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Jacobs Journal of Biotechnology and Bioengineering

Research Article

Structural Characteristics of *rocG* and *ure* Genes of a Novel Screened *Bacillus subtilis* natto Strain

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Received: 08-18-2014
Accepted: 10-14-2014
Published: 10-17-2014

Abstract

Natto is considered to be a health-enhancing food mainly because of nattokinase, which can lead to a mild enhancement of fibrinolytic activity in rats and dogs after oral administration. It has distinctive smell partly caused by the high amount of ammonia produced during the secondary fermentation. In order to decrease the production of ammonia by gene engineering, a novel *Bacillus subtilis* natto strain with a high nattokinase activity was isolated from natto products, and the structural characteristics of ammonia production-associated genes were analyzed.

The high identity of *rocG* and *ure* in amino acid sequences indicates the evolutionary stability of glutamate dehydrogenase and urease. The structural characteristics of the untranslated regions of *rocG* and *ure* suggest that the promoters of *rocG* and *ure* of *B. subtilis* are similar to the common microbial -35, -10 regions and σ^A (σ^{70}) promoters. The strong promoters that enhance the transcription efficiency of *rocG* and *ure* may be responsible for high levels of ammonia.

Keywords: *Bacillus subtilis* natto; *rocG*; *ure*; Structure Characteristics

Abbreviation

NK: Nattokinase;
UTR: Untranslated regions;
GDH: Glutamate Dehydrogenase;

Introduction

Natto is a traditional and popular Japanese soybean product obtained by fermentation by *Bacillus subtilis* natto. Natto has various bioactive properties, including prophylactic activity against venous thrombosis and osteoporosis, mainly due to the nattokinase (NK) which is produced during fermentation [1-3]. NK is a potential drug for thrombosis therapy and can be used as a dietary supplement to prevent thrombosis [4,5].

Other microorganisms that produce fibrinolytic enzymes have also been isolated from Japanese shiokara, Korea chungkook-jang [6], and Chinese douchi. Nevertheless, *B. subtilis* natto is still the most popular bacterium for production of NK due to its higher production and lower fermentation costs. Therefore, this *B. subtilis* with the high NK activity was of interest. Many NK-producing strains of *B. subtilis* natto have been identified, such as CCRC 14716, IAM 1028, IAM 1163, IAM 1232, NC2-1, NR-1, OK2, K-C3, B-12 and BN-1 [7-10] For example, forty five strains of *B. subtilis* were isolated from Thua nao, a traditional fermented soybean food in northern Thailand, and NK activity was found to vary from 1.0 to 9.6 U cm^{-3} among these strains [11].

Natto is popular in Japanese, but it is not so acceptable to people from other countries, due to its strong flavor.

One major issue that negatively influences the flavor of natto is the production of ammonia during the secondary fermentation. The major metabolic pathways of ammonia production in bacteria are decarboxylation, trans-deamination, transamination and degradation of urea. In *B. subtilis* natto, glutamate dehydrogenase (GDH) encoded by *rocG* gene is involved in both trans-deamination and transamination, while the urease encoded by *ure* gene is the key enzyme for degradation of urea. Ammonia production by *B. subtilis* natto was reduced by 50% when *rocG* was deleted [12,13], while, the ability to decompose urea was nearly lost when *ure* was deleted [13].

To obtain a genetically modified strain with no or reduced *rocG* and *ure* expression, characterization of these genes and cis elements in the promoter regions is needed. The aim of the present report was to characterize a novel strain of *B. subtilis* natto obtained from commercial natto products expressing high NK activity, and to analyze *rocG* and *ure* genes for further construction an engineered strain that produces less ammonia during natto fermentation.

Material and Methods

Strains and growth conditions

Four types of commercial Natto products, small grain and soybean natto (Marumiya, Kyushu, Japan), Xusong natto (Zhuhai Nishio Food Co. LTD, China), Yanjing natto (Yanjing zhongfa Biological technology Co., LTD., China) and Mito natto, (Sato Co., LTD., Japan), were purchased from a Japanese restaurant (Qibao, Shanghai, China) and Nextage Branch (Pudong, Shanghai, China). Luria-Bertani (LB) broth powder was purchased from BD Biosciences (USA).

E. coli JM109 was obtained from Takara (Dalian, China) and used as a host strain for cloning and sequencing. The plasmid pMD19-T Simple Vector (Takara, Dalian, China) was used for cloning and sequencing. *E. coli* strains were grown in LB medium supplemented with kanamycin. *B. subtilis* strains were grown in LB medium.

Isolation and screening of *B. subtilis* natto

To isolate *B. subtilis* natto, five grains of different varieties of natto were placed into conical flasks and extracted with 25 mL of sterile saline for 2h with votexing every 15min. The flasks were put into a boiling water bath for 15min to kill vegetative bacterial cells, and 5ml of the supernatant inoculated into 100ml of LB media and incubated at 37°C for 12h with shaking (220rpm). The cultures were streaked onto the LB agar plates and incubated at 37°C for 24h. Single colonies were isolated for enzyme activity assays.

NK activity assay

The isolated *B. subtilis* natto was further cultured in LB media at 37°C for 9.5h, and supernatants collected by centrifugation at 6,500×*g* for 5min (5810R, Eppendorf, Hamburg, Germany) at 4°C. Fibrinolytic activity of NK was determined by the fibrin plate method using urokinase as a standard [14]. Briefly, 10ml of 1.5 mg/ml bovine fibrinogen solution and 10ml of 1% agarose solution in Barbitol buffer (50 mM sodium barbitol, 90 mM NaCl, 1.7 mM $CaCl_2$, 0.7 mM $MgCl_2$, pH 7.75) were brought to 45°C in a water bath, followed by the addition of 10 µl thrombin solution (0.1 BP/µl). The solutions were mixed in a 100mm petri dish and allowed to stand for 30min at room temperature. Three wells were made on a fibrin plate using a steel gel puncture (0.5cm). Ten microliters of each culture supernatant was placed in the wells. The plates were incubated at 37°C for 18h. Two perpendicular diameters of the lysed zones on the fibin plate were measured and the fibrinolytic activity was determined according to the standard curve of urokinase. Each strain was tested in triplicate. Strains of *B. subtilis* with high NK activities were identified using API 50 (BioMerieux, Inc., Marcy 1'Etoile, France) assays.

DNA sequencing and analysis

To analyze the *rocG* and *ure* genes, the genes and their flanking regions of genomic DNA of the selected *B. subtilis* natto strains were amplified by polymerase chain reaction (PCR). Primers (*rocG*-F_2 5' -AGAGCAGAGGCATCTTCGACTTT - 3', *rocG*-R_2 5' -AAGTCTATCGCTTCAGCCGTGTC-3' and *ure*-$F_2$5'-AAAGTGTCGGAAGCCACGGGAGT-3', *ure*-$R_2$5'-CTTC

ATCAACAAACAGGCTTCCC- 3') were designed based on the published sequences of the *rocG* and *ure* genes of *B. subtilis* strain 168 [15]. Molecular techniques were performed as described by Sambrook et al [16]. PCR amplification was performed in a 25 µl reaction mixture with 0.5 U of Pyrobest DNA polymerase (Takara). The PCR product was purified (PCR clean-up kit, Generay) and cloned into the pMD19-T vector (Takara Bio, Japan) to yield pTRocG and pTURE. The obtained plasmids were transformed into *E. coli* JM109. To definitively ensure fidelity of the cloned gene, both strands of *rocG* and *ure* genes were sequenced using M13 forward and reverse primers (Sunny Bio, Shanghai, China). The *rocG* and *ure* gene coding regions as well as promoter and terminator elements were analyzed.

A phylogenetic tree of *rocG* and *ure* genes was constructed using B. subtilis subsp. subtilis str. 168, *B. subtilis subsp*. spizizenii str. W23, *B. subtilis* sp. JS, *B. amyloliquefaciens* DSM7, *B. subtilis* subsp. spizizenii TU-B-10, *B. subtilis* subsp. subtilis RO-NN-1, *B. amyloliquefaciens* TA208, *B. amyloliquefaciens* LL3, and *B. amyloliquefaciens* XH7 as reference strains. The *rocG* and *ure* gene sequences

obtained from the NCBI database were aligned using CLUSTAL. The poorly conserved 5' and 3' ends were discarded from the alignment. Rooted neighbour-joining phylogenetic trees were built based on the aligned sequences using the software Mega 5.05 [17].

Results and Discussion

Isolation of *B. subtilis* natto with high NK activity

To isolate strains with high NK activity, we used four kinds of natto consumed widely in China and Japan. We obtained 4 strains of *B. subtilis* natto confirmed by colony morphology, API 50 and molecular identification (data not shown). By the NK activity assay, it was determined that strain CGMCC 2801 possessed the highest NK activity (148.5 U/mL), which supports its potential for commercial use.

Analysis of *rocG* and *ure* genes and amino acid sequences

Sequences were aligned to the NCBI Nucleotide database, and results revealed that *rocG* of CGMCC 2801 comprises a 1,275 bp open reading frame coding 424 amino acids, while *ure* comprises a 2,397 bp open reading frame coding 798 amino acids.

Identity of proteins encoded by *rocG* and *ure* of CGMCC 2801 and 9 other strains were analyzed by Mega 5.05. Results are showed in Table 1. Results indicate that the amino acid sequences of GDH from CGMCC 2801 was more similar to *B. subtilis* than to *B. amyloliquefaciens*. The same trend was observed for the urease gene comparison.

Table 1. Amino acid identity between GDH and urease in CGMCC 2801 and other species.

Strains	Identity of GDH (%)	Identity of urease (%)
B. subtilis subsp. subtilis str. 168	99	99
B. subtilis subsp. spizizenii str. W23	98	98
B. subtilis subsp. spizizenii TU-B-10	99	99
B. subtilis subsp. subtilis RO-NN-1	99	99
Bacillus sp. JS	99	98
B. amyloliquefaciens DSM7	92	92
B.s amyloliquefaciens TA208	92	92
B. amyloliquefaciens LL3	92	92
B.s amyloliquefaciens XH7	92	92

Previous studies have reported that the coenzyme $NADP^+$ binding site of GDH was composed of residues 200 (R), 227-232 (RIIIQG), 251-253 (VIG), 302-304 (ILV) and 324-326 (IVV) [18]. The substrate-binding site was identified as residues 80-82 (VGP), 101 (L), 104 (W), 116 (Y), 154-156 (KDI), 196-203 (GSQGRETA), 326 (V), 354 (A), 355 (S) and 58 (P), which was supported by the crystal studies for other GDH enzymes (3ete.pdb) [18]. Through sequence analysis, the 251-253 amino acids of *B. subtilis subsp. spizizenii str*. GDH was determined to be VVG, with the 252th amino acid being valine rather than isoleucine as was observed in CGMCC 2801 and another 8 strains. For the $NADP^+$ binding site of GDH from *B. amyloliquefaciens* located at residues 324-326 (IIV), the 325*th* amino acid was isoleucine, while in CGMCC 2801 and another 5 strains, the 325th amino acid was valine. This showed that the coenzyme $NADP^+$ binding site of GDH from CGMCC 2801 was similar to *B. subtilis*.

The *ure* gene of *Actinomycetes, Helicobacter pylori, Klebsiella aerogenes, B. subtilis* and other microorganisms encodes urease. The nucleotide sequences of *ure* are different among various species, but the amino acid sequences maintain high conservation [19,20]. The identities of amino acid sequences of urease between CGMCC 2801 and another 9 strains were demonstrated to be above 90% (Table 1). This is consistent with the functional consistency of urease in different organisms [20] Amino acid identity of proteins encoded by *ure*A (1-105), *ure*B (106-229) and *ure*C (230-798) from CGMCC 2801 and from 9 other strains was determined to be 89.5%, 76.7% and 92.6%, respectively. Higher identity for *ure*C may be due to the fact that it contains the enzyme active site. The active site sequence of urease of *H. pylori* was reported to be MVCHHLDKSIKEDVQFA [21], while in CGMCC 2801 and the other 9 strains examined here, had a consensus motif of MVCHHLD (from position 549 to 555), EDV from 560 to 562 and F in position 564 (Figure 1). Therefore, we concluded that the active site of CGMCC2801 urease may be conserved within *Bacillus* spp. but differs between different genera.

Figure 1. Active site of urease from CGMCC 2801 and reference strains compared *H. pylori.*

The conserved *rocG* and *ure* amino acid sequences indicated evolutionary stability of GDH and urease. Therefore, non-genetic factors might have influences on the activities of GDH and urease.

Phylogenetic analysis

Phylogenetic trees were constructed using *rocG* and *ure* of 9 strains as references (Figure 2, Figure 3). The results showed that homology based on *rocG* between

CGMCC 2801 and *B. subtilis subsp. subtilis str.* 168 was the highest (99%). Homology between CGMCC 2801 and *subtilis subsp. spizizenii* RO-NN-1 was 94%, between CGMCC 2801 and *subtilis subsp. spizizenii* TU-B-10 was 93%. Homology between CGMCC 2801 and strains in group 2 and 3 were 92%-93% and 80%, respectively. All strains in group 3 were *B. amyloliquefaciens* (Figure 2).

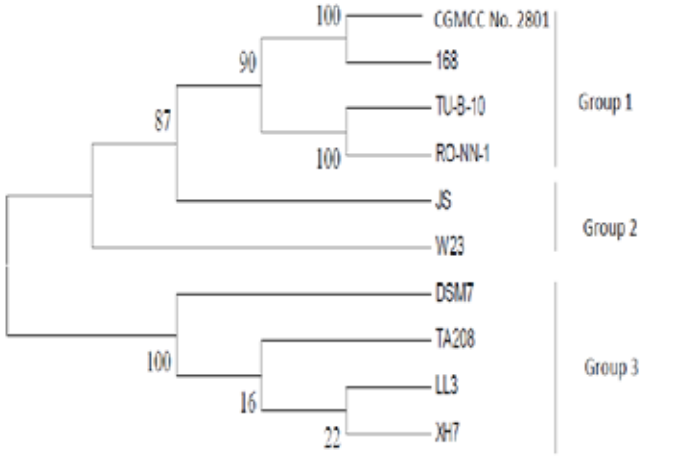

Figure 2. Phylogenetic tree based on the *rocG* genes of *B. subtilis subsp. subtilis str.* 168.

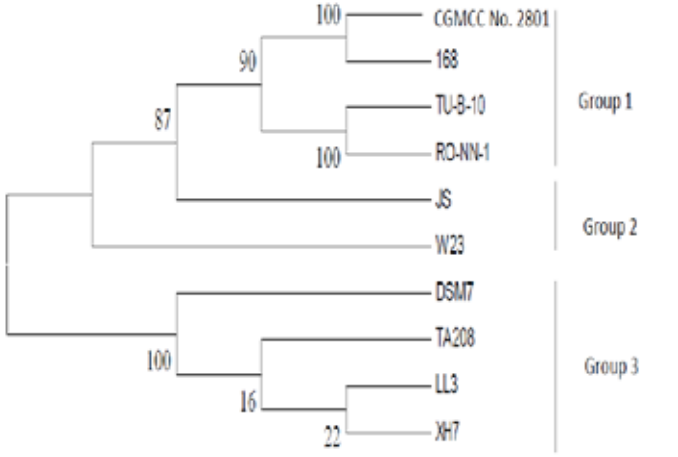

Figure 3. Phylogenetic tree based on the *ure* gene of *B. subtilis subsp. subtilis str.* 168.

Phylogenetic tree in Figure 3 showed that homology based on *ure* between CGMCC 2801 and *B. subtilis subsp. subtilis str*. 168 was 97%, which was the highest. Homology between CGMCC 2801 and *subtilis subsp. spizizenii* RO-NN-1 was 95%, between CGMCC 2801 and *B. subtilis subsp. spizizenii* TU-B-10 was 94%. Homology between CGMCC 2801 and strains in group 2 and group 3 were 92%-93% and 80%, respectively. All strains in group 3 were *B. Amyloliquefaciens*, it was the same as the phylogenetic tree of *rocG* gene.

The results of the phylogenetic analysis showed that evolutionary relationships of *rocG* and *ure* were consistent, and that CGMCC 2801 was more closely related to *B. subtilis* than *B. Amyloliquefaciens*. Homology of *rocG* and *ure* between CGMCC 2801 and *B. subtilis subsp. subtilis str*. 168 were respectively 99% and 97%, which was the highest among all the reference strains.

Analysis of promoters, terminator elements and transcriptional elements of *rocG* and *ure* genes

Compared to the sequencing results with the data in NCBI nucleotide database, we investigated the structural characteristics of the untranslated regions of *rocG* and *ure*, and found that that the promoters of rocG and ure of *B. subtilis* are similar to the common microbial -35, -10 regions and σ^A (σ^{70}) promoters as previously reported [22-23] (data not shown). It suggested that the strong promoters that enhance the transcription efficiency of *rocG* and *ure*, which may be responsible for high level of ammonia.

Conclusion

A novel *B. subtilis* natto strain with a high nattokinase activity of 148.5 U/mL culture solution was screened from natto products. The identity of *rocG* and *ure* gene based on amino acid sequences were analyzed, high identity of *rocG* and *ure* in amino acid sequences showed evolutional stability of GDH and urease. The structure characteristics of the untranslated regions sequences of *rocG* and *ure* suggested that promoters of *rocG* and *ure* in *B. subtilis* were respectively similar with common prokaryotic microorganism promoters -35, -10 and σ^A promoters. The strong promoters that enhance the transcription efficiency of *rocG* and *ure* may be responsible for high level production of ammonia.

Acknowledgment

This work is supported by the National Natural Science Foundation of China under Grant No. 31171737 and the State Oceanic Administration of China under Grant No. 201205031.

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