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Engineering and Production of Streptokinase in a *Bacillus subtilis* Expression-Secretion System

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Streptokinase is one of the major blood-clot-dissolving agents used in many medical treatments. With the cloned streptokinase gene (*skc*) available, production of the secreted streptokinase from various *Bacillus subtilis* strains was studied. The use of the six-extracellular-protease-deficient strain, WB600, greatly improved the production yield of the secreted streptokinase. A modified *skc* which has the original *skc* promoter and signal sequence replaced with the *B. subtilis* levansucrase promoter and signal sequence was also constructed. *B. subtilis* carrying either the wild-type or the modified *skc* produces streptokinase at a comparable level. Even with WB600 as the expression host, a C-terminally-processed streptokinase was also observed. Through region-specific combinatorial mutagenesis around the C-terminal processing sites, streptokinase derivatives resistant to C-terminal degradation were engineered. One of the derivatives showed a 2.5-fold increase in specific activity and would potentially be a better thrombolytic agent.

Streptokinase is a group of extracellular proteins produced by a variety of beta-hemolytic streptococci (21). It interacts with plasminogen to form a stoichiometric 1:1 complex which activates plasminogen to plasmin. Plasmin can then degrade the fibrin matrix of blood clots. Streptokinase has been widely used as a thrombolytic agent for the past 30 years (21). Its ability to induce reperfusion of the occluded coronary arteries and to reduce mortality has been firmly established. Recently, two international clinical trials, GISSI-2 (trial size, 12,490 patients) (8) and ISIS-3 (trial size, 41,299 patients) (12) resulted in conclusions that both streptokinase and tissue-specific plasminogen activator, a fibrin-specific thrombolytic agent from humans, appear equally effective and safe for use in routine conditions, and no significant differences in hospital mortality between these two agents were found.

It would be ideal to have a nonpathogenic expression system that can produce streptokinase in high quality and quantity since the original host is pathogenic and secretes several other toxins that complicate the downstream purification (7). The structural gene (*skc*) of streptokinase from *Streptococcus equisimilis* H46A has been cloned (17) and sequenced (19). By using the intact streptokinase gene including its promoter region, *skc* was expressed in various gram-negative and gram-positive hosts including *Escherichia coli* (17), *Proteus mirabilis* (16), *Streptococcus sanguis* Challis (13), *Streptococcus lactis* (16), and *Bacillus subtilis* (15). However, all of these hosts including *S. equisimilis* H46A produce at least two major forms of streptokinase. One is the intact mature streptokinase with a molecular mass of 47 kDa. The other is a 44-kDa degradation product. This 44-kDa form of streptokinase produced from *S. sanguis* has been purified. It is composed of a mixture of two streptokinases missing either 31 or 32 amino acid residues from the C terminus (13). The one missing the last 31 amino acids has retained almost the full activity of the intact streptokinase. The other one shows only 20% of the wild-type streptokinase activity. Further support of the importance of the C-terminal region of streptokinase is found in the deletion analyses by Malke et al. (18). Removal of 41 amino acids from the C terminus of streptokinase resulted in a streptokinase with only 14% of the wild-type streptokinase activity. Deletion of three extra amino acids (i.e., a deletion of 44 amino acids from the C terminus) generates an inactive streptokinase. Thus, production of intact streptokinase with full biological activity represents a major challenge. Although intact mature streptokinase can be produced intracellularly in *E. coli* (5, 24), an extra methionine was added to the N terminus of this protein. Furthermore, streptokinase has to be purified from many other *E. coli* intracellular proteins.

B. subtilis is an attractive host for streptokinase production since *B. subtilis* is nonpathogenic and capable of secreting extracellular proteins directly to the culture medium (1, 3, 4, 10, 23). However, secretion of at least seven extracellular proteases from *B. subtilis* severely reduces the production yield and intactness of the secreted foreign proteins including streptokinase (15). With our recent construction of a six-extracellular-protease-deficient strain, WB600, and the development of expression vectors (32, 34), we report the significance of applying this system to produce streptokinase. The use of the streptokinase promoter and signal sequence and the *B. subtilis* levansucrase (*sacB*) promoter and signal sequence to direct the expression of *skc* and the secretion of streptokinase was compared. To minimize the proteolytic cleavage of streptokinase at the C-terminal region, we modified sequences encoding the potential protease cleavage sites through a region-specific combinatorial cassette mutagenesis (26). Streptokinase derivatives resistant to C-terminal cleavage were produced in this expression system. Lys-386 (numbered according to the sequence of the mature streptokinase) was important for biological activity. One of the engineered streptokinases behaves as a superactive streptokinase with the specific activity 2.5 times higher than that of the wild-type streptokinase. This report represents the first successful case in developing superactive streptokinase analogs.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* DH5α (*supE44 ΔlacU169* [φ80 *lacZ*ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) and *B. subtilis* 168 (*trpC2*), DB104 (*his*

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*nprR2 nprA18 aprE*Δ3) (14), DB428 (*trpC2 nprA apr epr bpf*) (11), WB500 (*trpC2 nprA apr epr bpf mpr*) (34), and WB600 (*trpC2 nprA apr epr bpf mpr nprB*) (34) were used for routine transformation and expression studies. For preparing unmethylated plasmid DNA, *E. coli* GM2163 (*dam dcm supE*) (20) was used as the host. Superrich medium (9) containing 10 μg of kanamycin per ml was routinely used for the cultivation of *B. subtilis* strains carrying plasmids. For sucrose-induced expression, sucrose was added to the medium at a final concentration of 2% (wt/vol).

DNA manipulation. Chromosomal DNA isolation, restriction digestion, electroelution of DNA fragments, T4 polynucleotide kinase reaction, ligation, and DNA sequence determination were performed as described previously (31). For annealing oligonucleotides, each sequence was dissolved in Tris-EDTA buffer and adjusted to a final concentration of 1 nmol/μl. One microliter of each oligonucleotide was mixed with 1 μl of 10× annealing buffer (200 mM Tris-HCl [pH 7.6], 100 mM $MgCl_2$) and 7 μl of sterilized water. The sample was heated in a boiling bath for 3 min and then transferred to a 30°C water bath for 30 min and at room temperature for another 30 min.

Construction of pSK-1. The 2.5-kb *Pst*I fragment carrying the structural gene of streptokinase (*skc*) from *S. equisimilis* H46A (ATCC 12446) was cloned into pBR322 in an approach similar to that described by Malke and Ferretti (17). This plasmid is named pESK. To construct plasmid pSK-1, the 2.5-kb *Pst*I fragment from pESK was ligated to the *Pst*I-digested pUB18, a *B. subtilis* plasmid (33). One of the resulting plasmids was named pSK-1. Since the insert carries the *skc* promoter, the expression of *skc* is presumed to be under the control of this sequence element. However, the possibility of using other promoter-like sequences upstream of *skc*, if any, to direct the transcription of *skc* in *B. subtilis* is not excluded.

Construction of pSK-3. Restriction sites within *skc* that are important for the construction of pSK3 are shown in Fig. 1A. To place *skc* under the control of the *sacB* promoter and signal sequence, the first plasmid constructed was pUB-SP (Fig. 1B). This is a pUB18 derivative carrying the *sacB* promoter and the engineered *sacB* signal sequence (32). It was constructed by inactivating the unique *Hin*dIII site in the polylinker region by the fill-in reaction and the insertion of the *sacB* promoter and signal peptide cassette (a 570-bp *Eco*RI-*Pst*I fragment) from pIS568 (32). The second step involved the insertion of a 923-bp *Hin*dIII-*Pst*I fragment encoding the 3′ end region of *skc* into the *Hin*dIII-*Pst*I double-digested pUB-SP to generate pUB-CSK (Fig. 1B). To generate the mature form of streptokinase with an authentic N-terminal sequence after processing of the *sacB* signal sequence by signal peptidase, a pair of oligonucleotides encoding the signal peptidase cleavage site of the *sacB* signal sequence followed by the mature N-terminal sequence of streptokinase was designed (Fig. 1C). After annealing (see conditions described in "DNA manipulation"), the resulting product had a *Hin*dIII site at one end and an *Ava*II site at the other end. Two restriction enzyme sites, *Nru*I and *Pvu*I, were also introduced within this synthetic sequence. These annealed oligonucleotides were phosphorylated and then ligated to a 1.37-kb *Ava*II fragment isolated from *skc* (Fig. 1B). Since *Ava*II recognizes a degenerate sequence, the two *Ava*II sites on the 1.37-kb fragment have different sequences. The annealed synthetic DNA can ligate to only one of the two *Ava*II sites. The resulting ligated product was then digested by *Hin*dIII. A 760-bp *Hin*dIII fragment was gel purified and ligated to the *Hin*dIII-cut pUB-CSK. Clones with the *Hin*dIII fragment inserted at the correct orientation were determined by restriction digestion. One of these clones is named pSK-3. The DNA

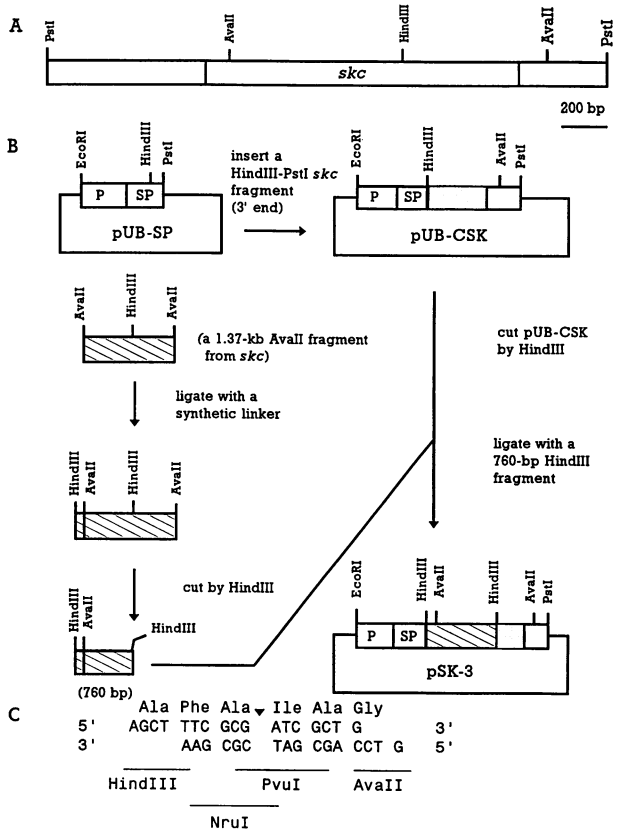

FIG. 1. (A) Restriction sites in the 2.5-kb *Pst*I *skc* fragment useful for pSK-3 construction. The central box represents the coding region for *skc*. (B) Construction of pSK-3. Abbreviations: P, *sacB* promoter; SP, *sacB* signal sequence. (C) Sequences of synthetic oligonucleotides designed to modify the sequence around the signal peptidase cleavage site so that restriction sites can be introduced and the secreted streptokinase with authentic N terminus can be produced.

sequence corresponding to the *sacB* signal sequence and the first *Ava*II site in *skc* was determined by sequencing.

Combinatorial cassette mutagenesis of streptokinase. Two restriction sites, *Bsm*I and *Bsa*BI, are properly located within the 3′ end region of *skc* and are ideal for combinatorial cassette mutagenesis (26). However, pUB18 has two *Bsm*I sites. Therefore, cassette mutagenesis cannot be performed directly with pSK-3. A 1.5-kb *Cla*I-*Pst*I fragment encoding the 3′ end of *skc* was isolated from pSK-3 and ligated to the *Cla*I- and *Pst*I-digested pKS plasmid (Bluescript II from Stratagene; this plasmid does not carry any *Bsm*I or *Bsa*BI sites) to generate pCP. This plasmid was digested with *Bsm*I and *Bsa*BI and ligated with the phosphorylated mutagenic primers (either set 1 or 2; Table 1). The ligated DNA was transformed to *E. coli* GM2163. Sixty transformants were randomly selected, and the sequence between *Bsm*I and *Bsa*BI was determined to identify the nature of mutation. Clones carrying proper mutations were digested by *Cla*I and *Pst*I to release a 1.5-kb fragment. These fragments were then exchanged with the corresponding *Cla*I-*Pst*I fragment from pSK-3 to generate pSKC-02, pSKC-19, pSKC-27, pSKC-32, and pSKC-52.

Streptokinase assays. Streptokinase activity was determined by two methods. The first method was radial caseinolysis in plasminogen-skim milk-containing agarose as described by Saksela (27). The square of the radius of each halo was used

TABLE 1. Sequences of mutagenic primers for combinatorial mutagenesis of *skc*

Primer	Sequence[a]	Mutants generated
Set 1	5′ AGC T(A,C)T CAT A(C,A)T TCT CA(T,A) GAT CAA 3′ 3′ GATCG A(T,G)A GTA T(G,T)A AGA GT(A,T) CTA GTT 5′ Ser Tyr/Ser His Thr/Asn Ser His/Gln Asp Gln	pSKC-02, pSKC-19 pSKC-52
Set 2	5′ AGC TCT CAT A(G,A)T (A,G)AT CAA GAT (C,A)AA 3′ 3′ GATCG AGA GTA T(C,T)A (T,C)TA GTT CTA (G,T)TT 5′ Ser Ser His Ser/Asn Asn/Asp Gln Asp Gln/Lys	pSKC-27, pSKC-32

[a] The *Bsm*I site is on the left, and the *Bsa*BI site is on the right.

for the estimation of the streptokinase activity. The second method is similar to that described by Castellino et al. (2) except that tosyl-glycyl-prolyl-lysine–4-nitroanilide acetate (Chromozym PL; Boehringer Mannheim) was used as the substrate. Streptokinase was mixed with plasminogen and preincubated at 37°C for 5 min. Substrate was then added, and the release of 4-nitroaniline was monitored at A_{405} with a Beckman DU65 spectrophotometer equipped with a constant-temperature cuvette chamber.

Other methods. Streptokinase from Sigma was gel purified and injected into two rabbits to prepare streptokinase-specific antibody as described previously (35). Western blot (immunoblot) analysis was performed as described by Towbin et al. (30). Samples for Western blot analysis were prepared as follows. Proteins in the culture supernatant were first precipitated by the addition of an equal volume of 40% (wt/vol) trichloroacetic acid. This step is important to inactivate any proteases. Samples were spun for 5 min in a microcentrifuge, and the pellet was resuspended in 0.3 ml of washing buffer (0.15 M Tris HCl [pH 8.8], 0.04% sodium dodecyl sulfate [SDS]). After the addition of 1.2 ml of cold acetone to each sample, the samples were kept at −20°C for 10 min and centrifuged at room temperature for 5 min. The pellet was dried in a SpeedVac concentrator and was then ready for Western blot analysis. An LKB ultrascan XL enhanced laser densitometer was used to semiquantify the streptokinase level from Western blots. For the N-terminal sequence determination, streptokinase was electroblotted onto an Immobilon membrane (22). The protein band was briefly stained and excised. The sequence was determined at the Microchemistry Centre, University of Victoria.

RESULTS

Construction of plasmid vectors (pSK-1 and pSK-3) that produce streptokinase with an authentic N terminus. To determine whether a *B. subtilis* promoter and signal sequence would be better than the native streptokinase promoter and signal sequence in directing the production and secretion of streptokinase in *B. subtilis*, two vectors were constructed. The first vector (pSK-1) carries the intact streptokinase gene (*skc*) including its promoter region. The second vector (pSK-3) carries the sequence encoding the mature portion of streptokinase fused to the *B. subtilis* levansucrase (*sacB*) promoter and signal sequence (Fig. 1B). The use of the *sacB* regulatory element allows the inducible expression of *skc* by sucrose since *sacB* is a sucrose-inducible gene. A special synthetic sequence (Fig. 1C) encoding the signal peptidase cleavage site and the authentic N-terminal sequence of the mature streptokinase was designed. The final construct (pSK-3) can generate mature streptokinase with an authentic N-terminal sequence if the signal sequence is processed properly by the *B. subtilis* signal peptidase.

Secretion of streptokinase with *B. subtilis* WB600 as an expression host. *B. subtilis* WB600(pSK-1) and WB600(pSK-3) were cultivated in superrich medium. Sucrose at a final concentration of 2% was added to one set of the WB600(pSK-3) cultures to induce the production of streptokinase. These strains shared an identical growth profile under the specified culture conditions. Streptokinase secreted to the culture medium was determined by monitoring the plasmin activity with Chromozym PL as the substrate. With the promoter and signal sequence derived from *skc*, WB600(pSK-1) produces streptokinase constitutively (Fig. 2). For WB600(pSK-3), which has the promoter and signal sequence derived from *sacB*, sucrose-induced production of streptokinase was observed. The secreted streptokinase activity reached the highest level 4 h after inoculation. By comparing the streptokinase-specific activity at the peak level with the two assay methods, streptokinase activity from WB600(pSK-3) is about 1.2- to 1.4-fold higher than that from WB600(pSK-1).

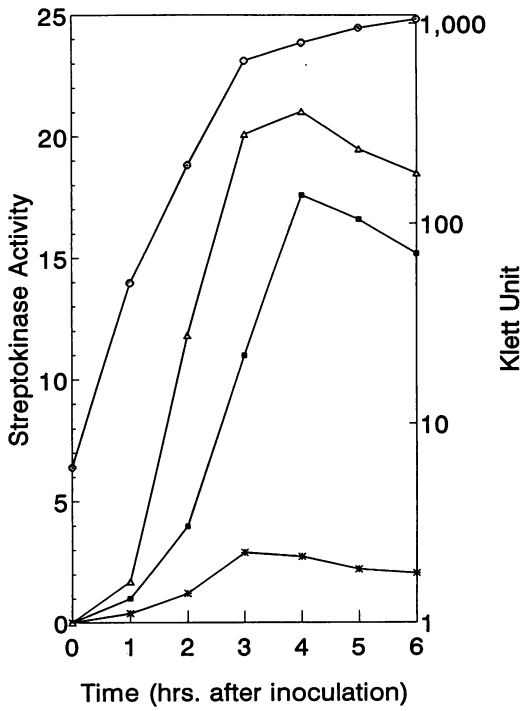

FIG. 2. Comparison of streptokinase production from WB600 (pSK-1) and WB600(pSK-3). Streptokinase activity from WB600 (pSK-1) (□) and WB600(pSK-3) in the presence (△) or absence (*) of 2% sucrose was determined by using Chromozym PL as the substrate. The growth curve (○) of these strains was monitored with a Klett-Summerson colorimeter.

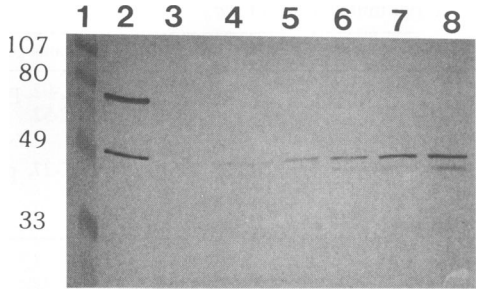

FIG. 3. Western blot analysis of streptokinase produced from various protease-deficient *B. subtilis* strains. Lanes: 1, prestained molecular weight markers with their molecular mass expressed in kilodaltons on the left of the figure; 2, streptokinase standard from Sigma; 3 to 8, streptokinase produced from *B. subtilis* 168(pSK-3) (14.4 μl of culture supernatant with a cell density of 0.714 at A_{600} was loaded), *B. subtilis* 168(pSK-3) (144.4 μl, 0.714 at A_{600}), DB104(pSK-3) (22.5 μl, 0.46 at A_{600}), DB428(pSK-3) (16 μl, 0.64 at A_{600}), WB500(pSK-3) (20 μl, 0.51 at A_{600}), and WB600(pSK-3) (16.3 μl, 0.63 at A_{600}), respectively. The volume of culture supernatant from each sample was adjusted to normalize the difference in cell density except for that in lane 4. Ten times more sample was applied to this lane. Both the volume of the culture supernatant and the cell density at A_{600} for each sample are shown above. A 68-kDa protein in lane 2 which cross-reacted with the antistreptokinase serum was bovine serum albumin (BSA). The commercially available streptokinase (from Sigma) contains BSA in a large quantity to serve as a stabilizer to minimize the proteolytic degradation of streptokinase by the contaminated proteases.

N-terminal sequence determination of the secreted streptokinase. To determine whether streptokinase produced from WB600(pSK-3) has the N-terminal sequence identical to the streptokinase produced from *S. equisimilis*, the sequence of the first five amino acid residues from the 47-kDa streptokinase was determined and found to match exactly that of the mature streptokinase produced from *S. equisimilis*. This indicated that the *sacB* signal sequence was processed properly by the *B. subtilis* signal peptidase.

Effect of using different protease-deficient strains on streptokinase production. Since streptokinase is shown to be sensitive to extracellular proteases produced from *B. subtilis* (15), we have systematically compared the production of streptokinase from wild-type and protease-deficient strains carrying pSK-3. For protease-deficient strains, we used DB104 (deficient in the two major extracellular proteases), DB428 (a four-extracellular-protease-deficient strain), WB500 (a five-extracellular-protease-deficient strain) and WB600 (a six-extracellular-protease-deficient strain) in this study. After normalizing the differences in cell density, the secreted streptokinase from these cultures was characterized by Western blot analysis. As shown in Fig. 3, the use of the protease-deficient strains greatly improved the production yield. No detectable intact streptokinase from *B. subtilis* 168(pSK-3) was observed (Fig. 3, lane 3) unless 10 times more culture supernatant was used in the analysis (Fig. 3, lane 4). No cross-reacting protein band was detected from the culture supernatant of the negative control, WB600(pUB18) (data not shown). The band intensity of each culture on the Western blot was compared by scanning through the laser densitometer. If the intensity of the 47-kDa streptokinase band (Fig. 3, lane 4) from *B. subtilis* 168(pSK-3) was set at 10 arbitrary units, the relative intensities of the streptokinase bands from DB104(pSK-3), DB428(pSK-3), WB500(pSK-3), and WB600(pSK-3) would be 17, 25, 52, and 85 units, respectively. Since 10-times-more sample was applied to lane 4 (Fig. 3), these data suggested that there was an 85-fold increase in intact streptokinase with WB600 as the expression host. By the activity assay with Chromozym PL as the substrate, there was a 15-fold increase in streptokinase activity with WB600(pSK-3) as the expression host [relative to the activity from *B. subtilis* 168(pSK-3); Table 2]. These results reflected that the degraded forms of streptokinase retained a certain degree of biological activity.

TABLE 2. Production of streptokinase by using various *B. subtilis* strains as hosts[a]

Strain	Sp act {[(ΔOD_{405}/min)/OD_{595}]/ml}[b]	Relative activity
168	0.49	1
DB104	1.36	2.8
WB428	2.06	4.2
WB500	4.25	8.6
WB600	7.44	15.2

[a] Activity was determined by using Chromozym PL as the substrate.
[b] OD_{405}, optical density at 405 nm.

Development of protease-resistant streptokinase mutants. Even with WB600 as the expression host, 10 to 20% of the secreted streptokinase was processed from the 47-kDa form to the 44-kDa form. This 44-kDa form of streptokinase was electroblotted to the polyvinylidene difluoride membrane, and the sequence of the first five amino acid residues from the N terminus was determined. It had the same N-terminal sequence as that of the 47-kDa streptokinase. This result demonstrated that the 44-kDa streptokinase was processed at the C-terminal region. Judging from the size, it is likely that the C-terminal 31 or 32 residues are removed in the same manner as that reported previously with *S. sanguis* as the expression host (13). Since chymotrypsin with a substrate specificity towards proteins with hydrophobic side chains can generate the 44-kDa form of streptokinase (13), it would be vital to determine whether changing several nonpolar or hydrophobic residues in the C-terminal region to either polar or charged ones can generate streptokinase derivatives that are resistant to C-terminal processing. Table 3 shows the C-terminal sequence of streptokinase with the two potential cleavage sites marked by arrows. The four hydrophobic residues (Table 3, residues 31 to 33 and 35, numbered from the C terminus) and a lysine residue (Lys-29, numbered from the C terminus) were converted to polar residues and glutamine, respectively, via two sets of combinatorial cassette mutagenic primers (Table 1). Five representative mutants (pSKC-02, -19, -27, -32, -52) were characterized by both Western blot analysis and activity determination. Supernatants from different cultures were normalized to have equivalent amounts of streptokinase for these analyses. Figure 4A shows that all mutants except pSKC-27 produce protease-resistant streptokinases as demonstrated by Western blot analysis. These protease-resistant streptokinases retained at least 90% of the wild-type specific activity (Fig. 4B and Table 3). Streptokinase produced from WB600(pSKC-27) showed a specific activity that is 2.5 times higher than that of the wild-type streptokinase (Fig. 4B and Table 3). A low percentage of this protein (10 to 20%) exists in a new processed form. This streptokinase has almost the same sequence as the one produced by WB600(pSKC-32). The only difference is that SKC-27 has a lysine residue at position 29 (numbered from the C terminus) and SKC-32 has a glutamine at the corresponding position (Table 3). Therefore, lysine at this position can directly or indirectly affect both the activity and the stability of streptokinase produced from *B. subtilis*.

TABLE 3. Relative specific activities of various streptokinases

Plasmid	C-terminal sequence of streptokinase and the corresponding nucleotide sequence[a]: 376 39	377 38	378 37	379 36	380 35	381 34	382 33	383 32 ↓	384 31 ↓	385 30	386 29	387[c] 28[d]	Relative sp act[b]
Wild type	E	N	A	S	Y	H	L	A	Y	D	K	D	1.0
	GAG	AAT	GCT	AGC	TAT	CAT	TTA	GCC	TAT	GAT	AAA	GAT	
pSKC-27					S		S	D	Q				2.5
					TCT		AGT	AAT	CAA				
pSKC-32					S		S	D	Q		Q		0.9
					TCT		AGT	AAT	CAA		CAA		
pSKC-52					S		T	S	Q		Q		0.9
					TCT		ACT	TCT	CAA		CAA		
pSKC-19					S		N	S	Q		Q		0.9
					TCT		AAT	TCT	CAA		CAA		
pSKC-02					S		N	S	H		Q		0.9
					TCT		AAT	TCT	CAT		CAA		

[a] The two arrows indicate the proteolytic processing sites.
[b] Streptokinase activity was determined by three independent measurements.
[c,d] The C-terminal portion of the streptokinase sequence is numbered from the N terminus and the C terminus, respectively.

Minor variations in electrophoretic mobility of these streptokinase derivatives were observed (Fig. 4A). Changes in sequence may affect the binding of SDS to these proteins and consequently the mobilities of these proteins in the SDS-polyacrylamide gel.

DISCUSSION

Streptokinase has been reported to be produced by many bacterial expression hosts including the pathogenic *S. equismilis* H46A, the natural streptokinase producer. All of these hosts produce a 44-kDa streptokinase degradation product to a different degree. This proteolytic processing causes a reduction in both the biological activity and the shelf life of this medically important protein. The appearance of the degraded streptokinase upon prolonged storage is fairly common (2). With the construction of the six-protease-deficient *B. subtilis* strain, WB600, we examined the possibility of applying this nonpathogenic expression-secretion system to produce streptokinase in high quality and quantity. Clearly, the use of the *B. subtilis sacB* promoter and signal sequence can produce streptokinase at a slightly higher level than the use of *S. equismilis skc* promoter and signal sequence (Fig. 2). The *sacB* signal sequence is processed correctly. Relative to wild-type *B. subtilis* 168, WB600 serves as a better production host because the production of intact streptokinase increases 85 times.

Although six chromosomal extracellular protease genes have been inactivated in WB600, this strain is still capable of secreting at least one minor protease encoded by *vpr* (28). This minor protease is a serine protease and may account for the generation of the 44-kDa streptokinase. Whether this protease has a chymotrypsin-like or a trypsin-like substrate specificity is not certain. Mutations within the C-terminal region of streptokinase would be an alternative to engineering protease-resistant streptokinases. To achieve this objective, all of the nonpolar residues forming the two C-terminal processing sites were converted to polar or charged residues (but not lysine or arginine) via combinatorial cassette mutagenesis. Two amino acid residues around the processing sites were also changed to make sure that the resulting streptokinase derivatives are resistant to C-terminal proteolytic processing mediated by proteases with chymotrypsin- or trypsin-like specificities. These two amino acids are tyrosine 380 and lysine 386. They were changed to serine 380 and glutamine 386 (except pSKC-27; Table 3), respectively. Although the three-dimensional structure of streptokinase is unknown, the structural and folding properties of streptokinase have been determined by various biophysical and biochemical approaches including nuclear magnetic resonance (29), Fourier transform infrared (6), and circular dichroism (25) spectroscopic studies. Combining these studies and the use of three predictive secondary-structure algorithms, the C-terminal portion (amino acid residues 376 to 387) of the streptokinase was suggested to be in a disordered structure (25). Furthermore, there are two histidine residues located around this region (His-358 and His-381). The pK_a values of these residues are in the range of 6.3, typical of that for a solvent-accessible histidine residue (29). Therefore, this region is likely to be exposed on the surface. These observations have two implications. (i) This C-terminal sequence would be accessible to protease. (ii) This sequence offers better tolerance to mutations since no particular secondary structure is required. Indeed, the generation of various protease-resistant mutants (Table 3) via combinatorial mutagenesis supports these predictions. At least 14 mutants (generated with the mutagenic primers shown in Table 1) were isolated. They all showed biological activity (about 90% of the wild-type streptokinase activity) comparable to that of the wild-type streptokinase. Characterization of streptokinase derived from pSKC-02, -19, -32, and -52 by Western blot analysis demonstrated that these forms of streptokinase were indeed resistant to the residual proteolytic activity from WB600. Although all mutants shown in Table 3 have the Tyr-380 (numbered from the N terminus) changed to Ser-380, this change is not essential to generate the protease-resistant streptokinases. Two mutants, pSKC-4 and -6, have sequences almost identical to those of pSKC-52 and -19, respectively. The only difference is that pSKC-4 and -6 have Tyr-380 instead of Ser-380. Streptokinases from these mutants behave identically to those from pSKC-52 and -19 in terms of both biological activity and protease resistance (data not shown).

Since a low percentage (10 to 20%) of the secreted streptokinase from WB600 is in a degraded form, the generation of intact streptokinase (from pSKC-02, etc.) should result in a 10 to 20% increase in specific activity. However, this is not the case. In fact, mutations introduced into this sequence slightly

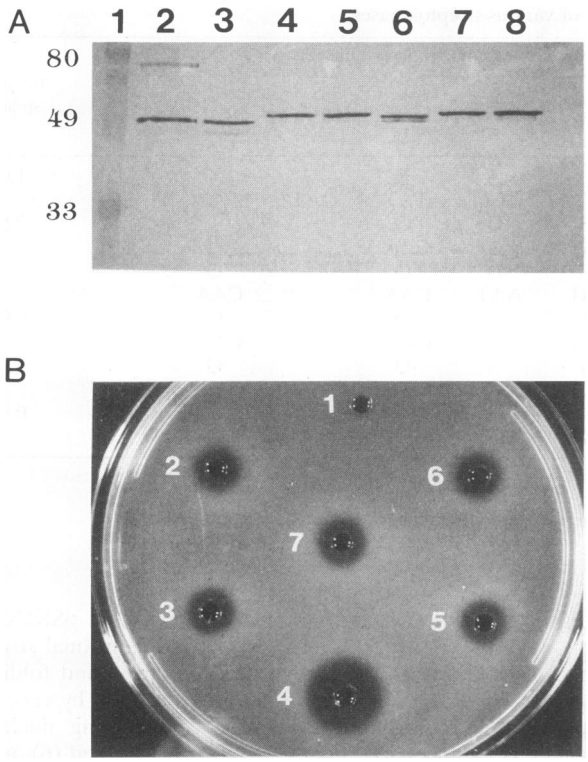

FIG. 4. (A) Western blot analysis of streptokinase and its derivatives. Lanes: 1 and 2, molecular weight standards and streptokinase standard, respectively; 3 to 8, streptokinase produced from WB600(pSK-3), WB600(pSKC-2), WB600(pSKC-19), WB600(pSKC-27), WB600(pSKC-32), and WB600(pSKC-52), respectively. The sample volume (14 to 25 μl) was adjusted so the same amount of streptokinase was present in each lane. (B) Streptokinase activity determined by the radial caseinolysis assay. The amounts of streptokinase in the individual wells were identical to those in the corresponding lanes shown in panel A. Wells: 1, WB600(pUB18); 2, WB600(pSKC-52); 3, WB600(pSKC-32); 4, WB600(pSKC-27); 5, WB600(pSKC-19); 6, WB600(pSKC-2); 7, WB600(pSK-3).

reduce the biological activity of these proteins. It suggests that the C-terminal region directly or indirectly plays a role in plasminogen activation. Comparing the sequence of pSKC-32 with that of pSKC-27 demonstrates the importance of lysine 386, which is located at the 29th position from the C terminus. SKC-27 has retained Lys-386 at that position while SKC-32 has lysine 386 changed to glutamine. SKC-27 shows a 2.5-fold increase in specific activity relative to that of SKC-32. The higher specific activity of this streptokinase may be a result of a higher binding affinity of this molecule to plasminogen or a higher catalytic activity to activate plasminogen. A small percentage of this derivative was in a degraded form. Judging from the size of this degraded protein, less than 31 amino acid residues were removed from the C terminus. This degraded streptokinase was likely to retain most of the biological activity. To our knowledge, the pSKC-27 mutant is the first superactive streptokinase mutant reported. It may allow the use of less streptokinase to achieve the same degree of blood clot lysis in medical applications. The reduction in the therapeutic streptokinase dosage may help minimize side effects induced by this protein.

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