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Engineering *Deinococcus radiodurans* into biosensor to monitor radioactivity and genotoxicity in environment

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Based on a genetically modified radioresistant bacteria *Deinococcus radiodurans*, we constructed a real time whole cell biosensor to monitor radioactivity and genotoxicity in highly radioactive environment. The enhanced green fluorescence protein (eGFP) was fused to the promoter of the crucial DNA damage-inducible *recA* gene from *D. radiodurans*, and the consequent DNA fragment (P*recA-egfp*) carried by plasmid was introduced into *D. radiodurans* R1 strain to obtain the biosensor strain DRG300. This engineered strain can express eGFP protein and generate fluorescence in induction of the *recA* gene promoter. Based on the correlation between fluorescence intensity and protein expression level in live *D. radiodurans* cells, we discovered that the fluorescence induction of strain DRG300 responds in a remarkable dose-dependent manner when treated with DNA damage sources such as gamma radiation and mitomycin C. It is encouraging to find the widely detective range and high sensitivity of this reconstructed strain comparing with other whole cell biosensors in former reports. These results suggest that the strain DRG300 is a potential whole cell biosensor to construct a detective system to monitor the biological hazards of radioactive and toxic pollutants in environment in real time.

Deinococcus radiodurans, biosensor, eGFP, RecA, genotoxicity, DNA damage, ionizing radiation, mitomycin C

With increasing awareness of the consequences of radioactive environmental pollution in such as DNA damage, genome instability, disease and cell death[1], there is a growing need for reliable tools for rapid assessment of the potential radioactive and toxic effects of environmental samples. The use of genetic engineered microorganisms that can detect both radioactivity and genotoxicity becomes an alternative environmental monitoring technology *in situ*[2,3]. Engineered *Escherichia coli* were widely employed as a biosensor to detect the genotoxicity[4−7]. However, high radioactive exposure will decrease the viability of most microorganisms, and eventually jeopardize the application of them as effective biosensors to detect chemical hazards and other genotoxicity in environment.

The bacterium *Deinococcus radiodurans* is characterized by its extreme resistance to ionizing radiation, UV-ray, desiccation and a variety of DNA damaging agents, and its highly effective DNA repair system without lethality and mutagenesis[8−10]. *D. radiodurans* is able to grow continuously at 60 Gy/h[11] or survive acute irradiation doses of 15 kGy[12]and its extreme radioresistance makes it one of the leading candidates for bioremediation of radioactive waste sites[11,13]. In this study, considering that eGFP fluorescent reporter shows more advantages than other reporter systems[14], we try to express the eGFP fluorescence protein in *D. radiodurans* cells and constructed a genetic engineered strain as a biosensor to monitor the extent of radioactivity and genotoxicity in real time.

To determine if eGFP can be expressed and develop

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visual fluorescence as endogenous marker, two promoters were respectively fused with *egfp* gene and cloned to *D. radiodurans* by shuttle vector. We show that the genetic manipulated strains DRG100 and DRG300 can express eGFP and development fluorescence under its own *groEL* gene promoter (P*groEL*) and DNA damage-inducible gene *recA* promoter (P*recA*) respectively. We assayed the correlation between the fluorescence intensity and expression level of RecA protein in strain DRG300 recovering from irradiation. Based on the constant correlation, we further illustrate the cellular stress responses of the strain DRG300 following exposure to different doses of ionizing radiation and different concentrations of mitomycin C in real time, which may help assess the potential applications of the *D. radiodurans* biosensor in radioactive environment.

1 Materials and methods

1.1 Bacterial strains, plasmids, media and growth conditions

D. radiodurans R1 and *E. coli* JM109 were used in this study. Shuttle vectors pRADZ3 was a gift from Cox M (University of Wisconsin). *D. radiodurans* cultures were grown at 32℃ in TGY broth (0.5% Bacto tryptone, 0.1% glucose, 0.3% Bacto yeast extract) with aeration or on TGY plates supplemented with 1.5% agar and *E. coli* at 37℃ in LB broth (1.0% Bacto tryptone, 0.5% Bacto yeast extract, 1.0% NaCl) or on LB plates solidified with 1.5% agar. When necessary, antibiotics were added in an appropriate ampicillin at 50 μg/mL for *E. coli*, chloramphenicol at 3 μg/mL for *D. radiodurans* grown on solid and liquid media, respectively. Transformations of *E. coli* were performed using the general $CaCl_2$ technique. *D. radiodurans* cells were transformed by using the modified $CaCl_2$ technique as described previously[15].

1.2 Construction of recombinant plasmids and strains

To define whether the *egfp* gene can be expressed and developed to fluorescence in *D. radiodurans* cells, a shuttle vector pRADG (carrying the promoter of *groEL* gene in *D. radiodurans*, P*groEL*) and a control plasmid pRADGW (without P*groEL*) were constructed. Two *egfp* gene fragments carrying different restriction enzyme sites (*Spe* I-*Bam*H I and *Bgl* II-*Bam*H I sites) were obtained by performing PCR amplification from the template plasmid pEGFP (BD ClonTech) using following primers: F1, 5′-AGCTACTAGTGATGGTGAGCAAGGGCGAG-3′ (*Spe* I); F2, 5′-CATAGAGATCTATGGTGAGCAAGGGCGAG-3′ (*Bgl* II); R12, 5′-ACGTGGATCCTCAGAGCATATGTGCCTTGTACAGCTCGTC-3′ (*Bam*H I). The DNA fragments were digested with the relevant enzymes respectively and then cloned into the corresponding sites of plasmid pRADZ3, forming pRADG and pRADGW (Figure 1).

Besides, a DNA fragment (approximately 2.3 kb) carrying the putative *recA* gene promoter (P*recA*) in the operon *cinA-ligT-recA*[16] by PCR amplification from *D. radiodurans* genome using the following primers: PrecAF, 5′-CATGAGATCTGAAGGATAAAAGCCTCCATCACAGCG-3′ (*Bgl* II); PrecAR, 5′-CTTCACTAGTT-

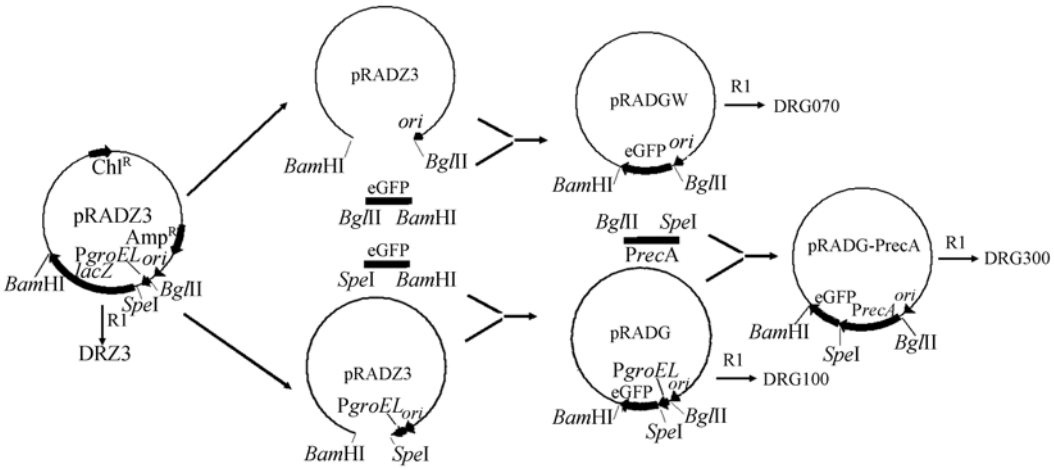

Figure 1 Construction of recombinant vectors and tranformants, restriction sites relevant for the construction of the vectors.

TGGTGGCGTCCTTGCTCATGGGTG-3′ (*Spe* I). The resulting PCR product was also digested with *Bgl* II and *Spe* I and then cloned into the *Bgl* II-*Spe* I sites of plasmid pRADG. This resulting plasmid was designated pRADG-PrecA (Figure 1).

Plasmids pRADZ3, pRADW, pRADG, and pRADG-PrecA were introduced into wild type strain *D. radiodurans* R1, respectively. The transformants obtained by chloramphenicol resistance selection were designated as *D. radiodurans* strains DRZ3, DRG070, DRG100, and DRG300 respectively (Figure 1).

1.3 EGFP protein expression and visual fluorescence in *D. radiodurans*

D. radiodurans strains DRZ3, DRG070, DRG100 and DRG300 were incubated to exponential phase (A_{600} is about 0.8) in TGY supplemented with 3 μg/mL of chloramphenicol and spread on a glassslide and examined with a fluorescence (Leica MPS30, Germany) or laser confocal microscope (Zeiss LSM510, Germany), respectively.

1.4 Correlation between fluorescence intensity of DRG300 and RecA expression level in *D. radiodurans* exposed to irradiation

D. radiodurans strain DRG300 was grown at 32℃ to exponential phase and used for fluorescence intensity assay and strain DRZ3 carrying pRADZ3 was used for RecA Western blotting analysis. Two strain cultures were irradiated at room temperature for 1 h with ^{60}Co γ-rays at 4 kGy. After treatment, the irradiated cells were post-incubated for 0, 0.5, 1.5, 3 and 6 h, and collected respectively. The strain DRZ3 sample was then suspended in a phosphate buffer and disrupted with a sonicator at 600 W output for a total of 5 min on ice and the debris was removed by centrifugation (16000×*g*, 20 min). Supernatant proteins were determined using the Bradford protein dye assay[17], and the quantities of RecA protein in the supernatant were measured by Western analysis following the standard protocol. In briefly, after electrophoresis, proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia Biotech, Buckingham Shire, England) and incubated with rabbit RecA antiserum (laboratory stock). Alkaline phosphatase-conjugated anti-rabbit IgG antiserum (Amersham Pharmacia Biotech) was used as secondary antibody. Anti-GroEL Western blotting bands were used for sample loading control. Chemiluminescent signals on the PVDF membrane were visualized and quantified by using the Gel Imager System (Bio-Rad Laboratories, Hercules, CA).

Each DRG300 sample was photographed and used to fluorescence intensity assay. Fluorescence analysis was carried out according to the approach[6] with slight modification. Briefly, fluorescence intensity of cell cultures was monitored using a fluorescence spectrometer (Shimadzu, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm. *D. radiodurans* strain DRG070 was used as the baseline sample to zero instruments. Raw fluorescence values were expressed in the instrument's arbitrary relative fluorescent units. Triplicate measurements were obtained for each sample. The data reported below include the specific fluorescence intensity (SFI, the raw fluorescence intensity divided by the optical density at 600 nm at each time point). For each stress tested, we also calculated the relative fluorescence induction factor RFI_t, which is equal to the relative fluorescence RF_t/RF_0 divided by the relative optical density A_t/A_0. $RFI_t = (RF_t \times A_0)/(RF_0 \times A_t)$, where RF_t is the raw fluorescence of the culture treated with DNA-damaging stress, RF_0 is the raw fluorescence of the control without stress, A_t is the optical density at 600 nm of treated culture and A_0 is the optical density of the control.

1.5 Treatment with DNA damage stress

To explore the feasibility of using P*recA-egfp* biosensor of *D. radiodurans* for monitoring radioactivity and toxicity, cultures of modified *D. radiodurans* R1 strain DRG300 was grown to exponential phase in TGY and then shifted to two kinds of stress, γ-irradiation and mitomycin C. During the former treatment, cell culture in exponential growth phase was suspended in 10 mL sodium phosphate buffer, and 1 mL culture was irradiated at room temperature for 1 h with γ-rays at several different dose levels. Dose levels were adjusted by changing the distance of the samples from the γ-rays source. After the treatments, the suspensions were post-incubated for different time and used for photograph and fluorescence intensity assay. Triplicate measurements were obtained for each sample. For the latter treatment, strain DRG300 exponential growth phase was challenged with different concentrations of mitomycin C at room temperature for 1 hour followed by post-incubation at 32℃ for 0.5, 1.5, 3, and 6 h. These treated samples were photographed and used for fluorescence intensity assay. Triplicate measurements were obtained for each sample.

2 Results and analysis

2.1 Fluorescence development of eGFP in *D. radiodurans*

To determine whether the exogenous *egfp* gene could be expressed under the promoter of the *groEL* gene of *D. radiodurans*, *D. radiodurans* strains DRG100 and DRG070 were incubated to exponential phase to be used for Western blot analysis with GFP antibody. By means of Western blot analysis, we confirmed that the *egfp* gene was successfully expressed in strain DRG100 under normal growth condition, while no detection of any expression of eGFP in strain DRG070 (Figure 2(a)). Fluorescence imaging analysis showed that all DRG100 cells displayed green fluorescence at an excitation wavelength of 488 nm and an emission wavelength of 535 nm and fluorescence foci were distributed through the cell (Figure 2(b)); while DRG070 cells were not detected fluorescence (Figure 2(b)), indicating that *egfp* was successfully expressed and visualized under the induction of the P*groEL* in *D. radiodurans*.

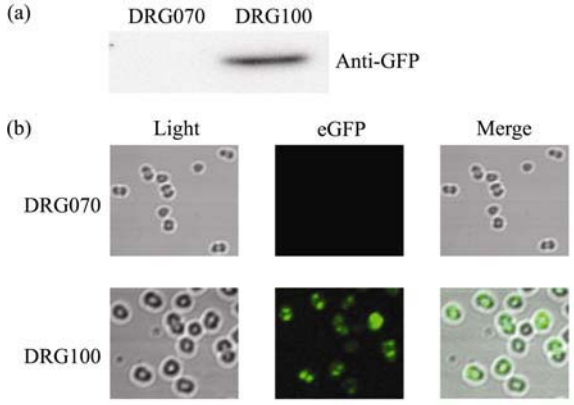

Figure 2 Expression of eGFP. (a) Western blot of eGFP protein in strain DRG100 with pRADG and strain DR070 with pRADW; (b) eGFP fluorescence detection of strain DRG100, strain DR070 as control.

2.2 Correlation between fluorescence intensity and RecA abundance

To establish correlation between the fluorescence intensity of eGFP in strain DRG300 and the RecA expression level in *D. radiodurans* cells, we analyzed the fluorescence intensity of strain DRG300 and carried out RecA protein Western blotting analysis in strain DRZ3 following exposure to 4 kGy γ-radiation (Figure 3(a)). The Western blot analysis confirmed that there was an increase in RecA expression in strain DRG300 following ionizing irradiation and it reached to a maximum value at post-incubation for 3 h, and then decrease rapidly (Figure 3(b)). The result was consistent with transcription analysis[18]. Meantime, a significant increase in the SFI was observed for stain DRG300 after exposure to 4 kGy γ-radiation (Figure 3(b)). Then the fluorescence intensity reached to a plateau and/or decreased slightly. According to Figure 3(b), the RecA protein profile was almost identical to the eGFP fluorescence profile until post-incubation for 3 h, after which the amount of RecA protein decreased quickly while the eGFP SFI decreased slowly. The difference was probably due to RecA proteolytic sensitivity, while eGFP could be stable in *D. radiodurans*. These results suggest that eGFP tag is suitable for studying gene expression differences and the promoter activity owe to its instantaneous without remarkable proteolytic in cells.

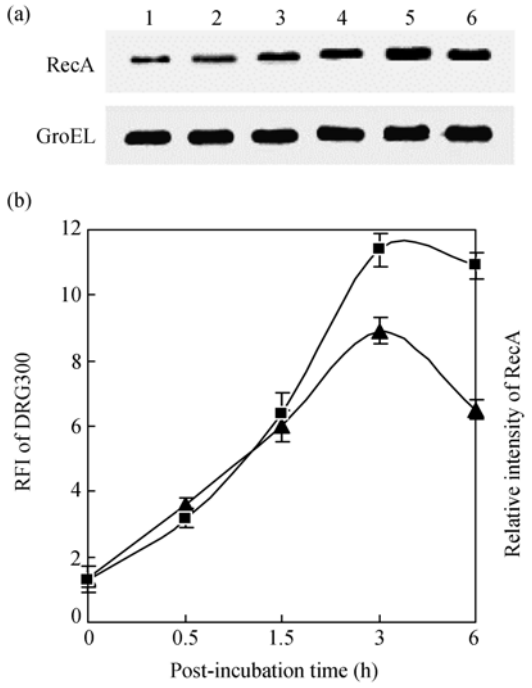

Figure 3 Correlation between fluorescence intensity and Western blot about RecA expression. (a) Western blot analysis of RecA expression in DRZ3 following exposure to 4 kGy γ-radiation. Lanes 2—6, samples taken 0, 0.5, 1.5, 3 and 6 h after irradiation, respectively; Lane 1, sample taken before γ-radiation. Anti-GroEL bands were used for sample loading control. (b) Relative expression level in RecA wild type R1 (▲) following exposure γ-radiation (Western blot) correlated with EGFP (RFI) (■).

2.3 Cellular response to γ-radiation

γ-irradiation can produce hundreds of double strand break (DSB) which is the most lethal form of DNA damage. The genome-wide transcription profile and proteomic analysis of *D. radiodurans* cells after treatment with irradiation indicated that γ-irradiation is an efficient inducer of homologous recombination repair

protein RecA[19,20]. Here, we evaluated the γ-irradiation dose-response profile of fluorescence intensity in strain DRG300. The results show that the induction of fluorescence began with no delay following exposure to different dose of γ-irradiation and almost increased most after post-incubation for 3 h, and then decreased slowly (Figure 4(a) and (d)). After exposure to γ-irradiation, the fluorescence response was non-linear for low dose

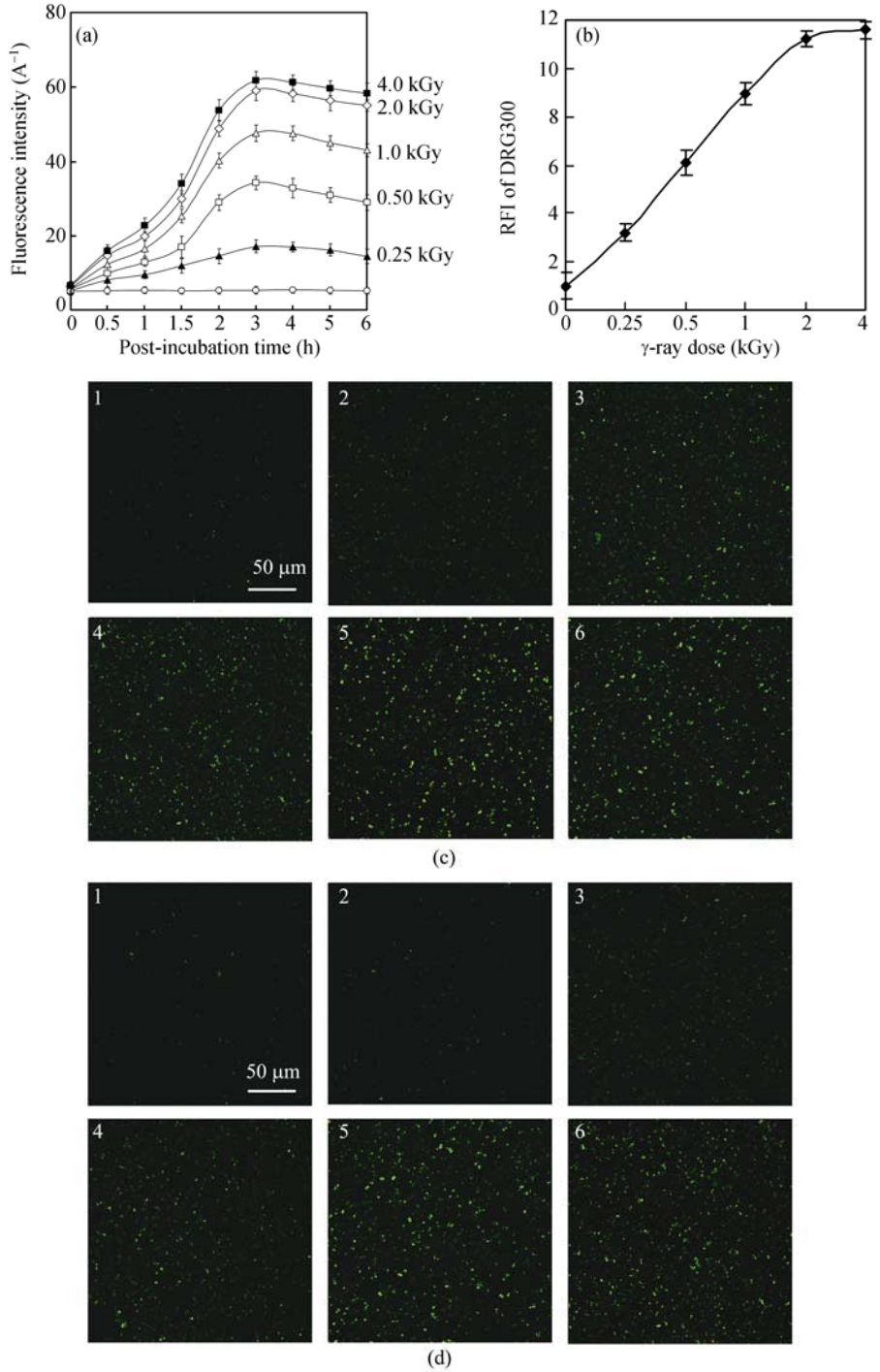

Figure 4 Strain DRG300 (cellular stress) response following stress to irradiation. (a) Dose-response curves for fluorescence intensity of DRG300 cells following exposure to 0.25 (▲), 0.5 (□), 1 (△), 2 (◆) and 4 kGy (■) γ-radiation, respectively. Samples were taken post-incubation for 0, 0.5, 1, 1.5, 2, 3, 4, 5 and 6 h, respectively, after exposure to γ-radiation. (○) non-irradiated to γ-radiation. (b) eGFP response profiles of strain DRG300 following to different dose γ-radiation. (c) Microscopic imaging of DRG300 cells following exposure 0.25 (2), 0.5 (3), 1 (4), 2 (5), and 4 kGy (6) γ-radiation, samples were taken post-incubation for 3 h, (1) non-irradiated, used as control. (d) Confocal micrographs of strain DRG300 irradiated with a dose of 4 kGy and post-incubated for 0 (2), 0.5 (3), 1.5 (4), 3 (5) and 6 h (6), and non-irradiated DRG300 used as control (1).

γ-irradiation (data not shown), while the fluorescence intensity was linear at all times with different dose ranging from 0.25 to 2 kGy, and the level of induction of fluorescence reached a plateau for dose more than 4 kGy (Figure 4(b) and (c)). These results indicate that the fluorescence efficiency increases linearly with a wide range of inducer dose, leveling off at a higher dose, approximate 12 folds of the background.

2.4 Cellular response to mitomycin C

To further characterize the fluorescence response to chemical genotoxins, the biosensor strain DRG300 allowed the detection of a concentration-dependent response for mitomycin C. The fluorescence response was non-linear for low concentrations (data not shown); the fluorescence intensity was linear at all times with different doses ranging from 0.001 to 1 μg/mL (Figure 5). Highest induction occurred after exposure to 10 μg/mL in our test. At higher concentrations, the fluorescence induction curve still remains constant due to cytotoxic effects. Viability of cells also significantly diminished at higher concentrations of mitomycin C, which resulted in significantly reduced fluorescence intensity.

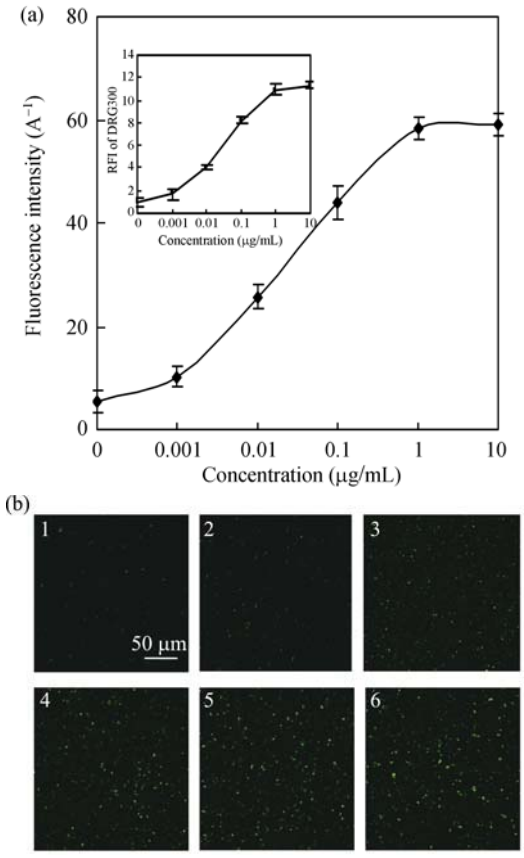

Figure 5 Strain DRG300 response following stress to mitomycin C. (a) eGFP response profiles of DRG300 cells following stress to different concentrations mitomycin C; (b) microscopic imaging of DRG300 cells following treatment with 0.001 (2), 0.01 (3), 0.1 (4), 1 (5) and 10 μg/mL (6) mitomycin C, samples were taken post-incubation for 3 h, (1) non-treated, used as control.

3 Discussion

So far, lots of microorganisms have been genetically engineered to detect and detoxify the pollutants[2,3]. However, those microorganisms are sensitive to extreme conditions, such as high radioactivity. Therefore, it is necessary to develop to a novel biosensor which can survive in a high radioactive area. Recently, the extremely radioresistant bacteria *D. radiodurans* and *D. geothermalis* have been engineered to reduce organic solvents and uranium[11,21]. While a fast and efficient method to detecting the radiation waste is still in searching, our work exhibits a potential worker in the field. Here, we constructed fluorescence tag system to explore its potential usages in living *D. radiodurans* cells.

In the present work, the enhanced green fluorescent protein eGFP was employed to be a reporter. Compared to the bioluminescence, the fluorescent proteins are more suitable for a real time monitoring system[2]. Our results show the *egfp* gene can be expressed and developed fluorescence under induction of the promoters (P*groEL* and P*recA*) of *D. radiodurans*. And the biosensor DRG300 based on P*recA-egfp* fusion displayed a potential ability in detecting biological effect of ionizing radiation in real time. The dose of ionizing radiation or the concentration of mitomycin C can be estimated by calculating the eGFP fluorescence intensity from a fluorescent microscope.

Promoters of genes involved in DNA repair SOS response, such as *recA*, *recN*, *uvrA*, *alkA*, and so on, have been fused to a reporter gene to assay the genotoxicity[2] and the promoters influenced sensitivity of bioassay[4,5,7]. In this study, we applied the putative *recA* gene promoter (P*recA*) in the operon *cinA-ligT-recA* of *D. radiodurans*[16] to be the response sensor, and we investigated the stress response of the strain DRG300 to two different DNA damage resources (γ-radiation and mitomycin C). Our results showed that fluorescence of DRG300 exhibits a dose-dependent response to ionizing radiation and concentration-dependent stress to mitomycin C under testing conditions, respectively, both responding profiles displayed a remarkable linear trend at

appropriate doses/concentrations scope. The similarity of two profiles may be due to the fact that both irradiation and mitomycin C can produce DNA damage and induce the expression of RecA protein. The biosensor DRG300 can detect a wide range of dose of γ-ray irradiation from 250 to 4000 Gy, while it can detect a wide range of concentration of mitomycin C from 0.001 μg/mL (3 nmol/L) to 1 μg/mL (3 μmol/L). The fluorescence system reported here established a feasible detection system for monitoring the extent of radioactive and toxic pollutants in environment in real time using P*recA-egfp* biosensor. It may detect a lower concentration if some membrane proteins are disrupted as previous did in *E. coli*[7].

Following quantity of RecA protein based on Western blot of *D. radiodurans* cells verified the strong correlation between *recA* gene expression and the fluorescence intensity of eGFP dependent on the fusion of P*recA* and eGFP gene following exposure to irradiation. The consistency indicated that the eGFP fused to the promoter of a targeted gene is able to develop a much rapid and sensitive methodology enabling parallel monitoring of expression difference of genes or proteins in living cells independent to transcription and proteomics analysis *in vitro*.

Taking together, extraneous eGFP tag could be used as a powerful tool to guide *D. radiodurans* engineering efforts aimed at monitoring DNA damage facts (radiation, toxic, metal, cosmic ray and microgravity) in environment and their integrating applications with bioremediation. We are looking forward to realizing some exciting discoveries using an eGFP tool in the tightest "bug" exploitation in near future.

The article is contributed to the 50th anniversary of Institute of Nuclear-Agricultural Sciences, Zhejiang University.

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