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Heterologous Production of Streptokinase in Secretory Form in *Streptomyces lividans* and in Nonsecretory Form in *Escherichia coli*

Kim, Mi-ran[1], Yong-Hoon Choeng[1], Won-Jae Chi[1], Dae-Kyung Kang[2], and Soon-Kwang Hong[1]*

[1]*Department of Biological Science, Myongji University, Yongin 449-728, Korea*
[2]*Department of Animal Resources Science, Dankook University, Cheonan 330-714, Korea*

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The *skc* gene encoding streptokinase (SK) with a molecular mass of approximately 47.4 kDa was cloned from *Streptococcus equisimilis* ATCC 9542 and heterologously overexpressed in *Streptomyces lividans* TK24 and *E. coli* using various strong promoters. When the promoter for *sprT* [*Streptomyces griseus* trypsin (SGT)] was used in the host *S. lividans* TK24, a 47.4-kDa protein was detected along with a smaller hydrolyzed protein (44 kDa), suggesting that posttranslational hydrolysis had occurred as has been reported in other expression systems. The casein/plasminogen plate assay revealed that the plasmid construct containing the SGT signal peptide was superior to that containing the SK signal peptide in terms of SK production. Maximal production of SK was calculated to be about 0.25 unit/ml of culture broth, a value that was five times higher than that obtained with other expression systems using *ermE* and *tipA* promoters in the same host. When the *skc* gene was expressed in *E. coli* BL21(ΔDE3)pLys under the control of the T7 promoter, a relatively large amount of SK was expressed in soluble form without hydrolysis. SK activity in *E. coli*/pET28a-T7$_p$SK$_m$ was more than 2 units/ml of culture broth, even though about half of the expressed protein formed an inactive inclusion body.

Keywords: Streptokinase, *Streptococcus equisimilis*, *skc*, *Streptomyces*, *E. coli*

Streptokinase (SK) is a simple polypeptide of 415 amino acid residues without disulfide bonds that is secreted by Lancefield group C *Streptococcus*. SK lyses blood clots by converting the plasma zymogen plasminogen to the active fibrinolytic enzyme plasmin [9]. Thus, SK has been thought to play an important role in streptococcal virulence by facilitating the invasion of host tissues *via* proteolysis at the bacterial cell surface [1]. For practical purposes, SK has been widely used as a thrombolytic agent in the treatment of acute myocardial infarction because it is a potent activator of human plasminogen. SK cannot catalyze the proteolytic cleavages necessary to convert plasminogen to plasmin [3], a characteristic that differs from those of other plasminogen activators. Instead, SK forms complexes with human plasminogens, generating the proteolytic active site of the plasminogen moiety, and then converting the free plasminogen to plasmin by the hydrolysis of a specific peptide bond, such as Arg560–Val561 [22].

Recently, we constructed a new expression system composed of the *sprT* [*Streptomyces griseus* trypsin (SGT)] promoter and its two regulatory genes, *sgtR1* and *sgtR2* [5, 11, 25]. Although there have been many reports on the overexpression of SK, severe problems such as posttranslational proteolysis and formation of an insoluble inclusion body have also been indicated [4, 16]. In this study, the *skc* gene encoding SK from *Streptococcus equisimilis* ATCC 9542 [14] was expressed in our system, and the level of expression was compared with that in other systems using *ermE* and *tipA* promoters in *Streptomyces* and the T7 promoter in *Escherichia coli.*

*Corresponding author
Phone: +82-31-330-6198; Fax: +82-31-335-8249;
E-mail: skhong@mju.ac.kr

Materials and Methods

Bacterial Strains and Plasmids

Streptomyces lividans TK24 was obtained from the John Innes Institute, U.K.. *E. coli* BL21(ΔDE3)pLysS (Stratagene) and the cloning vector pET28a (Novagen) were used for overexpression. The *Streptomyces–E. coli* shuttle vector pWHM3-TR1R2 [18] and the strong expression vectors, pUWL201PW containing the *ermE* promoter [7] and pSEV1 containing the *tipA* promoter derived from pIJ4123 [24], were used for overexpression in *Streptomyces*.

Media and Culture Conditions

E. coli maintained on M9 minimal agar was routinely cultured in LB medium at 37°C with agitation [21]. *Streptomyces* strains were

maintained on R2YE plates (2% agar) and were grown in R2YE liquid broth at 28°C for the preparation of protoplasts and isolation of plasmid DNA [12].

Enzymes and Chemicals

Restriction endonucleases, T4 DNA ligase, and *Taq* polymerase were purchased from Takara Shuzo Inc., Japan. PCR primers were obtained from DyneBio Inc., Korea. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

DNA Manipulations

DNA preparation and manipulations were performed in *E. coli* using methods described by Sambrook and Russell [21]. DNA samples were digested with restriction endonucleases and ligated using T4 DNA ligase according to the supplier's recommendations. DNA digests were analyzed by horizontal agarose gel electrophoresis in TAE buffer [21].

Transformation Procedure

Competent *E. coli* strains were routinely prepared according to the frozen storage protocol, and transformations were performed as described previously [8]. *Streptomyces* protoplasts were prepared as described by Okanishi *et al.* [19]. The resulting protoplasts were transformed using the PEG-mediated transformation method, and transformants were selected by overlaying with 2.5 ml of 0.6% soft R2YE agar containing 25 μg/ml of thiostrepton [12].

Construction of Expression Vectors for *skc*

Various expression vectors for *skc* were constructed with different promoters (Fig. 1). First, a 432-bp fragment (EcoRI/NdeI) encompassing the *sprT* promoter and signal peptide, a 1,266-bp fragment (NdeI/NotI) encoding mature SK, and a 1,214-bp fragment (NotI/SphI) encompassing the *sgtR1* and *sgtR2* genes were amplified by PCR using the primers listed in Table 1. PCR products were ligated into pWHM3 digested with EcoRI and SphI, yielding pWHM3-$T_pT_{sp}SK_m$R1R2. pWHM3-$T_pSK_{sp}SK_m$R1R2 was constructed by ligation of a 318-bp fragment (EcoRI/NdeI) encompassing the *sprT* promoter, a 1,325-bp fragment (NdeI/NotI) containing the entire coding region for *skc* (signal and mature SK peptides), and a 1,214-bp fragment (NotI/EcoRI) encompassing the *sgtR1* and *sgtR2* genes. Another fragment containing the entire SK coding region but with different restriction enzyme sites (NdeI/EcoRI) was amplified and subcloned into pUWL201PW and pSEV1 containing the strong *Streptomyces* promoters *ermE* and *tipA*, respectively, to produce pUWL201PW-$ermE_pSK_{sp}SK_m$ and pSEV1-$tipA_pSK_{sp}SK_m$. The DNA fragment encoding mature SK was amplified with primers SKm-F and SKm-R (Table 1) and inserted into pET28a digested with NdeI and NotI, resulting in pET28a-$T7_pSK_m$. Restriction maps of the constructs are

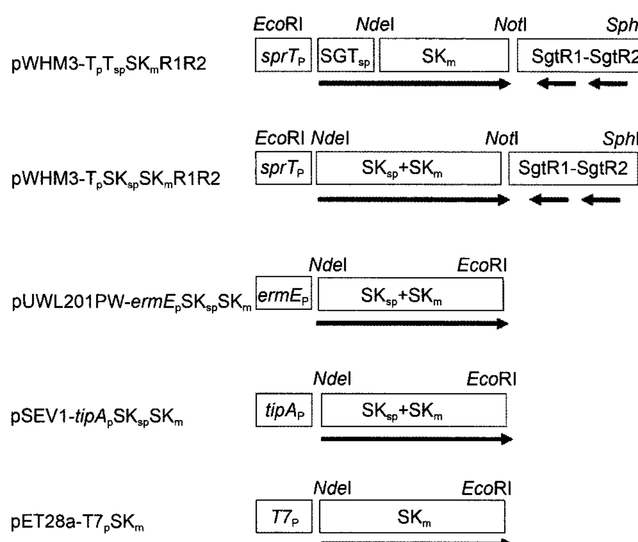

Fig. 1. Construction of expression vectors for the *skc* gene.
Arrows indicate individual ORFs. Restriction sites used for cloning are depicted. Promoters used for expression are represented by subscript p such as in $sprT_p$, $ermE_p$, $tipA_p$, and $T7_p$. SK_{sp} and SK_m stand for signal and mature SK peptides, respectively. SgtR1R2 indicates the positive regulatory proteins SgtR1 and SgtR2 for *sprT* expression.

Table 1. Primers used for PCR.

Primer	Oligonucleotide[a]
For cloning of $sprT_p$ and SGT signal peptide (SGT_{sp})	
$sprT_pSGT_{sp}$-F	5'-CGGCA***GAATTC***TAGGGCGGCCCGCCC-3' (*Eco*RI)
$sprT_pSGT_{sp}$-R	5'-AAT***CATATG***GACGGGGTTGGGGGCGG-3' (*Nde*I)
For cloning of *sgtR1* and *sgtR2*	
SgtR1R2-F	5'-CACGCT***GCGGCCGC***ACGTACCGGCA-3' (*Not*I)
SgtR1R2-R	5'-CCTC***GCATGC***CGACCCCTGCTCCACC-3' (*Sph*I)
For cloning of mature form of SK (SK_m)	
SKm-F	5'-GTC***CATATG***ATTGCTGGACCTGAGTG-3' (*Nde*I)
SKm-R	5'-T***GCGGCCGC***TGGTTATTTGTCGTTAG-3' (*Not*I)
For cloning of $sprT_p$	
$sprT_p$-F	5'-CGGCA***GAATTC***TAGGGCGGCCCGCCC-3' (*Eco*RI)
$sprT_p$-R	5'-***CATATG***GGATTGCCTTCTTTCGTGGG-3' (*Nde*I)
For cloning of signal peptide plus mature form of SK (SK_{sp}-SK_m)	
SK_{sp}-SK_m-F	5'-CC***CATATG***AAAAAGACAGCTATCGCG-3' (*Nde*I)
SK_{sp}-SK_m-R	5'-T***GCGGCCGC***TGGTTATTTGTCGTTAG-3' (*Not*I)

[a]Restriction enzyme sites introduced for subsequent cloning of DNA fragments are shown in italics; the corresponding restriction enzymes are shown in parentheses.

shown in Fig. 1. All recombinant plasmids were purified from *E. coli* and used for protoplast transformation of *Streptomyces*.

Sample Preparation from *Streptomyces*

Streptomyces transformants harboring each of the recombinant plasmids were grown in 100 ml of R2YE medium containing thiostrepton (25 μg/ml) in 500-ml baffled flasks at 28°C with vigorous shaking at 250 rpm. After 2 days of cultivation, 10 ml of culture broth was used to inoculate 100 ml of various liquid media in 500-ml baffled flasks maintained under the same conditions. Each day, 5 ml of culture broth was removed and centrifuged at 5,000 ×*g* for 10 min. The supernatant was fractionated with 80% saturated ammonium sulfate and the precipitate was used for measuring SK activity after dialyzing against SK assay buffer [100 mM K_2HPO_4/KH_2PO_4 (pH 6.0), 100 mM NaCl, and 10 mM L-lysine]. The cell pellet was disrupted by sonication and used to quantify cellular protein.

Sample Preparation from *E. coli*

The *E. coli* transformant was cultured in 50 ml of LB medium supplemented with kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml), in a 250-ml Erlenmeyer flask at 37°C and 200 rpm, to an OD_{600} of 0.5. IPTG (1 mM) was then added and the culture was allowed to grow for an additional 4 h at 37°C. Cells were harvested by centrifugation (5,000 ×*g*, 10 min), resuspended, and disrupted by sonication in disruption buffer [25 mM K_2HPO_4/KH_2PO_4 (pH 7.5), 300 mM NaCl, 20 mM imidazole, 0.2 mM $CoCl_2$, and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)]. Cell debris was removed by centrifugation and the protein solution was mixed with Ni^{2+}–nitrilotriacetic acid (NTA) agarose and left to stand for 1 h. The agarose was washed three times with the same buffer and the His_6-tagged protein was eluted with buffer containing 150 mM imidazole. The eluted protein was dialyzed in SK assay buffer and concentrated by ultrafiltration with a 30 kDa cutoff.

Protein Analysis

The protein concentration of the sample was measured using a Bradford protein microassay kit (Bio-Rad) with bovine serum albumin as the standard [2]. Protein samples were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli [15].

Determination of SK Activity by Casein/Plasminogen Plate Technique

The SK activity of the protein sample was estimated by comparison with a purified standard SK solution, using the casein/plasminogen plate technique [17]. Plates were overlayed with 9 ml of 50 mM Tris HCl (pH 8.1) and 150 mM NaCl containing 90 mg of agar, 100 μg of human plasminogen, and 1 ml of skim milk. Wells were cut in the agar and filled with 100–200 μl of samples. After incubation for a minimum of 3 h at 30°C, clear zones surrounding the wells indicated samples possessing SK activity.

Results and Discussion

Production of SK in *S. lividans* TK24

SK, with a molecular mass of approximately 47.4 kDa, is an extracellular protein that is produced by β-hemolytic streptococci groups. Because SK has been widely used as a thrombolytic agent in the treatment of acute myocardial infarction, its overexpression in various prokaryotic systems has been intensively studied. However, when the cloned gene for SK was expressed in *Streptococcus sanguis*, a peptide of about 44 kDa was generated by the posttranslational proteolysis of carboxyl-terminal residues [10]. In addition, it has also been reported that SK expressed in *E. coli*, *Bacillus subtilis*, *Proteus mirabilis*, and *Lactococcus lactis* [10, 13, 16, 17, 23] was present in mixed forms with sizes of 44 and 47.4 kDa.

To develop a better expression system for SK, various recombinant plasmids containing the *skc* gene under the control of strong *Streptomyces* promoters were constructed (Fig. 1). The plasmid pWHM3-TR1R2 has a strong promoter originating from *sprT*, encoding SGT as well as its two positive regulatory genes *sgtR1* and *sgtR2*. The present authors previously reported that *sgtR1* and *sgtR2* can stimulate *sprT* expression 5-fold in *S. lividans* TK24 [18]. The *skc* gene encoding SK with or without the signal sequence was subcloned in pWHM3 under the control of the *sprT* promoter, as described in Materials and Methods, and the resulting recombinant plasmids (pWHM3-TpTspSKmR1R2 and pWHM3-TpSKspSKmR1R2) were introduced into *S. lividans* TK24. When the culture broth of both types of transformants was concentrated and analyzed by SDS–PAGE, a protein band corresponding to 47.4 kDa was detected, which coincides with the expected molecular mass for SK (Fig. 2A). However, a smaller (44 kDa) protein assumed to be a hydrolytic product of SK was also detected, in agreement with other reports regarding several other expression systems. This result indicates that *skc* can be successfully expressed in the *Streptomyces* host–vector system, but the level of expression was quite low and posttranslational hydrolysis was unavoidable.

To evaluate SK activity, 10-fold concentrated samples of ammonium-sulfate-precipitated protein from the culture broth were subjected to the casein/plasminogen plate assay. *S. lividans* TK24/pWHM3-TpTspSKmR1R2 exhibited higher SK activity than did *S. lividans* TK24/pWHM3-TpSKspSKmR1R2 (Fig. 2B), suggesting that the use of the SGT signal peptide is preferred for SK secretion in the *S. lividans* host. Maximal SK activity was observed in the 8-day-old culture of *S. lividans* TK24/pWHM3-TpTspSKmR1R2; evaluation of the casein-hydrolyzed area revealed that this strain can produce as much as 0.25 unit/ml of culture broth (Fig. 2B).

The heterologous expression of *S. equisimilis* ATCC 9542 *skc-2* in *S. lividans* was previously reported by Pimienta *et al.* [20]. In that study, the SK structural gene was fused to the subtilisin inhibitor signal sequence of *Streptomyces venezuelae* (*vsi*) or to the xylanase C signal sequence of *S. lividans* (*xlnC*). SK could be successfully translocated *via* both systems in *S. lividans*, but the yield was about 30 times higher when it was fused to the Vsi

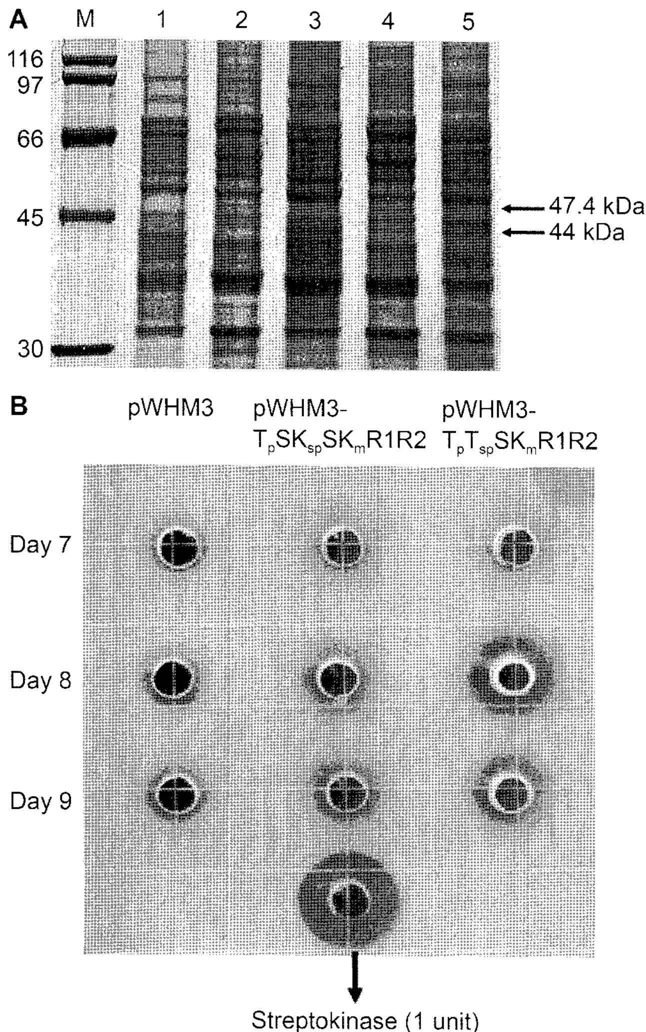

Fig. 2. SDS–PAGE (**A**) and SK activity assay (**B**) of the culture broth of *S. lividans* TK24 transformants.

A. Extracellular protein in *S. lividans* TK24 culture broth was precipitated with 80% ammonium sulfate and analyzed by SDS–PAGE. Lane M, molecular weight standards; lane 1, total extracellular protein from *S. lividans*/pWHM3 as the control; lanes 2 and 3, total extracellular protein from *S. lividans*/pWHM3-$T_pSK_{sp}SK_mR1R2$ after 8 and 7 days of cultivation, respectively; lanes 4 and 5, total extracellular protein from *S. lividans*/pWHM3-$T_pT_{sp}SK_mR1R2$ after 8 and 7 days of cultivation, respectively. Proteins with a molecular mass of 47.4 and 44 kDa are indicated by arrows. **B.** Casein/plasminogen plate assay for measuring SK activity (units/ml) of the transformants as a function of cultivation time. Protein samples prepared from 4 ml of bacterial culture broth were added to each well. Authentic SK (S3134; Sigma Chemical Co., U.S.A.) was used as the positive control.

signal peptide, which is translocated *via* the Sec pathway, versus the XlnC signal peptide, which is translocated *via* the twin-arginine translocation (TAT) pathway. Although the authors concluded that SK could be efficiently produced by their expression system, they were unable to detect SK activity in the culture broth or SK protein by SDS–PAGE of concentrated culture broth. A comparison with our results suggests that our expression system

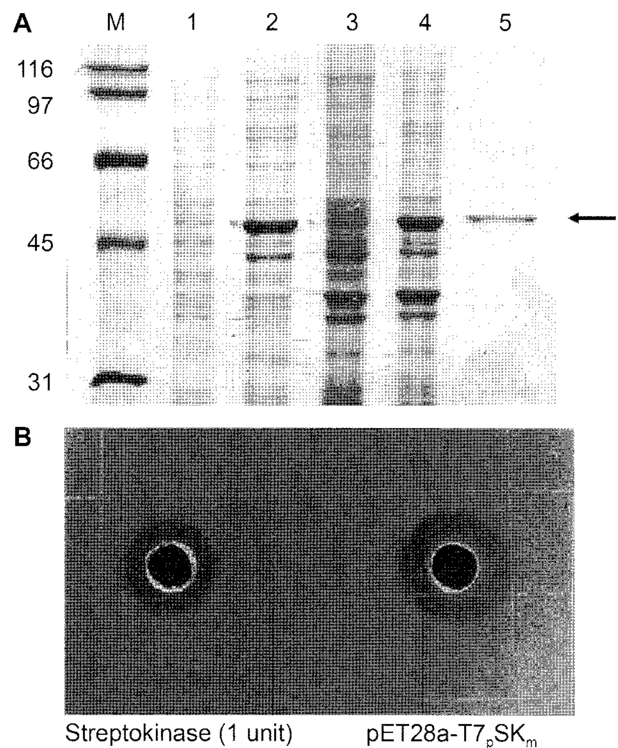

Fig. 3. SDS–PAGE (**A**) and SK activity assay (**B**) of *E. coli* BL21(ΔDE3)pLys overexpressing SK.

A. SDS–PAGE of the total cell lysate. *E. coli* cells were induced with IPTG and lysed by sonication, and then soluble and insoluble fractions were obtained by centrifugation. Lane M, molecular weight Standards; lanes d 1 and 3, total soluble and insoluble cellular proteins, respectively, from *E. coli*/pET28a as the control; lanes 2 and 4, total soluble and insoluble cellular proteins, respectively, from *E. coli*/pET28a-$T7_pSK_m$; lane 5, purified SK from *E. coli*/pET28a-$T7_pSK_m$. A protein with a molecular weight of 47.4 is indicated by the arrow. **B.** Casein/plasminogen plate assay for measuring SK activity (units/ml) of *E. coli*/pET28a-$T7_pSK_m$. Soluble fractions of total cell lysates prepared from 0.5 ml of bacterial culture were added to each well. Authentic SK was used as the positive control.

containing the *sprT* promoter and SGT signal sequence is superior for the production of SK. Pimienta *et al.* [20] also detected a 44-kDa degradation product along with the 47-kDa mature SK by ELISA during partial purification, a result that coincides with ours. In addition, they observed that SK activity reached a maximal level at 40 h of cultivation, and then sharply decreased to zero within 32 additional hours. In contrast, in our streptomycetes expression systems, SK production gradually increased until 8 days of cultivation, after which it gradually decreased, indicating that our systems are more stable.

In the present study, the DNA fragment containing the *skc* gene encoding the signal and mature SK peptides was also linked to other strong promoters that have been widely used in *Streptomyces*. However, use of the thiostrepton-inducible *tipA* promoter (pSEV1-*tipA*pSKspSKm) or the constitutive *ermE* promoter (pUWL201PW-*ermE*pSKspSKm) resulted in about 0.05 unit/ml of culture broth in *S.*

lividans, corresponding to one-half of the SK activity exhibited by *S. lividans*/pWHM3-TpSKspSKmR1R2. Owing to the low level of expression, the SK protein could not be detected by SDS–PAGE (data not shown).

Production of SK in *E. coli*

Because the level of SK expression in *Streptomyces* was not high enough to be satisfactory, an *E. coli* host–vector system was used for expression of *skc*. The *skc* gene encoding the mature SK peptide was cloned into pET28a to be transcribed from the T7 promoter, and then the recombinant plasmid (pET28a-T7$_p$SK$_m$) was introduced into *E. coli* BL21(ΔDE3)pLys. Total cellular protein was collected after IPTG induction and analyzed by SDS–PAGE. A relatively larger amount of the 47.4-kDa SK protein than was produced by *E. coli* was detected in the soluble and insoluble fractions of cell lysate obtained by centrifugation (Fig. 3A). The SK protein expressed with a C-terminal His-tag could be purified to homogeneity from the soluble fraction by Ni^{+2}-agarose affinity column chromatography.

To assess SK activity in the *E. coli* host–vector system, the soluble fraction of *E. coli*/pET28a-T7$_p$SK$_m$ total cell lysate was subjected to the casein/plasminogen plate assay. The diameter of the casein hydrolytic zone generated by a protein sample corresponding to the amount prepared from 0.5 ml of culture was significantly larger than that generated by 1 unit of authentic SK, suggesting that this strain can produce much more than 2 units of SK per milliliter of culture.

In this study, we constructed various expression systems for *skc* in *Streptomyces* and *E. coli* hosts. In the *Streptomyces* systems, the level of expression was much lower than expected. The effectiveness of the three promoters (*sprT$_p$*, *tipA$_p$*, *ermE$_p$*) used in this study has been verified in many previous instances, suggesting that they are not the main cause of the low level of expression in *Streptomyces*. Therefore, we tentatively suggest that the presence of many rare codons in the *skc* gene that were not adopted by *Streptomyces* genes could be a major reason for the low level of expression in the *Streptomyces* host. In fact, codons such as UUU, UCU, UUA, UAA, CUA, and AGA constitute less than 0.1% of the codons in *Streptomyces* genes, meaning that they are not generally adopted as the normal codons [11]. However, among the 436 SK codons, 25.2% (UUU), 11.5% (UCU), 27.5% (UUA), 2.3% (UAA), 20.6% (CUA), and 6.9% (AGA) are rare. The presence of these rare codons may reduce translational efficiency, resulting in a low level of SK expression. In general, it is known that most genes from *E. coli* or *Bacillus* cannot be expressed in a *Streptomyces* host because of biased codon usage. To evaluate this assumption, it would be necessary to change rare codons into common ones for expression in *Streptomyces* hosts, but the presence of many rare codons would make such an attempt too laborious. Conversely, some streptomycetes genes have been successfully expressed with improved efficiency in *E. coli* hosts fortified with rare codons, such as the *Streptomyces pristinaespiralis*-derived streptogramin-dependent repressor PIP in *E. coli* BL21(ΔDE3)pLysS [6]; such results may provide indirect evidence in support of our assumption.

In contrast with the *Streptomyces* systems, the *E. coli* BL21(ΔDE3)pLys host–vector system exhibited a significantly higher level of SK expression. Although proteolysis of SK

Table 2. Comparison of codon frequency of the *skc* gene with 100 *Streptomyces* genes.

[Triplet codon] [frequency/thousand] ([frequency/thousand in 100 *Streptomyces* genes][a])											
UUU	**25.2**	**(0.45)**	**UCU**	**11.5**	**(0.61)**	**UAU**	**29.8**	**(1.12)**	UGU	0.0	(1.06)
UUC	11.5	(27.51)	UCC	2.3	(21.07)	UAC	20.6	(21.13)	UGC	0.0	(7.72)
UUA	**27.5**	**(0.42)**	**UCA**	**6.9**	**(1.28)**	**UAA**	**2.3**	**(0.13)**	UGA	0.0	(2.59)
UUG	13.8	(2.50)	UCG	2.3	(14.73)	UAG	0.0	(0.48)	UGG	2.3	(15.21)
CUU	**6.9**	**(1.95)**	**CCU**	**18.3**	**(1.51)**	**CAU**	**13.8**	**(1.57)**	CGU	18.3	(6.02)
CUC	9.2	(36.73)	CCC	6.9	(23.19)	CAC	9.2	(22.67)	CGC	4.6	(37.24)
CUA	**20.6**	**(0.29)**	**CCA**	**20.6**	**(0.86)**	**CAA**	**34.4**	**(1.60)**	CGA	9.2	(3.04)
CUG	13.8	(53.57)	CCG	4.6	(29.17)	CAG	4.6	(24.47)	CGG	2.3	(31.19)
AUU	**27.5**	**(1.44)**	**ACU**	**22.9**	**(1.47)**	**AAU**	**20.6**	**(1.09)**	**AGU**	**11.5**	**(1.57)**
AUC	20.6	(29.88)	ACC	27.5	(42.75)	AAC	36.7	(22.38)	AGC	20.6	(14.57)
AUA	**9.2**	**(1.15)**	**ACA**	**18.3**	**(1.60)**	**AAA**	**59.6**	**(1.38)**	**AGA**	**6.9**	**(0.93)**
AUG	11.5	(16.20)	ACG	6.9	(19.79)	AAG	18.3	(22.19)	AGG	0.0	(4.26)
GUU	20.6	(2.24)	GCU	43.6	(3.04)	GAU	50.5	(2.98)	GGU	16.1	(7.91)
GUC	20.6	(44.93)	GCC	2.3	(76.54)	GAC	39.0	(60.59)	GGC	11.5	(58.44)
GUA	**9.2**	**(1.99)**	GCA	11.5	(5.60)	GAA	43.6	(10.41)	GGA	13.8	(8.13)
GUG	6.9	(31.13)	GCG	9.2	(45.25)	GAG	22.9	(48.55)	GGG	6.9	(16.52)

[a]The data for frequency/thousand in 100 *Streptomyces* genes is from Kieser *et al.* [12].

or formation of an inclusion body has been observed in many *E. coli* host–vector systems [4], our results clearly indicate that this *E. coli* system can produce at least 2 units of active SK per milliliter of culture without proteolysis. Other attempts to produce SK in *E. coli* were designed to secrete mature peptide using various signal peptides [14, 17]; however, our method of expressing SK within the cell seems to be one way to avoid proteolysis. Unfortunately, our SK activity assay is quite different from those described elsewhere, making a direct comparison of results impossible. On the other hand, the SK protein accounted for more than 50% of the total soluble protein, as determined by SDS–PAGE (Fig. 3A), suggesting that this system is superior to other *E. coli* host–vector systems previously reported. Moreover, there is much room for improvement in the efficiency of SK production by suppressing the formation of an insoluble inclusion body.

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