論文 / 著書情報
Article / Book Information

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Citation	Biosensors and Bioelectronics, Vol. 219, , 114793
Pub. date	2022, 10

Biosensors and Bioelectronics 219 (2023) 114793

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

The Patrol Yeast: A new biosensor armed with antibody-receptor chimera detecting a range of toxic substances associated with food poisoning

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ARTICLE INFO

Keywords:
Food safety
Immunosensor
Yeast
Open-sandwich immunoassay
Chimeric receptor
Transmembrane signal transduction

ABSTRACT

Baker's yeast is an attractive host with established safety and stability characteristics. Many yeast-based biosensors have been developed, but transmembrane signal transduction has not been used to detect membrane-impermeable substances using antigen-antibody interactions. Therefore, we created Patrol Yeast, a novel yeast-based immunosensor of various targets, particularly toxic substances in food. A membrane-based yeast two-hybrid system using split-ubiquitin was successfully used to detect practically important concentration ranges of caffeine and aflatoxins using separated variable regions of an antibody. Moreover, enterohemorrhagic *Escherichia coli* O157 was detected using a specific single-chain antibody, in which Zymolyase was added to partially destroy the cell wall. The incorporation of secreted *Cypridina* luciferase reporter further simplified the signal detection procedures without cell lysis. The methodology is more cost-effective and faster than using mammalian cells. The ability to detect various targets renders Patrol Yeast a valuable tool for ensuring food and beverage safety and addressing other environmental and technological issues.

1. Introduction

The contamination of foods with toxins such as mycotoxins, as well as viruses and bacteria affects human health and wellbeing. Among mycotoxins produced by fungi in deteriorated foods, aflatoxins produced by *Aspergillus flavus* are highly carcinogenic, and can cause liver cancer and even fatal incidents (Henry et al., 2002). Aflatoxin B1 or B2 produced by *A. flavus* is usually found in crops, such as corn and peanuts. They are quite stable and can easily contaminate food that is then consumed by livestock such as cattle (Rushing and Selim, 2019). Livestock animals could then produce aflatoxin M1 through metabolic reactions, which often result in contaminated milk (Marchese et al., 2018). Other important food contaminants include enterohemorrhagic bacteria, such as *Escherichia coli* (*E. coli*) O111 and O157 that can contaminate raw foods such as vegetables and meat and cause bloody diarrhea and other fatal symptoms in humans and other animals (Gally and Stevens, 2017). Traditional methods for analyzing these food contaminants include real-time polymerase chain reaction (PCR) (Spano et al., 2005), gas chromatography-mass spectrometry (GC/MS), and liquid chromatography-tandem mass spectrometry (LC/MS/MS) (Sontag et al., 2019), which require expensive instruments and trained personnel. Although immunoassays such as enzyme-linked immunosorbent assay (ELISA) have relatively simple requirements (Engvall and Perlmann, 1972), they still involve a series of operations that require skilled personnel. Nanomaterial-based sensors (Goud et al., 2018; Hossain et al., 2021; Mahmud et al., 2022) can also detect toxins and pathogens, but their manufacturing process is relatively complicated. Therefore, a rapid, convenient, and affordable analytical method is needed to reduce

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https://doi.org/10.1016/j.bios.2022.114793
Received 28 July 2022; Received in revised form 3 October 2022; Accepted 6 October 2022
Available online 11 October 2022

risk of food poisoning, especially in developing countries.

Cell-based biosensors offer an alternative approach to identifying food contaminants, especially when an antibody is the recognition unit. Much effort has been directed towards using antibody-based transmembrane (TM) receptors expressed on the plasma membrane of mammalian cells (Manhas et al., 2022; Scheller et al., 2018; Ueda et al., 2000) as biosensors, and a similar system has been developed in *E. coli* (Chang et al., 2018, 2021). As far as we can ascertain, such a system has not been established for yeast cells, probably because they have thick and stable cell wall. Nonetheless, many yeast-based biosensors have been developed because these well-studied eukaryotic species can withstand harsher environments than bacteria or mammalian cells while being safe for humans (Jarque et al., 2016). Moreover, yeast native or recombinant reporter enzymes can produce fluorescent or luminescent reporter signals (Välimaa et al., 2008). Many of these biosensors use a yeast two-hybrid (Y2H) system to study protein-protein interactions (PPIs), in which binding between bait and prey proteins activates gene regulatory factors that induce the expression of specific reporter proteins (Brückner et al., 2009). Among Y2H systems, a membrane-based yeast two-hybrid (MbY2H) system (Lentze and Auerbach, 2008) has been created that detects PPIs between membrane proteins. Bait and prey proteins are displayed on the yeast membrane and are connected by two split ubiquitin fragments with a TM domain in the MbY2H system (Thaminy et al., 2004). Triggered by the association between bait and prey proteins, split ubiquitin fragments form functional ubiquitin, which cleaves and releases a transcription factor to induce the expression of a reporter protein, such as β-galactosidase (Iyer et al., 2005).

Optimal concentrations of Zymolyase will render analytes that cannot penetrate yeast cell walls accessible to recognition units (antibody fragments) on cell membrane. After split ubiquitin complementation is triggered by analyte recognition, released transcription factor fusion (TF) will activate reporter enzyme gene circuits to provide a signal.

Here, we combined MbY2H with antibody-based chimeric receptors. Instead of native or engineered receptors (Chang et al., 2021), we found that using antibodies as affinity agents provided a simple programable platform that can detect a wide spectrum of targets. The Y2H system allows the choice of reporter and substrate systems to fit laboratory equipment. Yeast cells can be easily stored and cultivated, which would render yeast biosensors a durable practical tool under harsh storage and transformation conditions.

The underlying mechanism of small-molecule detection is based on the open-sandwich immunoassay principle (Ueda et al., 1996). In the absence of an antigen, affinity between two variable region fragments (V_H and V_L) of an anti-hapten antibody (Edelman et al., 1969) is too weak to form V_H/V_L complexes. However, affinity between two fragments can be significantly increased in the presence of antigens. This is a property of not only V_H and V_L but also of some nanobodies (V_HH) derived from heavy-chain antibodies from camelids (Hamers-Casterman et al., 1993) that lack light chains. Instead of V_H/V_L, the V_HH of the anti-caffeine heavy-chain antibody forms dimers in the presence of caffeine, thus revealing its presence (Sonneson and Horn, 2009). After replacing the bait/prey proteins with V_H/V_L or V_HH fragments and the erythropoietin receptor (EpoR) D2 domain, MbY2H can detect antigens by transmembrane signal transduction. In some cases, the EpoR D2 domain was used as a scaffold because it has been used successfully in mammalian systems (Ueda et al., 2000; Scheller et al., 2018).

For the detection of larger antigens, a single-chain variable fragment (scFv), formed by tethering V_H to V_L with a linker peptide (Huston et al., 1988), which retains antigen-binding activity like the original IgG (Peterson et al., 2006) was used. The scFv binds to multiple copies of epitope on the large targets and functions as a detector in the MbY2H sensor system under the principle of conventional sandwich immunoassay. In addition, breaking the cell wall using an optimal concentration of Zymolyase (lyticase) from *Arthrobacter luteus*, the Patrol Yeast is expected to detect large cell wall-impermeable antigens such as *E. coli*.

The proposed Patrol Yeast biosensors are novel MbY2H systems to identify various substances from small molecules to microorganisms irrespective of cell wall or membrane-permeability, and lead to food poisoning (Fig. 1). Patrol Yeasts detected caffeine and aflatoxins using the split-ubiquitin system. The secreted *Cypridina* luciferase reporter enzyme can also be integrated into this system for easier and safer high-throughput screening. The Patrol Yeast system could serve as a less labor-intensive tool to safely and economically identify food contaminants.

2. Materials and methods

2.1. Materials

Restriction enzymes were obtained from New England Biolabs Inc. (Ipswich, MA, USA). Chemical reagents were supplied by Fujifilm Wako Pure Chemicals (Osaka, Japan) unless otherwise stated. The monoclonal anti-aflatoxin (AFx) antibody 5A7 was generated by ARK Resource Co. Ltd., Kumamoto, Japan.

2.2. Plasmid construction

The plasmids pEF-BOS-BGPV_H-mEpoR and pEF-BOS-BGPV_L-mEpoR are pEF-BOS (Mizushima and Nagata, 1990)-based mammalian expression vectors for V_H-EpoR and V_L-EpoR, respectively. They have been created by replacing the V_H/V_L genes of HyHEL-10 in pME-V_H/V_L-ER (Ueda et al., 2000) with those of KTM219 (Lim et al., 2007), respectively, and allow BGP-dependent growth of Ba/F3 Pro-B cells (unpublished). A V_L(BGP)-EpoR DNA fragment was amplified using the primers Not-VLbgp_back and Sfi-EpoRTM_For from the plasmid pEF--BOS-BGPV_L-mEpoR. The yeast SUC signal sequence was first amplified by annealing the Spe-Suc_back and SUC_for primers. The resultant SUC sequence was fused with V_L(BGP)-EpoR by overlap extension PCR (OE-PCR) to obtain the SUC-V_L(BGP)-EpoR fragment. The V_H(BGP)-EpoR fragment was amplified from pEF-BOS-BGPV_H-mEpoR template using the SUR-VH-S1/SUR-VH-S2/SUR-VH-S3/Spe-Suc_back and Sfi-EpoRTM_For primers, then fused to the SUC sequence as described above, to obtain the SUC-V_H(BGP)-EpoR fragment. SUC-V_H(BGP)-EpoR and SUC-V_L(BGP)-EpoR were then inserted into pPR3-C (Dualsystems Biotech AG., Schlieren, Switzerland) and linearized with the restriction enzymes *Spe*I and *Sfi*I to construct pPR-V_H(BGP)-EpoR and pPR-V_L(BGP)-EpoR. The SUC-V_H(BGP)-EpoR and SUC-V_L(BGP)-EpoR DNA fragments were amplified using the primers Xba-Suc_back and Sfi-EpoRTM_For2 and inserted into a pBT3-C vector (Dualsystems Biotech AG.), which was linearized by *Xba*I and *Sfi*I restriction enzymes to construct pBT-V_H(BGP)-EpoR and pBT-V_L(BGP)-EpoR.

The V_HH(Caf) DNA fragment was amplified from pET-V_HH(Caf)-GS-GUS_{IV5}_KW using the primers NotVHHback and BamVHHfor (Su et al., 2019). V_HH(Caf) was then inserted into pPR-V_L(BGP)-EpoR and linearized by *Not*I and *Bam*HI to construct pPR-V_HH(Caf)-EpoR. The SUC-V_HH(Caf)-EpoR DNA fragments were amplified using the primers Xba-Suc_back and Sfi-EpoRTM_For2 and inserted into pBT-V_L(BGP)-EpoR linearized by *Xba*I and *Sfi*I to obtain pBT-V_HH(Caf)-EpoR. The SUC-V_HH(Caf)-ΔERD2 DNA fragment was amplified using the primer sets Spe-Suc_back and NheI_dERD2_For or Xba-Suc_back and NheI_-dERD2_For to remove the EpoR D2 domain while maintaining the TM domain. The SUC-V_HH(Caf)-EpoR was inserted into pPR-V_HH(Caf)-EpoR linearized with *Spe*I and *Nhe*I to generate pPR-V_HH(Caf)-ΔERD2, and the SUC-V_HH(Caf)-ΔERD2 DNA into pBT-V_HH(Caf)-EpoR linearized with *Xba*I and *Nhe*I to generate pBT-V_HH(Caf)-ΔERD2.

The monoclonal anti-aflatoxin (AFx) antibody 5A7 was generated by immunizing Balb/c mice with AFB2-keyhole limpet hemocyanin and screening by ELISA using AFB2-BSA. Binding to AFB1, AFB2, AFG1, AFG2, and AFM1 by this antibody was confirmed by competitive ELISA. The V_H and V_L region cDNAs were amplified from reverse-transcribed mRNA of hybridoma cells, using degenerate primer sets for mouse V_H

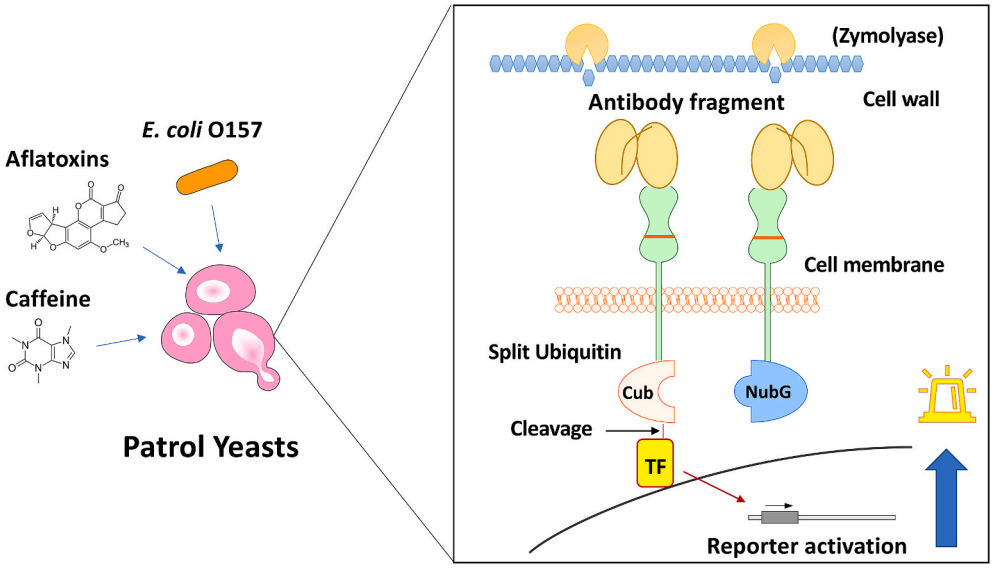

Fig. 1. Scheme of Patrol Yeast system. Optimal concentrations of Zymolyase will render analytes that cannot penetrate yeast cell walls accessible to recognition units (antibody fragments) on cell membrane. After split ubiquitin complementation is triggered by analyte recognition, released transcription factor fusion (TF) will activate reporter enzyme gene circuits to provide a signal.

(VH1-15 and JH1-4) and V_L (VK1-8 and JK1-5) (Hara et al., 2013). The amplified fragments were inserted into the pMD19 T-vector (Takara Bio Inc., Kusatsu, Japan), obtaining pMD19-5A7-V_H and pMD19-5A7-V_L, respectively.

The V_H(AFx)_1 and V_L(AFx)_1 DNA fragments were amplified using the primer sets VH_5A7_NcoI_Back/VH_5A7_Hind_For and VL_5A7_NcoI_Back/VL_5A7_Hind_For, with pMD19-5A7-V_H/pMD19-5A7-V_L as the templates, respectively.

To construct the inverse pair, V_H(AFx)_2 and V_L(AFx)_2 fragments were amplified using Not_VHback and Bam_VHfor and Not-VLback and Bam-VLfor primer sets, respectively, with the same plasmids as templates. Both fragments were inserted into linearized pPR-V_HH(Caf)-EpoR using *Not*I and *Bam*HI, to obtain pPR-V_H(AFx)-EpoR and pPR-V_L(AFx)-EpoR. The Spe-Suc_back and Sfi-EpoRTM_For2 primers were used to amplify the V_H(AFx)-EpoR and V_L(AFx)-EpoR DNA fragments, which were then inserted into pBT-V_HH(Caf)-EpoR linearized by *Sfi*I to construct pBT-V_H(AFx)-EpoR and pBT-V_L(AFx)-EpoR, respectively.

The mouse anti-O157 antibody 116-14 was generated by immunizing Balb/c mice with *E. coli* strain GTC03904 (O157; Division of Pathogenic Microbes, National BioResource Project, Gifu University, Gifu, Japan), and its specificity was confirmed by western blotting using several *E. coli* O strains expressing different O antigens (Fig. S1) (Yamasaki et al., 2016). The V_H and V_L region cDNAs were cloned by RT-PCR with mRNA extracted from corresponding hybridoma cells, using the degenerate 5′-primers MHV−2 (for V_H) and MKV−5 (Jones and Bendig, 1991) (for V_L) combined with fixed 3′-primers MHC-uni (for V_H) and mκ-GSP (for V_L), respectively. The DNA fragments were subcloned into pBlueScript II (Toyobo) and the V_H and V_L nucleotide sequences were determined. The V_H and V_L DNA fragments were then separately amplified with #O157V_H-Rev and #O157V_H-For, and #O157V_L-Rev and #O157V_L-For primers, respectively, and spliced by OE-PCR into a desirable scFv gene fragment. This was re-amplified using the primers Bam-VLO157for and Not-VHO157back using cloned scFv DNA as a template. The amplified ScFv(O157) fragment was then inserted into the pPR-V_HH(Caf)-EpoR plasmid linearized by *Not*I and *Bam*HI to construct pPR-ScFv(O157)-EpoR. We constructed pBT-ScFv (O157)-EpoR by amplifying ScFv(O157) DNA using the primers Hind-ScFvO157for and Nco_ScFvO157back, then inserting it into pBT-V_HH(Caf)-EpoR linearized with *Hin*dIII and *Nco*I.

To construct pLexA-CLY, the 8 × LexAOps LexA-binding sites and GAL1 promoter regions were amplified using LexA_SmaFor and LexA_Bam-back primers and pSH18-34 (Nova Lifetech Inc., Hong Kong, China) as a template. The amplified fragment was inserted into the multiple cloning site of the yeast Cluc reporter pCLY (ATTO, Tokyo, Japan) digested with *Bam*HI and *Sma*I using In-Fusion HD cloning kits (Takara Bio Inc.).

Supplementary Table 1 shows the sequences of the synthesized primers (Eurofins Genomics K.K., Tokyo, Japan).

2.3. Preparation of Patrol Yeasts to detect small molecules

The constructed plasmids were transformed into *S. cerevisiae* NMY51 (Dualsystems) using the Frozen-EZ Yeast Transformation II Kit (Zymo Research Corp., Irvine, CA, USA). The transformed yeasts were grown on selective agar media (Minimal SD Base with dropout supplement, Trp- and Leu-for the β-galactosidase system; Trp-, Leu-, Ura-for the CLuc system; Takara Bio Inc.) to generate single colonies carrying the plasmids of interest. Yeast was grown in the liquid selection medium containing 100 μg/mL ampicillin overnight at 30 °C.

2.3.1. Beta-galactosidase reporter system

Overnight yeast cultures diluted to OD_{600} = 0.3 were grown in selection medium with or without various concentrations of antigens. Yeast cultures were then incubated for 16 h at 30 °C. Thereafter, yeast cultures (2.5 mL) were adjusted to OD_{600} = 1.0, then centrifuged at 800×*g* for 2 min to obtain a precipitate. For the chemiluminescence assay, yeast precipitates were suspended in 500 μL of phosphate-buffered saline (PBS, pH 7.4) containing 2.5 mg/mL Zymolyase (100T; Nacalai Tesque, Kyoto, Japan), incubated for 1 h at room temperature, then disrupted for 10 s at 1 s intervals, 45% amplitude, 20 kHz on ice using a Q125 ultrasonicator (Qsonica LLC, Newtown, CT, USA).

2.3.2. CLuc reporter system

Yeast transformants were incubated in selection medium for ~48 h to reach OD_{600} = 5 (1 cm light path), then 25-μL portions were added to 3 mL of selection medium supplemented with 200 mM potassium phosphate buffer (pH 6.0). Yeast cultures were incubated with antigens at various concentrations for 25 h at 30 °C, then CLuc activity was measured.

2.4. Preparation of Patrol Yeasts to detect E. coli O157:H7

Shiga toxin-negative enterohemorrhagic *E. coli* O157:H7 (ATCC 43888) cultures were diluted to various concentrations based on OD_{600} measured with a V-730 spectrophotometer (Jasco, Tokyo, Japan), then colony-forming units (cfu)/mL were counted. A standard curve of the

linear relationship between OD_{600} and the cfu/mL of O157:H7 was derived and used to estimate the numbers of cfu/mL based on the OD_{600}.

Yeasts harboring pBT- and pPR-ScFv(O157) plasmids were grown in selection medium containing 100 μg/mL ampicillin at 30 °C overnight, centrifuged at 5000×*g* for 5 min, resuspended in selection medium containing Zymolyase (0.25 mg/mL) and ampicillin (100 μg/mL) to OD_{600} = 0.3, then incubated with gradient concentrations of *E. coli* O157:H7 for 16 h at 20 °C. A mixture of the cultured yeast and bacteria was centrifuged at 11×*g* for 10 min, then *E. coli* cells in the supernatant were removed. The pelleted yeast cells were sonicated as described above. *E. coli* strains producing Shiga toxin were autoclaved at 121 °C for 15 min in PBS, then specificity was determined at 10^7 cfu/mL.

2.5. Chemiluminescence assay

Extracts of Patrol Yeast containing the β-galactosidase reporter system were obtained by sonication and mixed with AttoGlow β-galactosidase chemiluminescence substrate (Michigan Diagnostics, Royal Oak, MI, USA) at a 1:1 ratio (v/v) for 45–60 min at room temperature. Chemiluminescence was assayed in Costar 96-well half-area white plates (Corning Inc., Corning, NY, USA). The intensity of chemiluminescence triggered by 0.2 M NaOH was measured using an AB-2350 luminometer (ATTO, Tokyo, Japan) with an 0.2-s gate and calculated as the sum of 30 measurements taken over 30 min.

Cultured Patrol Yeast (20 μL) harboring the CLuc reporter was mixed with 80 μL of CLuc substrate in the buffer provided in the CL-S1000 CLuc substrate kit (ATTO). Luminescence intensity was measured immediately at room temperature using an AB-2350 luminometer with a 0.2-s gate. Luminescence intensity was calculated as the sum of 30 measurements taken over 15 min. The OD_{600} of the yeast cultures for CLuc activity normalization was measured using an SH-1000 microplate reader (Corona Electric, Hitachinaka City, Japan) in transparent 96-well plates (200 μL/well) (Greiner Bio-One, Kremsmünster, Austria). Dose-response curves were fitted to the following four-parameter logistic equation: $y = d+(a-d)/(1+(x/c)^{\wedge}b)$, using ImageJ (Schneider et al., 2012) or GraphPad Prism7 (GraphPad Software Inc., San Diego, CA, USA). The limit of detection (LOD) was calculated as the concentration corresponding to the mean blank value plus 3 standard deviations (SDs) of the blank.

2.6. Flow cytometry

Yeast cells were collected by centrifugation at 700×*g* for 2 min and suspended in 600 μL of PBS containing 2.5 mg/mL Zymolyase and 0.1 mg/mL 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) (Wako, Tokyo, Japan). After incubation for 1 h at room temperature and centrifugation at 700×*g* for 2 min, the supernatant was discarded, then the cells were resuspended in PBS and measured using an SH-800 cell sorter (Sony, Tokyo, Japan). We measured 100,000 events per analysis. Products were excited at 488 nm and emission was detected using a 525/50 nm bandpass filter.

Patrol Yeast to detect *E. coli* O157 was incubated for 1 h with 2.5 mg/mL of Zymolyase to remove cell walls, then 10 nM biotinylated goat anti-mouse EpoR antibody (R&D Systems, Minneapolis, MN, USA) was incubated with the yeast lysate for 1 h at room temperature. The yeast cells were washed three times with PBS at 700×*g* for 2 min, and incubated for 1 h with 10 nM streptavidin-phycoerythrin to label yeast cells with EpoR on the membrane, and 0.1 mg/mL C_{12}FDG to trace β-galactosidase activity. The cells were washed three times with PBS (as above) and assessed by flow cytometry (FCM). Phycoerythrin (PE) was excited at 488 nm and emission was detected using a 585/30 nm bandpass filter.

3. Results and discussion

3.1. Patrol Yeast detected bone Gla protein (BGP)

The model small antigen septapeptide Bone Gla Protein (BGP)-C7 (derived from the C-terminal of human bone Gla protein or osteocalcin) was chosen as the target, and a Patrol Yeast was constructed to detect it. Transformed yeast cells were cultured with or without BGP-C7 antigen for 16 h at 30 °C, then β-galactosidase activity was measured. The V_H domain in pBT (bait) and V_L in pPR (prey) or V_L in pBT and V_H in pPR were constructed for the sensor proteins. Yeast cells harboring the plasmids were cultured and assayed using chemiluminescence. The results revealed a BGP-dependent increase in luminescence intensity in yeast cells harboring pBT-V_H(BGP)-EpoR and pPR-V_L(BGP)-EpoR (Fig. S2). Next, the Patrol Yeasts targeting food-related antigens were developed.

3.2. Patrol Yeasts detected caffeine

Construction of a Patrol Yeast to detect caffeine was attempted using the variable region of an anti-caffeine, single-domain antibody V_HH(Caf) to determine the feasibility of detecting small molecules such as caffeine and aflatoxins. Because the transmembrane (TM) signaling capability of sensor proteins is essential for detecting molecules outside cells, we used the EpoR D2 and TM regions to connect the V_HH and split ubiquitin inside the cells [pBT-V_HH(Caf)-EpoR and pPR-V_HH(Caf)-EpoR vectors]. Protein dimers that formed after exposing the V_HH detector to caffeine united the tethered split ubiquitins. These became functional and cleaved the artificial transcription factor LexA-VP16, triggering transcription of the β-galactosidase reporter gene. Variants without the EpoR D2 domain retained only the TM domain linking V_HH and the split ubiquitin proteins (Fig. 2A). The activity of β-galactosidase in the yeast lysate was higher after an overnight incubation with, than without caffeine (Fig. 2B). Moreover, the Patrol Yeast without the D2 domain increased the signal-to-background ratio by lowering the background signal (Fig. S3).

Yeast cells expressing sensor proteins were incubated overnight with various concentrations of caffeine, then disrupted by sonication to measure β-galactosidase activity. Higher caffeine concentrations resulted in more β-galactosidase activity (Fig. 2B), indicating that caffeine binding to the sensor proteins enhanced the β-galactosidase expression. The estimated EC_{50} was 1.4 μM, and the limit of detection (LOD) was 27 nM after fitting into a four-parameter logistic equation. The Patrol Yeast also detected the concentrations of caffeine in several Coca-Cola® beverages or caffeine-free Coca-Cola® spiked with caffeine (Fig. S4).

We analyzed β-galactosidase activity using the membrane-permeable substrate C_{12}FDG and FCM to determine reporter activation at the cellular level. Cells with increased fluorescence intensity detected by FCM indicated antigen-induced β-galactosidase activity (Fig. S5). The median fluorescence intensity of the yeast population increased 5-fold after incubation with higher caffeine concentrations.

3.3. Patrol yeasts detected aflatoxins

The V_HH(Caf) variable regions were replaced with $V_{H/L}$(AFx) of the anti-aflatoxin antibody in pBT-$V_{H/L}$(AFx)-EpoR and pPR-$V_{H/L}$(AFx)-EpoR to develop a Patrol yeast that detects aflatoxins. Thus, sensor proteins detect aflatoxins in a similar split-ubiquitin mechanism with the EpoR domain and the TM linker described above (Fig. 3A). The dose-dependence of Patrol Yeast harboring these plasmids was determined as chemiluminescence emission. Aflatoxins B1 and M1 activated the split-ubiquitin mechanism in the Patrol Yeast cells harboring pBT-V_H(AFx)-EpoR + pPR-V_L(AFx)-EpoR, which dose-dependently detected β-galactosidase activity (Fig. 3B and C). The estimated LODs for aflatoxins B1 (AFB1) and M1 (AFM1) were 0.003 and 0.033 nM after curve fitting, respectively, which were sufficiently low considering permissible

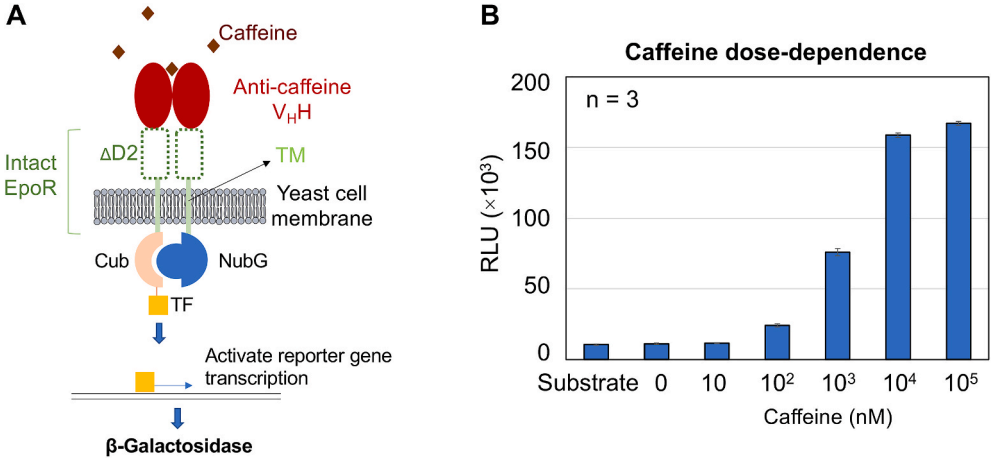

Fig. 2. Patrol yeasts detected caffeine. **A**, Scheme shows how Patrol yeast detects caffeine. Cub, C-terminal region of ubiquitin; TM, transmembrane; ΔD2, EpoR without D2 domain; NubG, N-terminal region of ubiquitin with I13G mutation; TF, transcription factor LexA-VP16. **B**, Chemiluminescence emission showing that Patrol yeast dose-dependently detects caffeine. Substrate, control without yeast lysate. Data are shown as means ± standard deviation. RLU, relative light units.

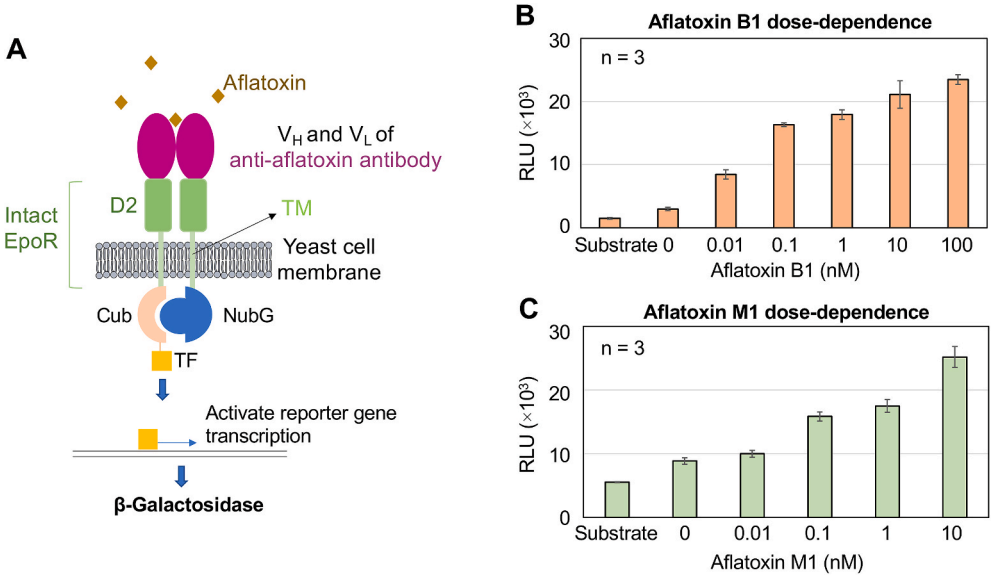

Fig. 3. Patrol yeast detected Aflatoxins. **A**, Scheme shows how Patrol Yeast detects aflatoxin. **B**, Patrol Yeast dose-dependently detected Aflatoxin B1 determined using chemiluminescence. **C**, Aflatoxin M1 dose-dependence. Substrate, control without yeast lysate. Data are shown as means ± standard deviation. Cub, C-terminal region of ubiquitin; D2, D2 domain of EpoR; V_H, variable region of heavy chain; NubG, N-terminal of ubiquitin with I13G mutation; RLU, relative light units; TF, transcription factor; TM, transmembrane domain; V_L, variable region of light chain.

aflatoxin contents in foods under the AFB1 worldwide and AFM1 European Union regulations of 3–64 and 0.15 nM, respectively (Clarke et al., 2014; Henry et al., 2002).

3.4. Patrol Yeast detected *E. coli* O157

Next, a system corresponding to that of enterohemorrhagic *E. coli* O157 was constructed to determine the ability of Patrol Yeast to detect

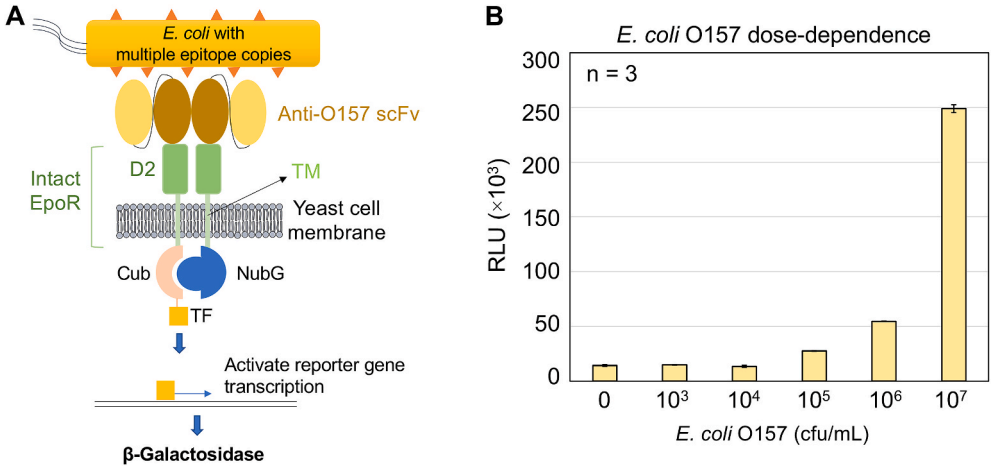

Fig. 4. Patrol Yeast detected *E. coli* O157. **A**, Scheme of how Patrol Yeast detected *E. coli* O157. **B**, Patrol Yeast dose-dependently detected *E. coli* O157:H7. Data are shown as means ± standard deviation. Cub, C-terminal part of ubiquitin; D2, D2 domain of EpoR; NubG, N-terminal part of ubiquitin with I13G mutation; RLU, relative light units; scFv, single-chain variable fragment of antibody; TF, transcription factor LexA-VP16; TM, transmembrane domain.

larger targets. Because the target was not a small molecule, we used the scFv of the anti-O157 antibody as the detector by replacing the V_HH(Caf) domain (Fig. 4A). Because one *E. coli* cell carries multiple copies of a specific sugar epitope on its outer membrane, scFvs should be combined to activate the ubiquitin mechanism and provide O157-dependent β-galactosidase activity. However, the yeast cell wall should be at least partially destroyed to allow O157 to reach the yeast cell membrane and emit the TM signal. First, the yeast was grown with various concentrations of live Shiga toxin-negative *E. coli* O157:H7. We finally detected O157:H7 dose-dependent β-galactosidase activity as chemiluminescence emission after optimizing Zymolyase digestion (Fig. 4B and S6a). Prolonged co-culture of the yeast and *E. coli* O157:H7 for 16 h at 20 °C with 0.25 mg/mL of Zymolyase resulted in intense signals. As expected, Zymolyase was essential to obtain O157-dependent signals (Fig. S6b).

The estimated LOD of O157:H7 detection was 2.5×10^4 cfu/mL. The specificity of Patrol Yeast for O157 was investigated using inactivated and autoclaved Shiga toxin-producing enterohemorrhagic *E. coli* O26, O111, and O157 isolates. Fig. S7 shows that the signals generated from live and heat-inactivated O157 were essentially the same after incubating Patrol Yeast in medium containing antibiotic ampicillin. The results showed that only the O157 strains generated a significant increase in luminescence signals, indicating that only specific binding between O157 and the Patrol Yeast generated β-galactosidase activity (Fig. S8).

We also assessed the ability of Patrol Yeast digested by Zymolyase to detect O157 as fluorescence emission. We eliminated interference from O157:H7 cells and visualized yeast cells harboring sensor proteins by labeling the EpoR domain of the sensor proteins on cell membranes with streptavidin/phycoerythrin (PE) and a biotin-conjugated anti-EpoR antibody. The FCM results showed increased red fluorescence from PE in all yeast groups harboring plasmids for sensor proteins, indicating sensor protein expression and integration into the membrane. The intensity of green fluorescence generated by β-galactosidase increased with higher concentrations of O157:H7 (Fig. S9). The increased ratio of the fluorescence intensity (green/red) corresponded to higher O157:H7 concentrations, indicating interaction between O157:H7 and sensor proteins on the cell membrane of the Patrol Yeast that activated β-galactosidase expression.

3.5. Patrol Yeasts with a secreted luciferase reporter system

We used a secreted *Cypridina noctiluca* luciferase (CLuc) reporter system (Yamagishi et al., 2006) to further widen the applications of the Patrol Yeast. The CLuc reporter has been included in yeast for several types of assays (Kanjou et al., 2007; Yamagishi et al., 2006). The plasmids, pBT-V_HH(Caf)-dERD2, pPR-V_HH(Caf)-dERD2, and pLexA-CLY were co-transformed into the yeast strain NMY51 to obtain a Patrol Yeast with secreted CLuc to detect caffeine. The LexA-VP16 artificial transcription factor liberated after caffeine recognition was predicted to trigger CLuc expression from the pLexA-CLY plasmid. The activity of CLuc can be directly detected in cultured yeast cells without a separation procedure. Fig. 5 shows that dose-dependent CLuc signals were found in the yeast cultures. Notably, CLuc activity was detected in yeast cultures containing 10 nM caffeine, which was not found in the β-galactosidase reporter system. After fitting into a four-parameter logistic equation, the estimated LOD of this system was 1.5 nM, which was one order of magnitude more sensitive than the β-galactosidase reporter with Atto-Glow substrate.

4. Conclusions

A novel yeast-based system was developed to detect toxins and microorganisms in food and beverages. Compared with other means of analyzing food contamination such as GC/MS/MS, yeast renders our new system inexpensive and more convenient as it is manageable and the technology for large-scale yeast production is mature. This is also an advantage over mammalian cell-based sensors that require expensive media and careful culture in a humidified atmosphere. Furthermore, the signal-on biosensor created for Patrol yeast led to rapid detection with good sensitivity. An organism-based sensor also reduces health and environmental risks during the detection process. Patrol yeast could detect multiple targets enabled by minor modifications to the sensor proteins. Caffeine detection was sufficiently sensitive for analyses of most common beverages, and sensitivity for aflatoxin detection also met mainstream food contamination regulations worldwide (Clarke et al., 2014; Henry et al., 2002), indicating the feasibility of Patrol Yeast in terms of food safety. The versatility of the Patrol yeast system was also shown by its ability to detect large targets such as *E. coli* O157. In addition to the established β-gal reporter, the secreted luciferase reporter (CLuc) further improved the feasibility of the Patrol Yeast system as it reduced the risk of generating aerosols and equipment requirements during practical operations.

One limitation of the Patrol yeast platform is that incubations are relatively longer than that in conventional analytical methods. However, incubations can proceed overnight which avoids hands-on time for sample analysis, and they take less time overall than mammalian systems. In conclusion, we believe that the Patrol Yeast system will contribute to the field of food safety as a sensitive, cost-effective, and manageable tool with which to detect and monitor a wide spectrum of targets.

Funding sources

This study was supported in part by the JST-Mirai Program (Grant

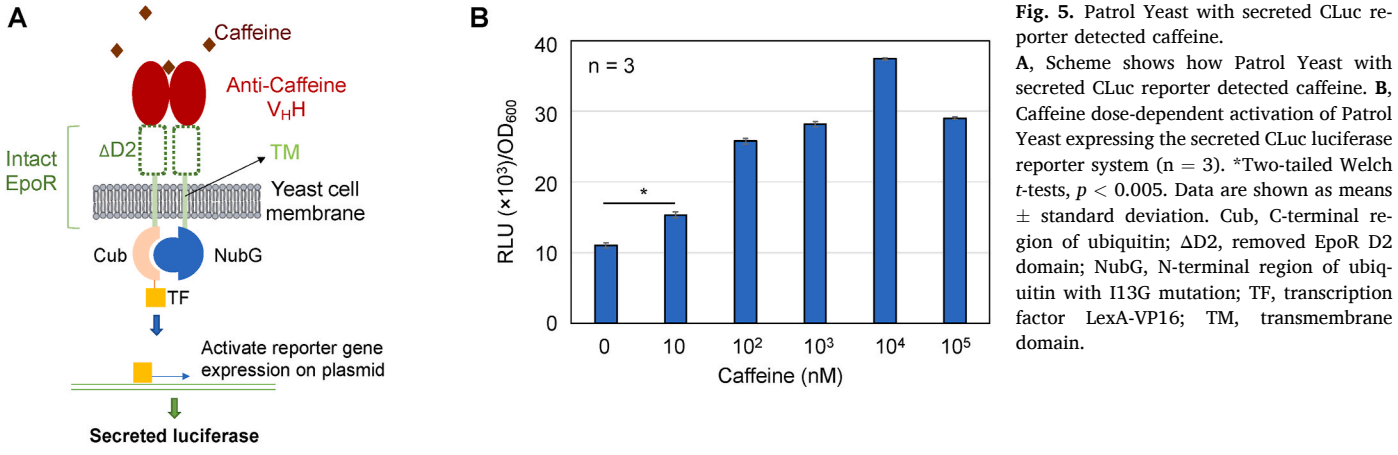

Fig. 5. Patrol Yeast with secreted CLuc reporter detected caffeine.
A, Scheme shows how Patrol Yeast with secreted CLuc reporter detected caffeine. **B**, Caffeine dose-dependent activation of Patrol Yeast expressing the secreted CLuc luciferase reporter system (n = 3). *Two-tailed Welch *t*-tests, $p < 0.005$. Data are shown as means ± standard deviation. Cub, C-terminal region of ubiquitin; ΔD2, removed EpoR D2 domain; NubG, N-terminal region of ubiquitin with I13G mutation; TF, transcription factor LexA-VP16; TM, transmembrane domain.

Number JPMJMI18D9) from the Japan Science and Technology Agency, Japan, by the JSPS KAKENHI (Grant Numbers JP18H03851 to H.U. and JP21K14468 to B.Z.) from the Japan Society for the Promotion of Science, and Nakatani Foundation for Advancement of Measuring Technologies in Biomedical Engineering.

CRediT authorship contribution statement

Jiulong Su: Investigation, Data curation, Writing – original draft. **Bo Zhu:** Visualization, Investigation, Validation, Formal analysis, Writing – original draft. **Akihito Inoue:** Investigation. **Hiroyuki Oyama:** Investigation. **Izumi Morita:** Investigation. **Jinhua Dong:** Investigation. **Takanobu Yasuda:** Investigation. **Yoshiko Sugita-Konishi:** Resources. **Tetsuya Kitaguchi:** Investigation, Supervision. **Norihiro Kobayashi:** Investigation, Supervision. **Shiro Miyake:** Resources, Investigation, Funding acquisition. **Hiroshi Ueda:** Conceptualization, Funding acquisition, Project administration, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank Jun Ishii, Yasuyuki Nakamura, and Akihiko Kondo at Kobe University, Japan, for the gifts of the pPR3-C/pBT3-C plasmids and the NMY51 yeast strain, Yuki Hirakawa and Tomomi Yamasaki at the Advanced Science, Technology & Management Research Institute of Kyoto (ASTEM) for providing the cells that produced the mouse anti-O157 monoclonal antibody 116-14, Yasunori Tanji at the Tokyo Institute of Technology for the *E. coli* O157:H7 strain, and Yoshio Iijima at the Kobe Institute of Health, Japan, for the *E. coli* strains producing Shiga toxin. We are also grateful to Chiaki Toyama and Yuki Kobayashi at the Tokyo Institute of Technology and Kenji Oonaka at Azabu University for their technical support, and the Division of Biomaterial Analysis, Open Facility Center, Tokyo Institute of Technology for the nucleic acid sequence analysis. W thank Editage Co. for English language editing. This work was performed under the Cooperative Research Program of "Network Joint Research Center for Materials and Devices" from MEXT, Japan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2022.114793.

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