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(∆DE3)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,SKm 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

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

Kim, Mi-ran¹, Yong-Hoon Choeng¹, Won-Jae Chi¹, Dae-Kyung Kang², and Soon-Kwang Hong¹*

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

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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.

MATERIALS AND METHODS

Bacterial Strains and Plasmids
Streptomyces lividans TK24 was obtained from the John Innes Institute, U.K.. E. coli BL21(∆DE3)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 NotI) encoding mature SK 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].

A 1,325-bp fragment (Ndel/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 (Ndel/EcoRI) was amplified and subcloned into pUWL201PW and pSEV1 containing the strong Streptomyces promoters ermE and tipA, respectively, to produce pUWL201PW-ermE, pUWL201PW-tipA, and pSEV1-ermE, pSEV1-tipA. The DNA fragment encoding mature SK was amplified with primers Skm-F and Skm-R and inserted into pET28a digested with Ndel and NotI, resulting in pET28a-T7,SKm. Restriction maps of the constructs are shown in Fig. 1.

**Table 1. Primers used for PCR.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide*</th>
</tr>
</thead>
<tbody>
<tr>
<td>sprT&lt;sub&gt;C&lt;/sub&gt;SGT&lt;sub&gt;C&lt;/sub&gt;-F</td>
<td>5’-CGGCAGAATTCTAGGGCGCCGGCGCCGGCC-3’ (EcoRI)</td>
</tr>
<tr>
<td>sprT&lt;sub&gt;C&lt;/sub&gt;SGT&lt;sub&gt;C&lt;/sub&gt;-R</td>
<td>5’-AATCATATGGACGGGTTGCGGCGG-3’ (NdeI)</td>
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<tr>
<td>For cloning of sprT&lt;sub&gt;C&lt;/sub&gt; and SGT&lt;sub&gt;C&lt;/sub&gt; peptides</td>
<td>5’-CGGCAGAATTCTAGGGCGCCGGCGCCGGCC-3’ (EcoRI)</td>
</tr>
<tr>
<td>sgtR1&lt;sub&gt;C&lt;/sub&gt;R2-F</td>
<td>5’-CACGCTGCGCAGCGCAGCTAACC-CA3’ (NotI)</td>
</tr>
<tr>
<td>sgtR1&lt;sub&gt;C&lt;/sub&gt;R2-R</td>
<td>5’-CCTCGCATGCCGACCCCTTGCTCCACC-3’ (SphI)</td>
</tr>
<tr>
<td>For cloning of mature form of SK (SK&lt;sub&gt;C&lt;/sub&gt;)</td>
<td>5’-GTGACATGGATGCTGGACCTAGTGGTGGTGCGG-3’ (NdeI)</td>
</tr>
<tr>
<td>SKm-F</td>
<td>5’-GCCGCGGCCGCGCTGGTGGTTGCTGGTAGG-3’ (NotI)</td>
</tr>
<tr>
<td>SKm-R</td>
<td>5’-GCCGCGGCCGCTGGTGGTTGCTGGTAGG-3’ (NdeI)</td>
</tr>
<tr>
<td>For cloning of sprT&lt;sub&gt;C&lt;/sub&gt; promoter</td>
<td>5’-CGGCAGAATTCTAGGGCGCCGGCGCCGGCC-3’ (EcoRI)</td>
</tr>
<tr>
<td>sprT&lt;sub&gt;C&lt;/sub&gt;-F</td>
<td>5’-CGGCAGAATTCTAGGGCGCCGGCGCCGGCC-3’ (EcoRI)</td>
</tr>
<tr>
<td>sprT&lt;sub&gt;C&lt;/sub&gt;-R</td>
<td>5’-CGGCAGAATTCTAGGGCGCCGGCGCCGGCC-3’ (EcoRI)</td>
</tr>
<tr>
<td>For cloning of signal peptide plus mature form of SK</td>
<td>5’-CCCATAGGAGAAAAAGACAGCTTCGCGG-3’ (NdeI)</td>
</tr>
<tr>
<td>SK&lt;sub&gt;m&lt;/sub&gt;-SK&lt;sub&gt;m&lt;/sub&gt;-F</td>
<td>5’-CCCATAGGAGAAAAAGACAGCTTCGCGG-3’ (NdeI)</td>
</tr>
<tr>
<td>SK&lt;sub&gt;m&lt;/sub&gt;-SK&lt;sub&gt;m&lt;/sub&gt;-R</td>
<td>5’-CCCATAGGAGAAAAAGACAGCTTCGCGG-3’ (NdeI)</td>
</tr>
</tbody>
</table>

*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 K₂HPO₄/KH₂PO₄ (pH 6.0), 100 mM NaCl, and 10 mM 1,4-dithioerythritol]. 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 OD₆₀₀ 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 K₂HPO₄/KH₂PO₄ (pH 7.5), 300 mM NaCl, 20 mM imidazole, 0.2 mM CoCl₂, and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)]. Cell debris was removed by centrifugation and the protein solution was mixed with Ni²⁺-nitrilotriacetic acid (NTA) agarose and left to stand for 1 h. The agarose was washed three times with the same buffer and the His₄-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-PAGE, a protein band corresponding to 44 kDa was detected, which coincides with the expected molecular mass of SK.

**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 mM NaCl, and 10 mM 1,4-dithioerythritol. The cell pellet was disrupted by sonication and used to quantify cellular protein.

**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-TPspSKmR1R2 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-TPspSKmR1R2 exhibited higher SK activity than did *S. lividans* TK24 [18]. The present study demonstrated that the SG1 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-TPspSKmR1R2; 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 Pimiento 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/
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 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-tipMspKspSKm) or the constitutive ermE promoter (pUWL201PW-ermEpSKspSKm) resulted in about 0.05 unit/ml of culture broth in S. lividans TK24 transformants.

**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_spSK_spSK_mR1R2 after 8 and 7 days of cultivation, respectively; lanes 4 and 5, total extracellular protein from S. lividans/pWHM3-T_spSK_spSK_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.

**Fig. 3.** SDS-PAGE (A) and SK activity assay (B) of E. coli BL21(ADE3)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_spSK_m; lane 5, purified SK from E. coli/pET28a-T7_spSK_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_spSK_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.
*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*skc*) was introduced into *E. coli* BL21(DE3)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*skc* 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*, *tipA*, *ermE*) 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* hosts. 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(DE3)pLysS [6]; such results may provide indirect evidence in support of our assumption.

In contrast with the *Streptomyces* systems, the *E. coli* BL21(DE3)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.

<table>
<thead>
<tr>
<th>Triplet Codon</th>
<th>[Frequency/thousand]</th>
<th>[Frequency/thousand in 100 <em>Streptomyces</em> genes]*</th>
<th><em>E. coli</em></th>
<th><em>S. lividans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>UUU 25.2 (0.45)</td>
<td>UCU 11.5 (0.61)</td>
<td>UAU 29.8 (1.12)</td>
<td>UGU 0.0 (1.06)</td>
<td>UGG 2.3 (15.21)</td>
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<tr>
<td>UUC 11.5 (27.51)</td>
<td>UCC 2.3 (21.07)</td>
<td>UAC 20.6 (21.13)</td>
<td>UGC 0.0 (7.72)</td>
<td>UGA 0.0 (2.59)</td>
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<tr>
<td>UUA 27.5 (0.42)</td>
<td>UCA 6.9 (1.28)</td>
<td>UAA 2.3 (0.13)</td>
<td>UGA 0.0 (2.59)</td>
<td>UGG 2.3 (15.21)</td>
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<td>UUG 13.8 (2.50)</td>
<td>UCG 2.3 (21.33)</td>
<td>UAG 0.0 (0.48)</td>
<td>UUC 2.3 (15.21)</td>
<td>UGG 2.3 (15.21)</td>
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<tr>
<td>CUU 6.9 (1.95)</td>
<td>CCC 18.3 (1.51)</td>
<td>CAU 13.8 (1.57)</td>
<td>CGU 18.3 (6.02)</td>
<td>CUG 9.2 (3.04)</td>
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<tr>
<td>CUC 9.2 (36.73)</td>
<td>CCC 6.9 (23.19)</td>
<td>CAC 9.2 (22.67)</td>
<td>CGC 4.6 (37.24)</td>
<td>CGG 2.3 (11.19)</td>
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<tr>
<td>CUA 20.6 (0.29)</td>
<td>CCA 20.6 (0.86)</td>
<td>CAA 34.4 (1.60)</td>
<td>CGA 9.2 (3.04)</td>
<td>CGG 2.3 (11.19)</td>
</tr>
<tr>
<td>CUG 13.8 (53.57)</td>
<td>CGG 4.6 (29.17)</td>
<td>CAG 4.6 (24.47)</td>
<td>CGG 2.3 (11.19)</td>
<td>CAG 4.6 (24.47)</td>
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<td>AUU 27.5 (1.44)</td>
<td>ACU 22.9 (1.47)</td>
<td>AUU 20.6 (1.09)</td>
<td>AGU 11.5 (1.57)</td>
<td>AGU 11.5 (1.57)</td>
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<td>AUC 20.6 (29.88)</td>
<td>ACC 27.5 (42.75)</td>
<td>AAC 36.7 (22.38)</td>
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<td>AGC 20.6 (14.57)</td>
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<tr>
<td>AUA 9.2 (1.15)</td>
<td>ACA 18.3 (1.60)</td>
<td>AAA 59.6 (1.38)</td>
<td>AGA 6.9 (0.93)</td>
<td>AGA 6.9 (0.93)</td>
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<td>AUG 11.5 (16.20)</td>
<td>ACG 6.9 (19.79)</td>
<td>AAG 18.3 (22.19)</td>
<td>AGG 0.0 (4.26)</td>
<td>AGG 0.0 (4.26)</td>
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<td>GAA 43.6 (10.41)</td>
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<td>GAG 22.9 (48.55)</td>
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</table>

*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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