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Improved Subtilisin YaB Production in *Bacillus subtilis* Using Engineered Synthetic Expression Control Sequences

JYH-PERNG WANG,[‡] CHUAN-MEI YEH,*[,‡] AND YING-CHIEH TSAI[§]

Department of Food Science and Biotechnology, National Chung-Hsing University, Taichung, Taiwan, Republic of China, and Institute of Biochemistry, National Yang-Ming University, Taipei, Taiwan, Republic of China

Alkaline elastase YaB, a favorable meat tenderizer, is an extracellular subtilisin-type protease produced by wild strain alkalophilic *Bacillus* YaB. The gene *ale* coding for subtilisin YaB with its own expression control sequence has been cloned and expressed in *Bacillus subtilis*, but at levels much lower than in the parental strain *Bacillus* YaB. This study investigates the influence of various expression control sequences including expression control sequences of *cdd* and *veg* from *B. subtilis*, a synthetic expression control sequence (SECS), and engineered synthetic expression control sequences (engineered SECSs) on the expression of subtilisin YaB in *B. subtilis*. The engineered SECSs were generated by using the Polymerase Chain Reaction; their UP element, Shine−Dargarno (SD) sequence, or both were different from those of the native SECS. The expression efficiencies of SECS and engineered SECSs were higher than those of expression control sequences of *ale*, *cdd*, and *veg*. Substitution of the SD sequence of SECS resulted in higher expression of subtilisin YaB than substitution of the UP element, whereas combined substitution of both gave the highest expression. These results demonstrate that engineering of SECSs is an approach for improving subtilisin YaB production in *B. subtilis*. Moreover, it is suggested that these enginnered SECSs could potentially be used to express homologous and heterologous proteins in *B. subtilis* at high level.

KEYWORDS: Subtilisin YaB; synthetic expression control sequence; *Bacillus subtilis*

INTRODUCTION

Alkaline elastase YaB, an extracellular serine protease produced by wild strain alkalophilic *Bacillus* YaB, has extensive homology with other subtilisins and is therefore known as subtilisin YaB (*1*). The enzyme is a good meat tenderizer because of its markedly superior specificity for elastin and collagen (*2*) and also has potential application in the preparation of bioactive peptides by enzymatic hydrolysis of soy protein (*3*).

In a previous study (*4*), the entire gene *ale*, coding for the pre-, pro-, and mature protein regions, with its own promoter and Shine−Dargarno (SD) sequence, was cloned into plasmid pHY300PLK. Using its own expression control sequence (ECS*ale*), the *ale* gene was not expressed in *Escherichia coli*, but was weakly expressed in *Bacillus subtilis* DB104 at a level much lower than in wild strain *Bacillus* YaB. By using *E. coli* bacterial T7 expression system, subtilisin YaB was expressed as inactive form (unpublished finding). To apply subtilisin YaB in the food industry and study its structure−function relationship, the use of *B. subtilis* as a host for the production of active subtilisin YaB or protein-engineered subtilisin YaB is favored over the use of *E. coli*.

B. subtilis, a generally recognized as safe (GRAS) bacterium, is widely used for the production of industrial enzymes that naturally occur in this organism and which are secreted into the supernatant in large amount (*5*). It is also an attractive host for the production of homologous and heterologous proteins for several reasons such as its ability to secrete proteins and ease of genetic manipulation and the fact that it is nonpathogenic and free of endotoxins and displays fast growth. *B. subtilis* is a suitable host for the expression of active subtilisin YaB, but the poor yield of subtilisin YaB in *B. subtilis* DB104 (*4*) limits its applications in the food industry. To increase recombinant subtilisin YaB production in *B. subtilis*, several strategies, such as improved transcriptional and translational efficiency of *ale* and the optimization of culture conditions, can be considered. To date, two methods have been used to increase subtilisin YaB production. One is the improvement of gene translational efficiency by changing the UUG initiation codon to AUG (*6*). Another is the enhancement of gene expression by replacement of the wild type ECS*ale* with a synthetic expression control sequence (SECS) (*7*). The SECS was constructed using the overlapping extension Polymerase Chain Reaction (PCR) and its sequence designed on the basis of the expression control sequence of the *B. subtilis veg* gene (ECS*veg*). The SECS

* Author to whom correspondence should be addressed [telephone +(886)-4-22840386, ext. 3190; fax +(886)-4-22853165; e-mail cmyeh@nchu.edu.tw].
‡ National Chung-Hsing University.
§ National Yang-Ming University.

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Table 1. Oligonucleotides Used in This Study

primer	sequence (5′–3′)[a]
UPF-1	CAATATGAATTCAGATCTAAATTAAAAATTTTTAAAAATTTTAAAAAAATTTGTTGACAAAAATGGGCTCGTGTGG
UPF-2	CAATATGAATTCAGATCTCACAAAAAAAGTGAGGATTTTTTTATTTTTGTATTGACAAAAATGGGCTCGTGTGG
UPR-1	CTCGGCTGCTTGTGCGATC
SDF-1	CAGAGGGAACAGGTATTGCAATTGTAGG
SDR-2	GATATACATATGTTTTCCTCCTTTGTCTGGAGCTCGCATCCACCTC
PvegF	CAATATAGATCTGAATTCTCCGTAATACGCTGACAAGAGAGAAAGG
PvegR	GAATTACATATGATCCACCTCACTACATTTATTGTACAACACGAG
P43F	CAATATAGATCTGACAAACATCACCCTCTTGCTAA
P43R	GAATTACATATGTACATTCCTCTCTTACCTATAATGGTACCG

[a] Restriction sites are underlined (GAATTC for *Eco*RI, AGATCT for *Bgl*II, and CATATG for *Nde*I).

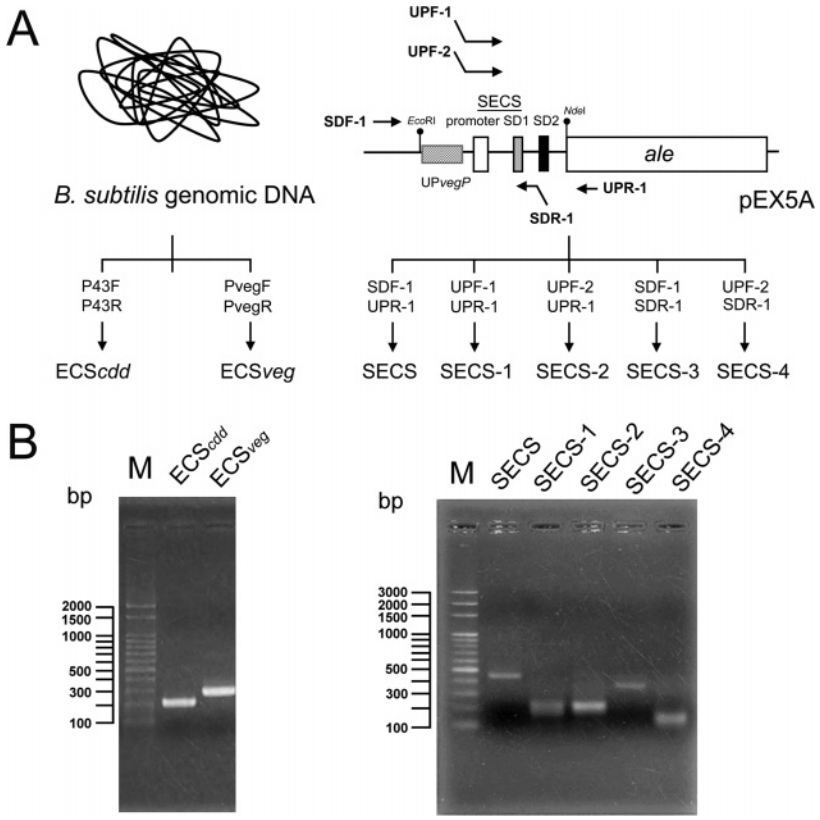

Figure 1. Amplification of expression control sequences: (**A**) schematic representation of the templates and primers used for generating various expression control sequences; (**B**) analysis of PCR-amplified expression control sequences by 2% agarose gel electrophoresis.

consists of consensus −35(TTGACA) and −10(TATAAT) hexamers, a TG motif, and two SD sequences from *veg* (SD1) and *ale* (SD2). The expression of secreted subtilisin YaB using this SECS was higher than that seen using the wild type ECS*ale*. Whether this SECS is superior to ECS*veg* has not been examined.

Previous studies have demonstrated that the core promoter sequence (*8*) and other structural elements, such as the UP element (an A+T-rich sequence located upstream of the −35 hexamer) (*9*) and SD sequence (*10*), influence the level of gene expression. In the present study, two well-studied constitutive expression control sequences from *B. subtilis*, the expression control sequence of the *B. subtilis* cytidine/deoxycytidine deaminase gene, *cdd* (ECS*cdd*, containing the P43 promoter) and ECS*veg* (*11*−*14*), were used to construct subtilisin YaB expression vectors, and the expression efficiencies of ECS*cdd*, ECS*veg*, and SECS were evaluated. In addition, the UP element and SD sequence or both of the SECS were engineered and the effects of their modification on the expression of subtilisin YaB examined. The objective of this study was to develop a new expression plasmid for the high-level expression of subtilisin YaB in *B. subtilis*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *E. coli* JM109 [*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(rK⁻mK⁺), e14⁻(*mcrA*⁻), *supE*44, *relA*1, Δ(*lac-proAB*)/F′ [*traD*36, *proAB*⁺, *lacI*ᵠ, *lacZ*ΔM15]] was used as the host strain for DNA manipulation, whereas *B. subtilis* DB104 (*his nprR2 nprE18 aprΔ3*) (*15*), deficient in two extracellular proteases, was used as the expression host. Luria−Bertani (LB) agar plates containing tetracycline (Tc; 25 μg/mL) were used for selection of transformants, and the same plates supplemented with 1.5% skimmed milk were used for detection of protease activity. All liquid cultures were grown in LB, pH 7.0, supplemented with Tc (25 μg/mL) at 37 °C with vigorous aeration (180 rpm).

Recombinant DNA and Genetic Manipulations. Enzymes were purchased from Promega (Madison, WI) and used as recommended by the supplier. The primers were purchased from Invitrogen Life

Technologies (Carlsbad, CA) (**Table 1**). *Pfu* DNA polymerase (MBI Fermentas, Hanover, MD) and Ex T*aq* DNA polymerase (Takara, Kyoto, Japan) were used for PCR amplifications. Electrotransformation of *E. coli* JM109 was carried out at a field strength of 12.5 kV/cm, capacitance of 25 μF, and resistance of 200 Ω using a Gene Pulser II electroporation apparatus (Bio-Rad, Hercules, CA). Electrotransformation of *B. subtilis* DB104 was carried out at a field strength of 8.75 kV/cm, capacitance of 25 μF, and resistance of 500 Ω, as described previously (*16*). Plasmid DNA was isolated using a Mini-M plasmid DNA extraction system (Viogene, Taipei, Taiwan). *B. subtilis* genomic DNA was isolated using a blood and tissue genomic DNA extraction system (Viogene). PCR products were purified using a PCR-M clean-up system (Viogene). DNA fragments were recovered from gels using a Gel-M gel extraction system (Viogene).

Construction of Subtilisin YaB Expression Vectors. Plasmid pEX600A (*17*) is a pHY300PLK derivative carrying the entire *ale* gene with its own expression control sequence (ECS*ale*). A 990 bp *Sal*I−*Xba*I fragment from pQE30 (Qiagen, Valencia, CA) containing the Lambda *t0* and *rrnB* T1 terminators was inserted into *Sal*I-/*Xba*I-digested PEX600A to generate pEX600T, selected to be the parental plasmid for the construction of variant expression vectors with different expression control sequences. SECS was amplified by PCR using pEX5A (*7*) as template and the SDF-1/UPR-1 primer set. For engineering the UP element and SD sequence of SECS, pEX5A was used as template in the PCR. Replacing the *vegP* UP element (UP_{vegP}) of SECS with the conserved UP element ($UP_{conserved}$) was achieved by using the UPF-1/UPR-1 primer set. Replacing the UP_{vegP} element of SECS with the *hagP* UP element (UP_{hagP}) was achieved by using the UPF-2/UPR-1 primer set. Replacing the SD2 of SECS with the optimal SD was achieved by using the SDF-1/SDR-1 primer set. Replacing the UP_{vegP} and the SD2 of SECS with the UP_{hagP} and the optimal SD was achieved by using the UPF-2/SDR-1 primer set. PCR reactions were performed with *Pfu* DNA polymerase by the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 1 min; 72 °C for 7 min. The PCR products were isolated from the agarose gel, digested with *Eco*RI and *Nde*I, and inserted into pEX600T digested with the same enzymes. The resulting plasmids were named $pSECS_{ALET}$, $pSECS\text{-}1_{ALET}$, $pSECS\text{-}2_{ALET}$, $pSECS\text{-}3_{ALET}$, and $pSECS\text{-}4_{ALET}$, respectively. The expression control sequences of the *cdd* gene (ECS*cdd*) and the *veg* gene (ECS*veg*) were amplified by PCR using *B. subtilis* genomic DNA as template with the primer sets P43F/P43R and PvegF/PvegR primer sets, respectively. PCR for amplifying ECS*cdd* was performed with Ex T*aq* DNA polymerase with the following conditions: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; 72 °C for 7 min. PCR for amplifying ECS*veg* was performed with Ex T*aq* DNA polymerase with the following conditions: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; 72 °C for 7 min. The PCR products were isolated from the agarose gel, digested with *Bgl*II and *Nde*I, and inserted into $pSECS\text{-}2_{ALET}$ digested with the same enzymes; the resulting plasmids were named $p43_{ALET}$ and pWV_{ALET}, respectively. All constructs were verified by DNA sequencing.

Enzyme Activity Assays. Extracellular production of subtilisin YaB was detected by the formation of a clear zone around the colony on a skimmed milk plate after incubation for 12 h at 37 °C. Caseinolytic activity was measured using casein as substrate and performed as described previously (*1*). One unit of enzyme activity was defined as the amount of enzyme required to release 1 μg of tyrosine per minute.

SDS-PAGE and Western Blot Analysis. Culture supernatants of *B. subtilis* transformants were collected after 60 h of culture and analyzed by SDS-PAGE and Western blot. Each supernatant was incubated with 1 mM (final concentration) phenylmethanesulfonyl fluoride (PMSF) to inhibit autolytic degradation of subtilisin YaB prior to analysis on SDS-PAGE and Western blot. Aliquots of 16 μL of each supernatant were mixed with 4 μL of 5× SDS-PAGE sample buffer and heat denatured at 100 °C for 5 min. SDS-PAGE was performed on 12.5% (w/v) polyacrylamide gels according to the method of Laemmli (*18*), using a minigel apparatus (model AE-6450; Atto, Tokyo, Japan). A prestained protein ladder (MBI Fermentas, Hanover, MD) was used for molecular weight comparison. Proteins present in the SDS−polyacrylamide gel were stained with Coomassie Brilliant Blue R-250. For Western blot analysis, proteins present in the SDS−polyacrylamide gels were transferred to a polyvinylidene fluoride Immobilon-P membrane (Millipore, Bedford, MA) with electroblotting buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 10% (v/v) methanol, pH 11] using an electroblotting system (model VEP-2, Owl, Portsmouth, NH) at 200 mA for 1 h. The membrane was then incubated with blocking solution [20 mM Tris-base (pH 7.4), 150 mM NaCl, 5% skimmed milk] for 1 h, and the proteins were immunodetected using the polyclonal rat antibody against subtilisin YaB, horseradish peroxidase-conjugated goat anti-rat IgG (H+L) (Pierce, Rockford, IL), and color development reagent [12.5 mL of TBS buffer (20 mM Tris-base, pH 7.4, 150 mM NaCl), 2.5 mL of methanol containing 2.5 mg/mL 4-chloro-1-naphthol, 50 μL 35% (v/v) hydrogen peroxide]. The relative amounts of proteins in the SDS−polyacrylamide gel and PVDF membrane were estimated with Gel-Pro Analyzer version 3.0 software (Media Cybernetics, Silver Spring, MD).

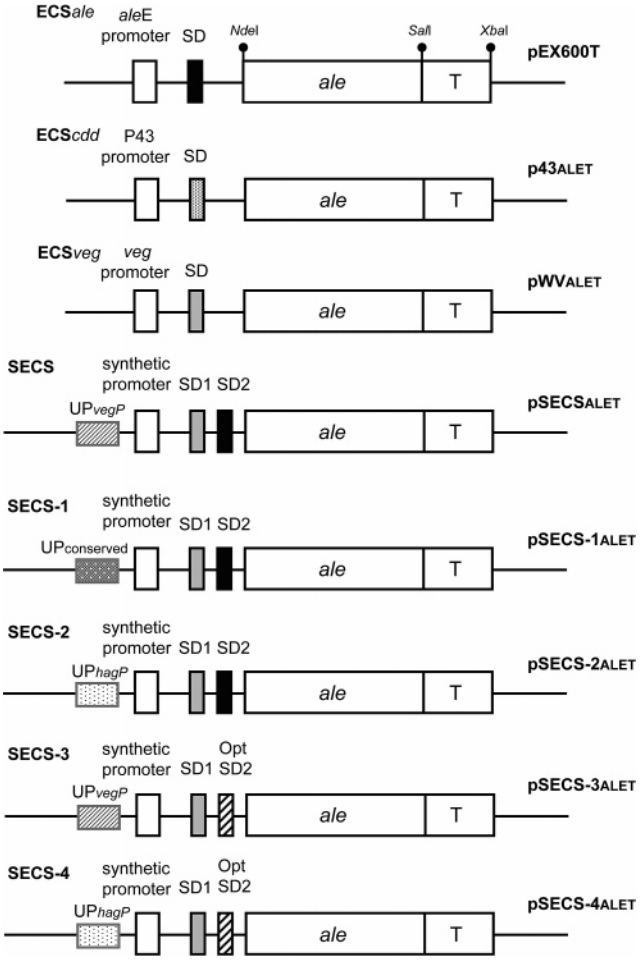

Figure 2. Schematic representation of the plasmids constructed in this study. T is a DNA fragment carrying the Lambda *t0* and *rrnB* T1 terminators. Opt SD2 is an optimal SD2 sequence.

Statistical Analysis. The data on the enzyme activity are expressed as means ± standard deviation, and difference among samples was determined by Duncan's multiple-range test using the Statistical Analysis System (SAS release 8.2) program (SAS Institute Inc., Cary, NC). A level of $P < 0.05$ was used as the criterion for statistical significance.

RESULTS AND DISCUSSION

Introducing Terminators Downstream of the *ale* Improves Subtilisin YaB Expression in *B. subtilis* DB104. The entire gene *ale* with its own ECS (ECS*ale*) has previously been inserted into an *E. coli*−*B. subtilis* shuttle vector pHY300PLK

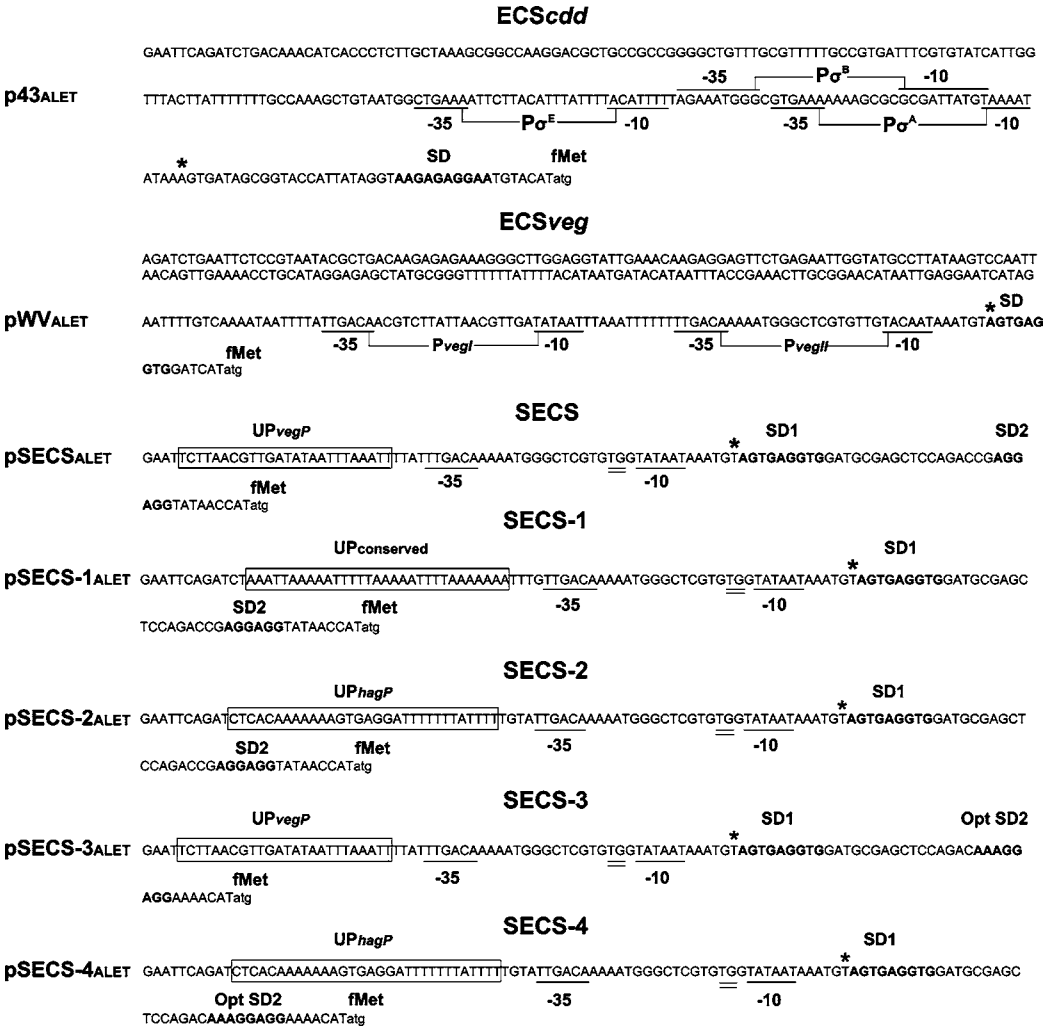

Figure 3. Nucleotide sequences of the upstream and downstream regions flanking the main transcriptional start sites of expression control sequences. The –35 and –10 regions (lines), transcription start sites (asterisk), SD sequences (bold), TG motif (double lines), and translational start sites (lower case letters) of these expression control sequences are shown.

(*17*). In the present study, a DNA fragment containing the Lambda *t0* and *rrnB* T1 terminators was inserted into the downstream region of *ale* in plasmid pEX600A to generate pEX600T. Lambda *t0* and *rrnB* T1 terminators, two well-characterized terminators, are reported to be utilized extremely efficiently in *B. subtilis* (*19*, *20*) and are valuable in the construction of expression plasmids for *B. subtilis*, because they not only reduce plasmid destabilization resulting from transcription readthrough but also increase the half-life of mRNA (*21*). In this study, it was found that expression of subtilisin YaB in *B. subtilis* DB104 was increased in the presence of terminators. In *B. subtilis* (pEX600T), the level of subtilisin YaB expression (138.74 units/mL) was significantly increased by 2.19-fold at 60 h when compared with that of the *B. subtilis* (pEX600A) (63.18 units/mL). Accordingly, the plasmid pEX600T was then used as the backbone for the construction of a series of subtilisin YaB expression vectors harboring various expression control sequences.

Amplification of Expression Control Sequences by Using PCR. Seven expression control sequences including ECS*cdd*, ECS*veg*, SECS, and SECS-1−4 were amplified from either *B. subtilis* genomic DNA or plasmid pEX5A (**Figure 1**). These expression control sequences were used to construct $p43_{ALET}$, pWV_{ALET}, $pSECS_{ALET}$, pSECS-1_{ALET}, pSECS-2_{ALET}, pSECS-3_{ALET}, and pSECS-4_{ALET}, respectively. A detailed description of construction of subtilisin YaB expression vectors is given under Materials and Methods. A schematic representation of subtilisin YaB expression vectors is shown in **Figure 2**. Nucleotide sequences of expression control sequences are shown in **Figure 3**. All expression plasmids were transformed into *B. subtilis* DB104. Using a skimmed milk plate assay, it was found that all transformants harboring subtilisin YaB expression plasmids were capable of producing subtilisin YaB (data not shown). After the expression of subtilisin YaB had been confirmed using the plate assay, subtilisin YaB caseinolytic activity was measured.

Expression Efficiency of SECS Is Superior to That of ECS*ale*, ECS*cdd*, and ECS*veg*. ECS*cdd* and ECS*veg*, two well-studied expression control sequences, have been frequently used to drive the expression of homologous and heterologous proteins in *B. subtilis* (*22*−*25*); ECS*cdd*, in particular, is reported to be a strong expression control sequence for *B. subtilis* (*26*). No comparison of the expression efficiencies of ECS*cdd*, ECS*veg*, and SECS has been made. In this study, these three expression control sequences were used for the construction of subtilisin YaB expression plasmids $p43_{ALET}$, pWV_{ALET}, and $pSECS_{ALET}$, respectively. Expression efficiencies of ECS*ale*, ECS*cdd*, ECS*veg*, and SECS were evaluated. Of the transformants harboring pEX600T, $p43_{ALET}$, pWV_{ALET}, or $pSECS_{ALET}$, that harboring $pSECS_{ALET}$ yielded the highest activity (837.29

Table 2. Extracellular Subtilisin YaB Activities of *B. subtilis* DB104 Transformants Harboring Various Expression Vectors

B. subtilis transformant	expression control sequence	subtilisin YaB activity[a] (units/mL)	fold[b]
DB104 (pHY300PLK)[c]		0	0
DB104 (pEX600T)	ECS*ale*	138.47 ± 4.27f	1
DB104 ($p43_{ALET}$)	ECS*cdd*	134.46 ± 8.55f	0.97
DB104 (pWV_{ALET})	ECS*veg*	272.75 ± 10.76e	1.97
DB104 ($pSECS_{ALET}$)	SECS	837.29 ± 6.91d	6.05
DB104 ($pSECS\text{-}1_{ALET}$)	SECS-1	803.08 ± 92.36d	5.80
DB104 ($pSECS\text{-}2_{ALET}$)	SECS-2	938.90 ± 42.38c	6.78
DB104 ($pSECS\text{-}3_{ALET}$)	SECS-3	1329.13 ± 53.13b	9.60
DB104 ($pSECS\text{-}4_{ALET}$)	SECS-4	1525.87 ± 31.53a	11.02

[a] Sixty-hour broth cultures of *B. subtilis* DB104 harboring various plasmids were centrifuged (10000*g*; 10 min) and the supernatants assayed for enzyme activity. Experiments were performed in triplicate, and each data point represents the mean value ± standard deviation; different letters (a–f) in a row indicate significant differences ($p < 0.05$). [b] Fold refers to the enzyme activity compared to that of *B. subtilis* DB104 (pEX600T). [c] *B. subtilis* DB104 (pHY300PLK) was used as negative control.

units/mL) after 60 h of culture (**Table 2**). Thus, the expression efficiency of SECS was higher than that of ECS*ale*, ECS*cdd*, or ECS*veg*.

SECS was designed on the basis of ECS*veg*; the −10 and −16 regions differ, being TATAAT and TG in SECS and TACAAT and TT in ECS*veg*, respectively. In addition, a transcriptional start site, two SD sequences, and a translational start site were incorporated into SECS. Our results show that SECS could be used to enhance secretory production of subtilisin YaB in *B. subtilis* DB104.

Engineering the UP Element and SD Sequence of the SECS Increases Production of Subtilisin YaB. Transcription, the first step and a key control point in gene expression, is initiated at a transcription start site and terminated at a transcription terminator. It is known that several structural elements such as the UP element, core promoter (−10 region, −35 region, and spacer region), and downstream sequence region affect transcription efficiency (*27*). The UP element, a DNA sequence rich in (A+T) located upstream of the −10 and −35 regions of some bacteria promoters, stimulates promoter activity by forming a docking site for the C-terminal domain of the RNA polymerase α subunit (αCTD). UP elements are independent promoter modules, as they can stimulate transcription when fused to other promoters (*9*, *28*, *29*).

In the present study, the UP element of SECS (UP_{vegP}) was modified to $UP_{conserved}$ and UP_{hagP}. The $UP_{conserved}$ nucleotide sequence was determined by examination of the −40 to −70 regions of 133 σ^A-dependent promoters (sequences not shown) from http://www.micro.cornell.edu/faculty/helmann/table1.html (*30*). The UP_{hagP} nucleotide sequence was from a previous study (*9*) and has been shown to stimulate transcription from both σ^D- and σ^A-dependent promoters in *B. subtilis* (*9*). Our data showed that the presence of UP_{hagP}, but not $UP_{conserved}$, in the SECS had a positive effect on subtilisin YaB expression (**Table 2**). The expression of subtilisin YaB in *B. subtilis* ($pSECS\text{-}2_{ALET}$) (938.51 units/mL) was 1.12-fold higher than that of *B. subtilis* ($pSECS_{ALET}$) (837.29 units/mL). A previous paper showed that modification of UP_{vegP} of P_{veg} to UP_{hagP} increased the expression of β-galactosidase by about 2.23-fold (*9*); however, the improvement in gene expression by UP_{hagP} was not as significant in our system. One possible explanation is that SECS is easily recognized by RNA polymerase and that replacement of UP_{vegP} with UP_{hagP} did not significantly increase the RNA polymerase−promoter interaction.

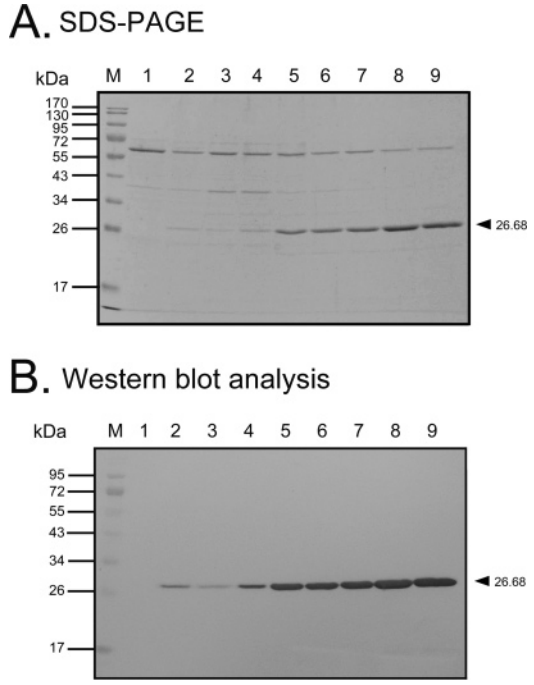

Figure 4. SDS-PAGE and Western blot analysis of extracellular subtilisin YaB produced by *B. subtilis* transformants harboring different plasmids: M, molecular weight markers; lane1, DB104 (pHY300PLK); lane 2, DB104 (pEX600T); lane 3, DB104 ($p43_{ALET}$); lane 4, DB104 (pWV_{ALET}); lane 5, DB104 ($pSECS_{ALET}$); lane 6, DB104 ($pSECS\text{-}1_{ALET}$); lane 7, DB104 ($pSECS\text{-}2_{ALET}$); lane 8, DB104 ($pSECS\text{-}3_{ALET}$); lane 9, DB104 ($pSECS\text{-}4_{ALET}$).

High-level expression is dependent not only on the efficiency of transcription but also on the efficiency of translation initiation (*31*). Factors influencing translation initiation include the SD sequence, the start codon, and the region between these two sequences.

Two SD sequences, SD1 (AGTGAGGTG) from *veg* and SD2 (AGGAGG) from *ale*, are present in SECS; the SD2 sequence is near the initiation codon and was selected to be engineered. The strong SD sequence for *B. subtilis* is AAAGGAGG, and the optimal spacing between the SD and initiation codon is seven nucleotides (*10*). We therefore engineered the SD2 sequence of SECS into AAAGGAGG, and the spacing between the SD and initiation codon was modified to be seven nucleotides. Our data showed that modification of the SD2 sequence and the spacing between the SD2 and initiation codon could indeed increase subtilisin YaB production (**Table 2**). The effect of SD2 modification on the expression of subtilisin YaB was more significant than that of UP element modification. However, the effect of SD1 on the expression of subtilisin YaB is unclear; we will investigate the effect of SD1 in the near future.

Combined transcriptional and translational modification of the SECS gave the highest expression efficiency. In *B. subtilis*, the subtilisin YaB expression achieved using pSECS-4 (1525.87 units/mL) was 11-fold higher than that using pEX600T (138.74 units/mL). The results of SDS-PAGE and Western blot analysis (**Figure 4**) also indicate that *B. subtilis* (pSECS-4) gave the highest amount of subtilisin YaB in the culture medium. On the basis of the band density of Western blot, the subtilisin YaB produced from *B. subtilis* (pSECS-4) was 11-fold over that of *B. subtilis* (pEX600T).

In conclusion, this study demonstrates that the SECS and engineered SECSs increase the production of recombinant subtilisin YaB in *B. subtilis* DB104. The expression efficiencies of SECS and engineered SECSs were higher than those of ECS*ale*, ECS*cdd*, and ECS*veg*. Both native SECS and engineered SECSs might be useful for expressing various protein-engineered subtilisin YaBs (*17*, *32*). In addition, these SECSs could potentially be used to express a wide variety of heterologous proteins in *B. subtilis* at high levels. Studies on the application of SECSs in the expression of homologous and heterologous proteins in *B. subtilis* and other *Bacillus* probiotics are in progress.

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