Original Article

Construction and Secretory Expression of β-Galactosidase Gene from Lactobacillus Bulgaricus in Lactococcus Lactis*

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Abstract

Objective This study is to examine the secretion effects of β-galactosidase in *Lactococcus lactis*.

Methods The *usp45* and β-galactosidase genes were cloned and inserted into plasmid pMG36e to obtain the recombinant plasmid pMG36e-usp-lacZ. This recombinant plasmid was transformed into both *Escherichia coli* DH5α and *L. lactis* MG1363. The enzyme activity, gene sequencing, SDS-PAGE and hereditary stability were assessed and studied.

Results The lacZ gene inserted into plasmids pMG36e-usp-lacZ was 99.37% similar to the GenBank sequence, and SDS-PAGE revealed an evident idio-strap at 116 KDa between L. lactis MG1363/pMG36e-usp-lacZ in both supernatant and cell samples. β-Galactosidase activity measured 0.225 U/mL in *L. lactis* pMG36e-usp-lacZ transformants, and its secretion rate was 10%. The plasmid pMG36e-usp-lacZ appeared more stable in MG1363.

Conclusion The authors concluded that these new recombinant bacteria well expressed and secreted β-galactosidase, indicating that the β-galactosidase expression system was successfully constructed, and this might provide a new solution for management of lactose intolerance specifically and promote the use of gene-modified organisms as part of the food-grade plasmid in general.

Key words: Gene constructs; Gene expression; Secretory expression; β-Galactosidase; *Lactococcus lactis*

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INTRODUCTION

Lactose intolerance (LI) is the inability to metabolise lactose due to lack of required lactase enzymes in the digestive system. Lactose intolerance can be found in many animals, including human beings, and it can cause osteoporosis, calcium deficiency and several other types of nutritional and health problems[1]. People suffering from LI have to avoid lactose-containing foods such as milk and milk products, which are generally considered as one of the important sources of nutrients for human beings, especially in developing countries, as they contain high-quality proteins and various minerals[2]. Some factors, such as age, ethnicity, heredity, and geographical environment are associated with LI[3]. However, its etiology remains poorly understood. It is estimated that the incidence of LI in China is 30.5% on average among children between 11-13 years and 55.1% among adults[4-5]. For

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these reasons, we conclude that LI may be one of the high-priority public health problems in China.

β-Galactosidase, an essential enzyme, is a disaccharide located on the mucous membrane brush border of the human intestine[6-7]. The endogenous β-galactosidase expressed in intestinal microbes is believed to help humans use lactose, at least in part[8]. Exogenous β-galactosidase is used to alleviate LI in subjects with lactase deficiency. Oral administration of β-galactosidase is inconvenient and not very effective because this enzyme cannot maintain its activity over long periods of time. Therefore, enhancement of β-galactosidase in human intestinal microbiota, together with selected probiotics may be a promising approach to LI management.

Fermentation with lactic acid bacteria (LAB) is considered a safe and natural bio-solution to reduce levels of lactose in milk. However, LAB currently used to prepare fermented milk can hydrolyse less than 20% of the lactose present in milk[9-10]. Most current probiotics that colonize in human intestine are LAB, particularly *Lactococcus spp*. One of the health-promoting effects of LAB is improvement of human lactose processing. In the dairy industry, *Lactobacillus delbrueckii subsp. bulgaricus* is one of the most important composite general starter cultures, but unfortunately it cannot colonize in human stomach, for it has a profound ability to express significant levels of β-galactosidase *in vitro*[25]. Therefore, construction of selected LAB with probiotic properties as a live delivery system for highly active β-galactosidase could be an effective approach towards solving the LI problem.

The purpose of the present study was to generate a new recombinant *L. lactis* with significant β-galactosidase expression by inserting the secretion signal peptide *usp45* upstream of the *lacZ* gene.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. E. coli were cultured in Luria-Bertani (LB) medium (both agar and broth) at 37 °C; L. lactis was cultured in Man Rogosa Sharpe (MRS) medium (both agar and broth) at 30 °C. Erythromycin (Sino-American Biotechnology Company, Beijing, China) was used at concentrations of 300 μg/mL for E. coli and 5 μg/mL for L. lactis. 40 μL of 20 mg/mL X-gal (TakaRa Biotechnology Co., Ltd., Dalian, China) and 4 μL of 100 mg/mL IPTG (isopropyl β-D-1-thiogalactopyranoside) (TakaRa Biotechnology Co., Ltd., Dalian, China) were plated in agar for screening positive recombinant colonies.

Genomic DNA Isolation and PCR Amplification of usp45 and LacZ

Genomic DNA was isolated from Lb. bulgaricus wch9901 with a Miniprep Bacterial DNA Kit (Watson Biotechnologies, Inc., Shanghai, China) and used as the template for PCR of lacZ. The primers lacZf2 and lacZr2 (Table 2) were used to amplify the lacZ gene. PCR was performed with a total volume of 100 μL containing 4U of Taq plus DNA polymerase (Tiangen, Tiangen Biotech Co., Ltd., Beijing, China), 1 μmol/L of each primer (Invitrogen Biotechnology Co., Ltd., Shanghai, China), 0.4 mmol/L 4dNTPs (Tiangen, Tiangen Biotech Co., Ltd., Beijing, China) and 2.5 mmol/L MgCl2 (Tiangen, Tiangen Biotech Co., Ltd., Beijing, China). Cycles consisted of a denaturation at 94 °C for 1 min, an annealing at 57 °C for 1 min, and an extension at 72 °C for 3 min.

Recursion PCR was used to amplify the usp45 gene. Four primers-uspf1, uspf2, uspr1, uspr2 were used for PCR. PCR was performed with a total volume of 100 μL containing 4U of Taq plus DNA polymerase, 1 μmol/L of uspf1 and uspr2 (Invitrogen Biotechnology Co., Ltd., Shanghai, China), 0.1 μmol/L of uspf2 and uspr1 (Invitrogen Biotechnology Co., Ltd., Shanghai, China), 0.8 mmol/L 4dNTPs and 2.5 mmol/L $MgCl_2$. Three different kinds of cycles were performed consecutively in the recursion PCR amplification: five cycles consisting of a denaturation at 94 °C for 30 s, an annealing at 41 °C for 30 s, and an extension at 72 °C for 40 s; five cycles consisting of a denaturation at 94 °C for 30 s, an annealing at 48 °C for 30 s, and an extension at 72 °C for 40 s; thirty cycles consisting of a denaturation at 94 °C for 30 s and an extension at 72 °C for 1 min.

Table 1. Bacterial Strains and Plasmids Used in This Study

Strain and Plasmid	Relevant Trait (s)	Source or Reference
Strains		
E. coli DH5α	supE44 Δlac U169 (ϕ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-l relA1	This laboratory
L. lactis subsp. lactis MG1363	Derived from L. lactis subsp lactis NCDO712 by plasmid curing	Prof. J. .Kok, Groningen University, Van de GM et al.[20-21]

(continued)

Strain and Plasmid	Relevant Trait (s)	Source or Reference
Lactobacillus delbrueckii subsp. bulgaricus wch9901	Isolated from Hua Xi yogurt	Ref.[12]
E. coli DH5α/ pMG36e-usp-lacZ	E. coli DH5α with pMG36e-usp-lacZ	This study
L. lactis MG1363/ pMG36e-usp-lacZ	L. lactis MG1363 with pMG36e-usp-lacZ	This study
Plasmids		
pMG36e	Emr; expression vector with P32 promoter, multiple cloning sites (MCF), and prtP translational terminator	Prof. J. Kok, Groningen University, Van de GM et al.[20-21]
pMG36e-usp-lacZ	Emr, expression of usp- wch9901β-galactosidase fusion protein	This study

Construction of pMG36e-usp-lacZ[11]

Both the 119 bp fragment of *usp45* and vector pUC18 were digested with *Bam*H Ⅰ and *Sac* Ⅰ (TaKaRa Biotechnology Co., Ltd. Dalian, China) and ligased by T4 ligase (TakaRa Biotechnology Co., Ltd. Dalian, China), to produce recombinant plasmid pUC18-usp. Then the *lacZ* gene from *Lb. bulgaricus* wch9901 was inserted into plasmid pUC18-usp and digested with *Bam*H Ⅰ and *Sal* Ⅰ (TaKaRa Biotechnology Co., Ltd. Dalian, China), producing the recombinant plasmid pUC18-usp-lacZ. *Usp-lacZ* fragment was obtained by digestion of plasmid pUC18-usp-lacZ with *Sac* Ⅰ and *Sal* Ⅰ and then the fragment was ligased with pMG36e digested by the same two restriction enzymes. The recombinant plasmid pMG36e-usp-lacZ was constructed.

Plasmid DNA Transformation

The plasmid was introduced into *E. coli* by standard $CaCl_2$ transformation and into *L. lactis* by electroporation by using an Eppendorf multiporator (Eppendorf AG, Hamburg, Germany) at a pulse voltage of 2 KV and a pulse time of 5 msec[11-13].

Identification of Positive Transformants

Positive transformants were identified by the color of the clones, *Sac* Ⅰ and *Sal* Ⅰ digestion, PCR analysis with the primers lacZf2 and lacZr2 (Table 2), and standard SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis)[11,14]. In order to confirm that the *usp-lacZ* gene had been inserted successfully, plasmid pMG36e-usp-lacZ was sequenced (Invitrogen Biotechnology Co., Ltd., Shanghai, China). DNAssist 2.0 was used to analyze the nucleotide sequence.

Enzyme Assay

The β-galactosidase activity of positive *E. coli* and *L. lactis* clones was determined by using *o*-nitrophenyl-β-D-galactopyranoside (Watson Biotechnologies, Inc., Shanghai, China) as the substrate at 30 °C in phosphate buffer at pH 7.0[15].

Table 2. Primers Used in This Study

Primers	Sequences
lacZf2	5′-GCGGGATCCTGAGCAATAAGTTAGTAAAAGAAA-3′
lacZr2	5′-GCGGTCGACTTATTTTAGTAAAAGGGGCTGAAT-3′
uspf1	5′-GCGGAGCTCAGAAGGGAAGAATTAGAAAATGAAAAA-3′
uspf2	5′-AATTAGAAAATGAAAAAAAAGATTATCTCAGCTATTTTAA TGTCTACAGTG-3′
uspr1	5′-CACCTGACAACGGGGCTGCAGCAGAAAGTATCACTGTA GACATTAAAAT-3′
uspr2	5′-GCGGGATCCCAGCGTAAACACCTGACAACGGGGCTGCA-3′

The number of β-galactosidase activity units of lysate per ml (U/mL) was taken to represent intracellular β-galactosidase activity, while the same of the supernatant to express extracellular β-galactosidase activity. The extracellular percentages of β-galactosidase units in the supernatant were used to express the secretion rate.

Hereditary Stability of Plasmid pMG36e-usp-lacZ[16]

A single clone of MG1363/pMG36e-usp-lacZ was inoculated in MRS broth containing erythromycin and cultured at 37 °C 220 r/min for 0, 2, 3, 4, 5, 6, 7, 8, and 10 h respectively. The bacterial counts after every incubation period were determined by the plate pouring method to obtain the growth curve. From this curve, the generation time and lag period were calculated by using the following formula: $G = (t\text{-}t_1)\ln_2/(\ln X\text{-}\ln X_0)$ (Where G is the generation time, t_1 the lag period, t the incubation time, X the number of bacteria, and X_0 the incipient number of bacteria)[17].

MG1363/pMG36e-usp-lacZ was continuously passaged through MRS broth with and without erythromycin. After every 5 generations, the rates of plasmid hereditary stability were determined, for which broth cultures were crossed on MRS plates without erythromycin and 100 clones were selected randomly from the plate. The selected clones were

then inoculated on MRS plates containing X-gal and IPTG with or without 5 μg/mL erythromycin. The rate of hereditary stability was defined by the number of blue clones on the MRS plate that contained X-gal and IPTG per 100 clones.

RESULTS

Identification of Positive Transformants

E. coli DH5α/pMG36e-usp-lacZ transformants in medium containing X-gal and IPTG appeared as blue colonies on LB plates containing erythromycin; *L. lactis* MG1363/pMG36e-usp-lacZ transformants formed blue colonies on MRS-lactose plate containing erythromycin, X-gal and IPTG.

A 119-bp length of usp45-amplified fragment is shown in Figure 1, and a 3 041-bp length of lacZ-amplified fragment is shown in Figure 2. The plasmid pMG36e-usp-lacZ was extracted and its digestion patterns are shown in Figure 3. *Usp-lacZ* gene fragments (3 148bp) were amplified from the recombinant plasmid pMG36e-usp-lacZ (Figure 4).

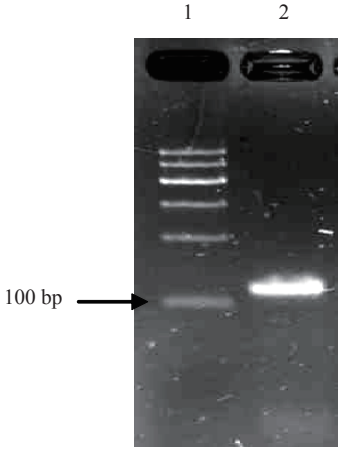

Figure 1. The PCR products of *usp45*. Lane 1: DNA molecular weight marker, Lane 2: PCR products of *usp45*.

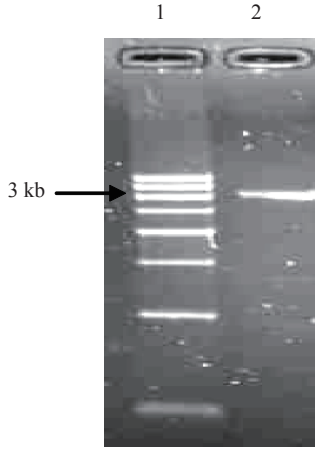

Figure 2. PCR products of *lacZ*. Lane 1: DNA molecular weight marker, Lane 2: PCR products of *lacZ.*

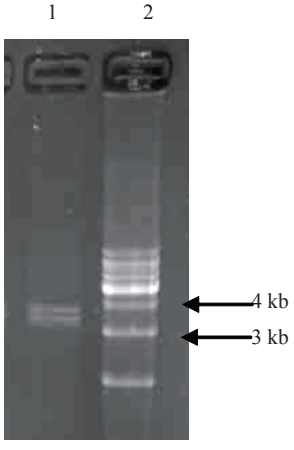

Figure 3. Restriction enzyme digestion fragments of recombinant plasmid. Lane 1: Sac Ⅰ /Sal Ⅰ digestion fragments of pMG36e-usp-lacZ; Lane 2: DNA molecular weight marker.

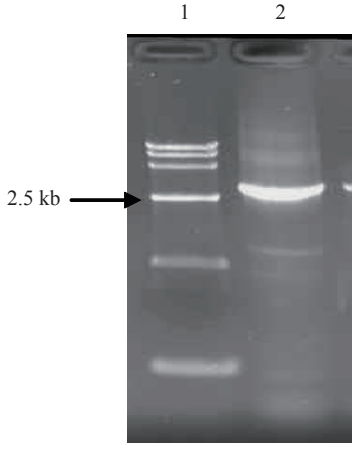

Figure 4. PCR amplification of pMG36e-usp-lacZ. Lane 1: PCR products of pMG36e-usp-lacZ, Lane 2: DNA molecular weight marker.

The *lacZ* gene inserted into plasmids pMG36e-usp-lacZ was 3 028 bp in length. Compared to the *lacZ* sequence from GenBank (M23530), pMG36e-usp-lacZ contained 19 base pair variants. In other words, it was 99.37% similar to the GenBank sequence.

SDS-PAGE (Figure 5) revealed an evident idio-strap at 116 KDa between *L. lactis* MG1363/pMG36e-usp-lacZ, as compared to *L. lactis* MG1363/pMG36e and *L. lactis* MG1363 in both supernatant and cell samples. This idio-strap was most evident in supernatant samples.

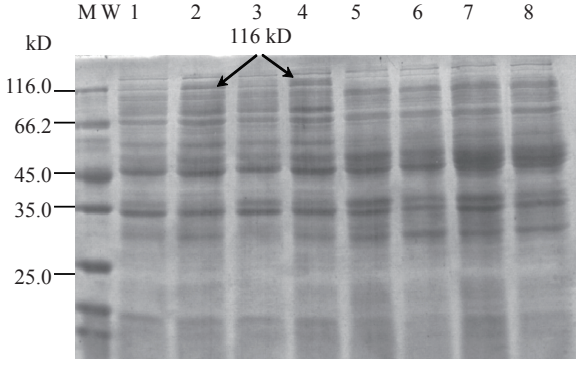

Figure 5. The SDS-PAGE of recombinant strains. MW: protein molecular weight marker, Lane 1/Lane 3: proteins from cell of *L. lactis* MG1363/pMG36e-usp-lacZ, Lane 2/ Lane 4: proteins from supernate of *L. lactis* MG1363/pMG36e-usp-lacZ, Lane 5: proteins from cell of *L. lactis* MG1363/pMG36e, Lane 6: proteins from supernate of *L. lactis* MG1363/pMG36e, Lane 7: proteins from cell of *E. L. lactis* MG1363, Lane 8: proteins from supernate of *L. lactis* MG1363.

Analysis of β-Galactosidase Activity in E. coli DH5α/ pMG36e-usp-lacZ and L. lactis MG1363/pMG36e-usp-lacZ Transformants

β-Galactosidase activity measured 0.142 U/mL in *E. coli* DH5α/pMG36e-usp-lacZ and 0.225 U/mL in *L. lactis* pMG36e-usp-lacZ transformants, and the secretion rate of *L. lactis* pMG36e-usp-lacZ was 10%. (Figure 6).

Hereditary Stability of Plasmid pMG36e-usp-lacZ

According to Figure 7, t1=2 h, G=0.92 h, 5G=4.6 h. Under erythromycin selective pressure, plasmid pMG36e-usp-lacZ appeared more stable in MG1363 (P<0.05).

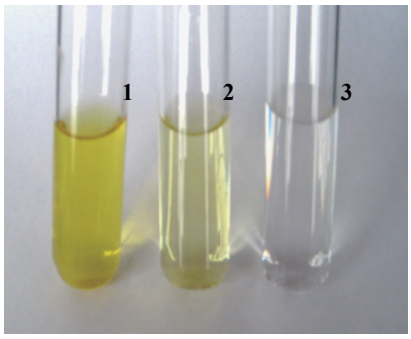

Figure 6. β-Galactosidase Activity. Tube 1: β-Galactosidase activity of *L. lactis* pMG36e-usp-lacZ transformants, Tube 2: β-Galactosidase activity of *E. coli* DH5α/ pMG36e-usp-lacZ transformants, Tube 3: blank control.

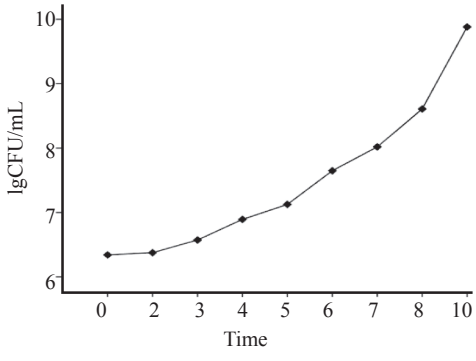

Figure 7. Growth curve of MG1363/pMG36e-usp-lacZ.

DISCUSSION

In recent years, a number of expression vectors have been developed to express and secrete proteins in LAB[18]. Expression vector pMG36e was constructed by Kok J and his colleagues in 1989. The plasmid pMG36e contains a strong promoter P32 and its partial downstream opens a reading frame from *L. lactis*, along with the multiple clone sites from pUC18, the terminator from prtP gene, a pWV01 replicon, and an anti-erythromycin gene. pMG36e can also replicate in several host strains, such as *E. coli*, *L. lactis*, and some other *Lactobacillus strains*[19]. pMG36e has been used successfully to express a number of lysozymes, such as dispase in *Bacillus subtilis* and superoxide dismutase in *E. coli* and in *L. lactis*[20-21].

L. lactis MG1363 is a well-known plasmid-free strain currently used as a delivery system because of its advantages in gene transformation, which

includes easy culturing and good repeatability. *L. lactis* MG1363 has neither β-galactosidase activity nor any tendency to interfere with β-galactosidase activity from other recombinant strains.

Gene *usp45*, which codes for the secretion protein of *L. lactis subsp. Lactis* MG1363, was first cloned by Van AM in 1990[22]. Its length is 81 bp and it codes for a polypeptide of 27 amino acids that can be secreted into the medium. It has been widely applied in expression and secretion research in *L. lactis*[23].

In order to avoid accidentally translating a product from the vector to the target protein, we used a non-fusion protein expression technique. In other words, instead of using the SD (shine-dalgarno) sequence of the vector, we used *lacZ* gene's own SD sequence. In addition, there was an ATGA sequence 9 bp downstream of *lacZ* gene's SD sequence which was able to guarantee the termination of the translation of the vector peptide while leaving the start of the translation of the target protein uninhibited. If the *lacZ* gene's SD sequence was not recognized by the host strain and the ATGA sequence was interpreted invalidly, the translation would have started from the ATG sequence of the plasmid and consequently, the *usp45* fusion β-galactosidase protein would not have been translated correctly.

The enzyme activity of *E. coli* DH5α/pMG36e-usp-lacZ was a little lower than that of *L. lactis* MG1363/pMG36e-usp-lacZ, possibly because of copy number variations (With the copy number of the plasmid pMG36e higher in *L. lactis*.)

Although the β-galactosidase activity of the *L. lactis* MG1363/pMG36e-usp-lacZ constructed in this study was lower than that of *Lb. bulgaricus* wch9901, with its β-galactosidase activity being 0.401 U/mL, the secretion rate of the enzyme was still increased from 3% to 10% by using these recombinant strains[24]. In addition, *Lb. bulgaricus* cannot colonize in human stomach even though these bacteria express significant levels of β-galactosidase in vitro. Therefore, these recombinant strains may be more helpful than original strains in terms of LI treatment.

The insertion of *usp45* resulted in decrease of β-galactosidase activity. One possible reason might lie in the fact that the distance between the *usp45* gene and the start code of *lacZ* was not ideal. The differences in the signal peptide C-terminus could possibly affect recognition and digestion. Although the enzyme activities of *E. coli* DH5α/pMG36e-usp-lacZ and *L. lactis* MG1363/pMG36e-usp-lacZ were lower, the secretion of recombinant plasmid pMG36e-usp-lacZ was higher than that of *Lb. bulgaricus* wch9901, indicating that the recombinant plasmid pMG36e-usp-lacZ was constructed successfully and appeared more stable in MG1363 under erythromycin selective pressure.

In the present study, the *usp45* signal peptide sequence was inserted upstream of the *lacZ* gene, and β-galactosidase was expressed successfully in *L. lactis*. The recombinant plasmid pMG36e-usp-lacZ appeared more stable in MG1363 under erythromycin selective pressure. This study demonstrates that a *L. lactis* β-galactosidase secretory expression system can be constructed successfully.

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