

Enhanced production of recombinant nattokinase in *Bacillus subtilis* by promoter optimization

Shu-Ming Wu · Chi Feng · Jin Zhong · Lian-Dong Huan

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Abstract Nattokinase (NK) is a health product for the prevention and potential control of thrombosis diseases. To explore the possibility of enhancing NK production in *Bacillus subtilis* by altering the promoter of NK gene (*PaprN*), we tested several methods. We substituted the wild-type -10 box (TACAAT) of *PaprN* with the consensus sequence (TATAAT) of σ^A -dependent promoters, mutated the original -35 box (TACTAA) to a partial consensus sequence (TACACA), and expressed *apnN* from two tandem promoters, respectively. The efficacies of these changes were monitored by fibrinolytic activity, SDS-PAGE, and northern blotting analyses. Fibrinolytic activity analysis showed that altering the -10 region of *PaprN* could increase NK production by 136%. This production is significantly higher than those reported in the literatures. Similar results were obtained in SDS-PAGE and northern blotting analyses. This engineered promoter was also able to enhance the expression of β -glucuronidase (GUS) by 249%. Partial alteration of the -35 element could slightly improve the production of NK by 13%, while two tandem promoters just had marginal effects on the production of NK. Our study showed that alteration of -10 or -35 elements in *PaprN*, especially -10 element, is an effective way to enhance the production of heterologous proteins in *B. subtilis*.

Keywords Nattokinase · Enhanced production · Promoter · *Bacillus subtilis* · Subtilisin NAT

Introduction

Nattokinase (NK, Subtilisin NAT, E.C. 3.4.21.62) was first identified in a Japanese traditional soybean food, natto. It is a highly active fibrinolytic enzyme secreted by *Bacillus subtilis* var. *natto* (Sumi et al. 1987) and exhibits 4-fold greater fibrinolytic activity than plasmin (Fujita et al. 1995). NK can hydrolyze thrombi efficiently as well as increase the production of thrombi-dissolving agents such as tissue-type plasminogen activator (t-PA) in vivo (Tai 2006). The fibrinolytic activity of NK can retain in blood for more than 3 h (Tai 2006), which makes it feasible for clinical use. Now NK is widely considered as a promising oral medicine for thrombolytic therapy.

Bacillus subtilis has many attractive features to serve as an expression host for heterologous protein production. During the past several years, considerable progresses have been made to engineer *B. subtilis* for the production of heterologous proteins at high levels. For nattokinase, several recent studies reported the expression of its gene (*apnN*) in recombinant *B. subtilis* strains (Liu and Song 2002). Several efforts have also been invested to increase the production of recombinant NK. Among these, the use of expression vectors with high structure stability (Chen et al. 2007a) or the elimination of nutrient limiting factors (Chen and Chao 2006) led to higher production of recombinant NK. Medium optimization using response surface methodology can also obtain high-level production of recombinant NK (Chen et al. 2007b).

In this study we performed experiments directed to increase expression of *apnN* and other genes (using *gusA* as the reporter gene) in *B. subtilis* by changing sequences of the -10 or -35 elements to the consensus sequences of σ^A -dependent promoters, or by making the genes expressed under the control of two tandem promoters of *apnN*. The

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efficacies of these optimization methods for enhancing the expression of NK and GUS (extracellularly and intracellularly expressed, respectively) were displayed.

Materials and methods

Reagents

Thrombin and urokinase were purchased from Chinese Medicine Testing Institute (Beijing, China). Reagents 4-methylumbellifery-D-glucuronide (4-MUG) and 4-methylumbelliferyl (4-MU) for β -glucuronidase (GUS) assay were from Sigma-aldrich. DIG Northern Starter Kit was from Roche Co. All other reagents were of the highest quality commercially available.

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study were listed in Table 1. *E. coli* strains were grown in Luria–Bertani (LB) medium with aeration at 37°C, and 100 μ g/ml ampicillin was added when required. *B. subtilis* strains were grown in NB broth (2% soya peptone, 2% glucose, 0.6% Na₂HPO₄, 0.1% NaH₂PO₄, 0.05% MgSO₄, 0.02% CaCl₂, pH7.0) with aeration at 37°C, and 30 μ g/ml tetracycline was added to select the strains harboring plasmids.

DNA manipulations

Genomic DNA from *B. subtilis* var. *natto* was isolated as described by Akamatsu et al. (2000). Plasmid constructs were electroporated into *B. subtilis* DB104 with high osmolarity as Xue et al. (1999) described. Site-directed mutagenesis was performed by the QuickChang Mutagenesis Kit (Stratagene) according to manufacturer's instructions.

Construction of recombinant plasmids

Primers used for the amplification of target DNA fragments are showed in Table 2. To clone the NK gene, 1.5 kb *aprN* including its promoter (*PaprN*), coding sequence (*aprN_CDS*), and terminator (*TaprN*) was amplified from genomic DNA of *B. subtilis* var. *natto* using primer 1 and primer 2. The amplified product was cloned into the *EcoRI/SalI* digested pUC18, yielding plasmid pUC305 (Table 1).

To get β -glucuronidase (GUS) gene, 1.8 kb *gusA* was amplified from pNZ8010 using primer 5 and primer 7. *PaprN* was amplified from pUC305 using primer 3 and primer 6. *PaprN* and *gusA* were ligated by SOE (splicing by overlap extension) (Horton et al. 1989) using primer 1

and primer 7. The resulting ligation product was cloned into the *EcoRI/KpnI* digested pUC18, yielding plasmid pSM101. *TaprN* was amplified from pUC305 using primer 8 and primer 2, and the resulting product was cloned into *KpnI/SalI* digested pSM101, yielding plasmid pSM102 (Table 1).

By using pUC305 as template, primer 10 and its reverse primer as mutant primers, site-directed mutagenesis was performed to change the –10 region (TACAAT) of *PaprN* to generate a –10 consensus region (TATAAT), yielding plasmid pSM103 (Table 1). This manipulation changed *PaprN* in pUC305 to *PaprN_M10*. Similarly, by using primer 9 and its reverse primer as mutant primers, –35 region (TACTAA) in pUC305 was modified to the more consensus sequence (TACACA), yielding plasmid pSM104 (Table 1), in which *PaprN* was altered to *PaprN_M22*. By using pSM102 as template, primer 10 and its reverse primer as mutant primers, site-directed mutagenesis was performed to change the –10 element (TACAAT) of *PaprN* to the consensus –10 region (TATAAT), yielding plasmid pMG10 (Table 1).

To construct the NK expression vector, the *aprN* expression cassette (*PaprN-aprN_CDS-TaprN*) including its promoter and terminator was digested from pUC305 as a 1.5 kb *EcoRI/SalI* fragment and inserted to pHY300PLK, yielding the expression vector pHY305 (Table 1, Fig. 1). Similarly, expression cassette *PaprN_M10-aprN_CDS-TaprN* harboring the –10 element mutant promoter (*PaprN_M10*) was digested from pSM103 as a 1.5 kb *EcoRI/SalI* fragment and was inserted into pHY300PLK, yielding the expression vector pHY10 (Table 1, Fig. 1). Expression cassette *PaprN_M22-aprN_CDS-TaprN* harboring the –35 element partial mutant promoter was digested from pSM104 as a 1.5 kb *EcoRI/SalI* fragment and was inserted into pHY300PLK, yielding the expression vector pHY22 (Table 1, Fig. 1). To express the nattokinase gene from two tandem promoters, *PaprN* and *PaprN_M10* were amplified by PCR from pUC305 and pSM103, respectively, and the resulting 200 bp fragments were digested by *EcoRI* and *BamHI*. These restriction sites were used to clone the promoters into the 5'-end of *PaprN-aprN_CDS-TaprN* and *PaprN_M10-aprN_CDS-TaprN* cassettes in pBlue-scriptSK+, respectively. Finally, the *PaprN-aprN_CDS-TaprN* and *PaprN_M10-PaprN_M10-aprN_CDS-TaprN* cassettes were inserted into *BamHI/SalI* digested pHY300PLK, yielding pSM108 and pSM109, respectively (Table 1, Fig. 1).

To construct the GUS expression vector, the *gusA* expression cassettes *PaprN-gusA-TaprN* and *PaprN_M10-gusA-TaprN* were digested from pSM102 and pMG10 as a 2.2 kb *EcoRI-SalI* fragment and inserted into pHY300PLK, yielding the expression vector pSM105 and pHG10, respectively (Table 1, Fig. 1).

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	References or source
<i>Strains</i>		
<i>E. coli</i> JM109	<i>RecA1 pupE44 endA1 hsdR17 gyrA96 relA1 thi</i> Δ (<i>lac-proAB</i>) F'[<i>traD36 proAB⁺ lacI^q lacZ</i> Δ M15]	ATCC 47076
<i>B. subtilis</i> var. <i>natto</i>	Producer of nattokinase	Spizizen, unpublished
<i>B. subtilis</i> DB104	<i>His nprR2 nprE18</i> Δ <i>aprA3</i> , a protease deficient host	Kawamura and Doi (1984)
<i>Plasmids</i>		
pBluescript SK+	3.0 kb, Ap ^R , cloning vector for <i>E. coli</i>	Stratagene
pUC18	2.7 kb, Ap ^R , cloning vector for <i>E. coli</i>	Yanisch-Perron et al. (1985)
pNZ8010	5.0 kb, Cm ^R , harboring <i>gusA</i>	de Ruyter et al. (1996)
pSM101	4.7 kb, Ap ^R , pUC18 with <i>PaprN</i> and <i>gusA</i>	This study
pSM102	4.9 kb, Ap ^R , pSM101 with <i>TaprN</i>	This study
pMG10	4.9 kb, Ap ^R , -10 box sequence (TACAAT) of <i>PaprN</i> in pSM102 was mutated to TATAAT	This study
pUC305	4.2 kb, Ap ^R , pUC18 with nattokinase gene (<i>aprN</i>)	This study
pSM103	4.2 kb, Ap ^R , -10 box sequence (TACAAT) of <i>PaprN</i> in pUC305 was mutated to TATAAT, yield <i>PaprN_M10</i>	This study
pSM104	4.2 kb, Ap ^R , -35 box sequence (TTGACA) of <i>PaprN</i> in pUC305 was changed to TACACA, yield <i>PaprN_M22</i>	This study
pHY300PLK	4.87 kb, Ap ^R , Tet ^R , a shuttle vector for <i>E. coli</i> and <i>B. subtilis</i>	Ishiwa and Shibahara (1985)
pHY305	6.3 kb, Ap ^R , Tet ^R , pHY300PLK containing nattokinase gene (<i>aprN</i>) and its promoter (<i>PaprN</i>)	This study
pSM106	6.2 kb, Ap ^R , Tet ^R , -10 box sequence (TACAAT) of <i>PaprN</i> in pSM105 was mutated to TATAAT	This study
pSM108	6.5 kb, Ap ^R , Tet ^R , pHY305 with an additional <i>PaprN</i> in 5'-end of <i>aprN</i>	This study
pHY10	6.3 kb, Ap ^R , Tet ^R , -10 box sequence (TACAAT) of <i>PaprN</i> in pHY305 was mutated to TATAAT	This study
pSM109	6.5 kb, Ap ^R , Tet ^R , pHY10 with an additional <i>PaprN_M10</i> in 5'-end of <i>aprN</i>	This study
pHY22	6.5 kb, Ap ^R , Tet ^R , -35 box sequence (TACTAA) of <i>PaprN</i> in pHY305 was mutated to TACACA	This study
pSM105	7.0 kb, Ap ^R , Tet ^R , pHY300PLK with <i>PaprN_gusA_TaprN</i>	This study
pHG10	7.0 kb, Ap ^R , Tet ^R , -10 box sequence (TACAAT) of <i>PaprN</i> in pSM105 was mutated to TATAAT	This study

Table 2 Primers used in this study

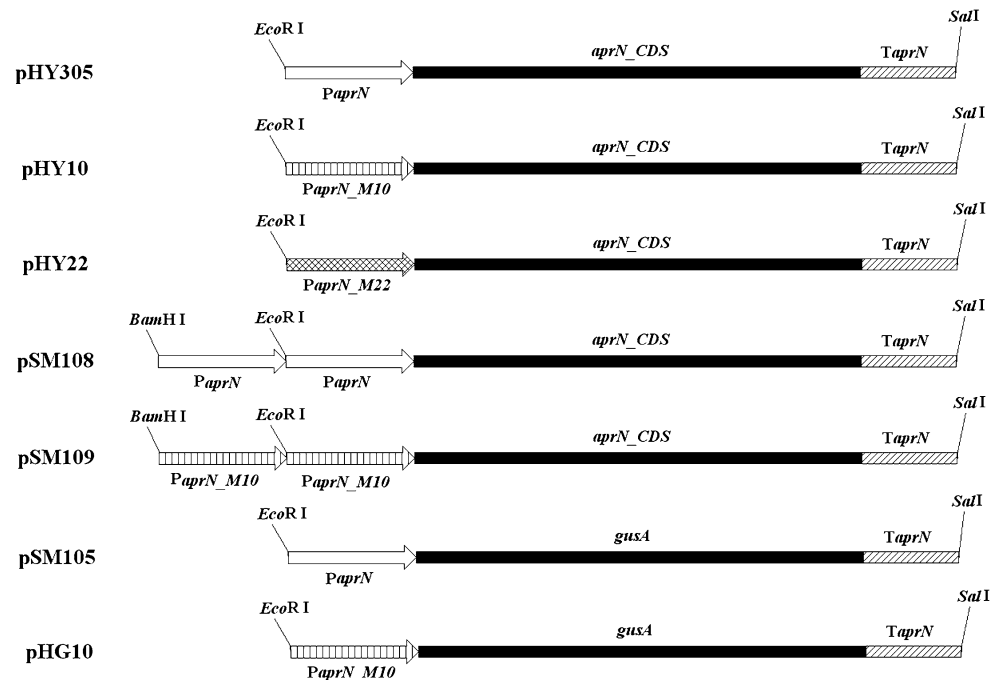
Primer	Sequence ^a (5'–3')	Description and location
Primer 1	GCGGAATTCGTATGAAAATAGTTA	<i>EcoRI</i> , upstream of <i>PaprN</i>
Primer 2	GTAGTCGACTCCGGTGCTTGTGAA	<i>SallI</i> , downstream of <i>aprN</i>
Primer 3	GCTCTAGAGGATCCGTATGAAAATAGTTA	<i>BamHI</i> , upstream of <i>PaprN</i>
Primer 4	GCGGAATTCCTTTACCCTCTCCT	<i>EcoRI</i> , downstream of <i>PaprN</i>
Primer 5	AGGAGAGGGTAAAGAATGTTACGTCTGTAG ^b	Upstream of <i>gusA</i> , can merged with 3'-end of <i>PaprN</i>
Primer 6	CTACAGGACGTAACATCTTTACCCTCTCCT ^b	Reverse complement with primer 5
Primer 7	CGGGGTACCCTATCATTGTTTGCCTCCC	<i>KpnI</i> , downstream of <i>gusA</i>
Primer 8	CGGGGTACCTAAAAAGAAGCAGGTTCT	<i>KpnI</i> , upstream of <i>TaprN</i>
Primer 9	CTCAAAAAAGTGGGTCTACACAAATATTATCCATC ^c	-35 Box mutant primer
Primer 10	TTATTCCATCTATTATAATAAATGCACAGAATAG ^c	-10 Box mutant primer

^a Relevant restriction sites are underlined

^b SOE overlaps between *gusA* and *PaprN* are showed in italics

^c Sequence different from the wild-type was show in boldface

Fig. 1 Physical maps of the expression cassettes in expression vectors constructed in this study. All expression cassettes are cloned into the *EcoRI/SalI* or *BamHI/SalI* digested pHY300PLK, respectively, and only the relevant restriction enzymes are showed. *PaprN*, *PaprN_M10*, and *PaprN_M22* are the wild-type, -10 mutant, and -35 mutant promoter of nattokinase gene (*aprN*), respectively. The elements *aprN_CDS* and *gusA* are the encoding sequences of *aprN* and GUS gene (*gusA*). *TaprN* is the terminator of *aprN*. The arrows indicate the direction of transcription



Construction of *B. subtilis* strains for nattokinase and GUS expression

All plasmid construction was carried out in *E. coli*, and the resulting plasmids were confirmed by sequence analyses. The expression plasmids were electroporated into *B. subtilis* DB104, to give strains for NK or GUS expression. The transformed *Bacillus* strains were confirmed by plasmid analyses.

Expression of recombinant nattokinase and GUS

B. subtilis strains harboring the appropriate expression vectors were used to produce NK or GUS. Cultures were established as follows: a single colony was inoculated into 5 ml NB broth supplemented with 30 µg/ml tetracycline and grew over-night at 37°C with shaking at 200 rpm. The overnight culture was 3:100 diluted into fresh media and grown at 37°C with shaking at 200 rpm for 84 h to express NK, or for 24 h to express GUS.

Fibrinolytic activity determination

Quantitative analysis of fibrinolytic activity was conducted by the fibrin plate method (Astrup and Mullertz 1952) using urokinase as a standard. Briefly, 10 ml 1.5 mg/ml bovine fibrinogen solution and 10 ml 1% agarose solution in Barbitol buffer (50 mM sodium barbitol, 90 mM NaCl, 1.7 mM CaCl₂, 0.7 mM MgCl₂, 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) into the agarose solution.

The fibrinogen and agarose solutions were mixed in a 100 mm petri dish and kept at room temperature for 1 h to form fibrin clots. Culture supernatant was obtained by centrifugation and diluted to 64-fold in Barbitol buffer. Holes were made on the fibrin plate and 10 µl of each enzyme solution was dropped in a hole. The plate was incubated at 37°C for 18 h. Two perpendicular diameters of the lyzed zone on the fibrin plate were measured and the fibrinolytic activity was determined according to the standard curve of urokinase.

Northern blotting analysis

For RNA preparation, a single colony was inoculated into 5 ml NB medium supplemented with 30 µg/ml tetracycline and grew overnight at 37°C with shaking at 200 rpm. The overnight culture was diluted (3:100) into fresh media and grown at 37°C with shaking at 200 rpm for 6 h to get the cells. Total RNA from *B. subtilis* was isolated using TRI-ZOL[®] Reagent. The isolation procedure was performed according to manufacturer's instructions. Northern blot was performed using DIG Northern Starter Kit according to the instructions of the manufacturer. NK gene (*aprN*) amplified from pUC305 and 16S rDNA amplified from *B. subtilis* DB104 were used as probes.

SDS-PAGE analysis

SDS-PAGE was performed with 5% (w/v) stacking gels and 12% (w/v) separating gels, and proteins were visualized by Coomassie Blue R-250 staining.

Fluorometric GUS assays

Extracts were prepared in GUS assay buffer and reactions were carried out as described by Jefferson et al. (1987) using 4-methylumbellifery-D-glucuronide (4-MUG, Sigma) as a substrate. The concentration of the fluorescent product, 4-methylumbelliferyl (4-MU), was determined with FLU-Ostar OPTIMA microplate reader (BMG Labtechnologies Ltd., Germany). The amount of protein in each extract was measured by the Bradford method using bovine serum albumin as standard.

Results and discussion

Cloning and sequence analysis of NK gene

The nattokinase gene (*aprN*) amplified from genomic DNA of *B. subtilis* var. *natto* was cloned into *EcoRI/SalI* digested pUC18 to form plasmid pUC305. Sequence analysis of the cloned *aprN* gene showed that it was 1472 bp long and composed of a 180 bp promoter (*PaprN*), a 1143 bp CDS (encoded 29 aa signal peptide, 76 aa pro-peptide, and 275 aa mature protein), and a 150 bp terminator (*TaprN*). The sequence of this gene was almost identical to that reported by Nakamura (NCBI Ac No.: S51909), differing in only 3 nucleotides (65- versus 65A, 81G versus 82A, and 578G versus 579A), while the predicted proteins were identical. Promoter region of *aprN* was showed in Fig. 2. The -10 element of *PaprN* was

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1  GTATGAAAAT  AGTTATTTTCG  AGTCTCTACG  GAAATAGCGA  GAGATGATAT  ACCTAAATAG
61  AGATAAATCA  TCTCAAAAAA  GTGGGCTAC  TAAAATATTA  TTCCATCTAT  TACAATAAAT
121 GCACAGAATA  GTCTTTTAAG  TAAGTCTACT  CTGAATTTTT  TAAAAGGAGA  GGGTAAAGAG

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Fig. 2 Sequence of promoter of nattokinase gene (*PaprN*). The -35 box of *aprN* is underlined, and the -10 box is showed with double underlines

TACAAT and the -35 element was TACTAA (Jan et al. 2001).

Effects of altering the -10 and -35 promoter elements of *aprN* on expression of NK

Compared with the consensus sequence of σ^A -dependent promoters, the -10 element (TACAAT) of *PaprN* had one nucleotide different from the consensus sequence (TATAAT) and the -35 element (TACTAA) has 4 nucleotides different from the consensus sequence (TTGACA) (Helmann 1995). Since these two elements were very important for the recognition of the α -subunit of the RNA polymerase (RNAP), we performed site-directed mutagenesis of the -10 and -35 elements of *PaprN*, respectively, to probe the effects of these alterations on expression of NK. The sequence of -10 element (TACAAT) was successfully mutated to the consensus form (TATAAT), which turned *PaprN* to *PaprN_M10*. The sequence of -35 element (TACTAA) was mutated to the partial consensus form (TACACA) and *PaprN* was changed to *PaprN_M22*. NK was expressed in *B. subtilis* DB104 under the control of *PaprN*, *PaprN_M10*, and *PaprN_M22*, respectively. The effects of these promoter alterations on the expression level of NK were monitored by fibrinolytic analysis using fibrin plate method and SDS-PAGE.

Fibrinolytic analysis results (Table 3, Fig. 3b) showed the production of NK in the control strain DB104 (pHY305) was 272 $\mu\text{g/ml}$, while that in the -10 mutant strain DB104 (pHY10) was 643 $\mu\text{g/ml}$. NK production of the -10 mutant strain was 136% higher than that of the control strain, suggesting that altered the -10 element sequence of *PaprN* could obviously improve the expression level of NK.

The production of NK in the -35 mutant strain DB104 (pHY22) was 13% higher than that in the control strain DB104 (pHY305), suggesting that mutating the sequence of -35 element of *PaprN* to the partial consensus sequence

Table 3 Nattokinase yield in different recombinant *B. subtilis* strains^a

<i>B. subtilis</i> strain	Description	Promoter	Promoter number	Activity ^b (U/ml)	Yield ^{b,c} ($\mu\text{g/ml}$)	Relative activity (%)
DB104 (pHY300PLK)	Negative control	None	0	4	0.00129	0.47
DB104 (pHY305)	Wild-type	<i>PaprN</i>	1	847	272	100
DB104 (pHY10)	-10 mutant	<i>PaprN_M10</i>	1	1999	643	236
DB104 (pHY22)	-35 partial mutant	<i>PaprN_M22</i>	1	957	308	113
DB104 (pSM108)	Wild-type, 2 promoters	<i>PaprN</i>	2	796	256	94
DB104 (pSM109)	-10 mutant, 2 promoters	<i>PaprN_M10</i>	2	1906	613	225

^a Fibrinolytic activities were detected by the fibrin plate method using urokinase as a standard and performed at 37° for 18 h, pH7.75 in barbital buffer

^b Values showed are the averages of six independent experiments with a standard deviation less than 10%, U is urokinase unit

^c Yield value is produced as activity divided by the specific activity, and the specific activity of NK is 3,109 U/mg (Wu et al. 2007)

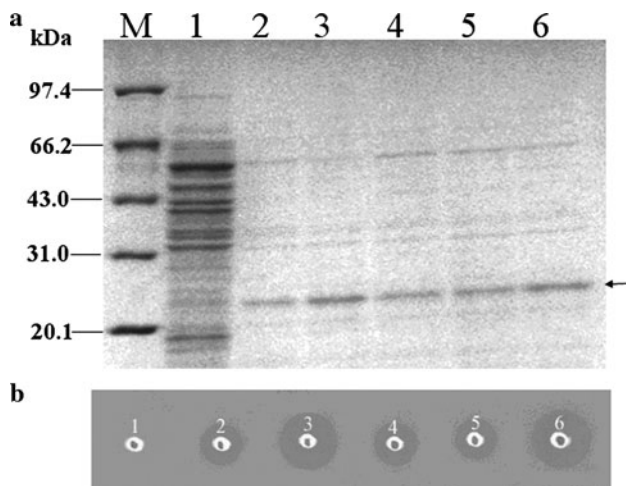


Fig. 3 Analyses of nattokinase production by recombinant *B. subtilis* strains. **a** SDS-PAGE analysis. **b** fibrinolytic activity analysis. Lane M, protein molecular weight marker; Nos. 1–6, *B. subtilis* DB104 harboring pHY300PLK (negative control), pHY305 (with wild type promoter), pHY10 (with –10 mutant promoter), pHY22 (with –35 mutant promoter), pSM108 (with 2 wild type promoters), pSM109 (with two –10 mutant promoters), respectively. Recombinant strains were cultured in fermentation medium for 72 h, following with supernatant culture and cells were separated by centrifugation. For SDS-PAGE analysis, 15 μ l supernatant culture was loaded to each lane. The arrow indicates the band of nattokinase. Fibrinolytic activities were detected by the fibrin plate method and done at 37° for 18 h, pH7.75 in barbital buffer. For each sample, 10 μ l 64-fold diluted supernatant culture was loaded to a hole in the fibrin plate

could slightly improve the expression level of NK. The result of SDS-PAGE analysis was consistent with that of fibrinolytic analysis (Fig. 3a).

Unfortunately we could not mutate it to the complete consensus sequence (TACTAA \rightarrow TTGACA) even after a long time effort. The presumable reason might be the hypertranscription from the consensus promoter leading to plasmid instability or the modified plasmid might be lethal to *E. coli*. However, a partial consensus element (TACTAA \rightarrow TACACA) we obtained slightly improved the yield of NK (13%).

Effects of two tandem promoters on expression of NK

Recombinant strains *B. subtilis* DB104 (pSM108) and DB104 (pSM109), which harbored two tandem wild-type and –10 box mutant promoters, respectively, were obtained to probe the effects of two tandem promoters on expression level of NK. Fibrinolytic activity analysis results (Table 3, Fig. 3b) showed the NK production of the two tandem wild-type promoter strain *B. subtilis* DB104 (pSM108) (256 μ g/ml) was 94% of that of the one wild-type promoter strain DB104 (pHY305), suggesting that two tandem wild-type promoters only had marginal effects on the expression level of *aprN*. The NK production of DB104

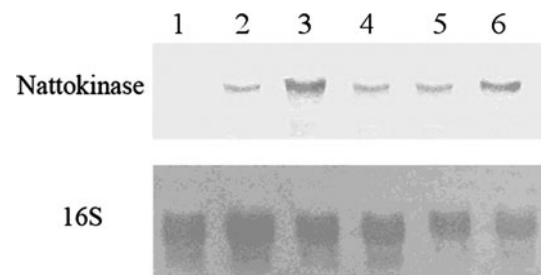


Fig. 4 Northern blotting analysis of the total RNA isolated from different recombinant *B. subtilis* strains. Lane 1–6, *B. subtilis* DB104 (pHY300PLK), DB104 (pHY305), DB104 (pHY10), DB104 (pHY22), DB104 (pSM108), DB104 (pSM109), respectively. 35 μ g total RNA is added to each lane. The polymerase chain (PCR) amplified DNA of *aprN* harbored in pUC305 was used as the probe. The 16S PCR amplified DNA was used as the loading control

(pSM109) (613 μ g/ml, harboring two tandem –10 element mutant promoters) was 95% of that of DB104 (pHY10) (643 μ g/ml, harboring one –10 element mutant promoter). This result suggested that two tandem –10 element mutant promoters also only displayed marginal effects on the expression level of *aprN* and could not further increase the production of NK. The results of SDS-PAGE analysis supported the above fibrinolytic results (Fig. 3a).

A few reports have showed that two or three tandem promoters could significantly improve the expression level of heterogeneous genes (Wei et al. 2002; Widner et al. 2000). In this study, the trials of two tandemly linked wild-type or –10 box modified promoters of NK gene did not obviously improved the expression level of NK in comparison to the control by one promoter alone. The result suggested that this strategy could not be a commonly effective method to increase the yield of heterogeneous proteins in bacteria.

Effects of altering the promoter sequence or two tandem promoters on the transcription level of *aprN*

In order to find the effects of altering the promoter sequence or the two tandem promoters on the transcription level, northern blotting analysis was used to determine the transcription levels of *aprN* in all the six strains. Northern blotting result (Fig. 4) showed that the *aprN* hybridization signal of *B. subtilis* DB104 (pHY10) was obviously stronger than that of the control strain DB104 (pHY305), suggesting that altering –10 element of promoter could obviously enhance the transcription level of *aprN*. Hybridization signals of DB104 (pHY22) and DB104 (pSM108) was similar to that of DB104 (pHY305), showing that partially altering –35 element of *PaprN* and two tandem wild-type promoters had not obvious effects on increasing the transcription level of *aprN*. Hybridization signals of DB104 (pSM109) was similar to that of DB104 (pHY10), indicating that two tandem –10 box

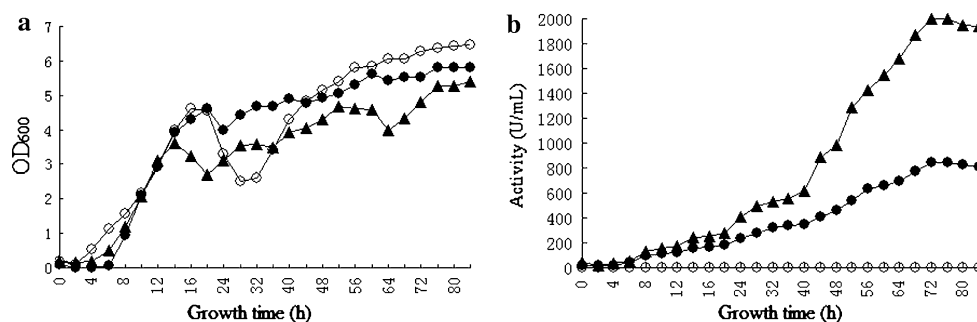


Fig. 5 Growth curves (a) and nattokinase expression profiles (b) of recombinant strains. The strains include *B. subtilis* DB104 (pHY300PLK) (open circle) which is the negative strain, DB104

mutant promoters (*PaprN_M10*) had not obvious effects on the transcription level of *aprN* in contrast to one alone.

Growth curve and NK expression profile of recombinant strains

Production and transcription analyses had showed that altering the -10 element to the consensus sequence could obviously enhance the expression level of NK. To understand whether this alteration affect the growth ability of host cell or the expression profile of NK or not, the growth curve and NK expression profile of three recombinant strain were determined. Growth curves showed that the growth biomass of the -10 mutant strain *B. subtilis* DB104 (pHY10) was slightly lower than that of the control strain DB104 (pHY305) after the late period of exponential phase (Fig. 5a). This suggested higher expression level of *aprN* might slightly decrease the ultimate growth biomass of *B. subtilis*. NK expression profile of *B. subtilis* DB104 (pHY10) was similar to that of DB104 (pHY305) despite the NK production of the previous strain was much higher (Fig. 5b), suggesting the expression characteristic of the -10 mutant promoter *PaprN_M10* was similar to that of the wild type promoter *PaprN*.

As that with the wild-type promoter, *aprN* with -10 mutant promoter was induced in a natural way at the transition stage when the maximum cell biomass is reached, minimizing the possibility of mutations and plasmid instability (Jan 2001). This way of *aprN* induction represents an important economical advantage and simplicity for industrial scales of fermentation. The promoter of *aprN* can also be applied to other genes of commercial relevance, with the advantage of avoiding the use of expensive elements for large-scale fermentation induction.

Effects of altering the -10 element sequence of *PaprN* on expression of GUS

Our study showed altering the -10 element region to consensus sequence can distinctly enhance the expression

(pHY305) (filled circle) which is the control strain harboring the wild-type *PaprN*, and DB104 (pHY10) (filled triangle) which harbors the -10 box mutant promoter of *aprN*. U is urokinase unit

Table 4 Analyses of β -galactosidase (GUS) production by recombinant *B. subtilis* strains

<i>B. subtilis</i> strain	Promoter	GUS activity ^a (U/ μ g protein)	Relative activity (%)
DB104 (pHY300PLK)	None, negative control	0.00034 \pm 0.000031	0.008
DB104 (pSM105)	Wild-type	42.3 \pm 1.57	100
DB104 (pHG10)	-10 mutant	147.9 \pm 12.33	349

^a Results are mean \pm SD of six determinations. One unit is defined as the amount of enzyme required to hydrolyze 1 nmol substrate in 1 min at 37°C

of secreted NK. This stimulated us to consider whether this engineered promoter had the same effect on the intracellular expression of heterogeneous genes. To answer this question, we selected *gusA* as the reporter gene. Recombinant strains DB104 (pSM105) and DB104 (pHG10), harboring wild-type and -10 box mutant *PaprN*, respectively, were obtained to probe the effect of the -10 element mutation on expression level of GUS. This effect was monitored by fluorometric GUS assays. The results (Table 4) showed GUS production of the -10 mutant strain DB104 (pHG10) was 147.9 U per micro gram protein, which was 249% higher than that of the control strain DB104 (pSM105). This result indicated that altering the -10 element to consensus sequence could obviously increase expression level of intracellular GUS.

Conclusions

It is an effective way to enhance the production of secretory NK by altering the -10 or -35 element of its promoter region. By changing the -10 element of the promoter, the expression level of nattokinase was improved to 643 μ g/ml, which is significantly higher than those reported in the literatures (Chen et al. 2007b). The modified

(–10 element) promoter also proved to be applicable to control the expression of other heterologous proteins. Since the engineered promoter is still naturally inducible like the wild-type one, it could be cost-effectively usable for the control of other genes with commercial values in large-scale fermentation.

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