Development of fluorescent
Escherichia coli for a whole-cell sensor of 2′-fucosyllactose

Jonghyeok Shin1,2, Myungseo Park1, Chakhee Kim2, Hooyeon Kim3, Yunjeong Park3, Chongjin Ban1,3, Jong-Won Yoon4, Chul-Soo Shin4, Jae Won Lee2,5, Yong-Su Jin2,5, Yong-Cheol Park6, Won-Ki Min7*, & Dae-Hyuk Kweon1,3,8*

2′-Fucosyllactose (2′-FL), a major component of fucosylated human milk oligosaccharides, is beneficial to human health in various ways like prebiotic effect, protection from pathogens, anti-inflammatory activity and reduction of the risk of neurodegeneration. Here, a whole-cell fluorescence biosensor for 2′-FL was developed. Escherichia coli (E. coli) was engineered to catalyse the cleavage of 2′-FL into L-fucose and lactose by constitutively expressing α- l-fucosidase. Escherichia coli ∆L YA, in which lacZ is deleted and lacY is retained, was employed to disable lactose consumption. E. coli ∆L YA constitutively co-expressing α- l-fucosidase and a red fluorescence protein (RFP) exhibited increased fluorescence intensity in media containing 2′-FL. However, the presence of 50 g/L lactose reduced the RFP intensity due to lactose-induced cytotoxicity. Preadaptation of bacterial strains to fucose alleviated growth hindrance by lactose and partially recovered the fluorescence intensity. The fluorescence intensity of the cell was linearly proportional to 1–5 g/L 2′-FL. The whole-cell sensor will be versatile in developing a 2′-FL detection system.

Human milk oligosaccharides (HMOs) are present in human breast milk and are closely associated with health benefits. HMOs act as decoys for pathogens (e.g., virus, bacteria, and protozoa) by inhibiting their ability to bind to the surface of epithelial cells1-4. Fucosylated oligosaccharides which are not found in bovine milk account for 50% of total HMOs. The HMOs 2′-fucosyllactose (2′-FL: Fuc-α1,2-Gal-β1,4-Glc) and 3-fucosyllactose (3-FL; Gal-β1,4-Fuc-α1,3-Glc), with a concentration range of 0.5–2 g/L, account for the largest portion of fucosylated HMOs5,6. Fucosylated HMOs circulate systemically, affecting the host immune response, the regulation of tumour metastasis, and resistance to bacteria, fungi, and other pathogens2,7,8. The concentration of 2′-FL in breast milk affects the ability to protect against vital systemic infections in nursing infant5. While low content of 2′-FL in breast milk has been associated with a higher rate of diarrhoea during lactation9,10, about 20% of human milk do not contain 2′-FL11. For these reasons, 2′-FL and 3-FL are spotlighted as nutraceutical and pharmaceutical ingredients; thus, a simple and visually measurable method is indispensable in evaluating their level in breast milk.

Various methods have been developed for the production of 2′-FL, including whole-cell biocatalysis12-20, enzymatic synthesis21,22, and chemical synthesis23,24. Regardless of the method used, a simple detection and quantification method is indispensable for the development of 2′-FL production. Quantification of 2′-FL has been mostly done using high-performance liquid chromatography (HPLC), high-pH anion exchange chromatography, and liquid chromatography–mass spectrometry (LC–MS)15,25,26. However, those types of equipment are time-consuming, labour-intensive, and expensive. Therefore, those methods are unlikely to be used in a high-throughput manner or for a brief test.

Recently, we developed 2′-FL quantification assays through two-step enzymatic reaction19 or one-pot reaction20 of fucosidase and fucose dehydrogenase, where 2′-FL concentration is spectroscopically readout by...
reduction of NADPH. In this study, we developed a visual detection method for 2′-FL using an *E. coli* strain expressing an α-L-fucosidase (FUC, E.C. 3.2.1.63) and a red fluorescence protein (RFP), which can be used in high-throughput applications. L-fucose was released from 2′-FL inside the bacterial cell by α-L-fucosidase, which specifically catalyses the hydrolysis of α1-2-linked L-fucopyranosyl residues from various oligosaccharides. L-fucose was then used for RFP synthesis (Fig. 1A). The *lacZ*-deficient *E. coli* strain was used to disable lactose consumption by cells. This engineered strain semi-quantitatively reported the 2′-FL content in milk in high-throughput by emitting fluorescence in a 2′-FL concentration-dependent manner.

**Results**

**Evaluating 2′-FL-dependent cell growth and fluorescence using a whole-cell biosensor.** A whole-cell biosensor, which grows and emits fluorescence only in the presence of 2′-FL, was designed (Fig. 1A). The plasmid pConFUC was constructed to constitutively express α-L-fucosidase under the control of the J23100 promoter (Fig. 1A). The 2′-FL can be hydrolysed by α-L-fucosidase in *E. coli* so that the amount of released L-fucose and lactose are proportional to 2′-FL concentration. The cells were engineered not to metabolize lactose by deleting their endogenous β-galactosidase gene (*lacZ*) while retaining *lacY* to import 2′-FL. Two *E. coli* BL21 (DE3) variants, ΔL YA and ΔL M15, with different levels of residual β-galactosidase activity, were cultured in R medium containing 2 g/L lactose. Both variants did not grow with lactose as a sole carbon source in a minimal medium for 24 h, whereas wild-type *E. coli* BL21 (DE3) grew well using lactose (Fig. 1B). The difference of growth rate of ΔL M15 and ΔL YA in lactose was marginal. For the strict restriction of lactose consumption, the *E. coli* ∆L YA strain which has much lower β-galactosidase activity was used in the following studies.

The active expression of α-L-fucosidase in *E. coli* ∆L YA was examined by analysing the 2′-FL cleavage in the soluble fraction of *E. coli* ∆L YA cell extracts. The cell extracts of wild type *E. coli* BL21 (DE3) and *E. coli* ∆L YA (Fig. S1A and Fig. 1C) did not digest 2′-FL. In contrast, 2′-FL was rapidly digested to release lactose and L-fucose by the cell extracts of *E. coli* BL21 (DE3) pConFUC (Fig. S1B) and *E. coli* ∆L YA pConFUC (Fig. 1D). These results suggest that α-L-fucosidase was actively expressed in *E. coli* regardless of *lacZ* deletion.

**2′-FL detection by the increase in fluorescence intensity.** *E. coli* ∆L YA or ∆L YA pConFUC were inoculated with R medium containing no carbon source, 2 g/L 2′-FL, 2 g/L lactose, or a mixture of 2 g/L 2′-FL.
and 2 g/L lactose. *E. coli* ΔL YA did not grow with any carbon source while *E. coli* ΔL YA pConFUC grew slowly in the presence of 2′-FL (Fig. 2A,B; Table S2) and did not grow using lactose as a sole carbon source. However, lactose did not affect the cell growth of *E. coli* ΔL YA pConFUC using 2′-FL as the carbon source (Fig. 2B).

*E. coli* ΔL YA pConFUC was co-transformed with pConRFP expressing a red fluorescence protein (RFP) under the control of the J23100 promoter. *E. coli* ΔL YA pConFUC/pConRFP (FLS1) cells had a long lag period of 4 days after their inoculation with R medium containing 2 g/L 2′-FL or a mixture of 2′-FL and lactose. Cells did not grow in R medium containing only lactose. RFP signal began to increase on the fifth day of bacterial inoculation in R medium containing 2′-FL (Fig. 2C). Although FLS1 showed a 2′-FL-dependent cell growth and a fluorescence increase, there was a few-days of long lag period. We thought that the cell was not ready to metabolize fucose even after 2′-FL cleavage to fucose and lactose.

**Figure 2.** Detection of 2′-FL through RFP fluorescence of *E. coli* strains. *E. coli* ΔL YA, ΔL YA pConFUC, and FLS1 strains were cultured in R medium, which contained lactose, 2′-FL or mixture of lactose and 2′-FL. Relative optical density (ROD) of (A) *E. coli* ΔL YA, (B) *E. coli* ΔL YA pConFUC, (C) Fluorescence intensity change (F/F₀) of RFP in FLS1 (*E. coli* ΔL YA pConFUC/pConRFP). Symbols denote the negative control (filled circle), 2 g/L 2′-FL (filled square), 2 g/L lactose (filled upward triangle), and 2 g/L lactose + 2 g/L 2′-FL (filled downward triangle). Results are the average of biological replicates (n = 2). Error bars represent standard deviations and are not displayed when smaller than symbol size.

**Adaptation of the whole-cell biosensor to L-fucose for faster response toward 2′-FL.** As the L-fucose metabolic pathway should be activated to use L-fucose as a carbon source, we tested whether the delayed response can be resolved by pre-adapting cells to L-fucose to shorten the lag period. Cells precultured in LB broth showed a long lag period lasting a day in R medium containing L-fucose (Fig. 3A). When *E. coli* ΔL YA was preadapted in R medium containing 10 g/L of L-fucose before inoculation for several rounds, the lag period was dramatically shortened, and the cells immediately entered the exponential growth phase upon inoculation (Fig. 3B). When adapted cells were precultured in the LB broth a second time, cell growth on the L-fucose was retarded similarly to the non-adapted cells (Fig. 3C). These results suggest that this fast-growing phenotype arose because of simple adaptation to the substrate.

*E. coli* ΔL YA pConFUC/pConRFP preadapted to L-fucose (denoted as FLS2) was cultured in R medium containing 2 g/L L-fucose. While *E. coli* ΔL YA lacking the plasmid for fucosidase did not grow in any media for 4 days, FLS2 showed exponential growth after a short lag period (< 8 h; Fig. 3D,E). Notably, the RFP fluorescence reached its maximal fluorescence within 10–20 h when FLS2 was grown in R medium containing 2′-FL (Fig. 3F). The fluorescence emission lasted for 4 days at a stable emission. Cells did not emit fluorescence using lactose as a sole carbon source and 2 g/L lactose present together with 2′-FL did not alter fluorescence intensity either. Although RFP intensity increased by only ~ 50%, these results suggest that RFP fluorescence was a relatively fast and reliable reporter of 2′-FL.

**Enhanced fluorescence intensity.** To quicken the 2′-FL detection and increase fluorescence intensity, the expression cassettes of RFP and fucosidase were recombined using different vectors. The small copy number (10–12) replicon p15A was replaced by the high copy number (20–40) replicon ColA for RFP expression (Fig. 4a). The plasmid pET-ConFUC was also constructed by replacing the kanamycin resistance cassette of pConFUC with the ampicillin resistance cassette of pET. *E. coli* ΔL YA strain was then transformed with the resulting plasmids pET-ConFUC and pColA-ConRFP. When *E. coli* ΔL YA pET-ConFUC/pColA-ConRFP (FLS3) was inoculated with R medium containing 2′-FL, fluorescence increased after a short lag phase (< 2 h; Fig. 4C). Furthermore, the maximum fluorescence intensity change was ~ 7 times higher than previous transformants containing pConRFP. The new whole-cell sensor not only exhibited much stronger fluorescence than the previous one the fluorescence intensity change was proportional to a 2′-FL concentration of 1–5 g/L (Fig. 4D and Fig. S2). The signal was strong enough to enable the visualization of the fluorescence (Fig. 4E).

**Reduced lactose toxicity by fucose adaptation.** We observed that the enhancement of fluorescence intensity by the new combination of expression cassettes was hindered by the presence of only 2 g/L lactose.
Indeed, media containing 50 g/L lactose or bovine milk supplemented with 2′-FL showed weak fluorescence intensity (Fig. 5B), indicating the hurdles imposed by lactose-induced cytotoxicity. E. coli symports lactose and protons through lacY, the lactose permease structural gene, and acidifies the cytoplasm via lactose transport. This acidification causes cytotoxicity followed by the induction of cellular acid shock, resulting in the reduction of proton motive force, intracellular ATP levels, and cell viability. As mentioned above, to accelerate fucose use, preadaptation to fucose was applied a second time to relieve the negative effects of cytoplasmic acidification by lactose. E. coli symports L-fucose and H+ by a L-fucose/H+ symporter, and the internalized cytoplasmic proton would induce E. coli to activate its acid resistance system and relieve cytoplasmic acidification. Therefore, preadaptation to fucose can be used not only to activate L-fucose metabolic pathway but also to enhance acid resistance by bacterial cells (Fig. 5A). Indeed, when ∆L YA pET-ConFUC/pColA-ConRFP was preadapted to fucose (denoted as FLS2), 2 g/L 2′-FL did not hinder the 2′-FL detection (Fig. 5C). Furthermore, pre-adaption to fucose enabled much larger fluorescence intensity change than the non-adapted cells, even in the presence of 50 g/L lactose (Fig. 5D,E). Pre-adaption no longer improved the fluorescence intensity when 2′-FL was added to bovine milk. However, the fluorescence intensity was still proportional to 2′-FL concentration (Fig. 5F). Both biosensors were still able to detect the biologically relevant concentration of 2′-FL mixed in bovine milk (Fig. S3).

Discussion

We designed and developed a functional E. coli whole-cell 2′-FL biosensor based on the growth-coupled red fluorescence emission produced after the cleavage of 2′-FL by recombinant α-L-fucosidase, and we demonstrated that the biosensor could quantify 2′-FL. As the working principle of the biosensor, 2′-FL enters E. coli ∆L YA perhaps via lacY and then is hydrolysed into L-fucose and lactose by the recombinant α-L-fucosidase in the cytosolic space. Because lactose is an abundant disaccharide in milk, it can be used after hydrolysing into glucose and galactose by β-galactosidase in the wild-type E. coli. Therefore, endogenous lacZ was deleted completely (∆L YA) or partially disrupted (∆L M15). We confirmed that both mutants did not grow in lactose medium at all, and there was no red fluorescence emitted in ∆L YA in the absence of a carbon source (Fig. 1B). Among the E. coli mutants,
the ΔL YA strain was chosen as the backbone strain for the transformation with the α-L-fucosidase originating from Xantomonas manihotis. The α-L-fucosidase was active in E. coli cytoplasm and efficiently cleaved the α(1→2) L-fucose branched site in the trisaccharide 2′-FL and hydrolysed it into lactose and L-fucose (Fig. 1D). L-fucose could be consumed, even though small amount, by E. coli cells extracts suggesting that L-fucose can be used as a sole carbon source. The expression of recombinant α-l-fucosidase and complete deletion of lacZ allowed cell growth in 2′-FL-containing media (Fig. 2B).

After 2′-FL hydrolysis inside the E. coli cell, L-fucose, a hexose, is metabolized into dihydroxyacetone phosphate and L-lactaldehyde by the sequential actions of a permease, an isomerase, a kinase, and an aldolase. Aerobically, L-lactaldehyde is oxidized in two steps to pyruvate using NAD-dependent lactaldehyde dehydrogenase and flavin-linked lactate dehydrogenase, and thus channelling all carbons from L-fucose into the central metabolic pathways involved in ATP and amino acid synthesis. Preadaptation to fucose is likely to accelerate cell growth and RFP production because this pathway is activated.

Lactose causes cytotoxicity in several ways. When lactose is transported into a cell that cannot metabolize lactose, the cellular membrane is damaged, and the membrane potential is disrupted by the so-called lactose-killing effect. Lactose can induce cytoplasmic acidification as it is transported into the cytoplasm with a proton through the lactose permease. The resulting acidification of the cytoplasm induces cellular acid shock and reduces

Table 1. List of strains and plasmids used in this study.

| Strain/plasmid | Relevant description | References |
|----------------|----------------------|------------|
| E. coli Top10 | F−mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7,697 galU galK rpsL (StrR) endA1 nupG | Invitrogen |
| ΔL YA | BL21 star (DE3) ΔL Tn7::lacYA (lacZ deleted) | 13 |
| ΔL M15 | BL21 star (DE3) ΔlacZYA Tn7::lacZΔM15 (lacZ disrupted) | 16 |
| FLS1 | ΔL YA harbouring pConFUC, pConRFP | This study |
| FLS2 | Fucose-adapted FLS1 | This study |
| FLS3 | ΔL YA harbouring pET-ConFUC, pColA-ConRFP | This study |
| FLS4 | Fucose-adapted FLS3 | This study |
| pConFUC | J23100 constitutive promoter/express α-L-fucosidase/kanamycin resistance/pBR322 replication origin | This study |
| pET-ConFUC | J23100 constitutive promoter/express α-L-fucosidase/ampicillin resistance/pBR322 replication origin | This study |
| pConRFP | J23100 constitutive promoter/express RFP/ampicillin resistance/p15A replication origin | This study |
| pColA-ConRFP | J23100 constitutive promoter/express RFP/kanamycin resistance/pColA replication origin | This study |
proton motive force, intracellular ATP levels, and cell viability. Our results show that cell preadaptation to fucose might allow to overcome lactose acidification. Because fucose is also transported with a proton, it is likely that the acid response system of E. coli can be activated during bacteria exposure to fucose.

To the best of our knowledge, this is the first demonstration of a simple and easy quantification method of 2′-FL using a whole-cell biosensor (Table S3). Our biosensor might be applicable for high-throughput screening applications using 96- or 384-well microplates. It has the potential to improve process development, colony selection, quality management, 2′-FL kit development and other steps involved in the production 2′-FL.

Material and methods

Chemicals and materials. 2′-FL was purchased from AP Technology (Suwon, Korea). L-Fucose was purchased from Carbosynth (Compton, Berkshire, UK). Lactose, trace elements for Riesenberg medium (R medium) and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Strains and plasmids. The list of strains and plasmids used in this study are listed in Table 1. The gene encoding α-l-fucosidase from Xanthomonas manihotis was synthesized from IDT (Coralville, IA, USA)29,35. The synthesized gene was cloned into the pET28b expression vector (Invitrogen, Carlsbad, CA, USA). Next, T7 promoter of pET28b was substituted with the J23100 promoter (BBa_J23100, https://parts.igem.org/Promoters/Catalog/Anderson) to construct pConFUC. pBbA5aRFP was purchased from Addgene (Addgene_35280)36. The lac UV5 promoter of pBbA5aRFP plasmid was also substituted with the J23100 promoter to construct pConRFP that constitutively expressed RFP. To construct pET-ConFUC, the kanamycin resistance gene of pConFUC was substituted for the ampicillin resistance gene amplified from pETduet (Novagen). pColA-ConRFP was constructed by inserting expression regions from pConRFP (Promoter-RBS-CDS) into pColAduet (Novagen).

Determination of 2′-FL cleavage by α-L-fucosidase. For the 2′-FL cleavage assay, cells harbouring the pConFUC plasmid were cultured in a 1 L baffled flask containing 200 ml of Luria–Bertani (LB) medium (1%
tity of 0.01 N H₂SO₄ was used as the mobile phase at a flow rate of 0.6 ml/min and 50 °C. All experiments were performed in triplicate.

Detection of 2'-FL from other carbon sources. *E. coli* cells were pre-cultured in LB medium at 37 °C by shaking at 250 rpm for 16 h. Pre-cultured cells were harvested and washed three times with sterilized phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂SO₄, 1.8 mM KH₂PO₄, pH 7.4). Cells were inoculated into R medium composed of 4 g/L (NH₄)₂HPO₄, 13.5 g/L KH₂PO₄, 1.7 g/L citric acid, 1.4 g/L MgSO₄, and 10 mL/L trace metal solution, i.e., 10 g/L FeSO₄, 2.25 g/L ZnSO₄, 1.0 g/L CuSO₄, 0.5 g/L MnSO₄, 0.23 g/L Na₂B₄O₇, 2.0 g/L CaCl₂, and 0.1 g/L (NH₄)₆Mo₇O₂₄, containing various carbon sources. OD and fluorescence intensity were measured during time course (Molecular Devices, Sunnyvale, CA, USA).

Analytical method. Cell concentration was measured by observing the OD at 600 nm. The fluorescence intensity of RFP was measured using the excitation at 584 nm and emission at 615 nm. The OD and fluorescence intensity were measured using the spectrophotometer (Spectramax M2, Union City, CA, USA). Obtained values in the presence of 2'-FL were divided by the values measured in the absence of 2'-FL to calculate relative optical density (ROD) and relative fluorescence unit (RFU). Concentrations of lactose, L-fucose, and 2'-FL after 2'-FL cleavage were measured by using HPLC (Waters Corporation, Milford, MA, USA) equipped with the Rezex ROA-Organic Acid H⁺ column (Phenomenex, Torrance, CA, USA) and a refractive index (RI) detector. A quantity of 0.01 N H₂SO₄ was used as the mobile phase at a flow rate of 0.6 ml/min and 50 °C. All experiments were performed in triplicate.

Statistics. No statistical method was used to determine the sample size in advance. No random method or blind test was used in the experiment and interpretation of results. Numerical analyses were performed using Graph Pad Prism (Graph Pad Software, San Diego, CA, USA).

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

J.S., J.W.L., Y.-S.J., W.-K.M., and D.-H.K. designed research. J.S. performed the experiments. J.S., M.P., C.K., H.K., Y.P., C.B., J.-W.Y., C.-S.S., J.W.L., Y.-S.J., Y.-C.P., W.-K.M. and D.-H.K. analyzed the data. J.S., W.-K.M., and D.-H.K. wrote the manuscript.

Competing interests

The authors declare no competing financial interest.

Additional information

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Correspondence and requests for materials should be addressed to W.-K.M. or D.-H.K.

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