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ARTICLE

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

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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,

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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'-TGAGGCTGCTGTTTC AGC-3'; Leu_menBfull_R: 5'-GATCATACCCCGTATT CC-3') 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-

Table 1. Microbial strains and plasmids

Strains or Plasmids	Description	Reference or Source
Strains		
Leuconostoc mesenteroides CJNU 0147	Isolate from a kimch	Eom and Moon, 2010
Bifidobacterium lactis BL 750	Target bacterium for BGS activity	Culture systems
Bifidobacterium longum FI10564	Target bacterium for BGS activity	Moon *et al.,* 2009
Escherichia coli DH5α	*supE44 ΔlacU169 (Φ80lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook and Russell, 2001
Plasmids		
pGEM-T Easy vector	TA cloning vector	Promega
pGEM-T::menB	*menB* gene amplicon from *Leu. mesenteroides* CJNU 0147 was inserted into pGEM-T Easy vector	This work
pCW4	Lactic acid bacteria-*E. coli* shuttle vector, Em^r	Park *et al.,* 2004
pCW4::menB	*menB* gene (*Not*I digest) from pGEM-T::menB was inserted into pCW4 (*Not*I site)	This work

ison, USA) and transferred to *E. coli* DH5α. Using a universal primer (M13_F: 5'-GTTTTCCCAGTCACGAC-3') from the vector sequence, the insert containing *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 *menB* gene fragment digested with *Not*I restriction endonuclease was inserted into pCW4 digested with the same enzyme. The recombinant DNA pCW4::menB was finally transferred to electro-competent *Leu. mesenteroides* CJNU 0147 using the same method presented in a previous paper for *Lactobacillus* (Moon *et al.,* 2008).

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

Glycerol stocks (-76°C) for *Leu. mesenteroides* CJNU 0147, CJNU 0147 (pCW4), and CJNU 0147 (pCW4::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 *menB* gene transcription, a slot blot analysis was done. Total RNAs from samples were extracted by a commercial kit (RNeasy 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 *menB* gene from *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 *Leu. mesenteroides* CJNU 0147 (pCW4::menB)

For bifidogenic growth stimulation (BGS) activity test, MRS cultures of *Leu. mesenteroides* CJNU 0147, CJNU 0147 (pCW4), and CJNU 0147 (pCW4::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 *Bifidobacterium lactis* BL 750 or *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 *menB* gene from *Leu. mesenteroides* CJNU 0147

Using the primer set (Leu_menBfull_F and Leu_menB full_R), a DNA fragment (1,190 bp) including *menB* gene encoding DHNA synthase from *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 database (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*, *Latobacillus 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.

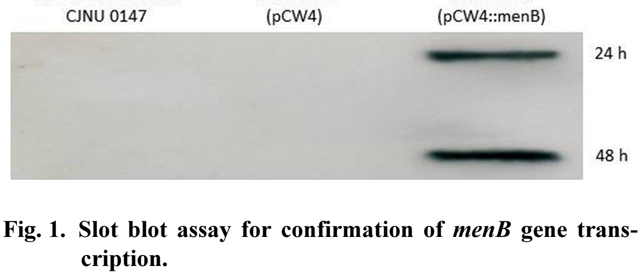

Fig. 1. Slot blot assay for confirmation of *menB* gene transcription.

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

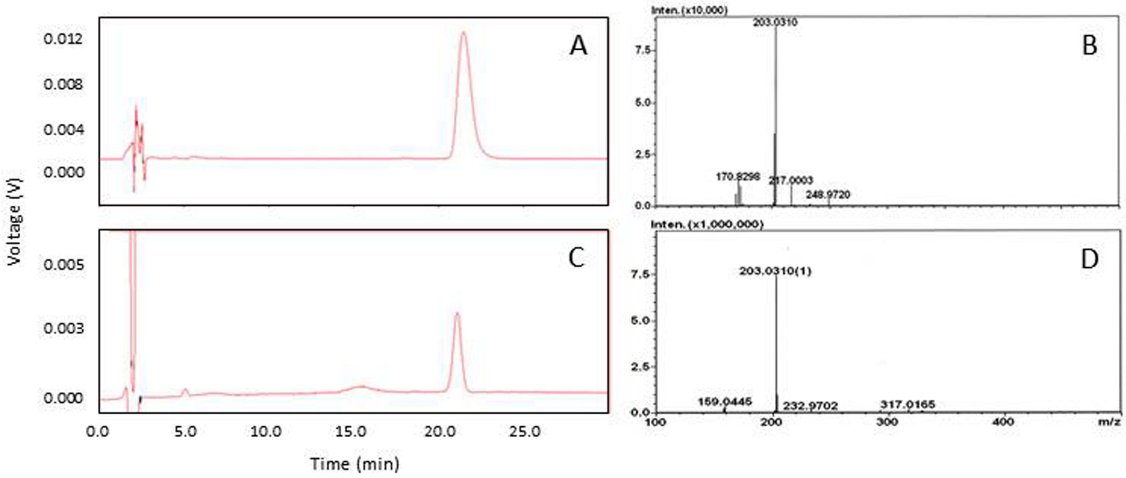

Fig. 2. HPLC chromatogram for a sample fraction corresponding to DHNA peak and LC-MS data. A, HPLC chromatogram for standard DHNA (10 ppm); B, MS spectrum for standard DHNA; C, HPLC chromatogram for the sample fraction; D, MS spectrum for the sample fraction.

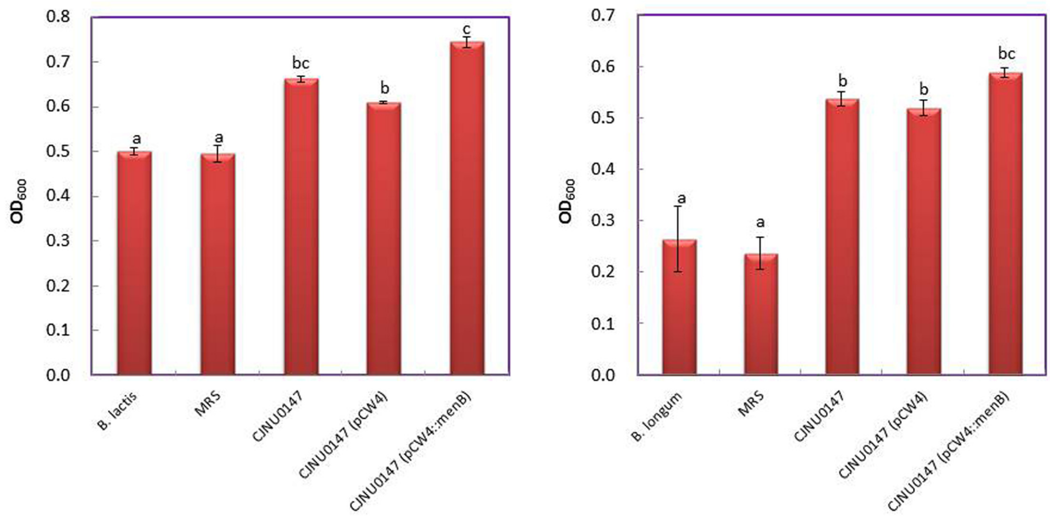

Fig. 3. BGS activities of samples for *Leu. mesenteroides* CJNU 0147, CJNU 0147 (pCW4), and CJNU 0147 (pCW4::menB). *B. lactis and B. longum*, Only the cells were inoculated in 5 mL RCM broth; MRS, cells plus 100 µL MRS broth as a control; others, cells plus 100 µL of culture supernatant. Values are shown as mean±SD (n=3) and means having different letters are significantly different by Duncan's multiple range tests (p<0.05).

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 dextransucrase 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 non-digestible 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 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 pharmaceutical 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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