Development of Bile Salt-Resistant *Leuconostoc citreum* by Expression of Bile Salt Hydrolase Gene

Seung Kee Cho¹, Soo Jin Lee¹, So-Yeon Shin¹, Jin Seok Moon¹, Ling Li¹, Wooha Joo¹, Dae-Kyung Kang², and Nam Soo Han¹*

¹Brain Korea 21 Center for Bio-Resource Development, Division of Animal, Horticultural, and Food Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea
²Department of Animal Resources Science, Dankook University, Cheonan 31116, Republic of Korea

Probiotic bacteria must have not only tolerance against bile salt but also no genes for antibiotic resistance. *Leuconostoc citreum* is a dominant lactic acid bacterium in various fermented foods, but it is not regarded as a probiotic because it lacks bile salt resistance. Therefore, we aimed to construct a bile salt-resistant *L. citreum* strain by transforming it with a bile salt hydrolase gene (*bsh*). We obtained the 1,001 bp *bsh* gene from the chromosomal DNA of *Lactobacillus plantarum* and subcloned it into the pCB4170 vector under a constitutive P710 promoter. The resulting vector, pCB4170BSH was transformed into *L. citreum* CB2567 by electroporation, and bile salt-resistant transformants were selected. Upon incubation with glycodeoxycholic acid sodium salt (GDCA), the *L. citreum* transformants grew and formed colonies, successfully transcribed the *bsh* gene, and expressed the BSH enzyme. The recombinant strain grew in up to 0.3% (w/v) GDCA, conditions unsuitable for the host strain. In *in vitro* digestion conditions of 10 mM bile salt, the transformant was over 67.6% viable, whereas only 0.8% of the host strain survived.

**Keywords:** *Leuconostoc citreum*, probiotics, bile salt hydrolase

*Leuconostoc* is an important genus of lactic acid bacteria (LAB) that plays a major role in maintaining the quality of fermented milk, dairy products, vegetables, and meats [3, 8, 12]. *Leuconostoc* species are used as starter cultures for commercial food products, such as *L. mesenteroides* subsp. *cremoris* for *viili* in Finland [14], various *Leuconostoc* species for *kefir* in many countries, and *L. mesenteroides* DRC for *kimchi* in Korea [9]. However, despite its important role, *Leuconostoc* sp. is not regarded as a probiotic because of its low colonization of the large intestine, mainly due to the absence of resistance to acid and bile salt.

Probiotics are “live microorganisms which when administered in adequate amount confer health benefits to the host” [11]. A variety of microorganisms, typically food-grade LAB, have been evaluated for their probiotic potential and are applied in a variety of food products or therapeutic preparations [24]. Microorganisms used in probiotic products generally contain lactobacilli and bifidobacteria [27]. Before a probiotic can benefit human health, it must fulfill certain criteria: it must survive passage through the upper gastrointestinal tract and arrive alive at its site of action, and it must be able to function in the gut environment [25].

Food-grade expression systems must avoid antibiotic resistance markers because such markers risk the transfer of antibiotic resistance to the human intestinal microbiota [6, 21]. Several potential selection markers have been developed that fulfill the food-grade requirements and avoid the use of any harmful or toxic substances [16, 23]. Resistance markers used in food-grade approaches can be classified, based on the method of selection, into dominant or complementation selection markers [7, 20].

In this study, we attempted to construct a bile salt-resistant *L. citreum* strain that can survive in the small intestine. We cloned the bile salt hydrolase gene (*bsh*) from *Lactobacillus plantarum* and expressed it in *L. citreum* using the pCB4170 vector. To improve the BSH activity, we also employed the strong P710 promoter from *L. mesenteroides*
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ATCC 8293 for the expression of BSH [4]. The characteristics of the transformant strains were investigated by in vitro tests. The bacterial strains and plasmids used in this study are listed in Table 1. L. citreum was routinely grown at 30°C in MRS medium (Difco, Detroit, MI, USA), whereas other LAB were grown at 37°C in MRS medium. Escherichia coli was cultured in Luria-Bertani (LB) medium with vigorous shaking at 37°C. Ampicillin (50 µg/ml) and chloramphenicol (10 µg/ml) were used for E. coli and LAB, respectively.

Restriction enzymes, T4 DNA ligase, CIAP (calf intestinal alkaline phosphatase), and Ex Taq polymerase were purchased from TaKaRa (Kyoto, Japan). A molecular weight standard of DNA was obtained from Bioneer Co. (Daejeon, Korea). Ampicillin, chloramphenicol, and glycodeoxycholic acid sodium salt (GDCA; G9910) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of reagent grade. Lactobacilli MRS broth and LB broth were purchased from Difco. Oligonucleotide primers were synthesized on an automated DNA synthesizer by Bioneer Co.

To obtain the bsh gene, the genomic DNA of Lb. plantarum was isolated. The bsh gene was amplified from the genomic DNA by PCR using the prepared primer sets (GenBank Accession No. AL935262). The purified PCR products were digested with SalI and PstI, extracted from a sliced gel, and ligated into SalI- and PstI-digested pCB4170 vectors, respectively. All of the above ligation mixture was transformed into CaCl2-competent E. coli MC1061 cells, respectively. Transformants were grown on LB medium containing ampicillin (50 µg/ml) at 37°C. Recombinant plasmids were extracted, and the resulting construct was designated pCB4170BSH (Fig. 1).

RNA was isolated from exponentially growing cells (OD600nm 0.3–0.4). Two volumes of RNA Protect (Qiagen, CA, USA) were added, and total RNA was isolated using an RNeasy Mini Kit. DNA was removed by digestion using

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Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Genotype/relevant features</th>
<th>Source</th>
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<tbody>
<tr>
<td>E. coli MC1061</td>
<td>Ffrr(araD139 ara-leu)7679 (lacIPOZYA)X74 galUl galK hsdR2 mcrB1 rpsL (SmR)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>L. citreum CB2567</td>
<td>Wild-type strain isolated from kimchi</td>
<td>KACC91348P</td>
</tr>
<tr>
<td>Lb. plantarum</td>
<td>Wild-type strain; template of bsh gene</td>
<td>KCTC 3104</td>
</tr>
<tr>
<td>pCB4170</td>
<td>E. coli-Leuconostoc shuttle vectors, AmpR, CmR</td>
<td>This study</td>
</tr>
</tbody>
</table>

KACC, Korean Agricultural Culture Collection; KCTC, Korean Collection for Type Cultures.

Fig. 1. Construction of pCB4170BSH vector harboring the bile salt hydrolase gene (bsh).
the RNase-Free DNase Set (Qiagen) at 37°C for 20 min. Total RNA (1 µg) was reverse-transcribed with 100 U of CycleScript Reverse transcriptase using 100 pmol random hexamer primers according to the manufacturer’s instructions (Bioneer).

The transcription levels of bsh were compared by real-time PCR using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The phosphoketolase gene (pho), a single-copy gene from the *L. citreum* CB2567 chromosome, was used as the reference gene (GenBank Accession No. NC010471). A 120 bp fragment of the pho gene was amplified with primers pho-F (5’-ACACAACTAA CGTCAATGGATG-3’) and pho-R (5’-CCITCAAGCCAACCTTCAGC-3’), and a 166 bp fragment of the bsh gene was amplified with primers bsh-F (5’-TCAAATAGCACACCCCAAAA-3’) and bsh-R (5’-TGCCACTCTCTGTCTGCATC-3’). The primers (0.05 pmol each) were added to a master mix containing 10 µl of iQ SYBR Green Supermix (Bio-Rad), 2 µl of cDNA, and RNase-free water in a final volume of 20 µl. Total RNA preparations were used as negative controls to verify the absence of chromosomal DNA in the cDNA libraries. The pho gene was used as a reference, and chromosomal DNA was used as a positive control. Melting curve analysis and amplicon size determination were performed to verify amplification of the appropriate transcripts. Transcription levels were quantified according to the method described by Pfaffl [22]. The threshold cycle number (Ct) of target reference genes was determined using the iQ5 Optical System Software (Bio-Rad) and used for further analysis.

To investigate heterologous expression of bsh in *L. citreum*, the plasmid pCB4170BSH was introduced by electroporation with chloramphenicol selection. BSH activity was examined by a bile salt plate assay as described by Dashkevich and Feighner [5]. Briefly, overnight cultures grown in MRS broth were streaked on MRS agar plates supplemented with 0.3% (w/v) GDCA. BSH activity was indicated by precipitate halos of deconjugated bile acid around active colonies. A bile salt survival assay was also performed to investigate the resistance of *L. citreum* to GDCA. Briefly, overnight cultures were inoculated (1%) into fresh MRS broth containing 0–0.5% (w/v) GDCA. Cell growth was measured by viable cell count.

In addition, the tolerance of *L. citreum* harboring pCB4170BSH against bile salts was determined using a simulated intestinal fluid (SIF) electrolyte stock solution (6.8 mM KCl, 0.8 mM KH₂PO₄, 85 mM NaHCO₃, 38.4 mM NaCl, 0.33 mM MgCl₂(H₂O)₆, 8.4 mM HCl, and 0.6 mM CaCl₂(H₂O)₆) with 10 mM GDCA [18]. Cells from all strains grown overnight in MRS broth were harvested by centrifugation, washed, and resuspended in 2 ml of the SIF electrolyte stock solution with 10 mM GDCA. After incubation for 0, 60, or 120 min at 30°C, viable cells were counted by standard plate counting. Measurements were performed in triplicate, and the mean values are reported.

To construct an expression vector for *Leuconostoc* sp., bsh was amplified from the template *Lb. plantarum* genome, and the generated PCR fragment was subcloned into the pCB4170 vector containing a constitutive P710 promoter (Fig.1). The constructed plasmid was transformed into *L. citreum* CB2567. Colonies carrying pCB4170BSH were selected on an MRS agar plate containing chloramphenicol.

The transcription level of bsh in *L. citreum* CB2567 harboring pCB4170BSH was determined by RT-qPCR analysis. Fig. 2 shows that bsh was expressed in the transformant at a higher level than that of pho (housekeeping gene). When the host and transformant strains were cultured in medium containing bile salt, growth of the wild-type strain was obviously inhibited at GDCA concentrations as
low as 0.1% (w/v). In contrast, the recombinant strain harboring pCB4170BSH sustained normal growth in MRS containing 0.1% (w/v) GDCA and could grow in the presence of 0.3% (w/v) GDCA. Therefore, 0.3% (w/v) GDCA can be used to screen transformants harboring the \( bsh \) gene. Furthermore, the agar plate containing 0.3% bile salt clearly showed sustained growth of only \( L. \) citreum CB2567 harboring pCB4170BSH, indicating that resistance to GDCA can be used as a food-grade selection marker (Fig. 3).

The bile salt resistance of \( L. \) citreum was examined to investigate whether it can survive in the small intestine environment. The cell viability of strains exposed to 10 mM GDCA for 120 min is shown in Table 2. After exposure for 60 min, the viable cell numbers of transformant decreased to 8.84 log CFU/ml, and the transformant was still viable (8.77 log CFU/ml) after 120 min exposure. The number of viable transformant cells was higher than that of the host strain, which decreased to 6.90 log CFU/ml at 60 min (Fig. 4). This result reveals that the recombinant strain was over 67.6% viable after incubation with 10 mM bile salt in \textit{in vitro} digestion conditions, whereas only 0.8% of the host strain survived.

Several genes or gene clusters have been used as selection markers for construction of food-grade expression systems: D-xylose catabolism of \textit{Lb. pentosus} [23], sucrose transporter system of \textit{Pediococcus pentosaceus} [16], nisin immunity of \textit{Lactococcus lactis} [26], lactacin F immunity of \textit{Lb. acidophilus} [1], purine biosynthesis of \textit{Lc. lactis} [7], alanine racemase of \textit{Lb. plantarum} [20], and glutamate racemase of \textit{Lc. lactis} [2]. Yin \textit{et al}. [28] also demonstrated that the \( bsh \) gene from \textit{Lb. plantarum} was functional in lactobacilli, permitting simple selection of transformant cells in the presence of bile salt. In our study, the \( bsh \) gene was newly used to provide bile salt resistance to bile-sensitive \( L. \) citreum to make it a potential probiotic. Indeed, our results show that expression of \( bsh \) was effective to confer bile tolerance to \( L. \) citreum and, at the same time, it was useful for selection of transformants.

In order to improve the BSH expression level in \( L. \) citreum, we also employed the strong P710 promoter from \textit{L. mesenteroides} ATCC 8293. Transcriptomic analysis using microarrays revealed that genes of \textit{Leum}_209, 710, and

**Table 2.** Survival of wild-type \textit{L. citreum} CB2567 and the transformant harboring pCB4170BSH after incubation in 10 mM bile salt for 60 and 120 min.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Initial mean counts (Log CFU/ml)</th>
<th>Mean counts after 60 min (Log CFU/ml)</th>
<th>Mean counts after 120 min (Log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{L. citreum} CB2567</td>
<td>9.08 ± 0.01</td>
<td>6.90 ± 0.01</td>
<td>6.94 ± 0.01</td>
</tr>
<tr>
<td>\textit{L. citreum} CB2567 harboring pCB4170BSH</td>
<td>8.94 ± 0.01</td>
<td>8.84 ± 0.01</td>
<td>8.77 ± 0.03</td>
</tr>
</tbody>
</table>

All values indicate the mean ± standard deviation.
1694 were expressed most strongly and constitutively in L. mesenteroides ATCC 8293 in both glucose- and sucrose-MRS media [4]. When the three promoter sequences (p209, p710, and p1694) were fused to the promoterless β-gal gene in pCB4170 and expressed in L. citreum CB2567, p710 resulted in the highest level of β-galactosidase activity, showing a strong and constitutive transcription of the heterologous gene. Annotation using NCBI BLAST analysis revealed that Leum_710 is an integral membrane protein and its functional information was not yet known.

The genome sequences of several Leuconostoc spp. have been recently determined, with growing industrial attention: L. mesenteroides ATCC 8293 [17], L. citreum KM20 [15], L. lactis [19], and L. gascomitatum [13]. However, few molecular-level manipulations have been performed on this genus and this is due to the absence of efficient tools for genetic engineering works. In this study, the pCB4170 vector was employed to express gene bsh in L. citreum, after size minimization of the pCB42 plasmid as described by Eom et al. [10]. The pCB42 plasmid was first obtained from L. citreum CB2567 and it was capable of replicating in various LAB, including Lb. plantarum, Lb. reuteri, Le. lactis, Streptococcus thermophilus, Weissella confusa, and Oenococcus oeni. Our results reveal that vector pCB4170 is applicable to construct food-grade transformants of the above LAB cells. In particular, W. confusa and O. oeni are important for food fermentation, and no appropriate tools are developed for genetic engineering. It is supposed that the expression of bsh by using pCB4170 can be further performed in various bile-sensitive LAB to give bile tolerance.

In this study, the bsh gene from Lb. plantarum was cloned and expressed in BSH-deficient L. citreum CB2567. The recombinant strain acquired the ability to deconjugate GDCA, revealing a strong correlation between the presence of the bsh gene and resistance to GDCA. Heterologous expression of the bsh gene in L. citreum CB2567 was under the transcriptional control of the P710 promoter. The recombinant strain acquired resistance to GDCA and grew in the presence of up to 0.3% (w/v) GDCA. In conclusion, gene bsh not only acted as a potential selection marker in L. citreum but also conferred probiotic properties.

Acknowledgments

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References

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