Characterization of a *Lactococcus lactis* promoter for heterologous protein production

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**ABSTRACT**

Constitutively active promoter elements for heterologous protein production in *Lactococcus lactis* are scarce. Here, the promoter of the PTS-IIC gene cluster from *L. lactis* NZ3900 is described. This promoter was cloned upstream of an enhanced green fluorescent protein, GFPmut3a, and transformed into *L. lactis*. Transformants produced up to 13.5 μg of GFPmut3a per milliliter of log phase cells. Addition of cellobiose further increased the production of GFPmut3a by up to two-fold when compared to glucose. Analysis of mutations at two specific positions in the PTS-IIC promoter showed that a ‘T’ to ‘G’ mutation within the –35 element resulted in constitutive expression in glucose, while a ‘C’ at nucleotide 7 in the putative cre site enhanced promoter activity in cellobiose. Finally, this *PTS-IIC* promoter is capable of mediating protein expression in *Bacillus subtilis* and *Escherichia coli* Nissle 1917, suggesting the potential for future biotechnological applications of this element and its derivatives.

1. Background

Lactic acid bacteria (LAB) are diverse Gram-positive bacteria that convert fermentable carbohydrates to lactic acid. They have been certified by the European Food Safety Authority as “safe microorganisms for use in food production” [1]. Many strains of LAB are utilized as starter cultures of dairy products for improvement of flavor and texture [2,3]. The use of LAB as probiotics has been widely reported [4]. Probiotics, as defined by the World Health Organization (WHO) are, “live microorganisms that when administered in adequate amounts confer a health benefit to the host” [5]. Prevention and treatment of gastrointestinal disorders as well as maintenance of normal intestinal flora are often associated with ingestion of probiotic LAB, such as *Lactococcus lactis*, *Bifidobacterium lactis*, *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus reuteri*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus acidophilus* and *Streptococcus thermophilus* [6–9].

Improvements in cell engineering technology have extended the potential of *L. lactis* as a biotherapeutic agent. A myriad of recombinant “food-grade” strains of *L. lactis*, including several auxotrophic strains for environmental containment, are now commercially available [10]. This, coupled with the development of well-established expression vectors [10–13] had resulted in multiple publications reporting on the use of *L. lactis* for production of heterologous enzymes [14–16] and for mucosal delivery of multiple biological mediators [17–20].

Most commercially available *L. lactis* expression systems utilize inducible promoters for heterologous protein production. These systems are optimal when the expressed protein is toxic, or interferes with host cell metabolism. However, they are often not viable when an inducer needs to be added to cells that are present in inaccessible locations. In these cases, constitutive expression of the heterologous protein at a level that is not metabolically taxing to the host cell is preferred. The *pepN* promoter, an endogenous promoter which regulates expression of an aminopeptidase in *L. lactis* [21,22], and is utilized commercially in the constitutive expression vector pNZ7021 [23]. Several studies have reported on the generation of synthetic promoter libraries that drive constitutive gene expression in *L. lactis* [24,25]. Expression from the
best of these synthetic promoters is comparable to the amount of protein produced by L. lactis in an optimized inducible (NICE) system [24].

Endogenous promoters of genes that play essential roles in nutrient metabolism, cell survival and growth in L. lactis may potentially serve as candidates for driving expression of heterologous proteins. While most bacteria utilize glucose as their primary source of energy, many are capable of metabolizing other complex sugars [26]. Cellobiose is a plant-derived β-glucoside resulting from the hydrolysis of cellulose by cellulase and consists of two glucose molecules linked together by a β(1,4) bond. The transport and metabolism of this molecule is dependent on components of the cellobiose-specific phosphotransferase system (PTS) in L. lactis [27,28]. When glucose or other rapidly metabolized carbon sources are present, the genes within the cellobiose operon are repressed by the binding of a catabolite control protein to the cis-acting catabolite-responsive element (cre) at the promoter region of this operon. Mutations at this element resulted in the significant up-regulation of the cellobiose-specific PTS IIC component (ptcC) and phospho-β-glucosidase (celA) in L. lactis. Expression of this protein minimally affected the growth characteristics of EGFP-transformed L. lactis compared to wild-type. When cellobiose was utilized as the sole carbon source in the culture medium, a two-fold increase of the PTS-IIC promoter activity was observed. Site-directed mutagenesis of the −35 box and cre further enhanced marker activity in response to cellobiose. In addition, the PTS-IIC promoter was constitutively active in B. subtilis and E. coli Nissle.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Four E. coli strains were used in this study: Top10 (Life Technologies) for routine cloning, Stellar™ (Clontech) for In-Fusion cloning, NEB5α (New England Bioslabs) for site-directed mutagenesis, and Nissle 1917 for assessment of promoter functionality in a probiotic Gram negative bacteria. Each of the E. coli strains was propagated in Luria-Bertani (LB) broth at 37 °C with constant agitation. The LB broth was supplemented with appropriate antibiotics (50 μg/ml carbenicillin or 150–200 μg/ml erythromycin) for selection of E. coli transformants depending on the plasmids used for transformation. L. lactis NZ9000 (MoBiTec) was propagated at 30 °C without agitation in M17B broth (Life Technologies) supplemented with 0.5% glucose or with 0.5% cellobiose. B. subtilis 1012 (ATCC) was cultured in LB broth at 37 °C with constant agitation. Erythromycin (5 μg/ml) was added to the culture media for selection of transformed L. lactis or B. subtilis cells. Cell growth was monitored and measured as optical density at 600 nm (OD600) using a Thermo Spectronic BioMate 3 spectrophotometer.

2.2. Isolation and cloning of promoters from L. lactis genes

Nucleotide sequences of the L. lactis PTS-IIC promoter and the NADH oxidase (noxE) promoter were retrieved from the NZ9000 genome database on the National Center for Biotechnology Information (NCBI) server. The noxE promoter, which is constitutively active in L. lactis MG1363 [23], was included in the study as a positive control for comparative promoter analysis. Polymerase chain reaction (PCR) primers for the amplification of PTS-IIC and noxE promoters are listed in Table 1. L. lactis NZ9300 genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega) and served as template in PCRs for the amplification of the PTS-IIC and noxE promoters. Amplified products were sub-cloned into the pGEM-T vector (Promega), and verified for orientation by restriction enzyme digestions.

2.3. Generation of expression plasmids pTRKH3-cepGFPmut3a and pTRKH3-noxEpGFPmut3a

The gene encoding GFPmut3a, a green fluorescent protein variant [32], was excised from pAD43-25 [33] by XbaI/HindIII double digestion, and subcloned into pBlueScript II (KS +) (Agilent Technologies). The PTS-IIC or noxE promoter were excised from pGEM-T with SacI/SpeI, and inserted upstream of GFPmut3a. The promoter-EGFP cassettes were subcloned by In-Fusion PCR cloning (New England Biolabs) into the E. coli/L. lactis shuttle vector pTRKH3 for expression. pTRKH3 was obtained by releasing the ermGFP cassette from plasmid pTRKH3-ermGFP [34], a gift from Michela Lizier (Addgene plasmid #27169), with BamHI/Sall. Primers used for PCR are listed in Table 1.

2.4. Transformation of bacterial cells

Chemically competent cells of different E. coli strains were transformed using standard protocols [35]. Transformation of L. lactis was achieved by electroporation according to Holo and Nes [36]. B. subtilis was transformed using a protoplast protocol as described by Chang and Cohen [37].

2.5. GFPmut3a fluorescence detection and measurement

GFPmut3a fluorescence from transformed bacteria were firstly visualized under a Zeiss Axioskop 2 Plus fluorescence microscope. The fluorescence from 100 μl of bacterial cultures at an optical density of between 0.5 and 0.8 were measured in 96-well assay plates. Quantification of fluorescence was performed on a SpectraMax M2e microplate reader (Molecular Devices) at excitation and emission wavelengths of 480 nm and 520 nm respectively. Since GFPmut3a fluorescence is quenched by a decrease in pH during L. lactis growth [22], cells were pelleted and then equilibrated in phosphate buffered saline (PBS) prior to fluorescence measurement. All measurements were performed in triplicate and repeated at least three times.

2.6. Enzyme-Linked immunosorbent assay (ELISA)

L. lactis pellets were re-suspended in PBS and lysed by sonication on ice. The cleared lysates were used to quantify GFPmut3a expression against standard curve of recombinant GFP (Alpha Diagnostic International) that ranged from 0.1 to 10 ng. Briefly, 100 μl of diluted (1X, 10X and 100X) cleared lysate or recombinant GFP diluents were captured onto Costar 96-well plate(s). After blocking and washing, a 1:5000 dilution of rabbit anti-GFP antibody (Life Technologies) was added to each well. The plates were incubated at 37°C for 1 h, and washed three times with prior to the addition of 1:5000 dilution of goat anti-rabbit IgG conjugated with hors eradish peroxidase (Promega) for colorimetric detection with TMB substrate. Quantitation was determined at an absorbance of 450 nm on a SpectraMax M2e microplate reader. All reactions were performed in triplicate and repeated at least 3 times.

2.7. Site-directed mutagenesis

Site-directed mutagenesis was performed to determine the consequences of single base mutation on the cre and/or −35 elements of the PTS-IIC promoter. The Q5 Site-Directed-Mutagenesis Kit (New England Biolabs) was utilized in conjunction with a Kinase-Ligase-DpnI (New England Biolabs) reaction according to manufacturer’s instructions. Reactions were performed on the parental pTRKH3-cepGFPmut3a plasmid with one of nine forward primers containing the desired mutations and a common reverse primer, cepAp-SDM_R (Table 2). Primers were designed using the NEBaseChanger server (http://nebasechanger.neb.com/). All site-directed mutations were sequence-verified prior to introducing into NZ3900 cells.
expression from both lines of the sole carbon source. The micrographs in Fig. 1 show GFPmut3a expression plasmids were designated pTRKH3-promoter [23]. Both promoters were subcloned upstream of the gene to ascertain promoter activity, two methods were employed. Initially, expression was compared to that of the native NADH oxidase (celAp) [29]. To these results confirm constitutive expression from both the noxE promoter [23] and PTS-IIC promoters [29]. To assess promoter activity, two methods were employed. Initially, measurement of GFPmut3a fluorescence from the two L. lactis lines was determined by plate assay where the GFP specific fluorescence at 520 nm was corrected for cell number density measured at 600 nm. As seen in Fig. 2, activity from the PTS-IIC promoter is approximately 3-fold greater compared to the native noxE promoter. This level of activity is comparable to that of the "fine-tuned" B6 version of the noxE promoter [25]. Subsequently, direct ELISA was utilized to quantitate the amount of GFPmut3a expressed. When grown to an OD600 of 0.72, the amount of GFPmut3a produced by the pTRKH3-celApGFPmut3a harboring cell line was calculated to be 13.5ug/ml.

The activity that is seen from the PTS-IIC promoter may be a consequence of its improved transcriptome architecture: it has a putative −10 sequence (TATAAT) that is an exact match of the consensus −10 (Pribnow box) region while its −35 sequence (TTGCTT) is very similar to the consensus −35 sequence (TTGACA) [38]. Further, the spacer length between the −10 and −35 elements is one nucleotide away from optimal. The mismatches in the putative −35 element have been reported to boost transcription frequency [39,40].

### 2.8. Statistical analyses

All the statistical analyses were performed using two-tailed paired student’s T-test on GraphPad Prism 6 (GraphPad Software Inc).

### 3. Results and discussion

#### 3.1. The PTS-IIC promoter drives higher gene expression compared to the noxE promoter in L. lactis

The ability of the PTS-IIC promoter to drive heterologous gene expression was compared to that of the native NADH oxidase (noxE) promoter [23]. Both promoters were subcloned upstream of the gene encoding GFPmut3a [32] into the pTRKH3 shuttle vector [34]. These expression plasmids were designated pTRKH3-celApGFPmut3a and pTRKH3-noxE-GFPmut3a, respectively. L. lactis NZ9000 transformed with either construct were cultured in M17 medium containing glucose as the sole carbon source. The micrographs in Fig. 1 show GFPmut3a expression from both lines of L. lactis. These results confirm constitutive expression from both the noxE [23] and PTS-IIC promoters [29]. To assess promoter activity, two methods were employed. Initially, measurement of GFPmut3a fluorescence from the two L. lactis lines was determined by plate assay where the GFP specific fluorescence at 520 nm was corrected for cell number density measured at 600 nm. As seen in Fig. 2, activity from the PTS-IIC promoter is approximately 3-fold greater compared to the native noxE promoter. This level of activity is comparable to that of the “fine-tuned” B6 version of the noxE promoter [25]. Subsequently, direct ELISA was utilized to quantitate the amount of GFPmut3a expressed. When grown to an OD600 of 0.72, the amount of GFPmut3a produced by the pTRKH3-celApGFPmut3a harboring cell line was calculated to be 13.5ug/ml.

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#### 3.2. Overexpression of GFPmut3a does not significantly impact growth of transformed L. lactis

The growth of both transformed NZ9000 lines were compared to untransformed cells to assess the burden of heterologous protein expression. Untransformed NZ9000 reached an optical density (OD600) of 0.5 at 8h. In comparison, cells harboring either pTRKH3-celApGFPmut3a or pTRKH3-noxE-GFPmut3a reached the same optical density at 9 and 11h, respectively (Fig. 3). These results suggest that expression of a heterologous protein driven by the PTS-IIC promoter has a minimal impact on the growth of L. lactis. Specific activities of the noxE promoter may account for the delayed growth of pTRHK3-noxE-GFPmut3a transformants.

#### 3.3. Cellobiose enhances promoter activity of celAp and noxEp in L. lactis

Endogenously, the PTS-IIC promoter regulates expression of cellobiose-specific phosphotransferase system IIC component and beta glucosidase, celA. The promoter contains a putative catabolite responsive element (cre) that is modulated by catabolite control protein A (ccpA). In the presence of glucose, ccpA binds to cre, and suppresses expression of genes involved in metabolizing other sugars such as cellobiose [26,41]. The ability of cellobiose to regulate the activity of the PTS-IIC promoter was examined. When cultured in media containing cellobiose, the activity of PTS-IIC promoter in L. lactis NZ9000 increased by almost 2-fold compared to its activity in glucose. Surprisingly, a similar level of induction was observed from the noxE promoter (Fig. 4). Subsequent examination of this latter promoter sequence revealed the presence of a putative cre site approximately 32 nucleotides upstream of the −35 element (Fig. 5).

#### 3.4. Mutations in PTS-IIC promoter and associated effects

The cre consensus sequence is shown in Fig. 5. Two point mutations, at nucleotide (nt) 7 and nt 12 of this element had been identified in the promoter region of the PTS-IIC gene in L. lactis NZ9000 when compared to L. lactis MG 1363 [26]. These two mutations, a ‘C’ to ‘T’ substitution at nt 7, and a ‘T’ to ‘G’ substitution at nt 12, are responsible for the constitutively active status of the PTSIIC operon in L. lactis NZ9000 [26]. Furthermore, the PTS-IIC cre overlaps with the putative −35 element (Fig. 5) in L. lactis. In these experiments, the point mutations were sequentially replaced using site directed mutagenesis to further ascertain if they affect promoter modulation by glucose and cellobiose (Table 2).

The ‘G’ point mutation at nt 12 of the PTS-IIC cre is located within the putative −35 element. In initial experiments, this mutation was held constant while changes were made to the point mutation to nt 7. Replacement of the ‘T’ at nt 7 with either ‘G’, ‘C’ or ‘A’ resulted in no significant changes in constitutive GFPmut3a expression when the cells were grown in glucose. These results would suggest that the ‘G’ mutation at the −35 element is responsible for the constitutive expression of this promoter. It is likely that this point mutation facilitates the binding

| Table 1 | List of primers used in the project. |
|----------------|--------------------------|
| Name | Sequence (5’−3’) | Restriction site | Reference |
| celAp.F | AGAGATCTAATTATGACAAATTGTGACAGGG | BglII | This study |
| celAp.R | TTAGGATCCGTTTGACAGCTCTTACCTTTTT | BamHI | This study |
| noxEp.F | GGGAGATTCCTTGGAGCAGACTTACTTGTG | BglII | [25] |
| noxEp.R | GATGGATCCACTAATAGGTCTCCTTTA | BamHI | This study |
| Inf-celApGFP_F | CCCTGCTCTGAGGATGATGCTTCTTTTATATGACATTTTTTG | This study |
| Inf-celApGFP_R | TTAGGATCCGGTTGAACAGTCTCCTTTTACTTTT | This study |
| Inf-noxE-GFP_F | CCCGTCCTGTAGGATGATGCTTCTTTTATATGACATTTTTTG | This study |
| Inf-noxE-GFP_R | TTAGGATCCGGTTGAACAGTCTCCTTTTACTTTT | This study |
| Inf-GFPmut3a_R | AAGGGATCCGTCGACAGTTGACATTTTTTGATTTT | This study |
| celAp-SDM_R | TTTTTTTTATATGACATTTTTTG | This study |
| celAp-SDM,F1 | AGAACTGCGTCTTTTCTTTT | This study |
| celAp-SDM,F2 | AGAAGCTGCTTTTCTTTT | This study |
| celAp-SDM,F3 | AGAAGCTGCTTTTCTTTT | This study |
| celAp-SDM,F4 | AGAAGCTGCTTTTCTTTT | This study |
| celAp-SDM,F5 | AGAGATCTAATTATGACAAATTGTGACAGGG | This study |
| celAp-SDM,F6 | AGAGATCTAATTATGACAAATTGTGACAGGG | This study |
| celAp-SDM,F7 | AGAGATCTAATTATGACAAATTGTGACAGGG | This study |
| celAp-SDM,F8 | AGAGATCTAATTATGACAAATTGTGACAGGG | This study |
| celAp-SDM,F9 | AGAGATCTAATTATGACAAATTGTGACAGGG | This study |

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of primary sigma factors. In the presence of cellobiose, ‘A’ and ‘G’ substitution at nt 9 boosted GFPmut3a expression by 1.6-fold. Reversion to the native L. lactis MG 1363 state, or replacement with a ‘C’ at this position increased GFPmut3a production by 3.4-fold in the alternate sugar source. This is higher than the 2-fold increase seen with the parental PTS-IIC promoter from L. lactis NZ9000/NZ3900. A ‘C’ at this position is part of the cre consensus sequence, suggesting that this position may have a significant influence on the release of the ccpA repressor from this cre.

In the next set of experiments, the ‘C’ at nt 7 of the cre was held constant, while changes were made to nt 12. When the ‘G’ at this position was changed to ‘C’, ‘T’ or ‘A’, the promoter became silent in the presence of glucose. These results would confirm earlier observations that the ‘G’ within the –35 element, or at nt 12 of the cre, is required for constitutive expression from this promoter. GFPmut3a expression was induced for each of the constructs tested, again, confirming that the ‘C’ at nt 7 is critical for activation in the alternate sugar source.

Finally, a series of substitutions were made to nt 9 while the ‘T’ at nt 12 of the native L. lactis MG 1363 cre promoter was held constant. Replacement of the ‘C’ at nt 9 (silent promoter) with any other nucleotide resulted in approximately 60 RFU/OD600 of GFPmut3a production when the cells were grown in glucose. The ‘leakiness’ of these promoters in the presence of glucose further affirms the importance of the ‘C’ at this position of the cre. It is possible that this position plays a critical role in the binding of the ccpA repressors to the cre. A mutation at this position may have modified the on and off rates of the repressor slightly, allowing for leaky expression of the marker protein. Cellobiose induces expression from each of these constructs by approximately 3-fold.

These results suggest that the constitutive activation of the PTS-IIC promoter in L. lactis NZ9000/NZ3900 is likely a result of the ‘T’ to ‘G’ mutation on nt 12 of the cre. In both NZ9000/NZ3900 and MG 1636 L. lactis strains, this portion of the cre overlaps with the putative –35 element on the PTS-IIC operon. A ‘G’ at this position may have inadvertently modified the –35 element, resulting in a stronger consensus binding site for sigma factors and RNA polymerases. Constitutive

| Promoter name | Mutations in cre site and –35 sequence (5’-3’) | Promoter description | Promoter activity (RFU/OD600) |
|---------------|-----------------------------------------------|----------------------|-------------------------------|
| celAp-SDM_F1  | agaaacgctTTCTT                                | silent state; native in MG1363 | glucose: 0.00, cellobiose: 15.99 (1.4) |
|               |                                              |                      |                               |
| celAp_NZnative | agaaactgctTGCTT                               | active state; native in NZ9000/NZ3900 | glucose: 431.89 (101.5), cellobiose: 830.50 (392.8) |
|               |                                              |                      |                               |
| celAp-SDM_F2  | agaaactgctTTCTT                               | newly derived, mutation in putative cre | glucose: 66.87 (3.3), cellobiose: 191.56 (8.8) |
|               |                                              |                      |                               |
| celAp-SDM_F3  | agaaacgcggTTCTT                               | newly derived, mutation in putative cre | glucose: 56.36 (5.9), cellobiose: 158.33 (7.0) |
|               |                                              |                      |                               |
| celAp-SDM_F4  | agaaacagcTTCTT                                | newly derived, mutation in putative cre | glucose: 62.47 (8.7), cellobiose: 126.15 (0.6) |
|               |                                              |                      |                               |
| celAp-SDM_F5  | agaaacgcgTTGCTT                               | newly derived, mutation in putative cre| glucose: 281.21 (8.7), cellobiose: 963.33 (30.6) |
|               |                                              |                      |                               |
| celAp-SDM_F6  | agaaacgcgTTACTT                               | newly derived, mutation in putative cre| glucose: 0.00, cellobiose: 26.76 (2.7) |
|               |                                              |                      |                               |
| celAp-SDM_F7  | agaaacgcgTTCCCT                                | newly derived, mutation in putative cre| glucose: 0.62 (1.9), cellobiose: 66.87 (2.9) |
|               |                                              |                      |                               |
| celAp-SDM_F8  | agaaacagcTGCTT                                | newly derived, mutations in both putative cre and -35 | glucose: 323.95 (18.0), cellobiose: 570.19 (44.2) |
|               |                                              |                      |                               |
| celAp-SDM_F9  | agaaacgcgTGCTT                               | newly derived, mutations in both putative cre and -35 | glucose: 296.55 (36.0), cellobiose: 491.90 (46.8) |

Table 2: Assessment on modulatory effects of L. lactis PTS-IIC promoter activity by point mutations in putative cre site and –35 element in the promoter. Two point mutations (highlighted in gray) in the putative cre (bold letters) and –35 element (capital letters) of the PTS-IIC (celA) promoter in L. lactis MG1363 and NZ9000/NZ3900 respectively. Promoter celAp-SDM_F1 is native to L. lactis MG1363. This promoter is inherently silent in the presence of glucose, but is induced by cellobiose. Promoter celAp-NZnative was isolated from L. lactis NZ9000/NZ9000. This promoter is constitutively active in glucose. New promoters were derived based on the sequence difference of these two promoters. Promoter activity was measured as relative fluorescence units (RFU) in 100 μl of cells grown to OD600 of 0.5–0.8. The activity reported for each construct is the average from three replicates, with the standard error of the mean in parenthesis.
promoters with this mutation produce between 280 and 432 RFU/OD600 of GFPmut3a regardless of the nucleotide on position 7 of the cre, though a pyrimidine may slightly enhance constitutive expression.

3.5. Functionality of L. lactis PTS-IIC promoter in other probiotic bacteria

Many studies have described *E. coli* and *B. subtilis* as cell factories for production of heterologous proteins. Both bacteria have also been used as probiotics. *E. coli* Nissle 1917, for example, is an active ingredient in the probiotic drug preparation, Mutaflor [42]. Probiotic preparations of *B. subtilis* spores are often distributed over-the-counter in Europe and in South East Asia [43–46]. We tested the functionality of both the PTS-IIC and noxE promoters in these two probiotics. The PTS-IIC promoter was shown to be functional and exhibited strong promoter activity as determined by the GFPmut3a fluorescence intensities observed in both *B. subtilis* and *E. coli* Nissle (Fig. 6). However, the noxE promoter was non-

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**Fig. 1.** The PTS-IIC promoter is constitutively active in *L. lactis* NZ3900 cells. Micrographs comparing the constitutive expression of GFPmut3a from *L. lactis* cells transformed with either pTRKH3-celApGFPmut3a (top) or pTRKH3-noxEpGFPmut3a (bottom). Left panel shows cells in bright field, while cells in the right panel were viewed through a FITC filter.

**Fig. 2.** Quantitation of GFPmut3a expression from *L. lactis* transformants. Relative fluorescence intensities from *L. lactis* transformed with either pTRKH3-celApGFPmut3a or pTRKH3-noxEpGFPmut3a were determined. Fluorescent activity was expressed as a ratio of relative fluorescent unit (RFU) per number of cells, as determined by OD600 of between 0.5 to 0.8. When compared in this manner to the noxE promoter, the PTS-IIC promoter appears to be at least three-fold more active.

**Fig. 3.** Overexpression of GFPmut3a does not significantly impact growth of transformed lines of *L. lactis*. The growth rates of *L. lactis* expressing GFPmut3a from either the PTS-IIC or the noxE promoter were compared to that of untransformed cells. Both transformed lines grew to an optical density of 0.5 within hours of the untransformed cells, suggesting that overexpression of GFPmut3a minimally interfere with cell growth.
The versatility of the PTS-IIC promoter may lie in the close similarity of its sequence to the consensus \(-35\) and \(-10\) sequence of prokaryotic promoters [39], suggesting the applicability of this promoter to both Gram positive and negative cells.

4. Conclusions

The \textit{L. lactis} PTS-IIC promoter, along with several derivatives, were cloned and validated for construction of a series of versatile expression vectors for efficient production of heterologous protein in probiotic bacteria including \textit{L. lactis}, \textit{B. subtilis} and \textit{E. coli} Nissle 1917. This progress is anticipated to enhance the engineering of probiotic bacteria for improving human and animal health.

Authors contributions

CEO and RD conceived and designed the study. CEO, QC and IH performed experiments. CEO analyzed data. CEO and QC wrote the manuscript. IH, AF and RD revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The dataset supporting the conclusions of this article are included within the article

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Competing interests

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