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Development of *Lactococcus lactis* encoding fluorescent proteins, GFP, mCherry and iRFP regulated by the nisin-controlled gene expression system

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Abstract

Fluorescent proteins are useful reporter molecules for a variety of biological systems. We present an alternative strategy for cloning reporter genes that are regulated by the nisin-controlled gene expression (NICE) system. *Lactococcus lactis* was genetically engineered to express green fluorescent protein (GFP), mCherry or near-infrared fluorescent protein (iRFP). The reporter gene sequences were optimized to be expressed by *L. lactis* using inducible promoter pNis within the pNZ8048 vector. Expression of constructions that carry mCherry or GFP was observed by fluorescence microscopy 2 h after induction with nisin. Expression of iRFP was evaluated at 700 nm using an infrared scanner; cultures induced for 6 h showed greater iRFP expression than non-induced cultures or those expressing GFP. We demonstrated that *L. lactis* can express efficiently GFP, mCherry and iRFP fluorescent proteins using an inducible expression system. These strains will be useful for live cell imaging studies in vitro or for imaging studies in vivo in the case of iRFP.

Keywords

fluorescent proteins; GFP; iRFP; *Lactococcus lactis*; mCherry; NICE; nisin-controlled gene expression; plasmids

Lactococcus lactis is a nonpathogenic Gram-positive lactic acid bacterium that generally is regarded as safe for human consumption; it is approved by the US Food and Drug Administration. Lactic acid bacteria are food-grade bacteria that have been used widely in

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the dairy industry, which confirms their lack of pathogenicity (Chamberlain et al. 1997, Nouaille et al. 2003). Scientific interest in *L. lactis* has increased owing to its safety profile and the fact that production of heterologous proteins using *L. lactis* does not produce endotoxins or lipopolysaccharides (Morello et al. 2008). The use of recombinant *L. lactis* that can deliver therapeutic molecules for treating infectious or gastrointestinal diseases as well as allergies has been well documented (Bermudez-Humaran et al. 2011, Pontes et al. 2011, Wells 2011). Therefore, *L. lactis* has a great potential for use in clinical settings (Bermudez-Humaran et al. 2005, Loera-Arias et al. 2014, Villatoro-Hernandez et al. 2008).

Expression of fluorescent reporter genes by bacteria has been used for many applications including gene expression analysis and imaging in vivo (Hoffman 2014, Yamamoto et al. 2011). The green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, is an extensively used reporter gene. Several improved mutant versions are now available (Chalfie 1995, Chudakov et al. 2010). Another reporter gene is mCherry, which is an improved red fluorescent protein isolated from *Discosoma sp.*, with peak absorption and emission at 587 nm and 610 nm, respectively (Shaner et al. 2004, Garcia-Cayuela et al. 2012). Another potential reporter gene is iRFP, a new infrared fluorescent protein, which is an improved mutant version of RpB-phP2 bacteriophytochrome from *Rhodospseudomonas palustris* that can be excited at 690 nm; fluorescence is emitted at 713 nm (Filonov et al. 2011).

We report here the construction and characterization of three recombinants of *L. lactis* that express GFP, mCherry and iRFP and are regulated by the nisin controlled gene expression (NICE) system.

Material and methods

Bacterial strains and growth conditions

The *L. lactis* NZ9000 strain that we used earlier (Rangel-Colmenero et al. 2014), was grown in M17 medium (Becton, Dickinson Co., Franklin Lakes, NJ), supplemented with 1% (w/v) glucose (GM17) at 30° C without agitation. The plasmid, pNZ8048, is a shuttle plasmid that has the ability to replicate in both *L. lactis* and *Escherichia coli*. *Escherichia coli*, DH5 α strain, was grown in LB medium (Becton, Dickinson Co.), with vigorous shaking at 37° C. *E.coli* was used for subcloning steps and to facilitate the screening of recombinants.

Lactococcus lactis transformation

Synthetic constructions were designed to express GFP, mCherry and iRFP. All sequences were codon optimized and synthesized by GenScript (Piscataway, NJ). The sequences synthesized by GenScript were flanked by *NcoI* and *SpeI* restriction sites and cloned in the same restriction sites of the NZ8048 plasmid. Plasmid constructs carrying GFP or iRFP were established first in *E. coli*, DH5 α strain, then transferred to *L. lactis*, NZ9000, by electroporation (Holo and Nes 1989). After ligation with the pNZ8048 plasmid (Kuipers et al. 1998), the gene sequence of mCherry was transformed directly in *L. lactis* NZ9000 by electroporation. For selecting recombinants, *L. lactis* was plated in GM17 agar containing 10 μ g/ml chloramphenicol.

Plasmid DNA isolation and characterization

Plasmid DNA from *E. coli* DH5 α , was isolated using a Midiprep Kit (Qiagen, Hilden, Germany). Plasmid extraction from *L. lactis* was performed by conventional alkaline lysis using phenol/chloroform (Sambrook et al. 1989). Isolation of DNA from agarose gels was performed using a GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA). Because the coding sequences of the reporter genes were flanked by *Nco*I and *Spe*I, the clone DNA was digested by the restriction enzymes, *Nco*I and *Spe*I (New England Biolabs, Ipswich, MA), using 1% bovine serum albumin (BSA) and incubated overnight at 37° C. On the next day, plasmid DNA digestion was analyzed by agarose gel electrophoresis. Positive clones exhibited two DNA fragments, the plasmid DNA and the gene. For iRFP, we also used *Bgl*II and *Hind*III restriction enzymes under the same conditions.

Induction of reporter gene expression and visualization of GFP and mCherry

Cultures of recombinant strains of *L. lactis* were incubated until an optical density (OD 600 nm) of 0.6–0.8 was reached, then 10 ng/ml nisin (Sigma Aldrich, St. Louis, MO) was added to the cultures to induce expression of GFP or mCherry. Two hours after induction of gene expression with nisin, 1 ml of culture was centrifuged at 23,000 $\times$ g for 10 sec and re-suspended in 20 μ l phosphate-buffered saline (PBS). Then 7 μ l of bacteria suspension was placed on slides to visualize the expression of reporter genes using a Leica DM1000 fluorescence microscope with an N2.1 filter for mCherry at 587 and 610 nm for excitation and emission, respectively, and an I3 filter for GFP at 488 and 507 nm for excitation and emission, respectively.

iRFP visualization

After reaching an OD 600 nm of 0.6–0.8, *L. lactis* expressing iRFP was induced with 10 μ g/ml nisin for 6 h. One milliliter of culture then was centrifuged at 23,000 $\times$ g for 10 sec. A microcentrifuge tube containing the pellet was observed using an Odyssey Infrared Imaging System scanner (LI-COR, Lincoln, NE) at 700 nm.

Results

Schematic diagram of NICE inducible system used to regulate the expressions of GFP, mCherry and iRFP

Figure 1 shows the designed gene constructions of mCherry, GFP and iRFP. The gene sequences of GFP, mCherry and iRFP were obtained from GenBank under accession numbers, U73901, AY678264 and JN247409, respectively. The mCherry coding region was subcloned in-frame with the secretion signal peptide encoding sequence of the *usp45* gene of *L. lactis* and under control of the constitutive promoter, P59 (van der Vossen et al. 1987). For GFP and iRFP, the respective coding sequences were cloned upstream of the nisin-inducible promoter, pNis; the structure of these expression cassettes lacks the *usp45* gene.

Alternative strategy for mCherry cloning and expression by *L. lactis*

Because we observed that mCherry in the pNZ8040 plasmid that carried the *usp45* signal peptide sequence and the P59 promoter was toxic for *E. coli* DH5 α , the GenScript company

cloned all of the sequence in plasmid pJet1.2 (pJet1.2:SS-P59-mCherry) and transformed it into EPI400 *E. coli* to reduce potential toxicity, because the EPI400 strain produces a low copy number of plasmids. To clone the mCherry gene, we removed the P59 promoter and the *usp45* signal peptide sequence from pJet1.2-SS-P59-mCherry using *NcoI* restriction sites (Fig. 2). After the product was transformed in *E. coli* DH5 α , the plasmid obtained was designated pJet1.2-mCherry; it was nontoxic to *E. coli* DH5 α compared to the constitutive expression mCherry. The plasmid pJet1.2-mCherry was digested with *NcoI* and *SpeI*. The mCherry gene was purified and ligated into the pNZ8048 plasmid containing a nisin-inducible promoter to result in production of mCherry in the presence of nisin within *L. lactis* bacteria.

Characterization of plasmid constructs

GFP and iRFP ligation products within the pNZ8048 vector were transformed first in *E. coli*. The selected colonies were characterized using *NcoI* and *SpeI* restriction enzymes. As expected, DNA fragments of 750 bp (Fig. 3A) and 958 bp (Fig. 3C), respectively, were released. For the iRFP construct, an additional digestion was performed using the restriction enzymes, *HindIII* and *BglII*. The expected DNA fragments of 500 bp and 900 bp were removed (Fig. 3C). Each construction of pNZ8048-GFP or pNZ8048-iRFP then was electroporated into *L. lactis*.

The mCherry-coding sequence was inserted into pNZ8048 between the restriction sites, *NcoI/SpeI*. After digestion with the restriction enzymes *NcoI/SpeI*, an expected DNA fragment of 714 bp was released (Fig. 3B). pNZ8048-mCherry then was electroporated into the NZ9000 *L. lactis* strain. Recombinants were selected in M17-agar (1% glucose) plates containing chloramphenicol (10 μ g/ml). Single colonies were picked up and used to inoculate a starter culture of 2 ml in GM17 medium. Colonies then were cultured overnight at 30° C without agitation.

Expression of the reporter genes by *L. lactis*

Expression of GFP was evaluated by fluorescent microscopy at 488 nm excitation and 507 nm emission. No GFP expression was detected in non-induced *L. lactis*. By contrast, strong GFP expression was detected in induced *L. lactis* (Fig. 4A).

To allow the expression of mCherry, cultures of recombinant strains of *L. lactis* (OD 600 nm = 0.6–0.8) were induced with 10 ng/ml nisin. *L. lactis*-mCherry cultures turned light pink. Figure 4B shows a cell pellets of non-induced (- Nisin) and induced (+ Nisin) *L. lactis*. mCherry expression was evaluated by fluorescence microscopy at 587 nm excitation and 610 nm emission. No expression of mCherry was detected in non-induced *L. lactis* compared to strong mCherry expression detected in induced *L. lactis* (Fig. 4B). Neither *L. lactis*-GFP nor *L. lactis*-iRFP showed a visible change of color after induction and centrifugation.

We also compared induced *L. lactis*-iRFP and *L. lactis*-GFP as negative controls. At 6 h post-induction, 1 ml of culture was centrifuged at 23,000 $\times$ g and the cell pellet was scanned using the infrared imaging system scanner at 700 nm. Under these conditions, *L. lactis*-GFP

exhibited low intensity, whereas greater signal intensity was detected in *L. lactis*-iRFP (Fig. 4C).

Discussion

L. lactis can be engineered to possess different types of promoters: constitutive promoters such as P59 or inducible promoters such as pNis. The inducible NICE system is well-characterized and is used most often for *L. lactis*. To obtain recombinant *L. lactis* that expresses mCherry, GFP or iRFP, we first designed synthetic gene sequences. This was a worthwhile approach, particularly for *L. lactis*, because the expression of genes of other organisms depends on the codon usage and the distribution of rarely used codons. Therefore, codon optimization provides better protein yields than gene sequences with their intact codons. (Mierau and Kleerebezem 2005).

Constitutive promoters have the advantage of not requiring an inducer; however, constitutive expression of heterologous proteins sometimes may be toxic to the host cells (Bahey-El-Din 2012). Continuing high protein production could cause intracellular accumulation followed by aggregation or degradation of the protein in the cytoplasm, which could cause physiological stress, slow the growth of the cell and adversely affect its viability (Chamberlain et al. 1997, Nouaille et al. 2003).

Although inducible expression requires a relevant inducer, the amount of protein produced by the system usually is greater than constitutive expression (Bahey-El-Din 2012). This could be explained by the fact that the inducible system can achieve adequate biomass prior to target protein production, whereas constitutive promoters are limited by amino acid synthesis and/or higher energy demand due to recombinant protein production (Kim and Mills 2007).

We obtained the pNZ8048-GFP construction first, which was used to make the remaining constructions. For mCherry and iRFP cloning, we did not isolate the pNZ8048-GFP vector from an agarose gel, because this step usually requires large amounts of DNA and exposure to ultraviolet light may decrease ligation efficiency severely; therefore, the vector was only digested and dephosphorylated.

We experienced difficulties transforming *E. coli* DH5 α using the pNZ8048-P59/usp45-mCherry plasmid; this likely was due to plasmid-induced toxicity. Therefore, the P59 promoter and the *usp45* signal peptide sequence were replaced for the nisin-inducible promoter to avoid any potential toxicity.

When using *E. coli* for intermediate cloning steps, the resulting clones must be electroporated into *L. lactis*, screened and characterized again; this protocol is laborious and time-consuming. It has been recommended that plasmid construction be performed directly in *L. lactis*, because when *E. coli* is used as an intermediate host, it could decrease gene expression or even produce rearrangements in the plasmids that carry the coding sequences (Kuipers et al. 1998). We did not find alteration of gene expression, however, when *E. coli* DH5 α was used as an intermediate.

Transforming ligation products directly into *L. lactis* has some disadvantages. Isolating DNA from colonies requires the use of phenol and chloroform, which are toxic substances. In addition, the DNA obtained is of poor quality and is difficult to characterize. To avoid the use of phenol or chloroform and risk poor plasmid DNA quality for characterization, we took advantage of the fluorescent property of the reporter gene. mCherry is a red fluorescent protein, so selection of *L. lactis* expressing mCherry was straightforward, because under fluorescence microscopy, a bright red color appears instead of green when induced with nisin. In cases in which a large number of colonies must be screened, the selection of *L. lactis*-mCherry is simple. Colonies were picked up, cultured in a 96-well plate with nisin and the bright red expression in the case of mCherry or the absence of green fluorescence in the case of iRFP was observed using fluorescence microscopy. This technique reduced the time consumed by decreasing the number of colonies of *L. lactis* that had to be analyzed by DNA isolation. Also, exposure of personnel to phenol and chloroform was reduced.

We could not detect a bright signal for *L. lactis* that expressed iRFP using fluorescence microscopy, because the emission wavelength is too high for routine microscopy filters. Consequently, we used an LI-COR Odyssey infrared scanner. The LI-COR Odyssey is a near-infrared scanner that can detect fluorescence in the 700–800 nm range. A near-infrared fluorescent protein has advantageous optical properties, because absorbance due to lipids and proteins such as hemoglobin is negligible at infrared wavelengths (Shu et al. 2009). iRFP possesses excitation and emission wavelengths that have superior tissue penetration and a low nonspecific background signal compared to visible light fluorescent proteins.

Tumor growth in deeper tissues can be visualized by an infrared scanner in whole mice using implanted HeLa cells that express iRFP (Jiguet-Jiglaire et al. 2014). The iRFP signal remained detectable after fixation and tissue sectioning. These investigators also visualized metastatic sites easily using tumor cells transduced with a lentivirus that expressed iRFP (Jiguet-Jiglaire et al. 2014). Using iRFP enables visualization of tumors that was not possible with other fluorescent proteins. In addition, the size of the tumors can be measured even when they are too small for the use of calipers.

We have shown that *L. lactis* can express efficiently different reporter genes that can be detected by fluorescent microscopy or an infrared scanner. These strains could be useful for live cell imaging studies in vitro or for imaging studies in vivo in the case of iRFP. In addition, we have developed a simplified protocol for screening recombinant *L. lactis* using fluorescent reporter genes.

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Fig. 1. Schematic of synthetic constructs. The scheme represents the construction of GFP (A), mCherry (B) or (C) iRFP depicted by green, dark red and red arrows, respectively, under regulation of nisin-inducible promoter pNis. Not to scale.

Fig. 2.

Alternative strategy for cloning mCherry in pNZ8048. The strategy to clone and express mCherry under regulation of the pNis promoter follows. 1) Initially, mCherry was obtained in the plasmid, pJet1.2, with signal peptide and constitutive promoter, P59. 2). The signal peptide and constitutive promoter then were excised by digestion with the restriction enzyme, *NcoI*, and transformed further in *E. coli* DH5 α . 3). mCherry coding sequence was excised from pJet 1.2 using restriction enzymes, *NcoI* and *SpeI*, and inserted into pNZ8048, which already contained the pNis promoter.

Fig. 3.

Characterization of recombinant plasmids. A) One clone of pNZ8048-GFP was digested with the restriction enzymes, *NcoI* and *SpeI*. A 750 bp band was released, which corresponds to the GFP gene. B) Four clones of pNZ8048-mCherry were digested by the restriction enzymes, *NcoI* and *SpeI*. A 714 bp band was released, which corresponds to the mCherry gene. C) Four clones of pNZ8048-iRFP were digested as described in (A); a 958 bp band was released. Four clones of *pNZ8048:iRFP* were digested with *HindIII* and three clones were digested with *BglII*. The sizes of bands expected for *HindIII* were 580 bp and 3,520 bp. Bands for *BglII* were 900 bp and 3,200 bp.

Fig. 4.

Expression of fluorescent proteins expressed by *L. lactis*. A) GFP expression was evaluated using a Leica DM1000 fluorescence microscope with a I3 filter at 488 and 507 nm excitation and emission, respectively. B) mCherry expression was evaluated using a Leica DM1000 fluorescence microscope with an N2.1 filter at 587 nm and 610 nm excitation and emission, respectively. An induced (+ nisin) *L. lactis* mCherry cell pellet was pink, whereas non-induced (- nisin) was white. Expression of GFP and mCherry were observed at 100 × after induction for 2 h with nisin. C) Intensity levels obtained in cell pellets from *L. lactis* iRFP (+) induced with nisin for 6 h. *L. lactis* iRFP (-) non-induced, and *L. lactis* GFP (+) induced with nisin for 6 h were evaluated using an Odyssey Infrared Imaging System scanner. Reporter gene expressions were performed three times; one representative experiment is shown.