highest concentration tested may be related to leakage of cellular components where the GFP fluorescence may be reduced. This result is in contrast to other obtained using *lux* reporter (Bechor et al. 2002). DnaK reporter strain exhibited similar activation response to ethanol concentrations, which may be related to the effect on the stability of GFP protein in the presence of ethanol, in contrast to the observed behavior with the *lux* reporter (Van Dyk et al. 1994). Although, DnaK reporter strain is activated with protein-damaging agents and can be used in the characterization of molecules that renders either protein misfolding or degradation (Neidhardt and VanBogelen 1987). This behavior is observed in reporter systems at high concentrations of the stressing agent. RecA-delayed activation using the highest mitomycin concentration may be related to transcription inhibition caused by SOS response activation.

Reporter activation with other stimuli

We conducted reporter activation tests with all the stimuli used in the concentrations that render the highest activity with the cognate reporter strain (Fig. 1). As shown in Fig. 2, KatG reporter strain showed no activation upon addition of the noncorresponding stimulus in the time frame tested. DnaK reporter strain showed 50 % activation with SDS, which is expected since this detergent can induce protein misfolding. RecA reporter showed activation upon addition of ethanol and H_2O_2. Hydrogen peroxide is a well-known DNA-damaging agent (Imlay 2013), and we found activation of the RecA reporter after 30-min exposure to 4 mM (Fig. 2). Ethanol activation of the RecA promoter may be explained since it has heat-shock-like effect on protein folding (Neidhardt and VanBogelen 1987), affecting protein stability of enzymes involved in DNA replication and repair. Also, the metabolic by-products of ethanol can also induce oxidative DNA damage (Park et al. 2012). This mechanism can be ruled out since no activation of the KatG reporter strain was observed. There is limited evidence that ethanol is mutagenic (de Flora et al. 1984), and the RecA reporter plasmid is activated by ethanol, not to the extent observed with mitomycin but at the same activation level observed with H_2O_2. This observation needs further examination using different experimental approaches to determine the extent of DNA damage on cells exposed to different concentrations of ethanol.

Overall, the reporter strains displayed a specific response to the stimulus that caused the specific cell component damage to which they were designed to detect and limited cross-reaction was observed due to similarities in cell-damaging mechanisms among chemicals tested. These kinds of assays are important to determine the specific cellular component affected when testing compounds of unknown chemical nature or mechanism of action on cells.

Viability assay

FabA, RecA and DnaK reporter strains exhibited moderate activation without stimuli; this can be explained either by GFP accumulation during plate reading or by cell viability during exposure to the chemicals tested, rendering lower GFP production over time. To rule out the effect of cell viability upon exposure to the stimuli tested in the fluorometric readings, we used a cell viability test (Fig. 3 panel a). Cells incubated the same time as in the fluorometric assays were serially diluted and spot plated. As shown in Fig. 3 panel a, cell viability remained unchanged for H_2O_2, ethanol, mitomycin and SDS with its corresponding reporter strain. Therefore, the activation observed in control and experimental conditions is due to viable cells and not to cell debris or other cell components that may increase

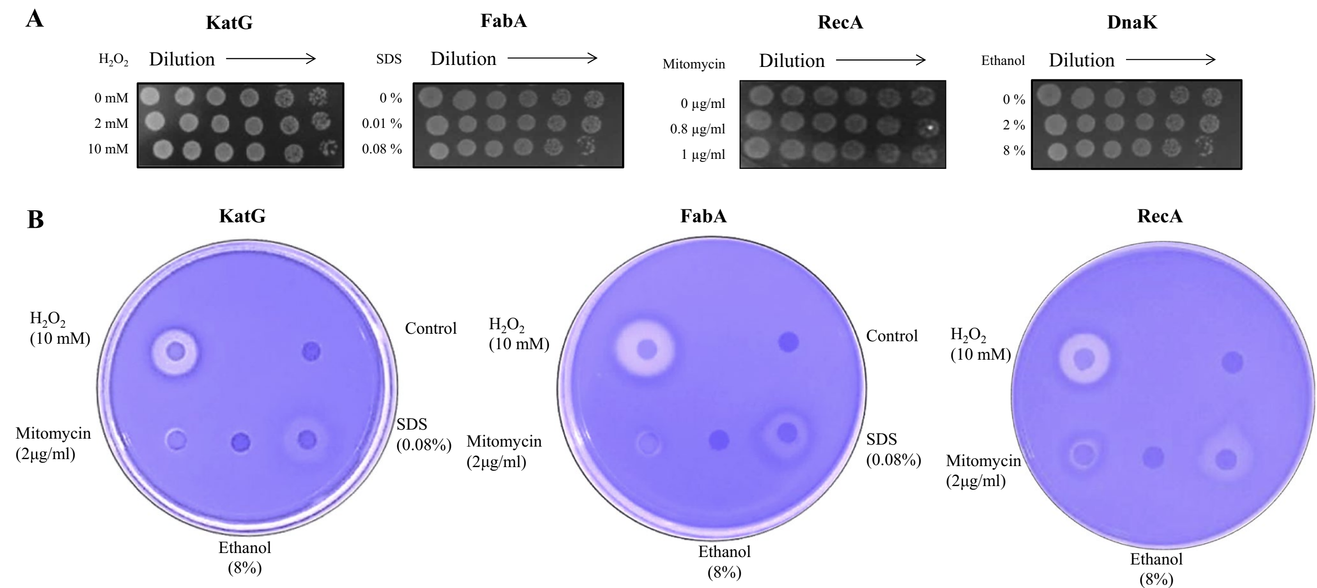

Fig. 3 Cell viability and qualitative expression analyses of the generated reporter strains. A representative experiment of each condition is shown. All experiments were done in quadruplicates. **a** Cell viability analyses of the reporter strains exposed to specific stimuli; incubation time for each strain was the same as in fluorometric analyses. **b** β-Galactosidase activity of KatG, FabA and RecA reporter strains

fluorescent signal. In control reactions, increasing fluorescent readings is likely to be related to GFP accumulation during the assay rather than unspecific activation.

Qualitative assays

Rapid and easy cell toxicity assays are needed to assess the presence of hazardous pollutants in soil and surface water systems. In this study, a fast and easy alternative assay based on β-galactosidase reporter was developed using KatG, FabA and RecA promoter sequences. Results are shown in Fig. 3 panel b. Strains harboring specific promoter–LacZ fusions were tested using biodisk tests (as described in Materials and Methods). We detected to types of halo, clear areas related to cell death due to the chemical used, differing from spot test since the stimulus is diluted once with LB and when cells are spotted on the plate is once more diluted in the plate media. In the biodisk assays the stimulus remains longer due to the paper filters and the volume used (10 μl). The second halo (deep blue color) observed corresponds to the activation of the reporter strain. Our results show that the reporter strains were activated by specific stimulus as in the fluorometric assays and showed cross-reactivity with certain stimuli also observed in the fluorometric assays. DnaK–LacZ reporter exhibited high background activity masking the activation halo (data not shown). As shown in Figs. 1 and 2, DnaK reporter strain had the highest GFP activity; this explains the high background activity for the LacZ reporter. The cross-reactivity shown by these reporter strains may be related to the longer exposure times than those used in the fluorometric assays. Quantitative measurements confirmed that activation took place upon the addition of the corresponding stimulus (data not shown). Cross-reactivity was found in KatG, FabA and RecA reporter strains with H_2O_2. For FabA, this cross-reactivity may be related to partial cell lysis and membrane lipid peroxidation (Imlay 2013). Activation of RecA reporter with H_2O_2 was observed since hydroxy radicals generate DNA lesions (Imlay 2013) and is in concordance with the result obtained in the fluorometric assays.

Qualitative reporter strains can be readily applied to screen the effect of several compounds on cell physiology at the same time and is prone to automatization. Qualitative reporter strains can readily be applied to environmental monitoring laboratories with limited equipment.

Conclusions

Reporter strain design needs no longer be cumbersome. Given the improvements in the present study, it can lead to automated portable devices that can render reliable and accurate results. In this study we developed two types of reporter strain assays that were capable of specific responses to different kinds of cell-damage-inducing agents. GFP-based reporter strains exhibited specificity and sensitivity toward the stimulus to which they were designed, except in those cases where the chemical used were capable of damaging the same target.

β-Galactosidase-based reporter strains also gave fast and reproducible results. These reporter strains can be used in less equipped laboratories and render equally reliable and fast results for environmental samples potentially contaminated with toxic compounds. Likewise, β-galactosidase-based reporter strains can provide information of several compounds at the same time and test for anti-microbial activity, primary mechanism of action, cross-reactivity in a single assay between several compounds, allowing the assessment of the potential hazardous effects of several compounds simultaneously.

Reporter strains can be used to study the bioavailability of toxic compounds to elucidate the primary mechanism of action of several anti-microbial agents and in the assessment of environmental pollution.

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Conflict of interest The authors declare no commercial or financial conflict of interest.

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