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Identification of the Genes Involved in 1-Deoxynojirimycin Synthesis in *Bacillus subtilis* MORI 3K-85

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1-Deoxynojirimycin (DNJ), a D-glucose analogue with a nitrogen atom substituting for the ring oxygen, is a strong inhibitor of intestinal α-glucosidase. DNJ has several promising biological activities, including its antidiabetic, antitumor, and antiviral activities. Nevertheless, only limited amounts of DNJ are available because it can only be extracted from some higher plants, including the mulberry tree, or purified from the culture broth of several types of soil bacteria, such as *Streptomyces* sp. and *Bacillus* sp. In our previous study, a DNJ-producing bacterium, *Bacillus subtilis* MORI, was isolated from the traditional Korean fermented food Chungkookjang. In the present study, we report the identification of the DNJ biosynthetic genes in *B. subtilis* MORI 3K-85 strain, a DNJ-overproducing derivate of the *B. subtilis* MORI strain generated by γ-irradiation. The genomic DNA library of *B. subtilis* MORI 3K-85 was constructed in *Escherichia coli*, and clones showing α-glucosidase inhibition activity were selected. After DNA sequencing and a series of subcloning, we were able to identify a putative operon which consists of *gabT1*, *yktc1*, and *gutB1* genes predicted to encode putative transaminase, phosphatase, and oxidoreductase, respectively. When a recombinant plasmid containing this operon sequence was transformed into an *E. coli* strain, the resulting transformant was able to produce DNJ into the culture medium. Our results indicate that the *gabT1*, *yktc1*, and *gutB1* genes are involved in the DNJ biosynthetic pathway in *B. subtilis* MORI, suggesting the possibility of employing these genes to establish a large-scale microbial DNJ overproduction system through genetic engineering and process optimization.

***Keywords*:** *Bacillus subtilis* MORI 3K-85, genomic DNA library screening, 1-deoxynojirimycin (DNJ), α-glucosidase inhibitor, gene cloning

1-Deoxynojirimycin (DNJ) is a polyhydroxylated piperidine alkaloid. These alkaloids can be considered as analogues of glucose in which the ring oxygen has been replaced by nitrogen. DNJ inhibits α-glucosidase, which hydrolyzes α-glucose residues within an oligosaccharide chain. α-Glucosidases are involved in a wide range of important biological processes. Therefore, the possibility of modifying or blocking these processes using DNJ as a glucosidase inhibitor has gained an increasing amount of interest related to cell biological and therapeutic applications, especially in relation to viral infections and diabetes (Asano *et al*., 2000; Watson *et al*., 2001).

DNJ has been shown to inhibit α-glucosidases I and II, which are involved in the *N*-linked glycosylation of secretory proteins (Asano *et al*., 2000; Dwek *et al*., 2002). *N*-linked oligosaccharides play important roles in the fate and functions of glycoproteins (Asano *et al*., 2000; Dwek *et al*., 2002). For example, *N*-glycosylation can assist in the folding of glycoproteins. Thus, prevention of the *N*-glycosylation process by an α-glucosidase inhibitor will cause some proteins to be misfolded and retained within the endoplasmic reticulum (ER). Because proper folding of key viral envelope glycoproteins are critical for the life cycle of viruses, such as the human immunodeficiency virus (HIV), hepatitis B virus (HBV), and bovine viral diarrhea virus (BVDV), DNJ has been regarded as a promising antiviral agent (Gruters *et al*., 1987; Fleet *et al*., 1988; Karpas *et al*., 1988; Mehta *et al*., 1998; Asano *et al*., 2000; Watson *et al*., 2001; Dwek *et al*., 2002; Jacob *et al*., 2007).

DNJ is also a potent inhibitor of various mammalian digestive α-glucosidases, such as sucrase, maltase and isomaltase, all of which are involved in the digestion of disaccharides in mammals. These enzymes are expressed on the surface of the epithelial cells of the brush border in the small intestine. Thus, α-glucosidase inhibitors such as DNJ can be used therapeutically in the oral treatment of the non-insulin-dependent (type II) diabetes mellitus (Yoshikuni *et al*., 1988; Asano *et al*., 1994, 2000; Watson *et al*., 2001; Jang and Rhee, 2004; Cho *et al*., 2008; Hwang *et al*., 2008; Kong *et al*., 2008; Schedel, 2008). In addition, it has been suggested that DNJ can be developed as a more efficient antidiabetic by chemical deriva-

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tization to improve retention in the small intestine (Asano *et al*., 2000; Watson *et al*., 2001; Schedel, 2008).

DNJ can be synthesized by removing the anomeric hydroxyl group of nojirimycin (Asano *et al*., 2000; Watson *et al*., 2001). DNJ has been isolated from the roots of mulberry trees and called moranoline (Watson and Nash, 2000; Asano *et al*., 2001). DNJ is also produced by several strains of *Bacilli* (Stein *et al*., 1984; Hardick and Hutchinson, 1993; Watson and Nash, 2000) and *Streptomyces* (Ezure *et al*., 1985; Hardick *et al*., 1991; Paek *et al*., 1997; Asano *et al*., 2000; Watson and Nash, 2000; Watson *et al*., 2001; Schedel, 2008). However, the enzyme or gene responsible for DNJ biosynthesis has not been identified.

For the first time, in this study we report the identification of the DNJ biosynthetic genes of *Bacillus subtilis* MORI 3K-85, which is a derivate of a naturally isolated DNJ-producing strain. Furthermore, we show that the recombinant *Escherichia coli* strain harboring DNJ biosynthetic genes can produce DNJ in the culture supernatant.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The *B. subtilis* MORI 3K-85 strain was selected after the γ-irradiation of *B. subtilis* MORI (KCCM-10450) at dose of 3 KGy. *E. coli* EPI 300 [F⁻ *mcrA Δ*(*mrr-hsdRMS-mcrBC*) Φ80*dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ*(*ara, leu*)7697 *galU galK λ⁻ rpsL* (Str^R) *nupG trfA dhfr*] (Epicentre, USA) and DH5α (*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lacZ ΔM15*) (Clontech, USA) were used as host strains for genomic library construction and gene cloning, respectively. CopyControl pCC1BAC Cloning-Ready Vector (Epicentre) and pEXT20 (Dykxhoorn *et al*., 1996) were used for genomic library construction and the induced expression of the operon, respectively. *E. coli* was cultured in LB broth at 37°C with shaking at 180 rpm. When necessary, chloramphenicol (12.5 μg/ml), ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) or isopropyl-β-D-thiogalactopyranoside (IPTG) (50 μg/ml) was added to the culture medium. The plasmids and chromosomal DNA were prepared using the GeneAll XPREP^{TM} Plasmid SV Mini kit (GeneAll biotechnology, Korea) and the Wizard Genomic DNA Purification kit (Promega, USA), respectively. Restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation were all carried out as described previously (Sambrook *et al*., 1989).

Construction and screening of the genomic DNA library

For chromosomal DNA extraction, the *B. subtilis* MORI 3K-85 strain was cultured in Difco^{TM} YM broth [Becton Dickinson (BD) and Company, USA] at 37°C with shaking at 180 rpm. The genomic DNA of *B. subtilis* MORI 3K-85 prepared using a Promega kit was partially digested with *Bam*HI and the DNA fragments were then ligated into the CopyControl pCC1BAC Cloning-Ready Vector. The ligation mixture was then transformed into TransforMax^{TM} EPI300^{TM} Electrocompetent *E. coli* cells by electroporation (Dower *et al*., 1988). The transformed cells were incubated for 1 h at 37°C with shaking at 220 rpm, and the aliquots were spread onto an LB agar plate containing chloramphenicol (12.5 μg/ml), X-Gal (40 μg/ml), and IPTG (50 μg/ml). After overnight incubation at 37°C, white colonies were selected and inoculated into LB broth containing chloramphenicol (12.5 μg/ml). The cells were incubated for 12 h at 37°C to determine the α-glucosidase inhibitory activity to screen clones harboring the DNJ biosynthetic genes of *B. subtilis* MORI 3K-85. Five positive clones showing α-glucosidase inhibitory activity were selected and their α-glucosidase inhibitory activities were compared. Among them, the clone C36-4, which exhibited the highest α-glucosidase inhibitory activity, was chosen for further analysis.

Sequence analysis

The recombinant plasmid isolated from the *B. subtilis* MORI 3K-85 genomic DNA library clone C36-4 was termed pCC1BAC-DNJbb, and the nucleotide sequence of the inserted DNA fragment was determined by Solgent (Korea). The resulting sequence information was used in a search of the GenBank at the National Center for Biotechnology Information (NCBI) using the BLAST program. An additional sequence analysis and a comparison were also performed using web-based programs (http://www.uniprot.org and http://gib.genes.nig.ac.jp). The promoter sequence and putative transcription factor binding sites on the upstream region of the DNJ biosynthetic genes were predicted with the web-based DBTBS (a database of *B. subtilis* promoters and transcription factors) analysis program (http://dbtbs.hgc.jp/) (Sierro *et al.*, 2008). The rho-independent terminator was predicted by ARNold, a web-based terminator finding program (http://rna.igmors.u-psud.fr/toolbox/arnold/) (Naville *et al*., 2011).

Subcloning and expression of DNJ biosynthetic genes

To identify the minimum set of genes required to direct DNJ biosynthesis in the heterologous host *E. coli*, various sizes of genomic DNA fragments from pCC1BAC-DNJbb were subcloned. First, a 5.56 kb *Hin*dIII fragment containing four putative genes was ligated into pCC1BAC, resulting in pCC1BAC-DNJhh. A 4.2 kb *Hin*dIII-*Nru*I fragment containing three genes was cloned into the *Hin*dIII site of pCC1BAC, resulting in pCC1BAC-DNJhn. To subclone the putative DNJ biosynthetic genes into the multi-copy expression vector pEXT20 under the IPTG-inducible P_{tac} promoter, a 3.3 kb DNA fragment containing *gabT1-yktc1-gutB1* genes was PCR amplified using pCC1BAC-DNJbb as template and the primer pairs DNJt1-N (5′-aaagagctcatgttg gtagtggggacta-3′, *Sac*I) and DNJt-C (5′-aaaggatcctacgcaaggtgaatgctg-3′, *Bam*HI). The PCR product was digested with *Sac*I and *Bam*HI and ligated with *Sac*I-*Bam*HI-digested pEXT20, resulting in pEXT20-DNJ0. Likewise, a 3.2 kb DNA fragment containing *gabT1-yktc1-gutB1* genes but starting from an ATG codon at 69 bp downstream from the GTG codon was PCR-amplified with pCC1BAC-DNJbb as the template and primer pairs DNJt2-N (5′-aaagagctcatggaaagaggtgaaggc-3′, *Sac*I) and DNJt-C. The PCR product was digested with *Sac*I and *Bam*HI and ligated with *Sac*I-*Bam*HI-digested pEXT20, resulting in pEXT20-DNJ1.

Induction of the expression of the *gabT1-yktc1-gutB1* operon

To investigate the effects of an increase in the gene dosage on the expression of the α-glucosidase inhibitory activity, the copy numbers of CopyControl pCC1BAC-based plasmids were induced using a copy number autoinduction solution according to the manufacturer's instructions. The relative amounts of the pCC1BAC control vector, pCC1BAC-DNJbb, and pCC1BAC-DNJhn isolated from a control culture and autoinduced cultures were compared by separation on 0.8% agarose gel by electrophoresis.

To determine the effects of the induced expression of the *gabT1-yktc1-gutB1* operon on DNJ production in *E. coli*, pEXT20-DNJ0 was transformed into the *E. coli* DH5α strain. The transformant was incubated in LB broth containing ampicilline (100 μg/ml) at 37°C with shaking at 180 rpm. To induce expression of the *gabT1-yktc1-gutB1*

operon cloned under the P_{tac} promoter, 0.1 mM IPTG was added to the exponentially growing cultures. Samples were taken after 4 h of induction and subjected to α-glucosidase inhibition assay. To determine the expression of the *gabT1-yktc1-gutB1* operon, whole cell extracts of the induced and uninduced cultures were separated on SDS-polyacrylamide gel by electrophoresis.

Analytic procedures

To screen the clones harboring recombinant vectors containing DNJ biosynthetic genes, the α-glucosidase inhibitory activities were measured. To prepare the α-glucosidase solution, 0.8% rat intestinal acetone powder (Sigma, USA) was dissolved in 0.1 M potassium phosphate buffer (pH 6.8) and the spun supernatant was collected. The culture broths of the genomic DNA library clones were heated at 100°C for 10 min. The supernatants were harvested by centrifugation (6,000×g, 10 min). The reaction mixture for the α-glucosidase inhibition assay was prepared by the addition of 5 μl of the boiled culture supernatant, 75 μl of 0.1 M potassium phosphate buffer (pH 6.8), and 50 μl of 12 mM *p*-nitrophenyl-α-glucopyranoside. Finally, 20 μl of 0.8% α-glucosidase solution was added into the mixture. After incubation at 37°C for 35 min, the reaction was terminated by the addition of 50 μl of 200 mM sodium carbonate and the absorbance at 405 nm was measured using a microplate reader (Molecular Devices, USA). To calculate the α-glucosidase inhibitory activity, the formula shown below was used. Clones having more than 20% of α-glucosidase inhibitory activity were selected for further analysis (Scofield *et al*., 1986; Cho *et al*., 2008).

$$\text{Inhibition (\%)} = \left\{ 1 - \frac{A_{405}\ \text{(inhibition)} - A_{405}\ \text{(control)}}{A_{405}\ \text{(enzyme)} - A_{405}\ \text{(blank)}} \right\} \times 100$$

To test the possibility of DNJ production in the supernatant of the selected clones, a thin-layer chromatography (TLC) analysis was performed by spotting 10 μl of culture broths onto Silica gel 60 F_{254} TLC plates (Merck, Germany) and separating the samples in a TLC chamber containing a solvent mixture of propanol-acetic acid-water (4:1:1) at room temperature. As the standard control, 0.5 μl of 10 mM DNJ (Sigma) was spotted under the same conditions. DNJ spots were detected by the spraying of a chlorine-*o*-tolidine reagent.

To identify the nature of the α-glucosidase activity inhibitor in the culture supernatant, the clone C36-4 was inoculated into 5 ml of LB medium in a 50 ml conical tube and incubated overnight at 37°C with shaking at 220 rpm. One milliliter of the overnight culture broth was inoculated into 500 ml of LB medium in a 1 L Erlenmeyer flask and incubated at 37°C with shaking at 220 rpm for 24 h. The culture broth was heated at 100°C for 10 min and centrifuged at 6,000×g for 10 min. The supernatant was concentrated to 50 ml using a vacuum evaporator (Büchi, Switzerland) to partially purify DNJ by two-step ion exchange chromatography using a previously described method (Asano *et al*., 2001; Cho *et al*., 2008).

Fifty milliliters of the concentrated supernatant was loaded into an ion-exchanger Amberlyst 15 (Sigma) column (100×10 mm, H^+). To remove the unbound part of the concentrated supernatant, the column was washed with 10 ml of deionized water. The column was eluted with 80 ml of 0.5 N NH_4OH and 1 ml fractions were collected. The eluate in each fraction was subjected to a TLC analysis to detect DNJ as described above. Active fractions were combined and concentrated to 4 ml by a vacuum evaporator. The concentrated eluate was loaded into a Dowex 1×2-100 (Sigma) column (100×10 mm, OH^-), and the column was eluted with 40 ml of deionized water. Again, active fractions were selected, combined, and concentrated to 4 ml using a vacuum evaporator.

For a qualitative determination of DNJ, the partially purified samples were labeled using 9-fluorenylmethyl chloroformate (FMOC-Cl) and were then analyzed via a HPLC analysis, as described previously (Kim *et al*., 2003; Cho *et al*., 2008). First, 10 μl of ion exchange fraction, 10 μl of 0.4 M potassium borate buffer (pH 8.5) and 20 μl of 10 mM FMOC-Cl in CH_3CN was mixed into Eppendorf tube and was allowed to react at 20°C for 20 min. The reaction was then terminated by the addition of 10 μl of 0.1 M glycine. To stabilize the DNJ-FMOC, 950 μl of 0.1% acetic acid was added into the reactant and then filtered through a 0.2 μm syringe filter (Nalgene, nylon filter). A 10 μl aliquot of the filtrate was injected into a Capcell Pak C_{18} MG HPLC column (φ: 4.60×250 mm) (Shiseido, Japan) and separated in a mobile phase of acetonitrile : 0.1% acetic acid (1:1, v/v) at a flow rate of 1 ml/min. The DNJ-FMOC fluorescence (excitation 254 nm, emission 322 nm) was monitored using a FL3000 fluorescence detector.

To confirm the isolation of DNJ via the molecular mass, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was conducted using an Agilent 1,200 series HPLC system and an Agilent 6410B system (Agilent Technologies, USA). Chromatographic separation was performed on an Agilent Epic HILIC-HC column (4.6×150 mm, 3 μm). The column temperature was maintained at 40°C and the sample injection volume was 2 μl. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) using gradient elution (0-0.1 min, 5% A and 95% B; 0.1-15 min, 80% A and 20% B; 15-20 min, 20% A and 80% B). It was delivered at a flow rate of 0.8 ml/min. Mass spectrometry was determined in positive mode by selected ion monitoring (SIM) and Scan mode. The mass spectrometer condition was set with a gas (N_2) flow rate of 35 L/min, a gas temperature of 320°C, a capillary voltage of 4 kV, and a fragmentor voltage of 150 V (Nakagawa *et al*., 2010).

Results

Construction of genomic DNA library of *B. subtilis* MORI 3K-85

In our previous attempt to isolate a new DNJ-producing bacterial strain, several hundred bacterial strains isolated from soil and traditional Korean foods were screened. *B. subtilis* MORI, which was isolated from Chungkookjang, a Korean traditional fermented soybean food, was identified as a new DNJ-producing strain (Kim *et al*., 2011). Subsequently, we

Table 1. Inhibitory activities against α-glucosidase by the culture broths of various selected clones

Clone	α-Glucosidase inhibitory activity (%)[b]
Control[a]	0.0
C5-26	20.0
C26-40	32.0
C36-4	48.0
C88-29	36.0
C91-31	38.0

[a] *E. coli* transformant harboring the empty CopyControl pCC1BAC Cloning-Ready Vector (Epicentre).
[b] The cells were incubated for 12 h at 37°C to determine the α-glucosidase inhibitory activity as described in the 'Materials and Methods' section.

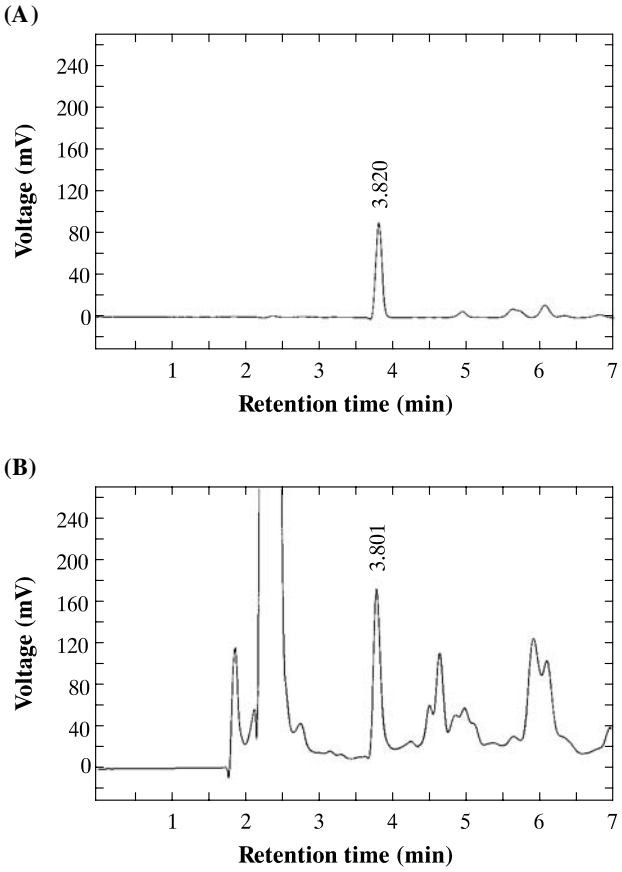

Fig. 1. Identification of DNJ in the culture broth of the recombinant *E. coli* strain harboring pCC1BAC-DNJbb. (A) HPLC chromatogram of standard DNJ, (B) HPLC chromatogram of the culture broth of *E. coli* strain harboring pCC1BAC-DNJbb (clone C36-4).

carried out random mutagenesis by γ-irradiation to improve the DNJ production of the *B. subtilis* MORI strain. As a result, we obtained a *B. subtilis* MORI 3K-85 strain whose DNJ production was increased by more than 10 fold compared to that of its parental strain *B. subtilis* MORI (manuscript in preparation). In this study, in an effort to isolate the genes involved in DNJ biosynthesis, a genomic DNA library was constructed by cloning the *Bam*HI digested genomic DNA of *B. subtilis* MORI 3K-85 into the CopyControl pCC1BAC Cloning-Ready vector followed by transformation into *E. coli* EPI300 competent cells. More than 20,000 transformants harboring the genomic DNA library vectors were selected and analyzed in terms of their inhibitory activities against α-glucosidase. Five clones, C5-26, C26-40, C36-4, C88-29, and C91-31, exhibited over 20% of α-glucosidase inhibitory activity (Table 1). Because clone C36-4 showed the highest α-glucosidase inhibitory activity of 48%, it was used for further characterization.

Identification of DNJ

First, to test whether this clone produced DNJ, the culture supernatant was analyzed by analytical TLC using a silica gel plate. It has been shown that the supernatant of clone C36-4 contains a substance which has an Rf value identical to that of the standard DNJ of 0.375 (data not shown). To investigate the identity of the α-glucosidase inhibitor further, the culture supernatant of clone C36-4 was subjected to two-step ion exchange chromatography for DNJ purification. When the fractions showing α-glucosidase inhibitory activity were pooled and analyzed by HPLC after being derivatized with FOMC-Cl, it was clearly shown that the fraction contained DNJ, which was detected at the same retention time (RT) of 3.8 min noted with the standard substance (Fig. 1).

Nucleotide sequence analysis of the genomic library clone conferring DNJ biosynthesis

To identify the genes that allowed the production of DNJ in *E. coli*, the size of the genomic DNA fragment inserted in the pCC1BAC-DNJbb isolated from the genomic DNA library clone C36-4 was determined by restriction enzyme digestion. It was found that the pCC1BAC-DNJbb contained a *B. subtilis* MORI 3K-85 genomic DNA fragment of about 10 kb (data not shown). The nucleotide sequences of the inserted DNA were determined, and the result showed that it contained a 10,086 bp DNA fragment flanked by *Bam*HI restriction sites at both ends. Based on the nucleotide sequence of this fragment, seven open reading frames encoding a polypeptide longer than 100 amino acid residues were predicted. When the entire nucleotide sequence was used to search the NCBI databases, two groups of genomic DNA stretches were identified to have strong homology with the insert of pCC1BAC-DNJbb. The sequence of the insert in pCC1BAC-DNJbb detected a homologous sequence with a similar length on the genome of *B. amyloliquefaciens* FZB42 (Chen *et al.*, 2007). According to the genome sequence information, in this region of *B. amyloliquefaciens* FZB42, seven ORFs were predicted to code for the following proteins, respectively: GlcP1 (404 aa, transmembrane transport), GabT1 (425 aa, transaminase), Yktc1 (316 aa,

Table 2. Experimentally defined gene functions in the *B. amyloliquefaciens* FZB42 which is matched with DNA sequence of the inserted DNA in the clone C36-4

ORF	Size (aa)	Protein name	Molecular function	Biological process
glcP1	404	GlcP1	Transmembrane transport	Unknown
gabT1	425	GabT1	4-Aminobutyrate transaminase activity and pyridoxal phosphate binding	Unknown
yktc1	316	Yktc1	Inositol or phosphatidylinositol phosphatase activity	Unknown
gutB1	348	GutB1	Oxidoreductase activity and zinc ion binding	Oxidation reduction
ybaR	434	YbaR	Integral to membarne and transpoter activity	Transmembrane transport
ybaS	327	YbaS	Bile acid:sodium symporter activity	Sodium ion transport
ybaA	210	YbaA	Unknown	Unknown

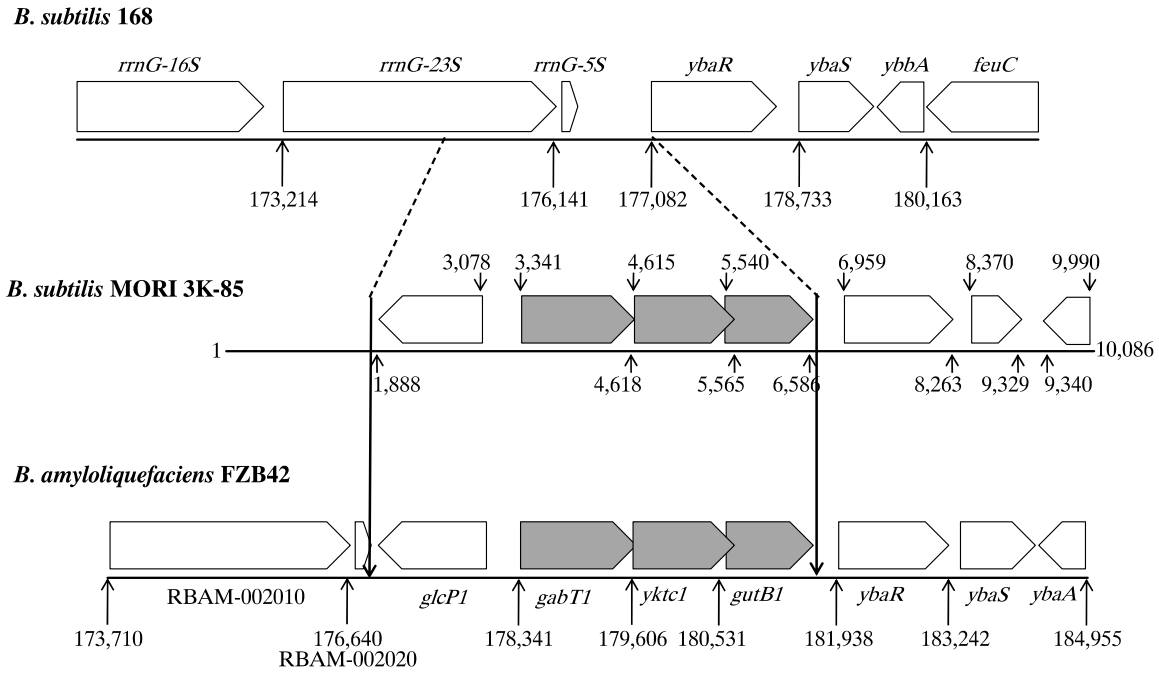

Fig. 2. Comparative organization of the DNJ biosynthetic genes of different bacilli strains. Homologous sequences of the putative DNJ biosynthetic genes of *B. subtilis* MORI 3K-85 were identified in the genome sequence of *B. amyloliquefaciens* FZB42, but they were absent in the genome sequence of *B. subtilis* 168. The shaded boxes represent *gabT1*, *yktc1*, and *gutB1* genes which share 86%, 78%, and 75% of their nucleotide sequence homologies between corresponding genes of *B. subtilis* MORI 3K-85 and *B. amyloliquefaciens* FZB42.

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   1  AAGCTTTTTACTCTGTTTGTATTTAGTTTTTTTACAATAAACCCATTTATTTCTATCATAACATATGAAGTTTGAATAGAAACCCCCCCAT
  91  GCAAACGTTAACAAATAGTTAACAATTTTTACTATTTATCTAGTTGACATTAATTTCTAGAATTTATACGATGATTAACGAGTTTTACAT
                    gabT1→
 181  ATTTTATGAATAGGGGTGTTGGTAGTGGGGACTAAAGAAATTACGAATCCAGACAGTTTGTATTACAAAGTGGATGACGTTGTCATGGAA
                      V  L  V  V  G  T  K  E  I  T  N  P  D  S  L  Y  Y  K  V  D  D  V  V  M  E

 271  AGAGGTGAAGGCATATATTTGTACGATTCAGAGGGCAATGAGTATATTGATTGTGCCTCTGCTACTTTCAACTTAAATTTGGGTTATACC
       R  G  E  G  I  Y  L  Y  D  S  E  G  N  E  Y  I  D  C  A  S  A  T  F  N  L  N  L  G  Y  T

 361  AATAAAGAAGTTATTGATACAGTCAAAGAACAGGCTGACCAGCTGATACACGTCACTTCTTCCTACCAAACCAACGCCGTTAATAAATTA
       N  K  E  V  I  D  T  V  K  E  Q  A  D  Q  L  I  H  V  T  S  S  Y  Q  T  N  A  V  N  K  L

 451  GCTGAAAAACTTGTAGAAATCTCCCCCCGACAATCTAACTAAAGTTCACCCTAAGGTTAGCAGCGGCTCTGCTGCGAATGAAGGAGCTATT
       A  E  K  L  V  E  I  S  P  D  N  L  T  K  V  H  P  K  V  S  S  G  S  A  A  N  E  G  A  I

 541  AAAATGGCTCAAAACTATTCTGGAAAAACAGATGTCATTTCTTTATTCCGAAGCCACCTTGGCCAAACGTATATGATGTCTGCGTTATCC
       K  M  A  Q  N  Y  S  G  K  T  D  V  I  S  L  F  R  S  H  L  G  Q  T  Y  M  M  S  A  L  S

 631  GGAAATTCATTTCGAAGAGAGCCATTCCCCCCTCAGTTTTCTTTTGGCTTACAGGTGCCTGACCCCTATTGCAACCGTTGTTTTTACAAT
       G  N  S  F  R  R  E  P  F  P  P  Q  F  S  F  G  L  Q  V  P  D  P  Y  C  N  R  C  F  Y  N

 721  CAGAAGCCAGATTCATGCGGAATGCTTTGTGTAGAAAGAATTAATGATTTTATTGAATATGCGAGTAACGGAAAAATTGCCGCGATGATT
       Q  K  P  D  S  C  G  M  L  C  V  E  R  I  N  D  F  I  E  Y  A  S  N  G  K  I  A  A  M  I

 811  ATTGAACCGATTTCCGGTAACGGAGGAAACATCGTTCCGCCTAAGGAGTACTTTAAGCAATTAAGAAAGCTCTGTGATGAGCATGATATT
       I  E  P  I  S  G  N  G  G  N  I  V  P  P  K  E  Y  F  K  Q  L  R  K  L  C  D  E  H  D  I

 901  GCACTTATTTTTGATGAAATTCAAACCGGATTTGGCCGGACAGGCAAGATGTTTGCGGCTGATTACTTTGATGTGAAACCGAATATGATG
       A  L  I  F  D  E  I  Q  T  G  F  G  R  T  G  K  M  F  A  A  D  Y  F  D  V  K  P  N  M  M

 991  ACTGTTGCAAAAGGATTGGGAGGCACGGGATTCCAGGTTGCCGCCACCCTCACAGAGGAAAAATACATGGGATTAGCCGGCCACAATCAC
       T  V  A  K  G  L  G  G  T  G  F  Q  V  A  A  T  L  T  E  E  K  Y  M  G  L  A  G  H  N  H

1081  TCTTTTACTTATGGCTCGAACGTGATGGCCTCGGCAGCAGCTTGTAAGACAATCGAAATCATGCAGCGGCCGGGCTTCTTAGAAAATGTA
       S  F  T  Y  G  S  N  V  M  A  S  A  A  A  C  K  T  I  E  I  M  Q  R  P  G  F  L  E  N  V

1171  ACAACTGTCGGGAATTACATTATGGATTCCTTAGAGCACATGAAGAAAGAATTCACATTTATTGCTGACGTCAGAGGCGTAGGTTTGATG
       T  T  V  G  N  Y  Y  I  M  D  S  L  E  H  M  K  K  E  F  T  F  I  A  D  V  R  G  V  G  L  M

1261  ATCGGTGTTGAAATTGTAAAAGAGAATAATGAGCGTGATGTAGAGCTGACCAATTACATTGCAAAACGGGCTATGGATTATGGGTTAATT
       I  G  V  E  I  V  K  E  N  N  E  R  D  V  E  L  T  N  Y  I  A  K  R  A  M  D  Y  G  L  I
```

Fig. 3. Continued

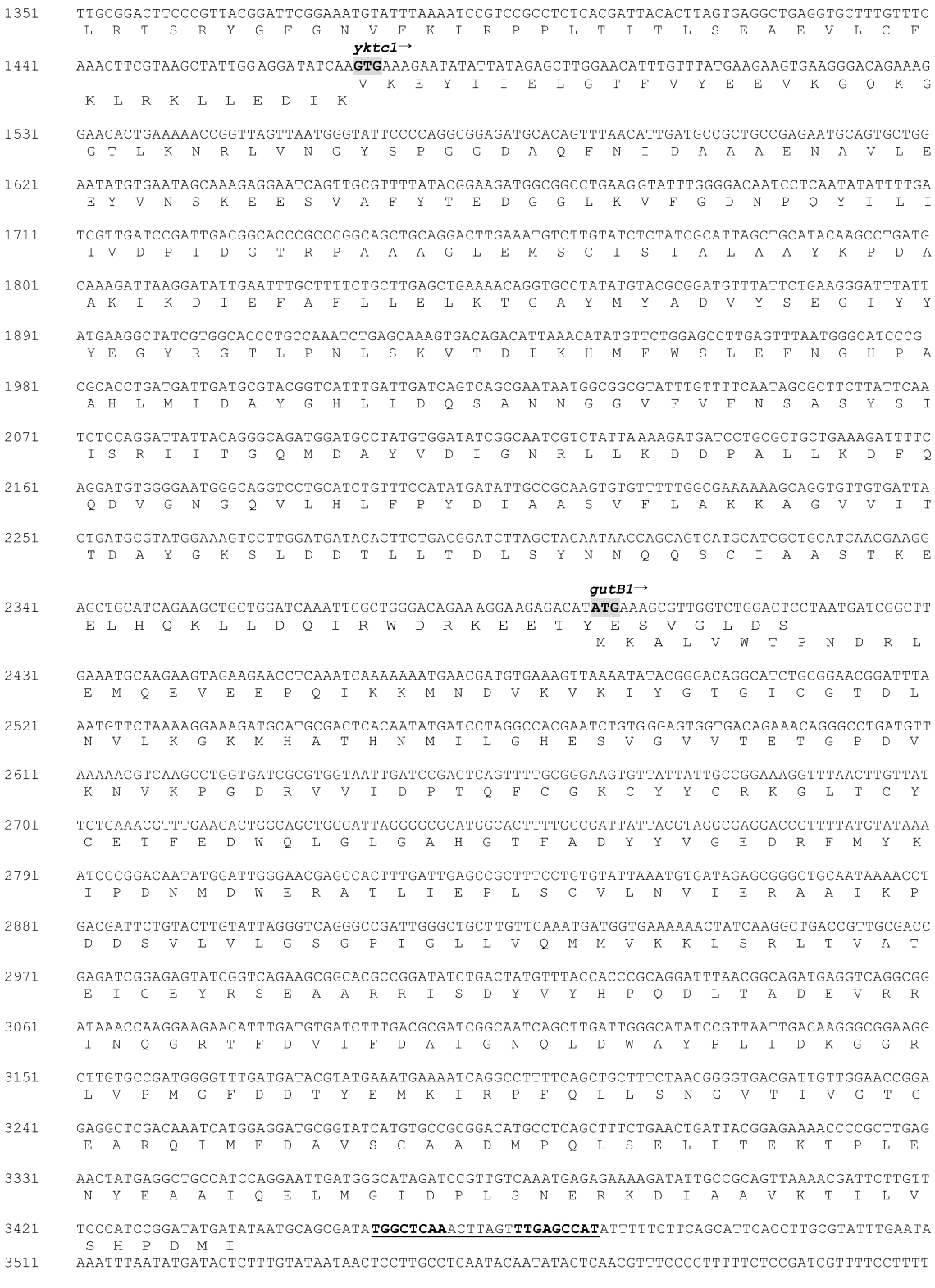

Fig. 3. The nucleotide sequence and deduced amino acid sequence of the DNJ biosynthetic genes from *B. subtilis* MORI 3K-85. The nucleotide sequence in the shaded box is the putative Sigma factor A (SigA) dependent promoter predicted by the web-based DBTBS (a database of *Bacillus subtilis* promoters and transcription factors) analysis program (http://dbtbs.hgc.jp/) (Sierro *et al.*, 2008). A putative CcpA binding sequence was identified upstream of the promoter (underlined italic letters). The putative ribosome binding sites and initiation codons of each gene are marked as boxed letters and bold letters in shaded boxes, respectively. The alternative ATG initiation codon for the *gabT1* gene is marked in bold letters. The rho-independent terminator (the underlined sequences where bold letters represent the stem region) was predicted by ARNOLD, a web-based terminator finding program (http://rna.igmors.u-psud.fr/toolbox/arnold/) (Naville *et al.*, 2011).

Table 3. Inhibitory activities against α-glucosidase by the culture broths of the selected clones harboring recombinant plasmids containing various sizes of DNA fragments

Type of DNA fragments (kb)	α-Glucosidase inhibitory activity (%)
pCC1BAC-DNJbb (10.07)	51.0
pCC1BAC-DNJhh (5.56)	71.7
pCC1BAC-DNJhn (4.20)	74.5

phosphatase), GutB1 (348 aa, oxidoreductase), YbaR (434 aa, transmembrane transporter), YbaS (327 aa, bile acid:sodium symporter), and YbaA (210 aa, unknown) (Table 2). In addition, it was noted that the sequence of the insert in pCC1BAC-DNJbb has the homology to genome sequence of *B. subtilis* 168. However, the homology pattern was different from that of *B. amyloliquefaciens* FZB42 (Chen *et al.*, 2007), and it could be divided into three regions depending on the homology to the *B. subtilis* 168 genome (Barbe *et al*., 2009). The first 2.3 kb sequence of the pCC1BAC-DNJbb insert has a homologous region in the genome of *B. subtilis* 168. However, the next 4.2 kb stretch of the pCC1BAC-DNJbb insert does not have any homologous sequence in the genome of *B. subtilis* 168. The last 3.6 kb stretch of pCC1BAC-DNJbb insert showed 93% identity to the region directly next to the first homologous region (Fig. 2). It is noteworthy that the *B. subtilis* 168 strain was not able to synthesize DNJ, whereas *B. amyloliquefaciens* FZB42 was able to synthesize DNJ. Thus, we closely examined the pCC1BAC-DNJbb sequences having a homologous region in the genome of *B. amyloliquefaciens* FZB42 but not in the genome of the *B. subtilis* 168 strain. This region was determined to have a putative operon consisting of the three open reading frames of *gabT1*, *yktc1*, and *gutB1* (Fig. 3). The nucleotide sequence homologies between the *gabT1*, *yktc1*, and *gutB1* ORFs of the *B. subtilis* MORI 3K-85 strain and those of *B. amyloliquefaciens* FZB42 were 86%, 78%, and 75%, respectively (data not shown). Interestingly, the nucleotide sequence of the *gabT1*, *yktc1*, and *gutB1* genes of *B. subtilis* MORI 3K-85 strain was identical to that of *B. subtilis* MORI strain (data not shown).

Identification of DNJ biosynthetic genes

To determine the gene sets required to confer the ability to produce DNJ to *E. coli*, the DNJ production of *E. coli* transformants harboring different sets of *B. subtilis* MORI 3K-85 genes from pCC1BAC-DNJbb were monitored. It was found that the *E. coli* transformant harboring subclone pCC1BAC-DNJhh containing *gabT1*, *yktc1*, *gutB1*, and *ybaR* genes shows α-glucosidase inhibitory activity. Furthermore, when the subclone pCC1BAC-DNJhn containing *gabT1*, *yktc1*, and *gutB1* genes was transformed, the resulting recombinant *E. coli* strain was also able to produce α-glucosidase inhibitory activity. As shown in Table 3, both transformants of pCC1BAC-DNJhh and pCC1BAC-DNJhn showed increased α-glucosidase inhibitory activity of about 70% compared to that of clone pCC1BAC-DNJbb. To confirm the production of DNJ by the recombinant *E. coli* strain harboring pCC1BAC-DNJhn, DNJ

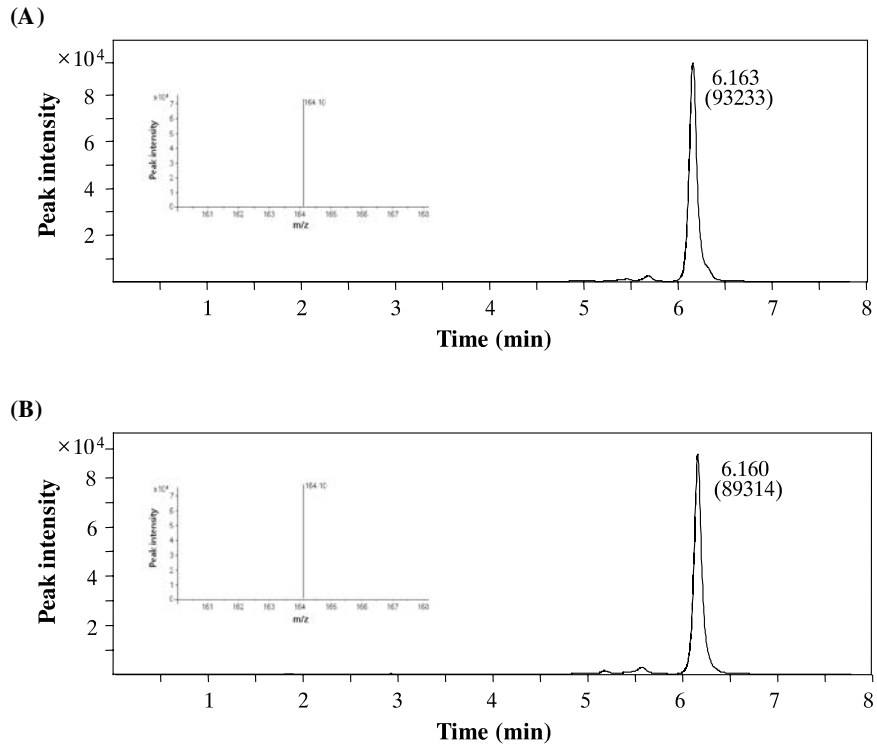

Fig. 4. Typical selected ion monitoring (SIM) chromatograms of DNJ. Standard DNJ (A) and the culture broth of *E. coli* transformant harboring pCC1BAC-DNJhn (B) were analyzed by LC-MS/MS. Mass spectrometry was determined in positive mode by SIM and Scan mode. Inserts represent the MS spectra of each sample.

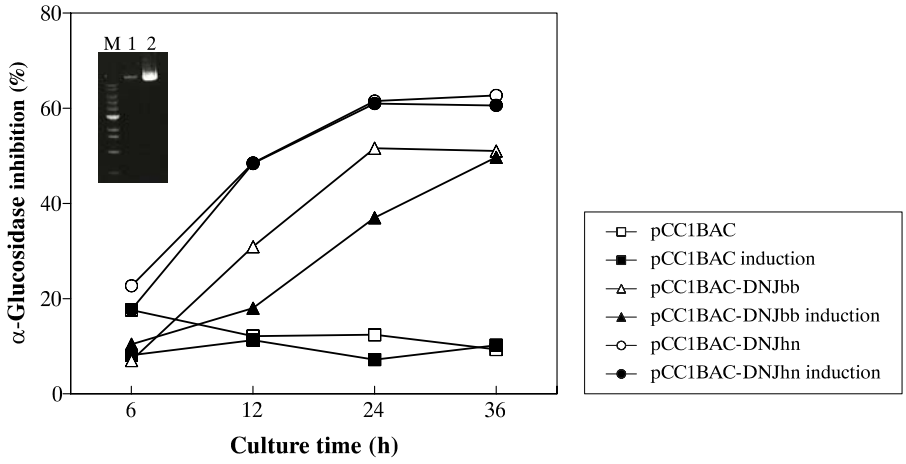

Fig. 5. Effects of the induced copy number of recombinant plasmids on the α-glucosidase inhibitory activities. *E coli* EPI300 strains harboring pCC1BAC (square), pCC1BAC-DNJbb (triangle), and pCC1BAC- DNJhn (circle) were cultured in the absence (open symbols) or presence of plasmid copy number induction (closed symbols). Samples were taken at the indicated time points and assayed for α-glucosidase inhibitory activity. In the insert, to confirm the plasmid copy number induction, pCC1BAC-DNJbb vectors prepared from the same volumes of uninduced (lane 1) and induced (lane 2) cultures were separated by electrophoresis on 0.8% agarose gel.

was purified by two-step ion exchange chromatography and analyzed by analytical LC MS/MS, as described in the 'Materials and Methods' section. It was found that DNJ existed in the culture supernatant at the same RT of 6.16 noted with the standard DNJ (Fig. 4). Furthermore, about 0.2 mg/L of DNJ was detected in the purified sample (data not shown). Thus, when considering the possible loss of DNJ during purification, it can be predicted that the culture supernatant of this transformant contained about 6 mg/L of DNJ. Taken together, our results suggest that the *gabT1*, *yktc1*, and *gutB1* genes of *B. subtilis* MORI 3K-85 are sufficient to lead to DNJ synthesis in the heterologous host *E. coli*.

Growth-phase-dependent synthesis of DNJ in recombinant *E. coli* strain

To investigate whether it is possible to increase DNJ production by inducing the expression of DNJ biosynthetic genes, different approaches were employed. Based on the property of the pCC1BAC vector system, whose copy number could be induced by treatment of the inducer, the α-glucosidase inhibitory activities of transformants harboring pCC1BAC-DNJbb or pCC1BAC-DNJhn were compared with and without copy number induction. We were not able to observe the expected increase in the α-glucosidase inhibitory activities upon copy number induction. It was rather found that the α-glucosidase

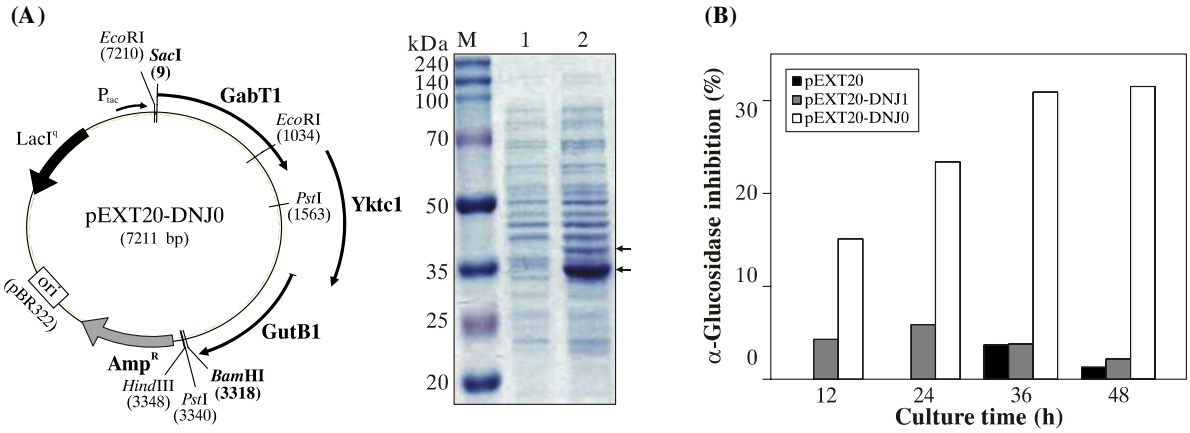

Fig. 6. Overexpression of DNJ biosynthetic genes. (A) DNJ biosynthetic genes, *gabT1*, *yktc1*, and *gutB1*, were cloned under the IPTG- inducible P_{tac} promoter in the medium copy number expression vector pEXT20. (B) *E. coli* DH5α strains harboring pEXT20 (lane 1) or pEXT20-DNJ0 (lane 2) were induced and whole cell lysates were separated on 10% PAGE. (C) α-glucosidase inhibitory activities in a culture broth of *E. coli* DH5α strain harboring, pEXT20 (black bars), pEXT20-DNJ1 (grey bars), or pEXT20-DNJ0 (white bars) were determined at the indicated times of cultivation.

inhibitory activities increased at the stationary phase of the growth (Fig. 5). To increase the expression of all three genes involved in DNJ biosynthesis further, the *gabT1-yktc1-gutB1* genes were cloned into the multi-copy expression vector pEXT20 under a strong IPTG-inducible P_{tac} promoter, resulting in pEXT20-DNJ0 (Fig. 6A). The *E. coli* DH5α strains harboring pEXT20-DNJ0 were induced by IPTG treatment, and whole cell lysates were separated on 10% PAGE (Fig. 6B). Some proteins have been clearly shown to be overexpressed by induction; however, the level of α-glucosidase inhibitory activity was not affected by IPTG induction but instead increased at the stationary phase of growth (Fig. 6C). To assess the possibility of utilizing the ATG codon, which is located 69 bp downstream from the originally predicted GTG codon, for translational initiation of the *gabTl* gene, the α-glucosidase inhibitory activity of the transformant harboring pEXT20-DNJ1 was determined, but no activity was detected, suggesting that the first 23 aa residues of the *gabT1* encoded protein is important for its activity.

Discussion

Based on isotope labeling studies, putative DNJ biosynthetic pathways have been postulated in several different organisms. In *Bacilli* and *Streptomyces*, it has been suggested that the precursor of DNJ biosynthesis, glucose, undergoes C2/C6 cyclization in which amination, oxidation, and epimerization played major roles (Hardick *et al*., 1992; Hardick and Hutchinson, 1993). In the higher plant dayflower *Commelina communis* (Commelinaceae), DNJ was proposed to be biosynthesized through a different route involving the C1/C5 cyclization of the original glucose molecule (Shibano *et al*., 2004). Despite detailed proposals for DNJ biosynthetic pathways in both bacteria and plants, no report is available on the genetics or enzymes of DNJ biosynthetic pathways.

In this study, for the first time, we isolated three genes, *gabT1*, *yktc1*, and *gutB1*, from the DNJ-producing *B. subtilis* MORI 3K-85 strain. When a recombinant plasmid harboring these genes was transformed into *E. coli*, the transformants produced DNJ into the culture medium. This suggests that the products of these three genes were enough to direct DNJ synthesis in the heterologous host *E. coli*. Further evidence of the involvement of these genes in DNJ biosynthesis was provided by the fact that these genes are missing in the genome of the DNJ non-producing *B. subtilis* 168, while homologous genes showing more than 75% of nucleotide sequence homologies were detected in the genome of the DNJ-producing *B. amyloliquefaciens* FZB42 strain. These genes were predicted to encode putative transaminase, phosphatase, and oxidoreductase, respectively. It is noteworthy that the putative DNJ biosynthetic pathway in *Bacillus subtilis var niger* was predicted to use a sugar-phosphate as an intermediate and involve amination and oxidation steps (Hardick and Hutchinson, 1993). According to the nucleotide sequence analysis, upstream of the SigA dependent promoter of the *gabT1-yktc1-gutB1* operon, a catabolite-responsive element (*cre*) has been predicted. It is well known that the expression of genes containing *cre* in *B. subtilis* is regulated by the carbon catabolite protein A (CcpA) transcription factor in response to carbon metabolism (Fujita, 2009). Interestingly enough, it has been reported that the maximal level of DNJ production in the *B. subtilis* DSM704 strain was observed at the onset of sporulation at the late stage of growth (Stein, 1984).

When DNJ biosynthetic genes were introduced into *E. coli*, DNJ production was observed clearly but only in a limited amount. The maximal level of production was only observed at the stationary phase of growth. Moreover, it was not possible to increase DNJ production further by inducing the copy number of the recombinant plasmid or by expressing these genes under the control of a strong inducible promoter. This may indicate that there is a limiting step to increase the level of DNJ production in recombinant *E. coli*, such as shortage of a substrate or insufficient expression of one of the DNJ biosynthetic genes in active form. In fact, the expression level of the *gabTl* gene was extremely low compared to those of other two genes and it could be due to its five rare Arg codons (AGA).

There are increasing demands for DNJ due to its potential to be developed as a new therapeutic based on its α-glucosidase inhibition activity. Although many reports describing the chemical synthesis of DNJ have been published, there remain several obstacles to be overcome before it can be put into large-scale production. For example, chemical synthesis requires a complicated process and lacks stereochemical selectivity (Schedel, 2008). Thus, to produce DNJ economically, it is likely necessary to employ a biotechnological fermentation process. The DNJ biosynthetic genes isolated in this study for the first time will allow an understanding of the physiological roles of DNJ as well as the fabrication of the bioprocesses for mass production of DNJ. Further studies regarding the biochemical properties of enzymes encoded by each gene and their substrates and cofactors will be required. In addition, to establish an economical production system using a heterologous host such as *E. coli*, it will be necessary to analyze and optimize the related metabolic pathways with molecular genetic and functional genomic tools.

Acknowledgements

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