Construction, Characterization, and Biosensing Potential of an SOS Inducible mCherry Based Reporter Plasmid

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ABSTRACT

Improved variants of mushroom coral derived monomeric red fluorescent protein have been considered as a reliable reporter protein for gene expression. One of these variants is the mCherry protein which is encoded by the *mCherry* gene. The aim of the present study was to use the SOS inducible expression of the *mCherry* gene for monitoring the unwanted presence of antibiotics in a given environment. For that purpose, the *mCherry* gene was amplified using PCR technique. Then the amplified DNA fragment was cloned in a previously described low copy plasmid that carried an SOS inducible promoter. This cloning resulted in the transcriptional fusion between the SOS inducible promoter and the *mCherry* gene. The induced expression of the *mCherry* gene from the resultant construct was analyzed in *E. coli* cells by using fluorescence microscopy. The biosensing characteristics of the red fluorescent cells were evaluated quantitatively by considering the red fluorescence as an early marker of DNA damage in the cells exposed to antibiotics such as ColE7, nalidixic acid and norfloxacin. Our data showed that the construct was capable of detecting the presence of DNA damaging antibiotics up to nanogram level in an aqueous environment. Our findings suggested that the construct may be useful in the development of future strategies for the controlling of dissemination of antibiotic resistance that occurs due to the contamination of environment with antibiotic residues.

INTRODUCTION

Microbe-derived biosensors are widely used in the environmental and medical diagnostics (Fahliyani *et al*., 2020). Their use in the monitoring of environmental contaminants is well documented (Bilal and Iqbal, 2019). The high sensitivity and reliability are the primary advantage of the biosensors. The development of biosensors provides a versatile, compact, flexible, and inexpensive way of the study of multiple environmental contaminants including antimicrobial agents (Bilal and Iqbal, 2019). A range of biosensors with a great potential of being used in the detection of contaminants in an environment has been reported so far. Among them, the majority of the recently developed biosensors has involved the engineered reporter genes (Ma *et al*., 2022). Given that the performance of microbe-derived biosensors is directly linked to the selection of reporter genes and their regulatory proteins (Gui *et al*., 2017), there is always a need for the development of a new and recent biosensor to be used as a biological screen.

The majority of toxicogenic materials present in the environment has ability to cause DNA damage, thus induce SOS response in a bacteria cell. Therefore, SOS inducible promoters have been used in many biosensors for the detection of environmental pollutant. One of the known SOS inducible promoters, the promoter of Colicin E9 (*ColE9p*) contains two overlapping SOS boxes (Bano *et al*., 2013). Any stress to the host bacterial cell causing DNA damage results in the induction of *ColE9p*. The inducing ability of *ColE9p* makes it a suitable indicator of DNA damage in the host cell. Therefore, the *ColE9p* can be exploited for the detection of any toxic agents in an environment if it regulates the expression of a reporter gene encoding a fluorescent protein. Among the reporter proteins, the monomeric red fluorescent protein (mRFP)

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from the *Discosoma* sp. has been modified to improve its efficiency. One of the improved variants of mRFP is mCherry protein which is encoded by *mCherry* gene (Shaner *et al.*, 2004). The mCherry protein folds faster than tetramer resulting its rapid maturation after transcription. mCherry absorbs and emits light between 540-590 nm and 550-650 nm, respectively (Shu *et al.*, 2006).

In this study we describe the construction of an SOS inducible *mCherry* based reporter plasmid which was found functional in *E. coli* K12 (JM83) cells. Therefore, the construct was characterized in *E. coli* cells using DNA damaging agents such as mitomycin C (MMC). The fluorescence from the red fluorescent *E. coli* cells, was the measure of the DNA damage caused by DNA damaging agent (toxin or mutagen) in their environment. The construct was then used to detect the presence of antibiotics such as nalidixic acid (NA), norfloxacin (Nor), and purified colicin E7 (ColE7) in a liquid environment. Our findings have suggested that the construct can be useful in the development of future strategies for controlling of the dissemination of antibiotic resistance that occurs due to the contamination of environment with antibiotic residues from different sources.

MATERIALS AND METHODS

Bacterial strains, growth conditions and media

The plasmids were maintained in *Escherichia coli* JM83 (F−ara_(lac-proAB) rpsL (Strr)[ϕ80 dlac_(lacZ) M15]thi) strain at 37°C (unless otherwise stated) with shaking using Luria-Bertani (LB) broth supplemented with antibiotics appropriate for the recombinant clones.

DNA manipulation techniques

The primers were supplied by Sigma-Aldrich (UK). All enzymes including restriction endonucleases, T4 DNA ligase and polymerase were purchased from New England BioLabs (UK). The extraction of plasmid DNA, agarose gel electrophoresis, and ligation of DNA fragments were carried out as described in Sambrook *et al*. (1989).

Amplification of the mCherry *gene*

A promoter less *mCherry* gene (Shaner *et al.*, 2004) was amplified using template DNA and a set of forward (SBM34) and reverse (SBM35) primers (Table I). To facilitate the cloning of amplified DNA fragment specific restriction sites were incorporated into the sequence of primers (Table I). The amplified DNA fragment (~ 0.71 kb) corresponding to the *mCherry* gene was subsequently digested with *Nde*I and *Hin*dIII to be ligated with the *Nde*I and *Hin*dIII digested pSBM6 to yield an SOS inducible mCherry based construct. The successful fusion between

ColE9p and the *mCherry* gene was confirmed by the nucleotide sequencing of the construct.

Table I. List of primers used in the present study.

Primers	Sequence (5'-3')	Restriction site
SBM34 (F)	GCG<u>CATATG</u>GTGAGCAAGG	*Nde*I
SBM35 (R)	GCG<u>AAGCTT</u>TACTTGTACAGCTCG	*Hin*dIII

SDS-PAGE analysis

Proteins were separated by SDS-PAGE in a 16% resolving gel using Tris/glycine buffer (Laemmli, 1970). 10µl of sample was loaded to run SDS-PAGE at 170V followed by the staining of gel with Coomassie blue dye. The size of protein bands was determined using protein standards (NEB, 10-175kDa range).

Fluorescence microscopic measurements

Fluorescence microscopic measurements were carried out with a Nikon labophot 2 fluorescence microscopes. For the microscopic observations, a pre-warmed glass slide was loaded with an aliquot of 10 µl of each sample and a cover slip was placed over the sample immediately. Images were photographed using Nikon digital camera (DXm1200) attached with the microscope and saved with Nikon -ACT-1 version 2 software.

Determination of biosensing abilities

A single colony of the red fluorescent biosensor cells was grown overnight in LB broth at 37°C. Next day, culture was diluted 100-fold into fresh broth and grown until OD_{600} reached 0.4. At this stage, a test concentration of a specific DNA damaging agent was added. After the treatment step, a volume of 200 µl of the exposed culture was poured into the wells of a pre-warmed 96-well microtiter plate (Nunc). Then, red fluorescence was assayed at different time points with a Tecan Infinite 200 microplate reader using the excitation/emission wavelength 535/595nm. The fluorescence was measured in relative fluorescence unit (RFU). The subtraction of any background fluorescence from the target cells was carried out using non fluorescent cells (*E. coli* JM83 cells with low copy number plasmid lacking the cloned *mCherry* gene). Dose response ratio was calculated using the maximum RFU obtained from the red fluorescent cells treated with any given concentration of DNA damaging agent minus the RFU obtained from the untreated cells serving as control at the same time point (60 min) divided by the RFU of the control cells at that time point (Vankemmelbeke *et al*., 2005).

$$\text{Dose response ratio} = \frac{\text{RFU(treated)t} - \text{RFU (untreated)t}}{\text{RFU (untreated)}}$$

RESULTS

Construction of the SOS inducible mCherry based plasmid

A recombinant plasmid carrying the *mCherry* gene under the control of an SOS inducible promoter in a low copy number plasmid was constructed. An amplified DNA fragment corresponding to the *mCherry* gene (Fig. 1A) was cloned into pSBM6 plasmid (Bano *et al*., 2022), at *Nde*I and *Hind*III site (Fig. 1B). This insertion resulted the replacement of the *gfpmut2* gene with the *mCherry* gene. Hence, the resultant construct carries *pColE9*: *mCherry* in the back bone of pACYC184 plasmid which is a low copy number plasmid (Fig. 1C). The SOS inducible property of *pColE9* makes it a useful tool for the detection of DNA damage in host cell. Therefore, the construct was introduced into *E. coli* JM83 cells in order to make red fluorescent cells. The cells were anticipated to sense the presence of genotoxin in their environment.
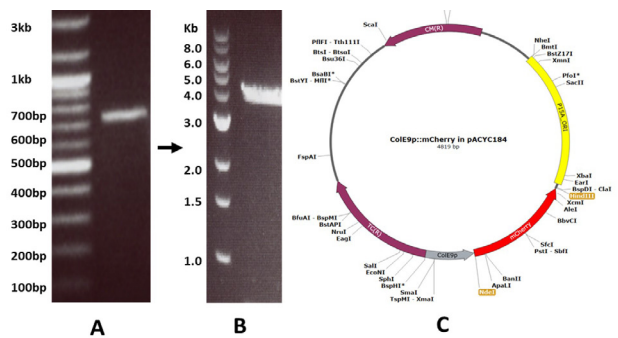

Fig. 1. Gel electrophoresis analysis of (A) the amplified mCherry gene of 0.7kb size. (B) pSBM6 with NdeI and HindII enzymes. (C) plasmid map of the resultant construct carrying mCherry under the regulation of SOS inducible promoter.

Characterization of the SOS inducible mCherry based construct

The construct was characterized by determining the expression of mCherry in *E. coli* cells in response to a well-known DNA damaging agent-MMC. For that purpose, the red fluorescent cells and the non-fluorescent cells were exposed to subinhibitory concentration of MMC (0.5μg/ml) for 2 h. SDS-PAGE analysis of the induced cells showed the appearance of a sharp band of mCherry protein suggesting that the expression of the mCherry protein is under the regulation of SOS system of host cell thus the uninduced cells have a very low level of the expression of mCherry (Fig. 2). Furthermore, the fluorescence microscopic observations of the MMC treated red fluorescent biosensor cells revealed that the red fluorescent cells were elongated, and the majority of cells

was fluorescent while untreated cells were normal in size and only a few cells were fluorescent (Fig. 3). The MMC induced elongation of *E. coli* cells are well described (Suzuki *et al*., 1967). Furthermore, the observed effects of MMC in the red fluorescent cells indicated that the expression of mCherry in the red fluorescent cells is under the regulation of an SOS response inducible promoter. Hence the cells could have abilities of biosensing DNA damaging agents in their environment.
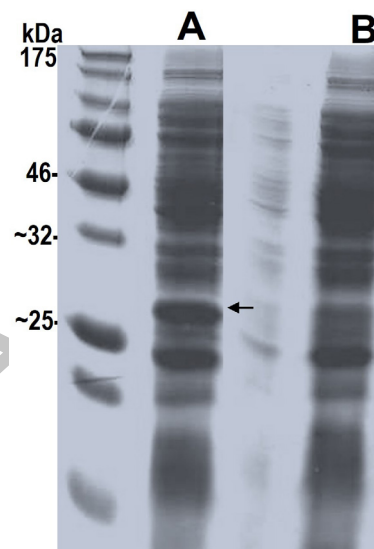

Fig. 2. SDS-PAGE analysis of whole cell proteins in the *E. coli* cells with SOS-inducible mCherry based construct induced with MMC for 2h (A) and uninduced cells (B).
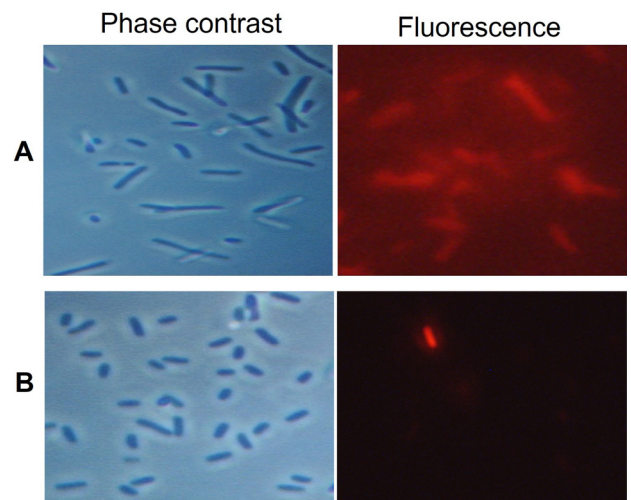

Fig. 3. Representative fluorescence microscopy images *E. coli* JM83 containing the SOS inducible mCherry based construct treated with MMC for two hour (A), and uninduced cells (B).

Biosensing abilities of the construct

To determine the ability of the red fluorescent cells to respond to different DNA damaging agents, the cells were exposed to NA, Nor and ColE7. Then the fluorescence was measured at different post exposure time points.
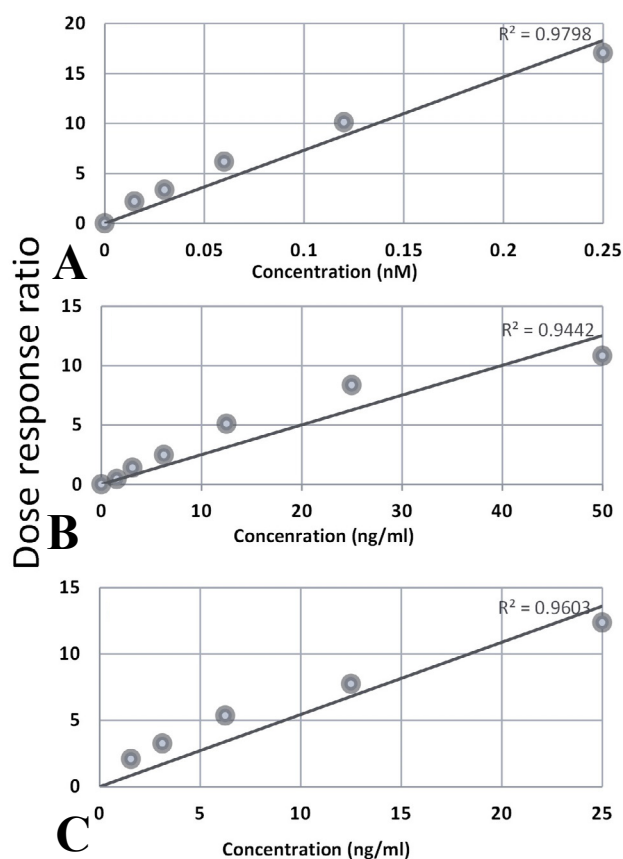

Fig. 4. A-C: Dose response ratio of fluorescence against ColE7 (A), NA (B) and Nor (C). The experiment was repeated twice in triplicates with similar results and a representative example is shown.

Detection of DNA-damaging toxins

ColE7 is a nuclease protein antibiotic that causes DNA damage in *E. coli* cells. The red fluorescent cells were exposed to a range of purified ColE7 (1nM-15pM), and the fluorescence, as an early marker of DNA damage, was detected. It was observed that the construct was able to detect the presence of ColE7 even in a very low quantity (data not shown). However, the dose response linearity curve was achieved up to 0.25nmol (Fig. 4A). No increase in RFU was observed from 0.5 nMol ColE7 albeit decline in the RFU was observed. This might have occurred due to the killing of the cells by the higher concentration of the toxin; thus, the fluorescence pattern was disturbed. The

production of the linear pattern from lower concentrations of ColE7 indicates about the more sensitivity of our construct in the detection of such genotoxins, if present in an aqueous environment.

Detection of DNA-damaging antibiotics

Similar response was observed from a range of concentration of two antibiotics, NA and Nor. The red fluorescent cells were very efficient in the detection of a range of the concentrations of NA and Nor antibiotics, while dose linearity was achieved up to 50 and 25 ng/ml, respectively (Fig. 4B, C). Fluorescence was detected at a very low concentration (up to 6.25ng/ ml).

DISCUSSION

In the present study, we describe the construction of an SOS inducible *mCherry* based plasmid and its characterization in *E. coli* cells which can be useful in the development of a red fluorescent protein-based biosensor. The fluorescent protein-based biosensors have an advantage of producing fluorescence without the addition of any exogenous substrate (Stiner and Halverson, 2002). Furthermore, the biosensing can be measured using fluorescence microscopy, fluorometry, flowcytometry and confocal microscopy. Among the fluorescent proteins, an improved variant of monomeric red fluorescent protein, mCherry, has been used as a reporter protein for the gene expression (Ransom *et al*., 2015). Although mCherry has been useful in its constitutive expression, the inducible expression of mCherry has been characterized recently (Kuduğ *et al*., 2019). Here, we created a construct that carries the fusion of *mCherry* gene with an SOS inducible promoter. The construct was functional in *E. coli* cells. We exposed the *E. coli* cells to MMC to characterize and validate the construct and to confirm that the construct is responsive to the DNA damaging agents. The post MMC treatment response was incredible. Therefore, the biosensing abilities of the construct were evaluated against antibiotics that cause DNA damage thus SOS response induction in *E. coli.*

Biosensing abilities of the SOS inducible *mCherry* based construct was promising as it allowed the red fluorescent cells to detect a very low level of DNA damaging agents within an hour of exposure. It was observed that the construct was very efficient to detect the agents in a low quantity while higher concentration did not show linearity in response. These observations occurred because the fluorescence was detected while the red fluorescent cells were exposed to DNA damaging agents. Apparently, the cells exposed to higher concentrations of the DNA damaging agents (ColE7, NA, and Nor) were

killed and the dose response linearity was not achieved. Our data is supported by a previous study where *lux* reporter could not detect higher concentration of DNase type colicin due to the killing of the cells (Vankemmelbeke *et al*., 2005). If the red fluorescent cells were exposed to the agents for a short time (less than lethal time), and then fluorescence was measured, it might have produced red fluorescence detected by fluorometry at a single point or by fluorescence microscopy.

The contamination of environment with antibiotic residues has now become a global issue (Ma and Zhai, 2020). Generally, wastewater from antibiotic manufacturing pharmaceutical industries, hospitals, animal husbandry, and households contribute to the increasing contamination of environment (water, food, soil, and other environments) with antibiotic residues which is obviously due to the increased consumption of the antibiotics (Manaia, 2017; Polianciuc *et al*., 2020). Consequently, the consumption of antibiotic residues by humans from food and water cause increasing antibiotic resistance and its dissemination (Manaia, 2017; Larsson and Flach, 2022). At one hand, the removal of the antibiotic residues itself is not an easy task. On other hand, the problem has been augmented due to the unavailability of an efficient routine monitoring system. Although, many detection methods and various biosensors have been described so far and many of them are currently in use, there is still need of different biosensors to face the issue of the increasing antibiotic contamination of environment, globally. The red fluorescent cells were sensitive enough to detect a very low quantity of NA and Nor in liquid medium which provides an easy method for the detection of antibiotics, particularly, those which block the bacterial DNA replication, thus induce SOS response in bacterial cell. However, the investigation of the detection ability of antibiotics directly from the environmental samples using our biosensing red fluorescent cells is our future research priority.

CONCLUSION

Our results showed that the SOS inducible mCherry based construct was functional in *E. coli* cells. The *E. coli* cells hosting the construct were capable of detecting the presence of DNA damaging antibiotics in a very minute amount. Our data suggests that the biosensing potential of the construct can be exploited for the detection of antibiotic contamination of environment. However, the investigations of the biosensing potential of the construct in real time using environmental samples need to be carried out.

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