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Highly sensitive and selective biosensor for a disaccharide based on an AraC-like transcriptional regulator transduced with bioluminescence resonance energy transfer

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KEYWORDS lactose, galactose, glucose, lactose-free, lactose-reduced, lactase, biosensor, milk, bioluminescence resonance energy transfer, low level

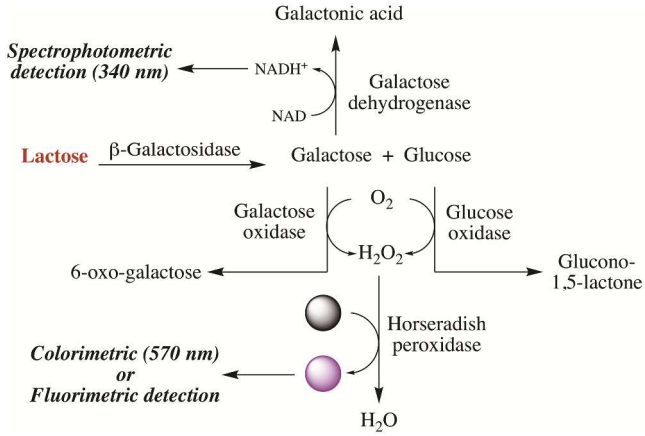
ABSTRACT: Sensitive and selective quantification of individual sugars in complex media is technically challenging and usually requires HPLC separation. Accurate measurement without the need for separation would be highly desirable. The measurement of trace levels of lactose in lactose-reduced milk exemplifies the problem, with the added challenge that trace lactose must be measured in the presence of ≈140 mM glucose and galactose, the products of lactase digestion of lactose. Biosensing is an alternative to HPLC but current biosensing methods, based on coupled-enzyme assays, tend to have poor sensitivity and complex biochemistry and can be time consuming. We explored a fundamentally different approach, based on identifying a lactose-specific binding protein compatible with photonic transduction. We identified the BgaR transcriptional regulator of *Clostridium perfringens*, which is highly selective for lactose, as a suitable ligand binding domain and combined it with a bioluminescence energy resonance transfer transduction system. This BRET-based biosensor showed a 27% decrease in the BRET ratio in the presence of saturating (1 mM) lactose. Using a five-minute assay, the half maximal effective concentration (EC_{50}) for lactose in PBS was 12 µM. The biosensor was 200 times more sensitive to lactose than to glucose or galactose. Sensitivity and selectivity were not significantly affected by the presence of 10% (v/v) dialyzed milk. The biosensor is suitable for direct determination of residual lactose in lactase-treated milk, with a limit of detection of 0.2 micromolar, 100 times below the most stringent lactose-free standard and without the need to remove fat or protein from the sample.

Saccharides are classified as mono, di, oligo or polysaccharides based on the number of five or six-carbon repeating units. Sugars, which are saccharides found in many foodstuffs include the disaccharides fructose in some fruits, sucrose in sugarcane and sugar beet and lactose in milk. Measurement of individual sugars can be important for food analysis as well as for some clinical purposes but is challenging because of the chemical similarities amongst saccharides, matrix interference and potentially low levels of the analyte. Therefore, accurate measurement of saccharides in complex and interfering matrices is usually only possible following removal of interfering components and chromatographic separation of different sugars. Refractive index detectors are commonly used as they are universal detectors, do not require sample derivatization and are quite widely available. HPLC with refractive index detection (HPLC-RI) typically has a limit of quantification of about 0.1 mM.[1] Commercial laboratories will only report disaccharide concentrations greater than to 3 mM.[2]

An alternative approach for quantifying saccharides is biosensing, based on the inherent selectivity of an enzyme that has evolved to metabolize such analytes. A common approach couples the enzymatic conversion of monosaccharides, such as galactose or glucose, by galactose dehydrogenase, galactose oxidase or glucose oxidase to the colorimetric or fluorometric measurement of a reaction product (Scheme 1).[3] This methodology has been extended for quantifying disaccharides, including lactose, sucrose, maltose and lactulose. The involvement of two or more enzymatic steps make these approaches complicated, liable to interference and cumulative errors and potentially time consuming. Specifically, biosensing based on galactose dehydrogenase, galactose oxidase or glucose oxidase cannot accurately measure low concentrations of disaccharides such as lactose, sucrose or maltose in samples that also contain their constituent monosaccharides. This highlights a fundamental problem with enzymes as recognition elements for trace analyte measurements.

Enzymes, by their nature, have evolved to trade-off affinity for their substrates (K_M) for high catalytic turnover (k_{cat}) and often tend to exhibit moderate affinity and selectivity. Furthermore, not all enzymes generate reaction products that can be directly measured, thereby necessitating the coupling of the analyte selective enzyme to downstream enzymes to generate an optically or electrochemically detectable product. In order to bypass these limitations we focused on binding proteins and receptors, instead of enzymes, as the analyte recognition element. Our rationale is that some binding proteins and recep-

tors have been subject to selection pressure for very high ligand affinity and/or selectivity. We sought to exploit these properties by coupling a saccharide binding protein to direct photonic transduction using changes in bioluminescence resonance energy transfer (BRET) efficiency.



Scheme 1. Approaches to lactose biosensing based on enzyme consortiums using β-galactosidase combined with galactose dehydrogenase, galactose oxidase or glucose oxidase and horseradish peroxidase. Modified from Jasti *et al*.[3]

Here, as a pertinent example of the problems described, we focus on the challenge of measuring trace amounts of lactose, the major sugar of bovine and human milk (4.5-7.0% (w/v) or 132-200 mM). Lactose-reduced milk and milk products are now widely produced commercially, both to support the expansion of dairy products to the majority of the human population who do not have the lactase persistence allele, as well as for consumers with a lactase persistence genotype who have an acquired secondary hypolactasia.[4] Lactose-reduced dairy products are generated by treating milk with β-galactosidase, which hydrolyses lactose to glucose and galactose. Various standards have been set for residual lactose by food regulatory authorities. Probably the most stringent objective threshold for 'lactose-free' milk is 300 µM or 0.01% (m/v) lactose, which is enforced in countries such as Denmark, Estonia, Finland and Norway.[5] The International Organization for Standardization[6,7] specifies HPLC-RI for the determination of lactose in milk and milk products. Coupled-enzyme assays are also commonly used to measure saccharide concentrations but, together with HPLC-RI, are considered either unsuitable or insufficiently sensitive for quantifying lactose in lactase-treated dairy products.[2,8]

The current work considers several lactose-binding proteins for their potential as analyte recognition elements for lactose biosensing, constructed RET-based biosensors and investigated their selectivity and sensitivity for lactose and non-target saccharides. We also investigated how a preferred biosensor for lactose performed under simulated real world use conditions.

Materials and Methods

Construction of RET proteins

The DNA sequence encoding BgaR (*C. perfringens*, strain 13) was codon optimized for expression in *E. coli* and synthesized commercially (GenScript, USA). The BgaR fragment was inserted between GFP^2 and RLuc8 sequences previously cloned into a pRSET vector (BioLabs, Australia), with an *N*-terminal histidine tag, using PstI/BstBI endonucleases.[9] Using PstI, the BgaR fragment was also inserted between the CFP and YFP sequences previously cloned into pRSET with an *N*-terminal histidine tag.[10] Integrity and orientation of clones were verified by sequencing.

Expression and purification of BgaR fusions

GFP^2-BgaR-RLuc8 (**1**) was expressed in *E. coli* BL21 DE3 (New England BioLabs). 50 mL of Lysogeny Broth supplemented with 2% (v/v) glucose and 100 µg/mL ampicillin was inoculated with a single colony and cultured at 37°C, 200 rpm until it reached an Abs_{600nm} of 0.8. 250 mL LB, supplemented with 100 µg/mL ampicillin, was inoculated to an Abs_{600nm} of 0.05 and cultured at 28°C, 200 rpm for 16 hours. CFP-BgaR-YFP was expressed in *E. coli* BL21 DE3 (New England BioLabs) according to published protocols, with the following modifications.[11] 200 mL LB supplemented with 100 µg/mL ampicillin was inoculated with a single colony and cultured at 28°C, 200 rpm for 48 hours. Cells were harvested and lysed using a homogenizer (Microfluidics M-110P, P = 20 000 psi). His_6-tagged proteins were purified using TALON® Superflow Metal Affinity Resin (Takara Clontech, Australia). Eluted proteins were dialyzed against Tris buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, pH 8.0) using a D-tube™ Dialyzer (Merck, 6-8 kDa MWCO). Purity was assessed by SDS-polyacrylamide gel electrophoresis (Fig. S-1). Protein concentrations were estimated by measurement of $Abs_{280\ nm}$.

BRET^2 measurements

Spectral scans were recorded with a SpectraMax M3 plate-reading spectrofluorimeter (Molecular Devices) in luminescence mode (20 nm increments) in white 96-well plates (Optiplate™-96, PerkinElmer). 1 µM of purified protein was used for all BRET assays, in a final volume of 100 µL, where the protein and analyte were diluted in phosphate-buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.3) or 10% (v/v) dialyzed full cream, lactose-free milk in PBS. The purified protein was incubated for 5 minutes at 30°C with the sugar or water. At the end of the incubation time, 5 µL of coelenterazine 400a in EtOH was added (to a final concentration of 17 µM) and the spectral scans were recorded immediately. BRET^2 ratio was calculated as the ratio of acceptor emission intensity at 500 nm to donor emission intensity at 420 nm. FRET measurements were carried out in a similar manner to the BRET^2 assays, with the following modifications. Spectral scans were recorded in fluorescence mode (λ_{ex} = 435 nm, 455 nm cut-off, 20 nm increments). FRET ratio was calculated as the ratio of acceptor emission intensity at 520 nm to donor emission intensity at 480 nm.

Full cream lactose-free milk dialysis

To remove low molecular weight components, particularly lactose, galactose and glucose, full cream lactose-free milk was dialyzed according to the following protocol. 20 mL of full cream lactose-free milk was dialyzed twice against 1 L of water at 4˚C for 90 minutes in a D-tube™ Dialyzer (Merck, 3.5 kDa MWCO). 1 mL aliquots of the dialyzed milk were frozen on dry ice and stored at -80˚C

Results and Discussion

We believe biosensors based on direct molecular recognition are well suited to address the need for a selective and sensitive tool to quantify small chemicals such as sugars in complex matrices. It is possible to transduce changes in protein structure or conformation into a photonic signal using Förster Resonance Energy Transfer (RET). RET is a non-

radiative transmission of energy between fluorescent molecules and, with luminescent proteins as donor, has been used for real-time ratiometric quantification of inter- and intramolecular protein interaction.[12] Amongst other factors, RET efficiency and strength is directly linked to the distance between and the relative orientation of the donor and acceptor.[13] Consequently, variation of the distance between RET pairs and its impacts on RET efficiency have been increasingly used for the design of probes and in assays studying protein-protein interactions.[12] Fluorescence Resonance Energy Transfer (FRET) is the most commonly used RET system with both the donor and acceptor moieties being fluorophores. In instances where the RET donor is a bioluminescent protein such as a luciferase enzyme, it is referred to as Bioluminescence Resonance Energy Transfer (BRET).[14,15] Moreover, BRET has been used successfully to detect protease activities,[10] to quantify µM-mM maltose in beer[9] and to detect fM levels of volatiles.[16]

Selection of biological recognition element

From literature reports, we identified a set of lactose binding proteins that exhibit lactose-dependent conformational change and are potentially able to measure lactose concentration through a quantifiable photonic output. The candidates were drawn from the repertoires of bacterial periplasmic nutrient binding proteins (PBPs), transcriptional regulators (TRs) and permeases. PBPs are a broad family of proteins involved in both chemotaxis and transport of small molecules such as sugars, vitamins and peptides. The binding of small molecules to PBPs stabilizes the closed conformation and allow signaling to membrane bound receptors specific to transport and/or chemotaxis.[17] TRs are a diverse class of proteins that have a DNA-binding motif and an effector-binding domain. Binding of metabolites or nutrients to the latter stabilizes a structural change, which regulates gene expression by modulating DNA accessibility. The major facilitator superfamily (MSF) of proteins comprises a broad range of evolutionary related membrane transport proteins found in bacteria, archaea and eukarya. Permeases of the MSF possess 12 or 14 transmembrane α-helical spanners and enable the transport of solutes including sugars, oligosaccharides, amino acids, nucleosides, organophosphate esters and metabolites through the membrane.[18]

Proteins of the lac operon

The consortium of expressed proteins forming the lactose operon of *E. coli* are regulated by the lac repressor, *lacI*. In the absence of lactose, the genes downstream of *lacI* are repressed, avoiding the expression of a metabolic pathway for a nutrient not available to the cell. When lactose is present, it is partially transgalactosylated to allolactose by β-galactosidase, which in turn binds to *lacI* and relieves the repression of the lactose metabolic pathway.[19] Since the actual effector for *lacI* is allolactose rather than lactose itself, *lacI* does not appear suitable for measuring lactose. The lac operon also encodes a lactose permease (*lacY*), an integral membrane protein that transports lactose from the bacterial periplasm across the cell membrane into the cell.[20] Although *lacY* directly binds lactose, work by Kaback *et al*[20] identified that the binding of galactopyranosides to the transporter could not be monitored with ensemble FRET carried out with a conventional spectrofluorometer but only by single-molecule FRET by alternating laser excitation spectroscopy. Consequently, *lacY* was not considered suitable as the biological recognition element for a BRET-based lactose biosensor.

Putative periplasmic lactose-binding protein of Agrobacterium radiobacter

The putative periplasmic lactose-binding protein from *A. radiobacter* (*ar*LBP)[21] was identified as a potential recognition element for lactose. Little is known about the specificity or function of this bacterial protein. Greenwood *et al.*[21] reported the binding protein-dependent active transport of lactose and of a non-metabolisable analogue of lactose in *A. radiobacter*. The isolated protein presented a high binding constant for lactose and based on sequence homology was identified as a putative lactose binding protein. We fused the sequence encoding *ar*LBP with the BRET donor and acceptor proteins *Renilla* luciferase (Rluc8) and GFP^2 at the *C*- and *N*-termini, respectively, taking care to exclude the signal sequence usually present at the *N*-terminal end of PBPs. Although BRET transmission was observed between the donor and acceptor of the fusion, we did not detect a change in BRET ratio in the presence of up to 100 µM lactose or galactose (data not shown). Although binding of lactose to *ar*LBP was observed previously, it is possible that the topology of *ar*LBP is unsuited for transducing conformational change by BRET.

Putative lactose-dependent TR of Clostridium perfringens

Since *lacI*, the TR from *E. coli,* binds allolactose rather than lactose we searched the literature for other known lactose-binding TRs. The search retrieved *bgaR*, which encodes a putative TR and sits upstream of a β-galactosidase gene in *C. perfringens*. The BgaR regulator was previously used by Hartman *et al.*[22] in a plasmid-based promoter system to regulate protein expression using lactose. BgaR therefore seemed to be a promising biological recognition element for lactose in a BRET biosensor.

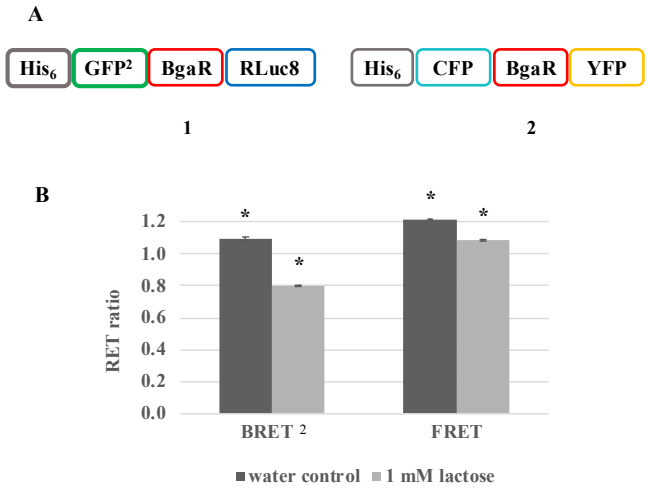


Figure 1. (A) Design of fusion proteins GFP^2-BgaR-RLuc8 (**1**) and CFP-BgaR-YFP: the BgaR protein is flanked with the BRET^2 donor RLuc8 at the *C*-terminus and the acceptor GFP^2 at the *N*-terminus GFP^2-BgaR-RLuc8 (**1**), or the FRET donor CFP at the *C*-terminus and the acceptor YFP at the *N*-terminus CFP-BgaR-YFP. (B) BRET^2 and FRET ratios (means ± SD, n = 3) of 1 µM of purified fusion protein GFP^2-BgaR-RLuc8 (**1**) or CFP-BgaR-YFP in the presence of water (dark grey bars) or of 1 mM lactose (light grey bars). BRET^2 scans were recorded following the addition of 17 µM coelenterazine 400a substrate. *$P < 0.0001$.

We expressed a fusion of the BgaR protein with BRET or FRET donor and acceptor polypeptides. BRET pairs have previously shown greater energy transfer when the luciferase (RLuc) donor is at the *C*-terminus and the GFP^2 acceptor at the *N*-terminus of biosensor fusions.[23] We designed and con-

structed the GFP^2-BgaR-RLuc8 (**1**) and CFP-BgaR-YFP fusions accordingly (Fig. 1A). RET ratios were measured for both the BRET^2 and the FRET fusions in the presence of water or 1 mM lactose. For both fusions, 1 mM lactose drove a decrease in the RET ratio. For GFP^2-BgaR-RLuc8 (**1**) the decrease was from 1.09 ± 0.01 to 0.800 ± 0.003 and, for CFP-BgaR-YFP, from 1.216 ± 0.004 to 1.089 ± 0.004. Therefore lactose caused a 27% drop in the RET ratio for GFP^2-BgaR-RLuc8 (**1**) compared with a 10% decrease for CFP-BgaR-YFP.

In an attempt to understand the differences between the FRET and BRET biosensor responses, we investigated the structure of BgaR protein. A homology model of BgaR, based on crystal structures of other AraC/XylS family members, was constructed using the Phyre2 algorithm (Fig. 2).[22] The DNA binding domain of BgaR is characteristic of members of the AraC/XylS subfamily of TRs, with two helix-turn-helix (HTH) motifs at the *C*-terminus (Fig. 2, red).[24,25] The *N*-terminal domain of the 279-amino acid BgaR protein has nine antiparallel β-strands (Fig. 2, yellow). Based on the homology model, we estimate the *N*- and *C*-termini of BgaR to be approximately 2.9 nm apart. With the previously determined radii of GFP-like proteins and *Renilla* Luciferase of 2.03 nm and 2.33 nm respectively,[26] we estimate the donor and acceptor separation of GFP^2-BgaR-RLuc8 (**1**) be 7.3 nm, and the separation between CFP and YFP in CFP-BgaR-YFP to be 7.0 nm. Consequently, the separation between donor and acceptor match the working range of BRET^2 transduction systems (3.8 nm - 11.3 nm)[26] better than that of FRET systems (2.4 nm - 7.2 nm).[23] The estimated chromophore-to-chromophore distance of 7.3 nm in GFP^2-BgaR-RLuc8 (**1**) predicts a RET efficiency of approximately 66% and is situated in the dynamic range of the Förster curve (Fig. S-2). Consequently, a small variation in the GFP^2/RLuc8 separation will result in a larger change in RET ratio than would occur in the equivalent CFP/YFP construct. The 7.0 nm donor-acceptor separation in the CFP-BgaR-YFP construct corresponds to a RET efficiency of 9%. The corresponding section of the Förster curve is relatively insensitive to variations in inter-chromophore distance (Fig. S-2). We therefore postulate that superior signal of the BRET construct is due to better matching of the BRET Förster distance with the molecular dimensions of the construct.

BgaR belongs to the helix-turn-helix (HTH) GntR superfamily of bacterial TRs. While they are characterized by their conserved *N*-terminal DNA-binding domain comprising a winged HTH motif (wHTH), TRs of the GntR superfamily show great heterogeneity within their effector-binding domains. Consequently, they are often subdivided based on similarities of the latter.[27] BgaR is a putative member of the AraC/XylS subfamily and to our knowledge, BgaR is the first example of this subfamily of TRs to have been converted into a biosensor. Members of the FadR subfamily have been incorporated as analyte recognition elements of FRET-based biosensors for metabolites such as lactate,[11] pyruvate[28] and trehalose-6-phosphate.[29] The FadR subfamily is the most well-studied with members characterized by the presence, in the effector binding domain, of a barrel-like structure composed uniquely of α-helices.

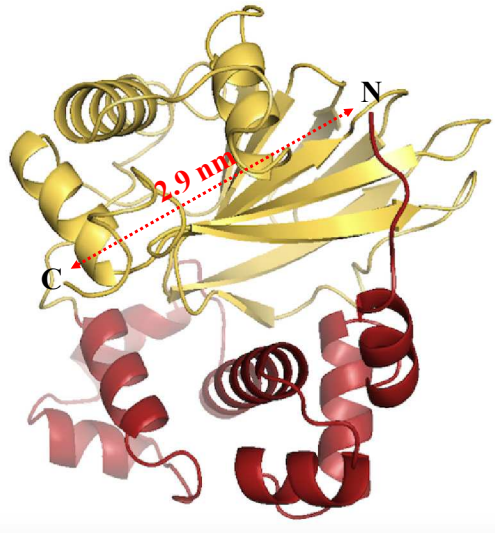


Figure 2. Cartoon diagram of the homology model obtained from the 279 amino acid BgaR TR from *C. perfringens*, generated with the Phyre2 algorithm.[30] The *N*-terminal effector-binding domain is showing in red and the *C*-terminal DNA-binding domain, in yellow.

Selectivity of BgaR investigated with BRET^2 reporters

When Hartman *et al*[22] developed a protein expression cassette under the control of the TR BgaR they reported that gene expression was upregulated in the presence of lactose but not glucose. However, the selectivity of BgaR towards other saccharides has not been characterized. The GFP^2-BgaR-RLuc8 (**1**) biosensor provides an opportunity to easily determine the broader ligand-specificity of BgaR. We tested a range of disaccharides that are structurally related to lactose (β-D-galactosyl-(1→4)-D-glucose), namely lactulose (4-O-β-D-galactosyl-D-fructose), melibiose (D-galactosyl-α(1→6)-D-glucose), maltose (4-O-α-D-glucosyl-D-glucose), cellobiose (4-O-β-D-glucosyl-D-glucose), trehalose (α-D-glucosyl-(1→1)-α-D-glucose) and sucrose (β-D-Fructosyl α-D-glucose), as well as the monosaccharides, galactose and glucose (Fig. 3). Of the sugars tested, lactose caused the largest decrease in BRET^2 ratio (27%, at 1 mM). 1mM lactulose decreased the BRET^2 ratio by 10%, whereas 1 mM of other disaccharides caused decreases in BRET^2 ratios of less than 2%. We address the potential interference of lactulose in a later section. The monosaccharides galactose and glucose reduced the BRET^2 ratio by 2% and 4%, respectively. Since GFP^2-BgaR-RLuc8 (**1**) exhibited the largest responses to lactose and lactulose, the affinity of the biosensor for each respective sugar was investigated further.

Characterization of GFP^2-BgaR-RLuc8 (1)

The response of GFP^2-BgaR-RLuc8 (**1**) to lactose and lactulose (in a 5 minute homogenous assay in PBS) was concentration dependent (Fig. 4). The response of GFP^2-BgaR-RLuc8 (**1**) to lactose was quasi-linear over almost 3 log units with an EC_{50} of 12 ± 1 μM and a limit of detection of 1 μM. The affinity of GFP^2-BgaR-RLuc8 (**1**) for lactulose was approximately 200 fold weaker, with an EC_{50} of 2.4 ± 0.2 mM. The limit of detection for lactulose was 0.1 mM i.e. 100 fold higher than for lactose. The lactulose response was quasi-linear over almost 2 log units.

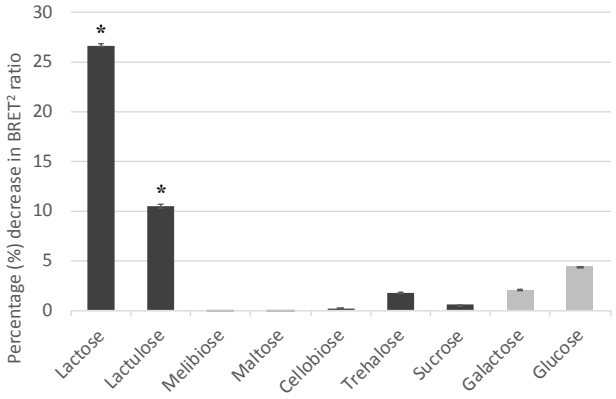


Figure 3. Response of GFP^2-BgaR-RLuc8 (**1**) to a range of di- and mono-saccharides, at 1 mM. BRET^2 ratios (mean ± SD, n = 3) were recorded following addition of 17 µM coelenterazine 400a to 1 µM of GFP^2-BgaR-RLuc8 (**1**) after incubation with the specified sugars for 5 minutes at 30˚C. BRET^2 ratios were normalized to the water response and expressed as percentages of decrease in BRET^2 ratio. *$P < 0.01$

The response of the GFP^2-BgaR-RLuc8 (**1**) to lactose in 10% (v/v) milk is concentration dependent with an EC_{50} of 21 ± 2 µM, linearity over almost 3 log units and a limit of detection of 1 µM (Fig. 4). The sensitivity of GFP^2-BgaR-RLuc8 (**1**) to lactose in 10% (v/v) milk and saturating concentrations of galactose and glucose (18-23 µM) is statistically different from that observed in PBS only (11-14 µM). However, the affinity of GFP^2-BgaR-RLuc8 (**1**) for lactose was not decreased dramatically by the presence of either 10% (v/v) dialyzed milk or high concentrations of glucose and galactose. This is probably due to the intrinsically high selectivity of the biological recognition element BgaR for lactose, but also due to the efficiency of the BRET^2 transduction mechanism in complex media. In BRET, the photons originate from a chemical reaction catalyzed by the luciferase and supplants the need to exogenously excite the RET donor through the media.

We also investigated the effects of measuring the lactose concentrations (13.9 mM to 0 mM) in simulated milk systems (Fig. 4, dashed line), while maintaining constant total masses of sugars according to [lactose]+([galactose+glucose])/2 = 13.9 mM (13.9 mM is equivalent to the total sugar concentration in unmodified 10% (v/v) whole milk). To achieve this, we dialyzed full cream, lactose-free milk against water to eliminate small molecules. The dialyzed milk was used to reconstitute a 10% (v/v) milk matrix with a range of precisely defined levels of lactose, galactose and glucose where [lactose]+[galactose+glucose]/2 = 13.9 mM. The ten-fold dilution factor was chosen to accurately simulate assay conditions when measuring lactose in samples at or below the 300 µM 'lactose-free' threshold, i.e. following lactase treatment.

Under these conditions, which closely mimic the situation in milk samples, the LOD for lactose was 0.2 µM (0.00003% w/v). The EC_{50} for lactose changed marginally under these conditions, from 12 to 21 µM, but the difference was not statistically significant at the p = 0.05 level. The EC_{50} for lactose is approximately 15 fold lower than the most stringent objective regulatory standard (0.01% w/v) for "lactose free" dairy products. The similarity of the log concentration-response functions in the presence or absence of 10% (w/v) full cream milk is remarkable because in the latter case, at lower concentrations of lactose, the measurements are made in the presence of 13.9 mM glucose and galactose. We attribute the strong ability of the biosensor to "ignore" potentially interfering substances to the intrinsically high selectivity of the biological recognition element BgaR. Secondary contributors to the biosensor's performance are likely the robust ratiometric nature of the BRET^2 transduction mechanism and the absence of an external source of illumination, which would cause light scattering and increase noise in a turbid medium such as milk, even when diluted tenfold.

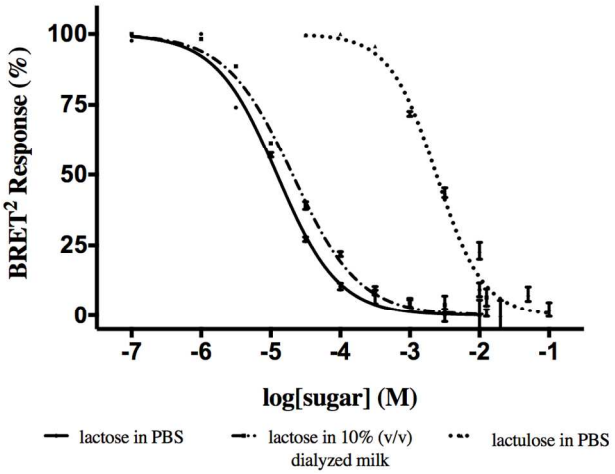


Figure 4. Log lactose/lactulose concentration dependence of GFP^2-BgaR-RLuc8 (**1**) response in PBS or 10% dialyzed full cream lactose-free milk. *Solid line*: lactose concentration dependence of GFP^2-BgaR-RLuc8 (**1**) in PBS. EC_{50} = 12 ± 1 µM, LOD = 1 µM. *Dashed line*: lactose-concentration dependence of GFP^2-BgaR-RLuc8 (**1**) in a defined carbohydrate milk matrix made of 10% dialyzed full cream lactose-free milk (mean ± SD, n = 3) with lactose, galactose and glucose added such that ([lactose]+[galactose+glucose]/2=13.9 mM). EC_{50} = 21 ± 2 µM, LOD = 0.2 µM. *Dotted line*: lactulose concentration dependence of GFP^2-BgaR-RLuc8 (**1**) in PBS. EC_{50} = 2.4 ± 0.2 mM, LOD = 0.1 mM. In all cases BRET ratios were measured with of 1 µM of GFP^2-BgaR-RLuc8 (**1**), by 5 minute incubation at 30°C, followed by addition of 17 µM coelenterazine 400a. Responses are plotted as the means (± SD, n = 3) of the percentage of maximal BRET^2 response in the absence of the ligand. In several cases error bars are too small to appear on the graphs.

The characterization of GFP^2-BgaR-RLuc8 (**1**) binding with lactose and lactulose highlights the intrinsic power of using binding proteins as analyte recognition elements for biosensing. The lactose binding TR, BgaR, used to construct GFP^2-BgaR-RLuc8 (**1**) yielded sensitivity in the low µM range, with the ability to discriminate between structurally related disaccharides, as emphasized by the 200-fold difference in EC_{50} observed between lactose and the second most potent sugar tested, lactulose. In contrast, if enzymes such as β-galactosidase (K_M = 5 mM for lactulose and K_M = 18.2 mM for lactose),[31] galactose dehydrogenase or glucose oxidase (K_M = 120 mM for glucose)[32] were to be used as biological recognition elements, the corresponding biosensors could be expected to have affinities in the low to mid-millimolar concentration range, based on the intrinsically lower affinities of enzymes

for their substrates. Moreover, discrimination between closely related analogues such as lactose and lactulose would likely not be achieved due to the broader selectivity characteristic of many enzymes.

Findings on BgaR structure and selectivity

Prior to this study, relatively little was known about the selectivity of the effector-binding domain of BgaR. Fusion of BgaR with the flanking $BRET^2$ donor-acceptor pair strongly suggests that conformational changes in the protein (Fig. 3) occur in the presence of lactose and, to some extent, lactulose. Our observations that D-glucose reduced the BRET ratio by only 4%, are consistent with observations of Hartman *et al*,[22] who found that a gene under the control of BgaR was upregulated in the presence of lactose, but not glucose. We might predict that Hartman *et al*'s system would also respond to mM concentrations of lactulose. In any case it has been demonstrated that BgaR exhibits very high specificity as well as sensitivity.

We obtained Hill coefficients of -0.98 for lactose and -1.29 for lactulose binding to GFP^2-BgaR-RLuc8 (**1**) from the non-linear regression of the respective dose response curves (GraphPad Prism 7.0d). Being close to unity, these results suggest non-cooperative binding of the sugars with BgaR. Moreover, saturating concentrations of either lactose (1 mM) or lactulose (13 mM) caused decreases of $BRET^2$ ratios of similar magnitude (27% and 25%, respectively). This suggests that, although the binding affinities of BgaR for lactose and lactulose differ, both sugars may bind at the same site through similar sets of interactions. AraC from *E. coli*, which is the archetype for BgaR based on sequence homologies, is well characterized. The crystal structure of liganded AraC shows each AraC monomer binding one molecule of its effector arabinose.[33,34] Our data are broadly consistent with BgaR following a similar binding model to AraC and other members of that family.

Estimation of lactose in whole milk

Engineering a biosensor to quantify an analyte in a defined buffer under controlled laboratory conditions is of itself a challenge but accurate biosensing under real world conditions can be even harder. In particular, complex and interfering sample matrices can complicate and degrade biosensor performance. We therefore tested the performance of GFP^2-BgaR-RLuc8 (**1**) in conditions simulating practical usage.

One use of a lactose biosensor would be to quantify lactose levels, which range from approximately 4.5 to 7.0 % (w/v), depending on species, in unmodified milk. We compared the lactose estimates obtained with GFP^2-BgaR-RLuc8 (**1**), calibrated against known amounts of lactose in PBS or 10% of a dialyzed milk matrix, as previously described, with two methods currently in commercial use, a coupled-enzyme lactose assay kit (BioVision) and HPLC-RI performed in a NATA accredited analytical laboratory. The sample for this test was whole pasteurized cow's milk purchased from a supermarket.

The EC_{50} of GFP^2-BgaR-RLuc8 (**1**) for lactose is 12 μM, i.e. approximately 10^4 -fold lower than the lactose concentration found in unmodified cow's milk. Consequently, we diluted the whole milk samples 3,200 fold in water prior to lactose estimation using GFP^2-BgaR-RLuc8 (**1**) and observed a 79 ± 1% decrease in the $BRET^2$ response, compared to water alone. This is equivalent to a lactose concentration of 49 ± 2 μM (Fig. 4, solid line; equation: 100/(1+10^(-4.909-X)-0.9841)), corresponding to 157 ± 6 mM lactose in the milk (Table 1). Using the manufacturer's protocol we estimated the original lactose concentration to be 129 ± 1 mM with a coupled-enzyme kit (BioVision). In this procedure, lactose is hydrolyzed by lactase to galactose and glucose, one of the monosaccharides is oxidized by either galactose or glucose oxidase to release one equivalent of H_2O_2 (Fig. S-3) which is indirectly quantified following its consumption by horseradish peroxidase to generate a quantifiable chromophore such as (or potentially) 10-acetyl-3,7-dihydroxyphenoxazine (Abs_{570nm}). A sample of the same milk was submitted to a NATA accredited laboratory for lactose estimation by HPLC-RI analysis. The laboratory reported a lactose concentration of 134 mM. In this case, no error value was reported.

Table 1. Comparison of lactose concentration in pasteurized whole cow's determined using GFP^2-BgaR-RLuc8 (**1**) and two independent methods. Note that the nutritional panel on the carton of milk stated a representative value for lactose of 137 mM.

	[Lactose] (mM)	[Lactose] (% w/v)
GFP^2-BgaR-RLuc8 (**1**)	157 ± 6	5.4 ± 0.2
Coupled-enzyme assay (BioVision)	129 ± 1	4.4 ± 0.03
HPLC-RI*	134*	4.6*

*No error quoted

Estimation of lactose in lactase-treated milk

A second possible scenario for practical use of a lactose biosensor is to measure lactose in different grades of lactase treated milk, characterized as "reduced lactose" or "lactose-free". Estimation of lactose in lactase-treated milk is challenging due to the low level of the analyte, the complexity of the milk medium and the presence of high levels of glucose and galactose that can interfere with the measurement of lactose itself. Food Standards Australia and New Zealand (FSANZ) specifies lactose-reduced dairy as containing no more than 0.3% (8.8 mM) lactose[35] whereas lactose-free products should contain "no detectable lactose",[35] a subjective, method-dependent definition. Some European authorities specify an objective threshold for lactose-free foods at 0.01% (w/v) (0.3 mM).[5]

Milk is a complex matrix comprising proteins and lipids each at concentrations of approximately 3% (w/v).[36] To minimize interference, analytical laboratories routinely precipitate fats and proteins from milk samples before analyzing the sugar content by HPLC or colorimetric coupled-enzyme assays. In addition to being time consuming and incurring extra cost, work-up of samples prior to analysis increases the risk of error due to yield variation and modification of sample volumes. The relative selectivity of biosensors, whether based on coupled-enzyme assays or binding proteins offers the potential advantage of directly estimating lactose in the milk matrix without any pre-treatment, other than dilution to meet the working range of the biosensor.

We used GFP^2-BgaR-RLuc8 (**1**) to estimate lactose concentration in a ten-fold dilution of commercially obtained full cream, "lactose-free" cow's milk. The $BRET^2$ response was decreased by 16%, equivalent to a concentration of 2.7 ± 0.1 μM (Figure 4 dashed line; equation y = (100/(1+10^(-4.686-

X)$^{-0.9065}$)), corresponding to 27 ± 1 µM lactose in the original milk sample (Table 2). We compared the result obtained with GFP^2-BgaR-RLuc8 (**1**) with those returned by the coupled-enzyme assays and HPLC-RI described previously.

Reputable analytical laboratories do not consider coupled-enzyme assays suitable for measuring residual lactose in lactase-treated milk.[8] The problem is that high levels of free galactose present in the milk due to the commercial lactase treatment must be subtracted from the total galactose resulting from the treatment with the lactase in the coupled-enzyme kit. Using the commercially available BioVision coupled-enzyme assay kit and the supplier's protocol we attempted to correct the galactose background by omitting to add lactase to a set of control samples. To ensure that the readings were within the linear range of the kit's calibration curve (Fig. S-3), we made a 4 x 10^{-3} -fold dilution of the full-cream lactose-free milk. The concentration of galactose in the control was estimated as 163 ± 2 mM (Table 2) and the total concentration of galactose and lactose was 159 ± 2 mM. Residual lactose is calculated by subtracting the control from the galactose + lactose. In this case, the formal result is a negative value for lactose, albeit the value is not statistically different from zero. We concluded that the residual level of lactose in the lactose-free milk was below the limit of detection of this method. This is consistent with the conclusion that such kits are unsuitable for quantifying residual lactose in lactase-treated milk.

Samples of the same full cream, lactose-free milk were submitted to a NATA accredited analytical laboratory for analysis by HPLC-RI. No lactose was detected, with a limit of detection of 0.1% (w/v) or approximately 3 mM (Table 2.). The limit of detection is dictated by the detector employed. While refractometric detectors lack sensitivity they are an attractive option to directly quantify saccharides and bypass the need to derivatize analytes. Research laboratories have published chromatographic methods with better sensitivities, but these protocols have not been adopted as standard methods, in part because they require specialist equipment, facilities and training.

Table 2. Comparison of lactose concentration in fresh "lactose free" full cream cow's milk using GFP^2-BgaR-RLuc8 (**1**) and two independent methods.

	[Lactose] (mM)	[Lactose] (% w/v)	[Galactose] (mM)
GFP^2-BgaR-RLuc8 (**1**)	0.027 ± 0.001	0.00092 ± 0.00003	Not Applicable
Coupled enzyme assay (BioVision)	Not Applicable	Not applicable	163 ± 2
HPLC-RI *	< 3	<0.1	124

*No error quoted

It appears from this analysis that the biosensor would be suitable for directly determining the concentration of residual lactose in lactose free commercial dairy products. However, since neither of the current methods commercially available in Australia was capable of measuring lactose below 3 mM (0.1% w/v) additional independent verification of the GFP^2-BgaR-RLuc8 (**1**) would be required. Bearing in mind this caveat, we note that the estimate of 27 µM for the particular sample tested with GFP^2-BgaR-RLuc8 (**1**) is well below the most stringent 0.01% (w/v) objective standard required by some food standards authorities.

Potential interference from lactulose in real world applications

For practical applications the relative sensitivity and selectivity of GFP^2-BgaR-RLuc8 (**1**) towards lactose and lactulose must also be taken into account. Lactose is the main carbohydrate found in raw milk.[36] Lactulose is an isomer of lactose resulting from heat treatment of lactose containing solutions, such as milk.[37] Lactulose has been proposed by the International Dairy Federation[38,39] as an indicator of milk damage caused by heat-treatment and as a criterion to distinguish between pasteurized milk ([lactulose]<10 µM), ultra-high temperature (UHT)-treated milk ([lactulose]<1.8 mM) and in-container sterilized milk ([lactulose]>1.8 mM).[40] The limit of detection of GFP^2-BgaR-RLuc8 (**1**) for lactulose (0.1 mM) is 10-fold higher than the levels found in pasteurized milk (10 µM), which means it could be used to determine any relevant level of lactose in lactase-treated pasteurized milk with negligible interference.

Lactulose concentrations in UHT milk (<1.8 mM) are comparable with the EC_{50} of GFP^2-BgaR-RLuc8 (**1**) for lactulose (2.4 mM) and therefore interference with lactose measurement would need to be considered in detail.

Due to the high levels of lactose in UHT whole milk (140 mM) and the extreme sensitivity of GFP^2-BgaR-RLuc8 (**1**), samples for analysis must be diluted by a factor of approximately 10^4. Such dilution would result in final lactulose concentrations of <1 µM, even in UHT treated products, i.e. well below the limit of detection of GFP^2-BgaR-RLuc8 (**1**). Therefore there appears to be no possibility of lactulose interfering with lactose measurements in UHT-treated whole milk.

In the case of UHT-processed "lactose-free" milk, lactase treatment may be performed prior to or following UHT processing. In the case where lactase is successfully incubated with milk before UHT processing, there will be negligible lactose present and therefore lactulose cannot be formed in a significant concentration. In the situation where lactase is added aseptically to UHT milk following heat-treatment, the available evidence[40] indicates that lactulose generated by the heat treatment would, like lactose itself, be hydrolyzed by lactases.

Therefore, although confirmatory testing would need to be performed, it seems unlikely that lactulose will affect lactose measurement in any likely commercial scenario for pasteurized or UHT treated dairy products.

CONCLUSION

We report identification of the lactose activated TR BgaR as suitable for the analyte recognition function in a novel genetically encoded BRET-based biosensor. The superior characteristics of BgaR, namely its high affinity and selectivity for lactose and its amenability to photonic transduction using the BRET^2 system, enabled the design of a selective and sensitive lactose biosensor. We demonstrated that BRET^2 was better suited than FRET to transduce the conformational change in BgaR. This is in line with similar results for a range of other biosensors where the analyte recognition element is a small globular protein. Fusion of BgaR with the flanking BRET^2 donor and acceptor pair allowed us to explore the selectivity of the TR towards mono- and disaccharides and supported the finding that it exhibits non-cooperative binding of lactose.

This is consistent with the stoichiometry of better-characterized members of the AraC/XylS family of TRs, to which BgaR has been assigned. The binding protein-based biosensors described here draw on the evolutionary adaptation of TRs, which may sensitively and selectively detect low or subtle changes in effector levels. This characteristic was highlighted by the low micromolar binding affinities calculated for GFP^2-BgaR-RLuc8 (**1**) and the selectivity it exhibited between lactose and structurally related disaccharides. Due to their function of modulating gene expression, using TRs as biological recognition elements of biosensors has the advantage of being easily engineered through directed evolution.[41]

GFP^2-BgaR-RLuc8 (**1**) was used to measure a trace concentration of lactose in lactase-treated milk within 5 minutes, at levels well below the most stringent regulatory standards. Although not tested by us, there is no reason to suppose that similar results could be obtained using significantly shorter incubation times. From a practical point of view, GFP^2-BgaR-RLuc8 (**1**) was simpler conceptually and in practice than the coupled-enzyme assay and, unlike the latter, was suitable for measuring trace lactose following lactase treatment of whole milk. It was also simpler, faster and more sensitive than HPLC-RI. By reason of its speed, sensitivity, selectivity and simplicity, the novel $BRET^2$-based GFP^2-BgaR-RLuc8 (**1**) has potential to be developed as a field-deployable method for monitoring and verifying lactase treatment of dairy products to the same standard as current gold standard methods used in dedicated analytical labs.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. SI (PDF)

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