Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–5, 2016

www.elsevier.com/locate/jbiosc

Paper-based colorimetric biosensor for antibiotics inhibiting bacterial protein synthesis

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Received 27 May 2016; accepted 22 July 2016
Available online xxx

Due to the presence of antibiotics in environmental water and their potential influence on the occurrence of antibiotic-resistant bacteria, development of a detection method suitable for the screening of environmental water for antibiotics is required. In this study, we developed a simple colorimetric paper-based biosensor based on a novel principle for the detection of antibiotics inhibiting bacterial protein synthesis, including aminoglycosides, tetracycline, chloramphenicol, and macrolides. This biosensor is based on the detection of a color change induced by β-galactosidase, which is synthesized on freeze-dried paper discs containing an *in vitro* transcription/translation system. When a water sample without antibiotics is applied to the paper discs, β-galactosidase can be synthesized, and it hydrolyzes a colorimetric substrate, resulting in a color change from yellow to purple. By contrast, in the presence of antibiotics, the color change can be hampered due to an inhibition of β-galactosidase synthesis. We investigated the effect of the incubation temperature and pH of water samples and confirmed that the paper discs showed the color change to purple in the ranges of 15–37°C and pH 6–10. We observed concentration-dependent color variations of the paper discs by the naked eye and further estimated detection limits to be 0.5, 2.1, 0.8, and 6.1 μg/mL for paromomycin, tetracycline, chloramphenicol, and erythromycin, respectively, using digitized pictures. The paper-based biosensor proved to detect 0.5 μg/mL paromomycin, spiked in real environmental water samples, by the naked eye.

[**Key words:** Resistant bacteria; Environmental water; Cell-free protein synthesis; Paper-based biosensor; Paromomycin; Tetracycline; Chloramphenicol; Erythromycin; β-Galactosidase; Chlorophenol red-β-D-galactopyranoside]

Emergence of antibiotic-resistant bacteria has been considered a global threat to human health (1). The excessive use and abuse of antibiotics have been considered to be one of the major causes of the occurrence and spread of resistant bacteria (2). Residual antibiotics in environmental water and their influence on the occurrence of antibiotic-resistant bacteria are becoming a particularly serious concern (3). A number of studies have demonstrated that the water environment is one of the most important habitats for bacteria in general and resistant bacteria in particular (4–6). The problem of the excessive use and abuse of antibiotics is much more serious in developing countries because of no or less regulation of antibiotic use (7). Therefore, the monitoring of antibiotic residues in environmental water, particularly in as many samples as possible and from various locations in developing countries, is crucial to facilitate further comprehensive studies aimed at the prevention of antibiotic resistance.

In general, antibiotics in water samples, including environmental water and wastewater, can be detected using liquid chromatography in combination with mass spectrometry (LC/MS) because of its high sensitivity and reliability (8–10). In developing countries, however, it is difficult to implement these instruments because of their high cost and complicated operation, need for well-trained personnel, and difficulty in applying them to preliminary screening. Based on these circumstances, there is a growing demand for developing a simple detection method for antibiotics in environmental water, i.e., a detection method appropriate for preliminary screening.

Biosensors have been expected to become appropriate tools for preliminary screening that could overcome the drawbacks of conventional methods. Generally, a biosensor is composed of a biological recognition element, which is combined with a suitable physicochemical transducer to convert the biological response to a measurable signal that is proportional to the concentration of the target analyte (11). More recently, patterned paper has been gaining considerable attention as a highly multiplexed platform for biosensors (12). Unique aspects of the paper platform-based biosensors are that they are low cost, easy to transport, easy to dispose, and flexible to design. A paper-based sensor was originally developed in the point-of-care clinical diagnostic field, but the paper-based sensor technology has been applied recently to other fields, including environmental analysis (13). Recently, novel paper-based sensors for a diverse range of environmental contaminants, including metals, pesticides, and bacteria, have been reported (14).

We herein describe a colorimetric paper-based biosensor based on a novel principle for the detection of one of the main antibiotic categories, i.e., antibiotics inhibiting bacterial protein synthesis,

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.07.015

such as aminoglycosides, tetracycline, chloramphenicol, and macrolides. The paper-based biosensor utilizes a system proposed by Pardee et al. (15), in which reporter proteins such as the green fluorescent protein or β-galactosidase (β-GAL) are synthesized on freeze-dried paper discs containing an *in vitro* transcription/translation (IVTT) system reconstituted from purified recombinant components necessary for *Escherichia coli* translation. The concept of the paper-based biosensor is that it detects the anti-translational activity of a group of antibiotics inhibiting bacterial protein synthesis through the color change induced by β-GAL synthesis on paper discs (Fig. 1). In the absence of antibiotics, β-GAL is synthesized and hydrolyzes a colorimetric substrate, changing the color of the paper discs from yellow to purple. By contrast, in the presence of antibiotics, inhibition of the β-GAL synthesis occurs, resulting in the inhibition of the color change. This paper-based biosensor can be a basis for a visual, convenient, and simple detection method for preliminary screening of environmental water for antibiotics inhibiting bacterial protein synthesis.

MATERIALS AND METHODS

Antibiotics Paromomycin sulfate was purchased from Sigma–Aldrich (USA). Tetracycline hydrochloride, chloramphenicol, and erythromycin were purchased from Nacalai Tesque (Japan). Stock solutions were freshly prepared by dissolving antibiotics in Milli-Q water in the case of paromomycin and tetracycline. Chloramphenicol and erythromycin were dissolved in ethanol, and subsequent dilution was done with Milli-Q water.

Preparation of DNA template for IVTT reaction A DNA template for β-GAL synthesis in the IVTT reaction was prepared by polymerase chain reaction (PCR) amplification from the pET21-based plasmid containing the *LacZ* gene sequence (16) using the T7F (5′-TAATACGACTCACTATAGGG-3′) and T7R (5′-GCTAGTTATTGCTCAGCGG-3′) primer set (17) with PrimeSTAR Max DNA polymerase (Takara, Japan). The PCR fragment contains the ribosome-binding site, T7-tag sequence, *LacZ* gene under control of the T7 promoter, and T7 terminator. The resulting PCR product was purified using the QIAquick PCR purification kit (Qiagen, Germany), and DNA concentrations were determined by absorbance at 260 nm.

Preparation of IVTT reaction The IVTT system used in this study was the PURExpress *In Vitro* Protein Synthesis Kit (E6800; New England Biolabs, USA). IVTT reactions were set up on ice according to the manufacturer's instruction. In a fluorometric assay, the reaction mixture (2 μL) was composed of 0.8 μL of solution A, 0.6 μL of solution B, 100 pg of the purified PCR product containing the *LacZ* gene (DNA template), and a 10 μM fluorescent substrate, Tokyo Green-β-GAL (TG-β-GAL) (Sekisui Medical, Tokyo, Japan). In a colorimetric assay, the reaction mixture (2 μL) was composed of 0.8 μL of solution A, 0.6 μL of solution B, 2 ng of the *LacZ* DNA template, and a 0.6 mg/mL colorimetric substrate, chlorophenol red-β-D-galactopyranoside (CPRG) (Sigma–Aldrich). An IVTT reaction without the DNA template or a β-GAL substrate was used as a negative control.

IVTT reactions on paper discs Paper discs (1442-055; Whatman, UK) were prepared using a 2-mm biopsy punch (15076; Ted Pella, USA). For fluorometric assays, 2-mm paper discs were placed into PCR tubes. Then, 2 μL of the IVTT reaction mixture was spotted onto the paper discs, and the discs were flash-frozen in liquid nitrogen and freeze-dried for at least 3 h. The freeze-dried paper discs were rehydrated with 2 μL of Milli-Q water and incubated at 37°C for 2 h in a Mx3000P real-time PCR system (Stratagene, USA). For colorimetric assays, 2-mm paper discs were placed on an adhesive plate seal (DC100PCR; Nippon Genetics, Japan) in a box. The process of spotting, freezing, and freeze-drying was performed as described for fluorometric assays. Two microliters of Milli-Q water, an antibiotic solution at the concentration specified, or a specific pH solution were spotted on the paper discs for rehydration. The specific pH solutions were prepared by adding NaOH to Milli-Q water. The adhesive plate seal with paper discs was placed in a humidified microarray chamber to prevent evaporation and incubated at specified conditions. In the case of testing the effect of temperature, the paper discs were prepared in PCR tubes and incubated at specific temperatures using a thermal cycler.

Detection of responses of paper-based IVTT reactions Fluorescence from the hydrolysis product of TG-β-GAL on paper discs was monitored over time by the Mx3000P real-time PCR system (Stratagene) using the FAM channel. The paper discs containing CPRG were air-dried at 37°C for 15 min after performing IVTT reactions and their digitized pictures were obtained using a scanner (GT-9800F, Epson, Japan) at a resolution of 1600 dots per inch. Then, color changes of paper discs were quantified using ImageJ. First, images were split into red, green, and blue (RGB) channels. Then, a mean of pixel intensities was measured within each paper disc region of the green channel. The value obtained from a paper disc that did not contain the DNA template was used for background subtraction. The limit of detection (LOD) was defined as $3 \times SD/S$, where S is the slope of a linear calibration curve, and SD is the standard deviation of the mean intensity of the blank. The slope was calculated using the data for the blank and 0.5 μg/mL for paromomycin, blank and 1.0 μg/mL for chloramphenicol, and blank and 5.0 μg/mL for erythromycin. The slope for tetracycline was calculated based on a regression line from data points for the blank, 0.5 and 5.0 μg/mL.

Preparation of real environmental water samples spiked with paromomycin Real water samples were collected from three different locations, a garden stream and a pond at Osaka University (Japan) and the Hau River (Vietnam). These samples were filtered through 0.2-μm filters (Kurabo, Japan) and spiked with paromomycin to reach a final concentration of 0.5 μg/mL. Two microliters of the spiked water samples was spotted onto the paper discs with CPRG, and the resulting color change was observed by the naked eye or quantified using the image scanner and ImageJ software as described above.

RESULTS AND DISCUSSION

Confirmation of β-GAL synthesis using IVTT reaction embedded into paper discs To construct a paper-based biosensor using IVTT reactions for antibiotics inhibiting bacterial protein synthesis, we initially examined whether the reporter gene can be expressed in the IVTT system on paper discs, as previously described by Pardee et al. (15). We embedded a commercial IVTT system reconstituted from purified recombinant components necessary for *E. coli* translation, including the *LacZ* DNA template and the β-GAL substrate TG-β-GAL, onto 2-mm paper discs, and then freeze-dried the discs (Fig. 1). The IVTT system we adopted was based on the PURE system technology (18). TG-β-GAL is a non-fluorescent substrate that emits fluorescence at 510 nm with an excitation at 490 nm when hydrolyzed by β-GAL (19). After adding 2 μL of Milli-Q water, the paper-based IVTT reactions showed an increase in fluorescence intensity, while those without the *LacZ* DNA template did not

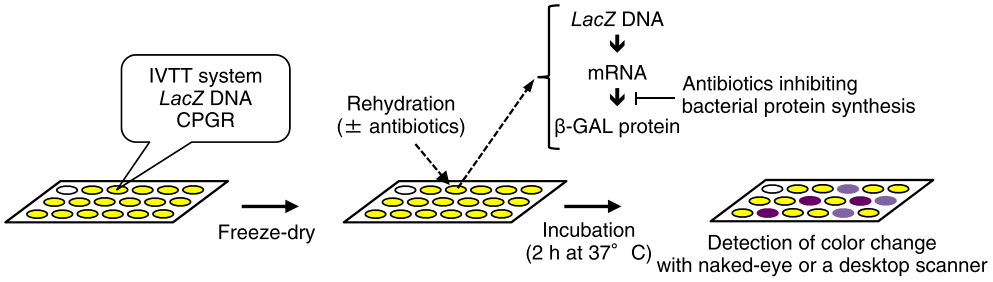

FIG. 1. Schematic of the colorimetric paper-based biosensor for the detection of antibiotics inhibiting bacterial protein synthesis. A mixture of an IVTT system, *LacZ* DNA template, and colorimetric substrate (CPRG) was embedded into paper discs, and the discs were then freeze-dried. The freeze-dried paper discs were rehydrated with water samples. In the absence of antibiotics, β-GAL is synthesized and hydrolyzes CPRG, resulting in the color change of the paper discs from yellow to purple. In the presence of antibiotics, β-GAL synthesis is inhibited, resulting in an inhibition of the color change. The color change was evaluated by the naked eye or using a desktop scanner with an image processing and analysis program (ImageJ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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(Fig. 2A), indicating the occurrence of β-GAL synthesis on paper discs.

Owing to the requirement for an expensive and complicated device to detect fluorescent signals of multiple samples, it is difficult to use the biosensor outside of the laboratory or in developing countries. To confer simplicity and portability to our paper-based biosensor, we also tested a colorimetric β-GAL substrate, which would enable the monitoring of β-GAL synthesis by the naked eye or using a low-cost imaging device such as a desktop imaging scanner. For this, we replaced the fluorometric substrate TG-β-GAL by the colorimetric substrate CPRG, which shows a color change from yellow (CPRG) to purple (chlorophenol red) when hydrolyzed by β-GAL. We confirmed by the naked eye that the paper discs incubated at 37°C showed a color change from yellow to purple after no longer than 2 h, while those without the *LacZ* DNA template did not show any color change (Fig. 2B). We also found that the color change could be quantified by digitizing paper disc images using a desktop scanner and measuring the mean pixel intensity within paper discs for green color channel images in the RGB color mode using an image processing and analysis program (ImageJ) (Fig. 2B). The paper discs used for the tests maintained their changed color for at least 50 days at room temperature (data not shown).

Effect of temperature and pH on color change of paper-based biosensor To investigate the range of temperatures at which the paper-based biosensor could function, other than 37°C, freeze-dried paper discs with IVTT reactions, activated with water, were incubated at 4°C, 15°C, or 25°C. At 25°C, the color change occurred after incubating for 2 h, similar to the incubation at 37°C (Fig. 2C). At 15°C, we observed a color change relative to the initial yellow color after 12 h of incubation and an almost complete color change, similar to that observed at 25°C or 37°C, after 24 h of incubation (Fig. 2C). In the case of 4°C, the paper discs retained the initial yellow color even after 24 h of incubation (Fig. 2C). These results suggest that our paper-based IVTT reactions could be utilized at various locations with different temperatures, at least in the temperature range from 15°C to 37°C, without a constant-temperature incubator, although the performance of the paper-based IVTT reactions might be different at different temperatures.

We also investigated the effect of pH of test water samples on the responses of the paper-based IVTT reactions because the common pH values of real water samples are in the range from 6.0 to 8.5 (20). As shown in Fig. 2D, the color change from yellow to purple was observed for water samples with pH values of 6.0–10, suggesting that the paper-based IVTT reactions could be applicable in the common pH range of real water samples.

Detection of antibiotics inhibiting bacterial protein synthesis using paper-based IVTT reactions To demonstrate their potential as a biosensor, we next tested whether the paper-based IVTT reactions could be utilized for detecting the presence of antibiotics inhibiting bacterial protein synthesis in water. Antibiotics inhibiting bacterial protein synthesis can be broadly classified into three classes, depending on their ribosome-binding sites, i.e., the 30S subunit (group 1), the peptidyl transferase center on the 50S subunit (group 2), and the peptide exit tunnel on the 50S subunit (group 3) (21). We focused on representative antibiotics from the three classes and selected tetracycline for group 1, chloramphenicol for group 2, and erythromycin (one of macrolides) for group 3. In addition, paromomycin, a particular aminoglycoside belonging to group 1, was selected because of its characteristic feature, i.e., paromomycin shows not only antibacterial activity (as other aminoglycosides) but also antiprotozoal activity. Because of its antiprotozoal activity, paromomycin has been used against parasite diseases caused by *Cryptosporidium*, *Entamoeba*, or *Leishmania* (22–24). These infectious diseases are known to occurred mainly in the developing countries (25). For example, visceral leishmaniasis is ranked second in mortality among parasitic diseases, and 90% infectious cases have occurred in developing countries (26).

The freeze-dried paper discs impregnated with the IVTT system, *LacZ* DNA template, and CPRG were rehydrated with 2 μL of antibiotic solutions at different concentrations (0.05–20 ng/μL) and incubated at 37°C for 2 h. As a result, the concentration-dependent inhibition of the color change from yellow to purple was observed for each of the antibiotics (Fig. 3A–D, upper panel). When the paper

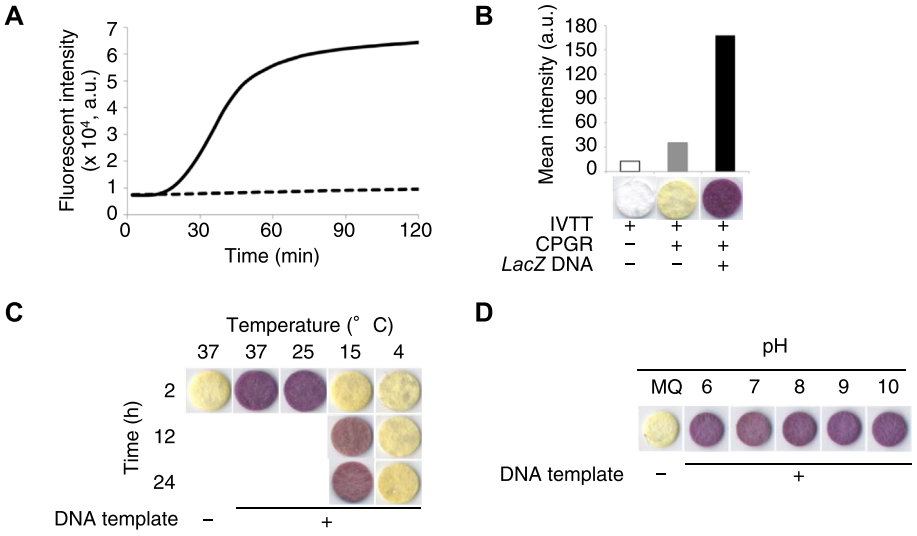

FIG. 2. Reporter protein synthesis on paper discs. (A) Representative time course data of the fluorescence intensity derived from the paper discs impregnated with an IVTT system and fluorescent substrate (TG-β-GAL) with (solid line) or without (dashed line) a *LacZ* DNA template. Fluorescence intensity was monitored using an Mx3000P real-time PCR system. (B) β-GAL-mediated color change of the paper discs impregnated with an IVTT system with/without CPRG and/or the *LacZ* DNA template. Representative paper discs are shown. The graph shows the mean pixel intensity within the paper discs of green color channel images in the RGB color mode. (C) Effect of the temperature on the color change of the paper-based biosensor. After being rehydrated with Milli-Q water, the paper discs were incubated at the indicated temperature. Representative images of the paper discs that were scanned at the indicated time are shown for each condition. (D) Effect of pH on the color change of the paper-based biosensor. A composite image of a paper disc, with the median value of five independent paper discs, is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

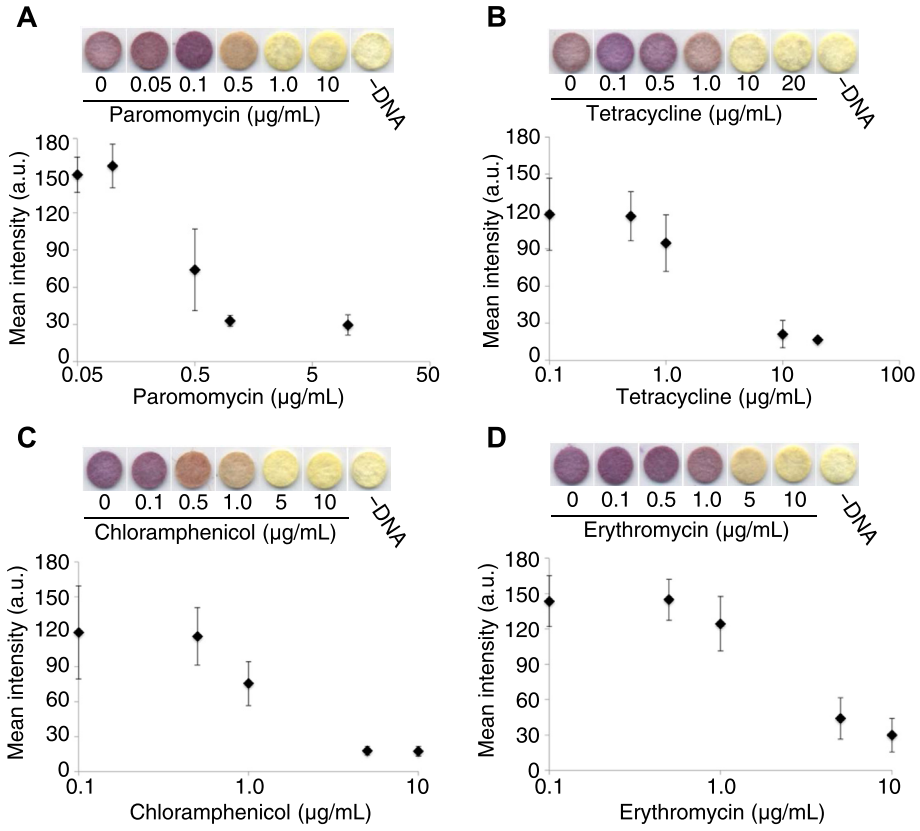

FIG. 3. Colorimetric responses of the paper discs to various concentrations of antibiotics inhibiting bacterial protein synthesis, including paromomycin (A), tetracycline (B), chloramphenicol (C), and erythromycin (D). A composite image of paper discs, with the median value of five independent paper discs, is shown for each sample. The −DNA sample contained no antibiotics and *LacZ* DNA template. The results are the means ± SD of the mean pixel intensities from five independent paper discs. The X-axis is shown in the logarithmic scale.

discs were scanned with a desktop scanner and their color changes were quantified using the ImageJ software (Fig. 3A–D, lower panel), we could estimate the LOD for each antibiotic. The LOD was calculated using the 3 × SD/S criteria (see Materials and methods), resulting in LODs of 0.5, 2.1, 0.8, and 6.1 μg/mL for paromomycin, tetracycline, chloramphenicol, and erythromycin, respectively. Antibiotics inhibiting bacterial protein synthesis work at different stages of mRNA translation, which probably influences the LOD values of specific antibiotics (21). These results showed the potential of our paper-based biosensor as a semi-quantitative biosensor in the case of observation by the naked eye or a quantitative biosensor when using a desktop scanner.

Analysis of real environmental water samples spiked with paromomycin A potential application of the colorimetric paper-based biosensor was evaluated using practical analysis of real environmental water samples spiked with 0.5 μg/mL of paromomycin (LOD of the biosensor for paromomycin). The water samples were collected from three different locations; two were from the campus of Osaka University in Japan, and one was from a river in Vietnam. The detection assay was carried out at 37°C. All of the environmental water samples containing 0.5 μg/mL of paromomycin, as well as Milli-Q water with the same concentration of the antibiotic, when applied to the paper discs, showed a moderate color change from yellow to purple. Importantly, the colors were distinguishable from those of the corresponding paper discs treated with the same water samples without paromomycin (Fig. 4). The presence of 0.5 μg/mL paromomycin in Milli-Q water and the three water samples was also detected by the naked eye at 25°C and 15°C (data not shown). These findings demonstrate that this paper-based biosensor has the potential for testing real environmental water samples.

There is a growing concern of the influence of environmental water contaminated with antibiotics on the occurrence and spread of antibiotic-resistant bacteria, which is closely linked to the abuse of antibiotics (1). In this proof-of-concept study, we successfully developed a simple colorimetric paper-based biosensor for the presence in environmental water of antibiotics inhibiting bacterial protein synthesis. Although further evaluation is required for other antibiotics inhibiting protein synthesis, our biosensor might enable the visual detection of a group of antibiotics inhibiting bacterial protein synthesis, using a single paper disc. This advantage is in contrast to the use of LC/MS or enzyme-linked immunosorbent

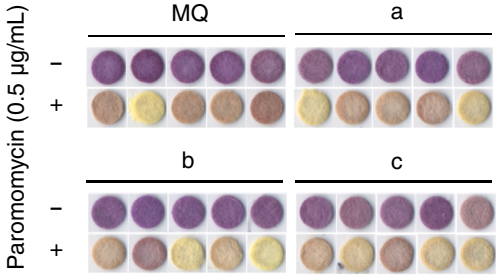

FIG. 4. Detection of paromomycin in spiked environmental water samples by the paper-based biosensor. Paromomycin was spiked at 0.5 μg/mL into Milli-Q water (MQ) or water samples collected from the Hau River in Vietnam (a), a garden stream (b), and a pond (c) at Osaka University in Japan. A composite image of 10 paper discs incubated at 37°C for 2 h in a humidified microarray chamber is shown.

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assays, for which the simultaneous detection of different classes of antibiotics typically presents a difficulty. Another advantage of the paper-based biosensor is its ease of use because the biosensor can be used by only adding a small volume of water samples and observed without any expensive supporting instruments or well-trained personnel. The IVTT system is not classified as genetically modified organisms; thus, the paper discs can be utilized outside of laboratories without a risk to the natural environment.

Our paper-based biosensor could have potential for practical application in a certain situation, e.g., a concentration of 20 mg/L of oxytetracycline (one of tetracycline) was detected in the Xiao River, China (27) because the river received treated wastewater from a pharmaceutical manufacturer. However, the sensitivity of our biosensor needs to be improved to determine the common concentration of antibiotic in wastewater or environmental water (28,29) and to widen its practical application. In terms of further improvement, the sensitivity of the biosensor might be improved by taking advantage of the PURE system (18), which constitutes the IVTT system we used in this study (i.e., PURExpress system). The PURE system is a reconstituted system obtained by combining components required for *E. coli* translation, and thus, it can be easily adjusted by changing components. An example of the strategies are utilization of ribosomes highly sensitive to antibiotics instead of *E. coli* ribosomes; ribosomes derived from different bacteria highly sensitive to antibiotics (e.g., *Staphylococcus aureus* NCTC 6571 or *Streptococcus pneumonia* ATCC 49619) (30); or ribosomes with mutations that increase their sensitivity to antibiotics. For example, the commercial PURExpress delta ribosome kit (E3313; New England Biolabs) would be useful for the utilization of an alternative ribosome.

Overall, we represented a novel concept of paper-based, simple, rapid, easy-to-use, and easy-to-dispose biosensors appropriate for the preliminary screening of antibiotics contaminating environmental water. However, there is still a need for further investigation of the future practical applications, e.g., examinations on the response of the biosensor for antibiotics other than those tested in this study or on the interference materials of the biosensor. Comprehensive monitoring of residual antibiotics in environmental water using our paper-based biosensor would provide important information for future studies that can contribute to the prevention of the occurrence of resistant bacteria in developing countries.

ACKNOWLEDGMENTS

We thank Dr. K. Pardee for his advices for the paper-based IVTT reactions and Dr. T. Matsuura for providing the plasmid DNA encoding β-GAL. The study was supported through a grant of Long-range Research Initiative (LRI) by Japan Chemical Industry Association (JCIA).

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