Communications

A Dual-Color Bacterial Reporter Strain for the Detection of Toxic and Genotoxic Effects

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To genetically engineer a bacterial whole cell dual-function toxicity/genotoxicity bioreporter system, a plasmid was constructed containing two independent fusions of stress-responsive promoters (of the recA and grpE genes) to green and red fluorescent protein reporter genes, respectively. An Escherichia coli strain harboring this plasmid exhibited distinct green fluorescence in response to the presence of the SOS inducing agent nalidixic acid, and red fluorescence in reaction to ethanol. The different fluorescent responses, which exhibited little or no overlap, were quantified by microtiter plate fluorometry, confocal microscopy, and fluorescence emission spectroscopy. Mutations in lexA and rpoH, which affected the E. coli SOS and heat shock systems, respectively, abolished the green and red fluorescence. Similar constructs may serve as biological entities in future whole-cell toxicity/genotoxicity biosensor systems.

1 Introduction

The traditional approach to the monitoring of toxic chemicals in the environment is based on chemical analysis; it allows accurate and sensitive determination of the exact composition of any sample, is essential for regulatory purposes, and is necessary for understanding the causes of the pollution and the means for its potential remediation. However, when the nature of the target compound is unknown, the array of analytical instrumentation necessary for a comprehensive analysis is complex, costly, and requires specialized laboratories. Furthermore, such analytical methodologies fail to provide data as to the bioavailability of pollutants, their effects on living systems, and their synergistic/antagonistic behavior in mixtures. As a partial response to these needs, a complementary approach is based on the use of living systems in a variety of environmentally oriented bioassays.

The biological systems used for such purposes range from live organism assays such as fish toxicity tests, via cell-based systems, to others based on sub-cellular components or enzymes. All of them share the same characteristic: they do not aim to identify the target chemicals, but rather quantify their toxic effects. A special position among test organisms utilized for such purposes is held by unicellular microorganisms, in particular bacteria. Their large population sizes, rapid growth rates, low costs and facile maintenance often make them a lucrative option for toxicity monitoring. An additional attractive feature is that bacteria can be genetically “tailored” to respond by a detectable signal to pre-specified changes in their environmental conditions (see [1–5] for recent reviews).

In recent years, genetically engineered microorganisms have played two parallel roles in the development of toxicity bioassays, which may be generally divided into “lights off” and “lights on” assays [1]. The former is an extension of the widely accepted bioluminescent Vibrio fischeri toxicity bioassay, based upon measuring the decrease in light emission by this bacterium as a function of sample concentration, following a short-term exposure [6]. The “lights on” approach is mostly based upon the molecular fusion of a reporter system to selected promoters of different stress-responsive genes. Bacterial strains have been described that sensitively detect the presence of either specific compounds or classes of chemicals such as naphthalene [7], heavy metals [8], dioxins [9] and endocrine disruptors [10] or report on the general toxicity of the studied sample [11]. In a similar manner, bacterial strains have also been developed for assaying genotoxicity, rather than “regular” toxicity. In these cases, the promoters serving as the sensing elements were selected from among DNA repair operons such as the SOS system [12–15]. The reporters in these examples (see [13] for a review) were either bacterial bioluminescence (lux-CDABE, [7–12], β-galactosidase (lacZ, [8]), or fluorescent protein genes such as Aquorea victoria gfp [14, 15].

There have been very few descriptions of the inclusion of two distinct reporters in a single organism. In an early report, Wood and Gruber [16] introduced two beetle luciferases, different in their light emission spectra, into E. coli. A similar study was reported in which two fluorescent proteins (green and yellow) were introduced into the same bacterial host species [16]. In both cases one of the reporters served as the responder to the analyte, and the other as an internal control. Mitchell and Gu [18, 19] have reported the construction of Escherichia coli sensor strains capable of reporting on oxidative stress by bioluminescence (a kat-G::luxCDABE fusion) and on genotoxicants by fluorescence (recA::GFPuv4). Ponomarev et al. [20] have described a triple-modality mammalian reporter gene for fluorescence, bioluminescence and nuclear whole-body noninvasive imaging.
In this communication we describe a double-labeled *E. coli* construct harboring, in a single plasmid, two inducible promoters fused to different fluorescent protein genes: recA::EGFP and grpE::DsRed Express. The recA promoter is a part of the bacterial SOS system and its activation is considered an indication of DNA damage hazards [12]. GrpE is a heat shock protein, induced by a broad spectrum of chemicals, and was shown to be an excellent indicator of toxic cellular stress [11]. Their combination, therefore, potentially allows an assay of both genotoxicity and “regular” toxicity (cytotoxicity) to be carried out by the same reporter organism.

2 Materials and Methods

2.1 Plasmid Construction

Plasmid pNHEX, containing the two transcriptional fusions, was based on plasmid pES2, harboring a recA::gfpuv fusion [14]. This plasmid was digested by *Mun*I and *Bam*HI, for excluding the *gfpuv* gene. A 847 bp fragment containing the *egfp* gene coding sequence was cut from pEGFP-1 (Clontech, US, #6994-1) with the same restriction enzymes and ligated to the now linear pES2, forming the plasmid *recA’::egfp*, 3539 bp in size.

A second plasmid, *grpE’::DsRed express*, was constructed by cloning a 570 bp fragment containing the *grpE* gene promoter into the multiple cloning site of the pDsRed Express-1 (Clontech, US, #6086-1). The *grpE* promoter region was amplified using the following PCR primers:

\[ 5’-ATATAGGAATTCGGCCCTTCCAGCACATCGGC-3’ \]
\[ 5’-CTAGCAGGTACCCTGAGCTTCGAGATTCGCAAC - 3’ \]

(positions –440 – –420 and +170 – +190, respectively, relative to the *grpE* transcription start point (NCBI, X07863)).

The PCR product, the plasmid restricted by *EcoRI* and *Bam*HI and the relevant fragments were then ligated to create the 4672 bp *grpE’::DsRed Express*.

The final plasmid pNHEX (4951 bp, see Fig. 1), containing the two transcriptional fusions (recA::EGFP and grpE::DsRed Express) was then constructed by ligating *preca’::egfp*, digested with *Sac*I and *Mun*I, with a 1412 bp fragment, harboring the *grpE’::DsRed Express* fusion, derived from *grpE’::DsRed Express* and restricted by the same enzymes.

The presence and orientation of both promoter-reporter fusions in the plasmid was verified by PCR amplification of selected segments, restriction analysis and sequencing.

2.2 Bacterial Strains, Growth and Assay Conditions

The *E. coli* strains employed in the course of the described study are listed in Tab. 1. Strain RFM 443, transformed with the new dual-function plasmid pNHEX, was named strain NHEX-R.

All strains were grown in LB (Luria-Bertani) broth in the presence of 100 μg/mL ampicillin. An overnight culture was diluted 1:100 and grown at 30 °C to the early logarithmic phase (OD600 = 0.2–0.3, approx. 10⁸ cells/mL). Bacterial aliquots (50 μL) were then added to duplicate wells of an opaque white microtiter plate containing the appropriate inducers (nalidixic acid and/or ethanol) at the desired concentrations in 50 μL LB. The plates were placed in a temperature controlled microtiter plate fluorimeter (Victor 2, Wallac) and fluorescence was measured at 10 minutes intervals for the duration of the experiment, using the excitation/emission filters 485/535 nm for EGFP, and 550/590 nm for the DsRed Express protein. The excitation emission wavelengths used were not necessarily the maximal values recommended by the manufacturer for each of the individual fluorescent proteins, but rather optimized combinations for avoiding interferences and cross activities.

Fluorescence intensities are reported in the instrument’s arbitrary relative fluorescence units (RFU). All experiments were carried out in duplicate and were repeated at least...
three times. Variations between duplicates and repeats did not exceed 7% and 17%, respectively.

Fluorescence emission spectra were measured using 1 mL of culture, resuspended in minimal medium M9 [24] to reduce background fluorescence, in a SLM spectrofluorometer (Spectronics Instruments, Urbana, IL) following induction by nalidixic acid or ethanol and excitation at either 488 nm or 550 nm, respectively.

2.3 Confocal Microscopy

Bacteria were scanned using a MRC1024 confocal microscope (BioRad, England), employing a 63X or a 40X oil immersion objective. Excitation was carried out at 488 nm or 514 nm; green emission was imaged using a 525+/–20 nm filter, and red emission was collected using a 580+/–16 nm filter. After scanning, images were processed using Image Pro (Media Cybernetics). Average cellular fluorescence was quantified for at least ten bacteria per scanned field.

3 Results

Fluorescence in E. coli strain NHEX-R was induced by two representative chemicals: ethanol, an accepted inducer of the heat shock response [11], and nalidixic acid (NA), known to induce the SOS system in E. coli. In the present system, these chemicals served as models for a general toxicant and a DNA damaging genotoxicant, respectively. As demonstrated in Fig. 2, the fluorescent response to ethanol (see Fig. 2A) was red (excitation: 550 nm; emission: 590 nm) while that to NA (see Fig. 2B) was green (excitation: 485 nm; emission: 535 nm). In both cases, fluorescence intensity was dose-dependent, with the response to ethanol maximal at around 6% and to NA at approximately 3 to 6 mg/L. These values are similar to those reported to induce the grpE and recA gene promoters in other E. coli systems [11, 12].

Confocal micrographs of cellular fluorescence following activation by the same inducers are displayed in Fig. 3, which also serves to demonstrate that under these conditions there was little or no cross reactivity between the inducers: ethanol and nalidixic acid induced, respectively, red and green fluorescence only. This is also exhibited in Fig. 4, which displays the average fluorescence intensity of the fluorescent cells. A further demonstration of the specificity of the fluorescent responses is provided by the fluorescence emission spectra in response to the two inducers (see Fig. 5). The low level of background fluorescence (see Fig. 4), observed also as fluorescent cells in the non-induced samples (see Fig. 3), may possibly be attributed to the natural heterogeneity of the cell population [25].

As also indicated by the fluorescence spectra in Fig. 5, the potential interference of DsRed to EGFP reading is small, but the opposite occurrence may be significant: the fluorescence assayed as emanating from induced DsRed may harbor a substantial component of EGFP. This limitation will need to be taken into account in future versions of the dual-color reporter. To ensure that the observed fluorescent responses were indeed due to the specific activation of the gene promoters fused
to the reporting genes, we have used plasmid pNHEX to transform two pairs of host strains (see Tab. 1), each of which was composed of a wild type strain and a mutant in the relevant regulatory circuit. Strain DM803 contains a lexA\textsuperscript{ind} mutation that encodes a noncleavable form of the LexA, the repressor of SOS-induced gene expression, thus allowing only basal transcription of the \textit{recA} gene; strain CAG9333 harbors a null mutation in its \textit{rpoH} locus, eliminating the expression of \textit{r}\textsubscript{32} and thus preventing transcription of \textit{grpE} along with other heat shock genes. As depicted in Fig. 6, fluorescence is induced as expected in the wild type strain of each pair but not in the mutant. In each of the mutants, induction of the fluorescence driven by the gene promoter not expected to be influenced by the mutation was, indeed, not significantly affected (data not shown).

Finally, Fig. 7 displays the response of strain NHEX-R to a combination of the two inducers, demonstrating that the two fluorescent reactions can occur and be analyzed simultaneously. The observed induction of DesRed under these conditions appeared to be transient, and measured DsRed levels dropped after peaking at approximately 3 hours from induction. We have no explanation of this phenomenon at this time. Additional experiments have indicated that while NA had no effect on the red fluorescent response to ethanol, in some mixture combinations ethanol had a certain inhibitory effect on the green fluorescent response to NA (not shown).

4 Discussion

In this communication, we describe a plasmid that allows the quantification of two types of deleterious environmental effects: cytotoxicity and genotoxicity. An \textit{E. coli} strain harboring this plasmid synthesized fluorescent proteins in two different colors, dependent upon the toxic mode of activity of the chemical it was challenged with. Ethanol, the model general toxicant in the present experimental system, is an inducer of the bacterial heat shock system, demonstrated to be activated by a broad spectrum of toxic chemicals [11]. Nalidix acid, a DNA intercalating agent, is similarly considered a model inducer of the bacterial SOS system, activated in response to potential DNA damaging agents. We have shown that strain NHEX-R responded to the presence of ethanol by synthesizing the red fluorescent protein (DsRed Express), while exposure to nalidixic acid induced the synthesis of the green fluorescent protein GFP. We have further demonstrated that there was little overlap, if at all, between the two responses.
We have thus validated the viability of our basic concept, a single plasmid dual reporter system. Quite clearly, further optimization is required to allow this construct to be used for environmental monitoring. Sensitivity should be enhanced, response times shortened, and cross-interferences should be investigated and minimized. Nevertheless, engineered plasmids of this type can play an important role in future whole-cell toxicity sensing systems, in which flexibility and multi-functionality will doubtlessly be important. Similar features may also be achieved by genetically engineering other types of dual- (or even multi-) reporter bacterial strains [18,19]. The ultimate embodiment of this principle will be achieved by whole-cell array biosensors, in which multiple reporter strains will be immobilized onto the surface of a single biochip, similarly to nucleotide strands in a DNA chip [26–28]. Even in such a whole-cell biochip, adding dual-functionality to the reporter strains will greatly enhance the biosensing capacity.

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