Construction of a Recombinant Leuconostoc mesenteroides CJNU 0147 Producing 1,4-Dihydroxy-2-Naphthoic Acid, a Bifidogenic Growth Factor

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Abstract

1,4-Dihydroxy-2-naphthoic acid (DHNA), a precursor of menaquinone (vitamin K2), has an effect on growth stimulation of bifidobacteria and prevention of osteoporosis, making it a promising functional food material. Therefore, we tried to clone the menB gene encoding DHNA synthase from Leuconostoc mesenteroides CJNU 0147. Based on the genome sequence of Leu. mesenteroides ATCC 8293 (GenBank accession no., CP000414), a primer set (Leu_menBfull_F and Leu_menBfull_R) was designed for the PCR amplification of menB gene of CJNU 0147. A DNA fragment (1,190 bp), including the menB gene, was amplified, cloned into pGEM-T Easy vector, and sequenced. The deduced amino acid sequence of MenB (DHNA synthase) protein of CJNU 0147 had a 98% similarity to the corresponding protein of ATCC 8293. The menB gene was subcloned into pCW4, a lactic acid bacteria - E. coli shuttle vector, and transferred to CJNU 0147. The transcription of menB gene of CJNU 0147 (pCW4::menB) was increased, when compared with those of CJNU 0147 (pCW4) and CJNU 0147 (-). The DHNA was produced from it at a detectable level, indicating that the cloned menB gene of CJNU 0147 encoded a DHNA synthase which is responsible for the production of DHNA, resulting in an increase of bifidogenic growth stimulation activity.

Keywords: 1,4-dihydroxy-2-naphthoic acid, Leuconostoc mesenteroides, DHNA synthase, menB gene, lactic acid bacteria

Introduction

One of the powerful trends in future might be probiotics, including lactic acid bacteria, in the functional food market worldwide (Foligné et al., 2013). Thus, many researchers as well as food microbiologists have focused on studying and understanding the role of probiotics in human health. Due to their efforts, huge amount of data for supporting scientific evidences for health-promoting effects of lactic acid bacteria have been produced. Most of the effects are categorized into: 1) control of pathogenic bacteria (Kanmani et al., 2013), 2) prevention of diarrhea (Pattani et al., 2013), 3) alleviation of constipation (An et al., 2010), 4) stimulation of immune response (Tsai et al., 2012), 5) prevention of cancer (Zhong et al., 2014), 6) improvement of metabolic disorders (Aggarwal et al., 2013), etc. Based on this classification, lactic acid bacteria were broadly used as probiotics. Besides lactic acid bacteria, bifidobacteria are also recognized as one of the beneficial bacteria for human health (Tojo et al., 2014). Bifidobacteria play similar roles as the lactic acid bacteria in the human gut, and normally reside in colon and effectively eradicate few pathogenic bacteria, including Escherichia coli O157:H7, by producing acetic acid as well as lactic acid (Fukuda et al., 2011). Unfortunately, they belong to strictly anaerobic bacteria (Marteau et al., 2001), indicating that they are very difficult to handle in atmospheric environments. Nevertheless, dairy companies are using some bifidobacterial strains as probiotics since they have a beneficial impact on human health (Chen et al., 2014). Prebiotics also have positive effects on human gut, but in different ways (Vandenplas et al., 2015). In general, they are non-digestible fibers which stimulate the growth of beneficial gut bacteria, including bifidobacteria, resulting in production of short chain fatty acids (SCFA) such as acetate, propionate, and butyrate (Johnson et al., 2015). Many prebiotic candidates have been developed and several have already been commercialized. Among them, inulin, galacto-oligosaccharide,
and fructo-oligosaccharide have been widely used (Rastall, 2010). Besides non-digestible carbohydrates as prebiotics, 1,4-dihydroxy-2-naphthoic acid (DHNA) is also known as a bifidogenic growth stimulator (Isawa et al., 2002). The DHNA are produced from a Propionibacterium freudenreichii strain for commercial use and a corresponding gene (menB) for the production was identified from the genome sequence of P. freudenreichii CIRM-BIA1 (Falentin et al., 2010). The biosynthetic pathway for menaquinone (vitamin K2) was well established in Escherichia coli (Young, 1975). An E. coli mutant, where menB gene was mutated, lost a function of conversion of o-succinylbenzoic acid to DHNA, and a menA gene mutant blocked the formation of demethylmenaquinone from DHNA. In several bacterial species including Mycobacterium phlei (Meganathan and Bentley, 1979), Micrococcus luteus (Meganathan et al., 1980), and Bacillus subtilis (Taber et al., 1981), the synthetic pathways have been investigated, but the information for lactic acid bacteria is very limited for this pathway. In our previous study, we isolated several strains which stimulated the growth of Bifidobacterium longum (Eom and Moon, 2010; Moon, 2009). One of the strains was Leuconostoc mesenteroides CJNU 0147; however, we were unable to detect DHNA from its culture supernatant by HPLC (high performance liquid chromatography) analysis. Nevertheless we found a corresponding gene (menB) for DHNA synthase as well as menA gene for demethylmenaquinone synthesis from a genome sequence of Leu. mesenteroides ATCC 8293 (GenBank accession no., CP000414). Our aim was to study if Leu. mesenteroides CJNU 0147 has the menB gene encoding a DHNA synthase, and whether the gene overexpression in the strain can affect DHNA production. In this study, we successfully cloned a menB gene from Leu. mesenteroides CJNU 0147, and the gene overexpression in the strain lead to the production of DHNA at a detectable level by HPLC analysis.

Materials and Methods

Microbial strains, plasmids, and culture conditions

Microbial strains and plasmids used in this study are listed in Table 1. E. coli DH5α was cultured in LB broth (10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract, pH 7.0) at 37°C with vigorous shaking (250 rpm). Leu. mesenteroides CJNU 0147 was cultured in MRS (De Man, Rogosa, and Sharpe) broth (Difco, Sparks, USA) at 37°C without shaking. Ampicillin at 100 µg/mL (Sigma-Aldrich, USA) was used for the antibiotic selection of recombinant E. coli, and 400 µg/mL and 5 µg/mL erythromycin were used for E. coli and Leu. mesenteroides CJNU 0147, respectively.

PCR cloning of menB gene from Leu. mesenteroides CJNU 0147

For cloning of menB gene from Leu. mesenteroides CJNU 0147 in which a putative promoter is included, a primer set (Leu_menBfull_F: 5'-TGAGGCTGCTGTTTCAAGC-3'; Leu_menBfull_R: 5'-GATCATACCCGTATTC3') based on the genome sequence of Leu. mesenteroides ATCC 8293 (GenBank accession no., CP000414) was designed. A commercial PCR premix kit (Bioneer, Korea) was used for amplification of the gene. PCR cycling condition was 95°C/5 min for predenaturation, 30 cycles of 95°C/30 s, 55°C/30 s, and 72°C/1 min, and 72°C/10 min for final extension. The PCR product (1,190 bp) was visualized with GelStar™ nucleic acid gel stain (Lonza, USA) under a UV transilluminator (UVP, Camlab, UK). The menB amplicon was directly ligated with pGEM-T Easy vector (TA cloning vector; Promega, Mad-
ison, USA) and transferred to \textit{E. coli} DH5α. Using a universal primer (M13_F: 5’-GTTTTCCAGTCACGAC-3’) from the vector sequence, the insert containing \textit{menB} gene was sequenced by a commercial company (Macrogen, Korea). Homology search for the sequence was done with BLAST (basic local alignment search tool) programs (blastn and blastp) at NCBI (national center for biotechnology information; http://www.ncbi.nlm.nih.gov/). The \textit{menB} gene fragment digested with \textit{NorI} restriction endonuclease was inserted into pCW4 digested with the same enzyme. The recombinant DNA pCW4::\textit{menB} was finally transferred to electro-competent \textit{Leu. mesenteroides} CJNU 0147 using the same method presented in a previous paper for \textit{Lactobacillus} (Moon et al., 2008).

**Comparison of the recombinant \textit{Leu. mesenteroides} CJNU 0147 (pCW4::\textit{menB}) with wild type, based on growth rate, \textit{menB} gene transcription, and DHNA production**

Glycerol stocks (-76°C) for \textit{Leu. mesenteroides} CJNU 0147, CJNU 0147 (pCW4), and CJNU 0147 (pCW4: \textit{menB}) were inoculated into 5 mL MRS broth and incubated at 37°C for 24 h. The cultures were subcultured into 200 mL MRS broth supplemented with 0.05% L-cysteine (Sigma-Aldrich) in an anaerobic jar (Oxoid, UK) with a GasPak EZ Anaerobe Container System (BD, Sparks, USA), at 37°C for 48 h. At specific times (0, 24, and 48 h) the cultures were sampled, and viable cell counts (Log CFU/mL) and pH were measured. For comparison of \textit{menB} gene transcription, a slot blot analysis was done. Total RNAs from samples were extracted by a commercial kit (RNAeasy kit, iNtRON biotechnology, Korea). Each 10 μg was bound to nitrocellulose membrane equipped with a slot blot kit (Bio-Dot® SF microfiltration apparatus, Bio-Rad, Hercules, USA). The blot membrane was hybridized with a probe for \textit{menB} gene from \textit{Leu. mesenteroides} CJNU 0147 which was labeled and detected using a ECL direct labeling and detection kit (GE Healthcare Life Sciences, UK) according to the manufacturer’s guide. For HPLC analysis of DHNA, each 20 mL of culture sample was extracted with the same volume of methanol, and freeze-dried (FD). The FD samples were diluted with a mixture (water: methanol = 1: 2), centrifuged at 6,000 rpm for 10 min, and filtered with syringe filters (0.45 μm, Millipore, Billerica, USA). The filtrates were injected for HPLC analyses using a C18 column (ACE 5 C18 column, 4.6 × 150 mm; Advanced Chromatography Technologies, UK). For mobile phase, a mixture [acetonitrile: methanol: water: acetic acid = 15: 25: 225: 0.1; pH 5.5 adjusted with 5% (w/v) ammonium hydroxide] was used. Other parameters were as follows: column temperature was 45°C; flow rate was 1 mL/min; sample injection volume was 20 μL; detection wavelength was 254 nm. For a standard curve, several concentrations (0, 0.125, 0.25, 0.5, 1.0, 5.0, and 10.0 μg/mL) of pure DHNA (Sigma-Aldrich) were used. For LC-MS (liquid chromatography-mass spectrometry) analysis, fractions for a peak corresponding to DHNA retention time were collected, concentrated, and subjected to LC-MS (LC: Prominance 20A apparatus, Shimadzu, Japan; MS: LCMS-IT-TOF system, Shimadzu). An XR-ODS LC column (3.0 × 75 mm, Shimadzu) was used; other parameters were as follows: column temperature was 45°C; injection volume was 20 μL; flow rate was 0.2 mL/min; mobile phase composition was the same as that of HPLC analysis.

**Bifidogenic growth stimulation activity of the recombinant \textit{Leu. mesenteroides} CJNU 0147 (pCW4::\textit{menB})**

For bifidogenic growth stimulation (BGS) activity test, MRS cultures of \textit{Leu. mesenteroides} CJNU 0147, CJNU 0147 (pCW4), and CJNU 0147 (pCW4::\textit{menB}) were centrifuged and filtered using syringe filters (0.45 μm, Millipore). Each 100 μL of filtrate was added to 5 mL RCM broth (Difco) where 2% (v/v) of culture of \textit{Bifidobacterium lactis} BL 750 or \textit{Bifidobacterium longum} FI10564 had been inoculated; they were incubated at 37°C for 10 h in an anaerobic jar supplemented with a GasPak EZ Anaerobe Container System (BD). Optical densities at 600 nm of 10-h cultures were measured and compared.

**Statistical analysis**

All experiments in this study were done in triplicate and data are represented as mean or mean±standard deviation (SD). A statistical software (SPSS v. 12.0; SPSS Co., USA) was used for Duncan’s multiple range tests for determining significance of difference (p<0.05).

**Results and Discussion**

**PCR cloning of \textit{menB} gene from \textit{Leu. mesenteroides} CJNU 0147**

Using the primer set (Leu\textunderscore menBfull\textunderscore F and Leu\textunderscore menBfull\textunderscore R), a DNA fragment (1,190 bp) including \textit{menB} gene encoding DHNA synthase from \textit{Leu. mesenteroides} CJNU 0147, was amplified and cloned into a TA cloning vector pGEM-T Easy vector (Promega). The nucleotide sequence of the gene was analyzed and deposited to GenBank data-
base (accession no., KT591869) of NCBI (National Center for Biotechnology Information). The amino acid sequence deduced from the menB gene nucleotide sequence was well matched with that of MenB protein from Leu. mesenteroides ATCC 8293 by 98%, indicating the menB gene was correctly amplified from Leu. mesenteroides CJNU 0147. For the expression of the menB gene in Leu. mesenteroides CJNU 0147, it was subcloned into pCW4 which was developed for E. coli-lactic acid bacteria shuttle vector. The plasmid vector worked in Leu. mesenteroides, Lactobacillus bulgaricus, and Lactobacillus plantarum (Park et al., 2004). The developed recombinant DNA pCW4::menB as well as pCW4 as a control vector, was successfully transferred to Leu. mesenteroides CJNU 0147.

Comparison of the recombinant Leu. mesenteroides CJNU 0147 (pCW4::menB) with wild type, based on growth rate, menB gene transcription, and DHNA production

To compare growth rate of the recombinant Leu. mesenteroides CJNU 0147 (pCW4::menB) strain with the wild type strain, the cells were incubated in MRS broth at 37°C for 48 h, and viable cell counts (Log CFU/mL) and pH were measured. There was no difference in growth rate and pH between the recombinant strain and wild type, indicating that the transformed plasmid pCW4::menB does not influence the growth of Leu. mesenteroides CJNU 0147 (data not shown). At the same time, we also checked transcription of the menB gene in the recombinant Leu. mesenteroides CJNU 0147 (pCW4::menB) at 24 h and 48 h post inoculation. A slot blot assay showed the menB gene was well transcribed in the recombinant strain, but was not detected in the transcripts from wild type strain and a control transformant Leu. mesenteroides CJNU 0147 (pCW4) (Fig. 1). The result signifies that the cloned menB gene is transcribed in its original host Leu. mesenteroides CJNU 0147, indicating that it is stable and independent from the genome of the host. In the entire genome sequence of Leu. mesenteroides ATCC 8293, only one copy of menB gene exists, which might be insufficient to be detected by the slot blot assay if the transcription level is low. Even though the menB gene overexpression was performed in the original host, to the best of our knowledge, this is the first reported case of recombinant menB gene expression in lactic acid bacteria. In the near future, the recombinant plasmid pCW4::menB will be transferred to other lactic acid bacterial genera, including Lactobacillus, Lactococcus, Enterococcus, Pediococcus etc., to generalize our theory. Overexpression of menB gene in the strain enabled us to expect a detectable production of DHNA from it. To confirm whether the recombinant Leu. mesenteroides CJNU 0147 (pCW4::menB) can produce DHNA at a detectable level, the prepared culture supernatant of the recombinant strain was subjected to HPLC analysis. We found a corresponding peak for DHNA from the sample, and the concentrations were 0.28 and 1.04 ppm at 24 h and 48 h, respectively, during cultivation in MRS broth, when evaluated against a standard curve (0.0-10.0 ppm) (data not shown). To further confirm if the peak on the chromatogram was for DHNA, LC-MS (liquid chromatography-mass spectrometry) analysis was done. The mass spectrum of the sample fraction corresponding to DHNA peak on HPLC chromatogram exactly matched with a standard DHNA (Sigma-Aldrich) by an m/z 203.03, which indicates a de-protonated DHNA ion [M-H]⁻ (Fig. 2). The results confirm that the recombinant strain can produce DHNA at a detectable level, which is caused by the recombinant plasmid vector pCW4::menB; a control recombinant strain harboring pCW4 did not produce detectable level of DHNA. At the same time, it was also confirmed that the cloned menB gene from Leu. mesenteroides CJNU 0147 encodes a DHNA synthase responsible for the synthesis of DHNA. The results in this study are basic and preliminary, and further studies using biochemical and bioengineering tools are required for their application to reach industrial fields.

Bifidogenic growth stimulation activity of the recombinant Leu. mesenteroides CJNU 0147 (pCW4::menB)

Since DHNA has earlier been reported to have BGS activity (Isawa et al., 2002), we tested the activity of Leu. mesenteroides CJNU 0147 (pCW4::menB). In our previous study (Eom and Moon, 2010), Leu. mesenteroides CJNU 0147 wild type had shown BGS activity, but the recombinant Leu. mesenteroides CJNU 0147 (pCW4::menB) presented higher BGS activity than that of the wild type (Fig. 3). The result indicates that overproduced DHNA...
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from the recombinant *Leu. mesenteroides* CJNU 0147 (pCW4::menB) might positively influence the BGS activity. Previous studies (Chung and Day, 2002, 2004) have presented that some *Leu. mesenteroides* strains can produce glucooligosaccharides which can be utilized by bifidobacteria and lactobacilli but not by potentially pathogenic bacteria such as *Salmonella* sp. and *Escherichia coli*. Gentio-oligosaccharides (GnOS), which were synthesized by *Leu. mesenteroides* NRRL B-1426 dextranu- crase using gentiobiose and sucrose as material sources, showed low degrees of digestibility (18.1% and 7.1%) by simulated human gastric juice (pH 1.0) and α-amylase (pH 7.0), respectively after 6 h. It presented growth stimulation activity for probiotics, including *Bifidobacterium infantis* and *Lactobacillus acidophilus*, and the activity was considerable to that of inulin (Kothari and Goyal, 2015). The GnOS also showed selective inhibitory effect on HT-29 cells (a human colon carcinoma), which indicates it can be used as an anti-cancer agent. Besides nondigestible oligosaccharides, there is limited information
for bifidogenic growth stimulation activators from *Leu. mesenteroides*. Most of research papers for DHNA as a bifidogenic growth stimulator have focused on the *P. freudenreichii* ET-3 strain (Isawa et al., 2002), except our research paper on the production of DHNA from *Lactobacillus casei* LP1 strain (Kang, 2015). Besides bifidogenic growth factor, DHNA has been described as an activator of the aryl hydrocarbon receptor which recognizes environmental xenobiotics and involves in the metabolism of its detoxification and involves in the metabolism of its detoxification and was recently highlighted as a regulator of inflammation leading to suppression of IBDs (Inflammatory bowel diseases) (Fukumoto et al., 2014). DHNA also presented an antimicrobial activity against *Helicobacter pylori*. It has been normally treated by triple therapy with amoxicillin, clarithromycin, and a proton-pump inhibitor but clarithromycin-resistant strains have been frequently appeared. DHNA could inhibit clinical *H. pylori* isolates resistant to the antibiotics by disrupting cellular respiration and ATP generation (Nagata et al., 2010). Therefore DHNA could also be a good pharmacological candidate for treatment of IBD, antibiotic-resistant pathogenic bacteria, etc. In this study, we constructed a recombinant *Leu. mesenteroides* using a putative menB gene originating from its wild type strain; the recombinant strain successfully produced DHNA at a detectable level and presented BGS activity, which indicates the cloned menB gene encodes a DHNA synthase and the gene overexpression enables the strain to strengthen its BGS activity.

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