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# Tripartite streptokinase gene fusion vectors for gram-positive and gram-negative procaryotes

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**Summary.** A specific 1,596 bp *Hin*cII fragment (*'skc*) from the chromosome of *Streptococcus equisimilis* contains an active streptokinase (SK) gene (*skc*) lacking, in addition to the expression signals, codons 1 through 39 of wild-type *skc* but retaining the remainder of the *skc* coding sequence together with the transcription terminator. Using this fragment as an indicator gene, we constructed two types of vectors which in appropriate hosts resulted in the synthesis of SK fusion proteins after insertional activation of *'skc*. The first type are open reading frame (ORF) vectors in which *'skc* was inserted into pUC18 out of frame with respect to *lacZ'*, thus conferring an SK-negative phenotype. Any DNA fragments representing ORFs inserted between the *lacZ'* expression signals and *'skc* such that the *skc* reading frame was restored resulted in the production of tripartite proteins which exhibited SK activity. The second type of vector, which functioned in both gram-positive and gram-negative bacteria, used the streptococcal *speA* expression and secretion signals in front of the ORF to activate *'skc* insertionally. Using a large fragment from the chymosin gene as the target sequence, the usefulness of these vectors for studying foreign gene expression in streptococci as well as Escherichia coli was demonstrated.

**Key words:** Gene fusion vectors – Tribrid streptokinase – Prochymosin – Secretion – *Streptococcus*

## Introduction

In recent years, gene fusions have been used to study a wide variety of biological phenomena. The lactose (*lac*) operon of *Escherichia coli* has been employed most extensively in these studies because a great amount of information is available regarding various aspects of this genetic system (Berman 1983; Koenen et al. 1985; Silhavy and Beckwith 1985; Silhavy et al. 1984). Although many of the methods used for constructing *lac* fusions are sufficiently general to be suitable for any target gene from *E. coli*, other genes from this organism have also been successfully employed and in certain cases found to provide adventages over the *lac* fusion techniques (Silhavy and Beckwith 1985). Nevertheless, it is fair to say that the majority of approaches rely on the molecular genetics of *E. coli*, and only sporadically has the technique involved other organisms and their genes.

We have used the truncated group C streptococcal streptokinase gene, *'skc*, as the indicator gene in an attempt to construct gene fusion vectors that function in both gram-negative and gram-positive bacteria including streptococci. Previous studies have shown that *skc* has features required for the construction of active hybrid genes (Malke et al. 1987). First, directed in vitro mutagenesis revealed that mature streptokinase (SK) may be deprived of at least 15 N-terminal amino acids without losing activity as a plasminogen activator. Second, the truncated *'skc* gene when put under the control of *lacPO* and fused to a stretch of DNA encoding the N-terminal hexapeptide of beta-galactosidase (*lacZ'*) was shown to specify a hybrid protein which still activated human plasminogen. In the present study, we describe plasmid vectors designed for cloning any open reading frame (ORF) in front of *'skc* and demonstrate the synthesis in *E. coli* and *Streptococcus* of chymosin-SK hybrid proteins with the plasminogen activator potential of SK and the antigenic identity of chymosin.

## Materials and methods

*Bacterial strains and plasmids.* The bacterial strains and plasmids used are described in Table 1. Prochymosin cDNA cloned in pBR322 (Liebscher et al. 1985) and shown to code for amino acids 6–365 of prochymosin was obtained from H. Liebscher. The prochymosin cDNA was recloned as a *Bam*HI–*Eco*RI fragment into pUC9. *E. coli* strains were grown aerobically in LB medium (Lennox 1955). *Streptococcus sanguis* and *S. lactis* were cultivated without agitation in Todd-Hewitt broth (Difco) and glucose-M17 medium (Terzaghi and Sandine 1975), respectively. For plating, the media were solidified with 1.5% agar and, if required, selective agents were added as follows: 100 μg/ml ampicillin; 10 μg/ml chloramphenicol; 10 μg/ml erythromycin; 15 μg/ml tetracycline.

*Recombinant DNA techniques.* For large scale plasmid isolation, CsCl-ethidium bromide density gradient equilibrium centrifugation was used according to Maniatis et al. (1982). The method of Holmes and Quigley (1981) was used for minipreparation of *E. coli* plasmids. Microscale plasmid isolation from *S. sanguis* and *S. lactis* was carried out as described by Friedrich and Lütticken (1984), except that cells were lysed with a *Streptomyces globisporus* muralytic

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**Table 1.** Bacterial strains and plasmids used

| Species | Strain | Plasmid | Relevant properties | Reference |
|---|---|---|---|---|
| *Escherichia coli* | JM101 | | *supE, thi, Δ(lac−proAB)* [F′, *traD36, proAB, lacI*$^q$ *ZΔM15]* | Yanisch-Perron et al. (1985) |
| | | pUC8 | Ap$^r$, *lacPOZ′* | Vieira and Messing (1982) |
| | | pUC9 | Ap$^r$, *lacPOZ′* | Vieira and Messing (1982) |
| | | pUC18 | Ap$^r$, *lacPOZ′* | Yanisch-Perron et al. (1985) |
| | HB101 | pACYC184 | Tc$^r$, Cm$^r$ | Chang and Cohen (1978) |
| | | pMF5 | Tc$^r$, *skc* | Malke and Ferretti (1984) |
| *Streptococcus sanguis* | Challis 6 | None | Competence developing | J. Ranhand |
| | | pSM6 | Em$^r$ | Laplace et al. (unpublished) |
| | | pLKM6181 | Em$^r$ | Laplace et al. (1987) |
| | | pSA32 | Em$^r$, *speA* | Weeks and Ferretti (1984) |
| *S. lactis* | MG1363 | | Lac$^-$, Prt$^-$ | Gasson (1983) |

enzyme (Schmidt et al. 1987) in a solution containing 0.05 M Tris, pH 8.0, 0.05 M EDTA, and 1% Triton X100. Digestion of plasmid DNA with restriction endonucleases (Boehringer), isolation of specific DNA fragments and their ligation by T4 DNA ligase (Boehringer), and agarose gel electrophoresis were performed by standard procedures (Maniatis et al. 1982) or as recommended by the supplier of the enzymes. The procedures of Maniatis et al. (1982), LeBlanc and Hassell (1976) and Kok et al. (1985) were used to transform *E. coli*, *S. sanguis*, and *S. lactis*, respectively, with plasmid and recombinant DNA. SK-producing colonies were detected by the casein-plasminogen overlay technique as described by Malke and Ferretti (1984).

*Detection and activity of hybrid proteins.* Whole cell extracts of *E. coli* obtained by sonication (Malke and Ferretti 1984) or cell-free culture supernatant fluids from streptococcal cultures were subjected to sodium dodecyl sulfate-polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE) according to Laemmli (1970). The gels were stained with Coomassie blue R250 or, after treatment with 2% Triton X100 and washing in water, overlayed with casein-plasminogen agarose (Malke and Ferretti 1984) to visualize bands with SK activity. Alternatively, the proteins were transferred to nitrocellulose membranes by electroblotting using the semidry technique of Kyhse-Anderson (1984), and the nitrocellulose was placed on the surface of the SK assay medium. The latter technique resulted in caseinolysis bands more distinct than those obtained by overlaying the polyacrylamide gels directly. For Western immunoblotting, the nitrocellulose membranes were blocked with nonfat dry milk according to Johnson et al. (1984) and subjected to the peroxidase technique for immunodetection of chymosin. The primary antibody was anti-calf chymosin raised in rabbits by intramuscular injection of electrophoretically pure chymosin obtained by affinity chromatography on histidyl-Sepharose (Amourache and Vijayalakshmi 1984) of commercial calf rennet (Hansen Laboratory, Copenhagen). As secondary antibody, goat anti-rabbit IgG conjugated with peroxidase was prepared using established procedures (Towbin and Gordon 1984). Non-chymosin-reactive rabbit serum was included as control in the immunodetection experiments. In addition, samples derived from isogenic strains lacking *skc* and/or prochymosin sequences capable of being expressed served as negative controls in the detection of hybrid proteins.

## Results

*Construction and characterization of* skc *fusion vectors*

The starting plasmid was pMF5 carrying the previously cloned and sequenced *skc* gene including its transcriptional and translational control signals (Malke and Ferretti 1984; Malke et al. 1985). The key element for the construction of hybrid genes was its 1,596 bp *Hin*cII fragment which contained a truncated *skc* gene (*'skc*) lacking, in addition to the expression signals, codons 1 through 39 of wild-type *skc* but retaining the remainder of the *skc* coding sequence together with the transcription terminator. When cloned in the unique *Sma*I site of pUC9 to form pKM1, the early *skc* region was replaced by DNA encoding the 5 N-terminal amino acids of beta-galactosidase including the in-frame translational start signal. The resulting *lacZ′−'skc* fusion gene specified, under the control of *lacPO*, a hybrid SK which retained the full potential to activate plasminogen. In addition, *E. coli* colonies carrying this recombinant plasmid gave a positive SK reaction without the requirement of breaking open the cells. Obviously, hybrid SK was consistently exported by an unknown mechanism even in the absence of a signal sequence in amounts sufficiently high to be readily detected by the formation of caseinolysis zones in the plate assay.

Based on the properties of this system, two types of vectors designed to specify tripartite hybrid streptokinases were constructed. The first type carried *'skc* in the *Hin*cII site of pUC18 to form pKM2. The multiple cloning site of pUC18 introduced a frame shift such that *'skc* was translated out of frame with respect to *lacZ′*. Accordingly, pKM2 specified an SK-negative phenotype.

The second type of vector, pKM3, also utilised *'skc* as indicator gene but carried a gram-positive promoter and a secretion sequence in front of the genes to be fused. These new elements came from the streptococcal pyrogenic exotoxin type A gene (*speA*) previously cloned and sequenced by Weeks and Ferretti (1984, 1986). To construct pKM3, the *Sal*I−*Hin*dIII fragment from pKM1 containing the first half of *'skc* was ligated to a *Hin*dIII−*Pst*I fragment containing the rest of the *skc* coding sequence, and cloned into pUC8 cut with *Sal*I+*Pst*I. In the resultant intermediary plasmid, the 5' portion of *'skc* was available as a *Sma*I−*Bst*EII fragment for subsequent constructions. In the second step, the *Hin*dIII−*Sau*3A fragment containing the *speA* promoter ($P_{speA}$) and the early coding region of *speA*

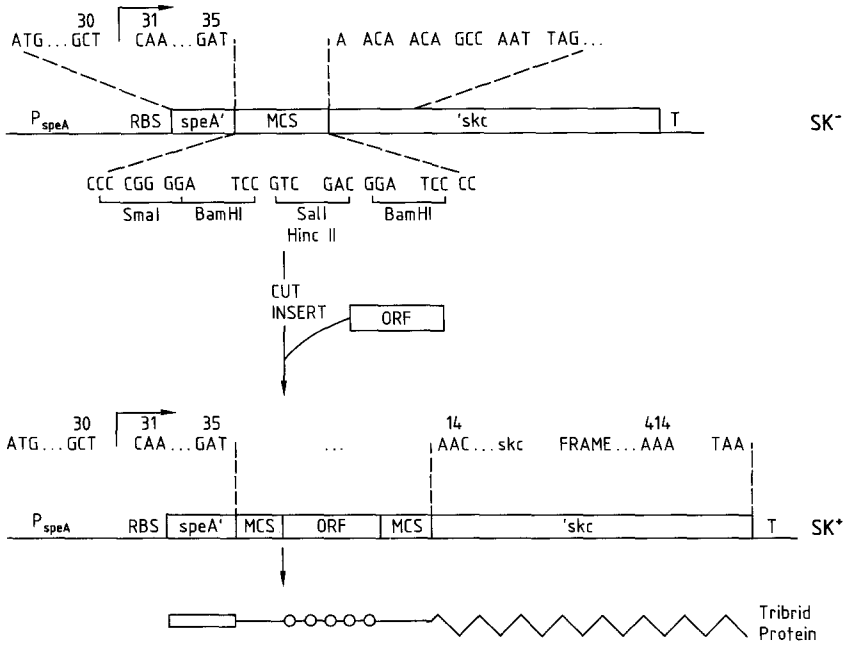


**Fig. 1.** Scheme for isolating *speA'*—ORF—*'skc* tripartite fusions using pUC9 as vector. $p_{speA}$, streptococcal pyrogenic exotoxin type A gene (*speA*) promoter; RBS, ribosome binding site; *speA'*, 5′ end of *speA*, MCS, multiple cloning site; *'skc*, truncated streptokinase gene; SK, streptokinase; T, *skc* transcription terminator; ORF, open reading frame. *Numbers* indicate codons for SPE type A and mature SK, respectively. The *bent arrow* indicates the SPE type A signal peptide cleavage site

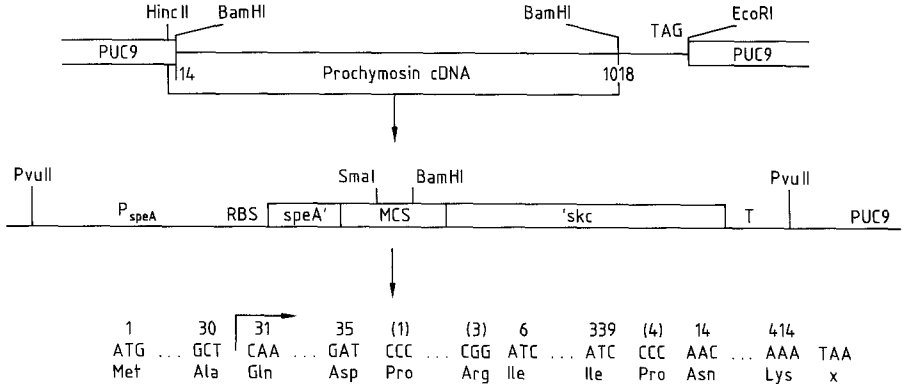


**Fig. 2.** Insertion of prochymosin cDNA coding for amino acids 6–339 into pKM3 to form pKMS3. Symbols are as in Fig. 1. *Numbers* in the upper construct refer to prochymosin cDNA nucleotides according to the numbering convention of Harris et al. (1982). *Numbers* in the nucleotide sequence indicate codons for SPE type A (1–35), prochymosin (6–339), mature SK (14–414), and codons formed by the multiple cloning site [(1)–(3), and (4)]

was isolated from pSA32 and cloned into pUC9 cleaved with *Hin*dIII + *Bam*HI. The resultant plasmid cut with *Sma*I + *Eco*RI was used to insert the above *Sma*I − *Bst*EII fragment containing the 5' portion of *'skc*, together with the *Bst*EII − *Eco*RI fragment from pKM1 containing the remainder of *'skc*. This resulted in pKM3 specifying an SK-negative phenotype (Fig. 1). Both pKM2 and pKM3 contained synthetic DNA corresponding to the multiple cloning sites of the pUC plasmids between the control elements for expression and *'skc*, thus allowing the in vitro insertion of target DNA between the key DNA pieces. If the target DNA represented an ORF of (3N + 1) nucleotides, *'skc* was realigned in either vector with the initiation codon and insertionally activated to produce a tripartite fusion protein having SK activity. The N-terminus of the tribrid proteins consisted of the N-terminal hexapeptide of beta-galactosidase in the case of pKM2 or, in the case of pKM3, the first 35 amino acids of the *speA* gene product (SPE type A), including 30 amino acids corresponding to the signal sequence. In the middle, the fusion protein contained the sequence encoded by the target DNA, and its C-terminus was formed by SK.

To assess the utility of these systems, particularly for inserting long target gene sequences, the 1,007 bp *Hinc*II-*Bam*HI fragment coding for amino acids 6 through 339 of prochymosin (Harris et al. 1982) was inserted into pKM3 opened by cutting with *Sma*I + *Bam*HI. In this configuration, the three component DNA sequences were aligned correctly for translation (Fig. 2). Accordingly, the resultant plasmid, pKMS3, when transformed into *E. coli* JM101 gave rise to SK-positive clones as detected by the plate assay. Furthermore, all transformant colonies tested (12) carried plasmids containing the expected prochymosin cDNA fragment as revealed by restriction analysis.

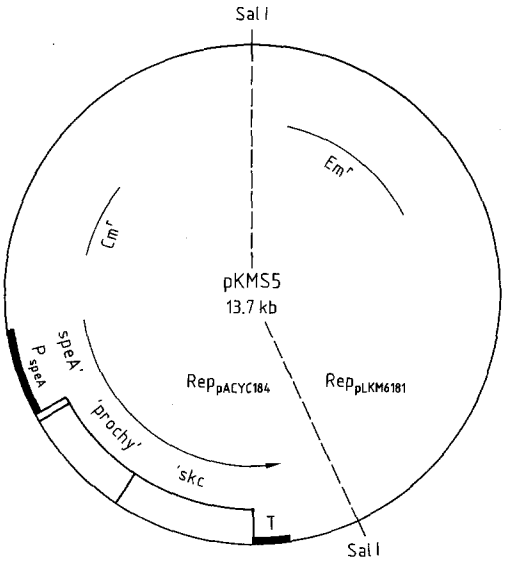


**Fig. 3.** Structure of pKMS5. Symbols are as in Fig. 1. The *arrow* indicates the direction of transcription of the tripartite fusion gene

*Detection of hybrid proteins*

The prototype vectors described above could replicate only in *E. coli*. To study the expression of the tribrid SK encoded in pKMS3 in both gram-positive and gram-negative hosts, a shuttle plasmid containing the relevant sequence configuration was constructed to provide replication functions for appropriate members of both groups of organisms. To this end, the complete transcription-translation unit contained in pKMS3 was removed in the form of a 3.8 kb *Pvu*II fragment and inserted into the unique *Eco*RV site of pACYC184. The resultant plasmid, pKMS4, was linearized with *Sal*I and fused with the *Sal*I-digested streptococcal vector plasmid pKLM6181 to form pKMS5 (13.7 kb; Fig. 3). The latter was transformed into *E. coli* JM101, *S. sanguis* Challis 6, and *S. lactis* MG1363 by selecting, respectively, chloramphenicol- and erythromycin-resistant colonies, all of which showed SK activity. A summary of the ORF vectors constructed in the course of this work is presented in Table 2.

The hybrid SK encoded in pKMS5 had a total of 774 amino acid residues with a calculated molecular weight of 87.5 kDa. Removal of the 30 amino acid residue signal peptide would result in a mature hybrid protein of 84 kDa. When sonicates of *E. coli* JM101 (pKMS5) and culture supernatant fluids of the two streptococcal strains carrying pKMS5 were subjected to SDS-PAGE and the gels were processed for visualizing bands with SK activity, an 84 kDa protein species capable of activating plasminogen to result in caseinolysis was detected in both *E. coli* and *S. sanguis* (Fig. 4). This activity was not consistently detected in samples derived from *S. lactis*. In addition to the 84 kDa band, *E. coli* and *S. sanguis* produced smaller proteins with SK activity, and so did *S. lactis* (Fig. 4). Presumably, these activities corresponding to molecular weights ranging from 40–46 kDa reflected proteolytic degradation of the 84 kDa species that occurred to different extents in the three organisms. In *S. lactis*, degradation appeared to proceed furthest, with most of the SK activity being associated with protein cleavage products.

Since in the prochymosin component of the tribrid protein the 26 C-terminal amino acid residues of the prochymosin molecule were missing, no chymosin activity was detectable in the samples. In fact, control gels processed in the absence of plasminogen failed to show any traces of caseinolysis at the end of the short incubation period (1–3 h at 37° C) adequate to visualize SK activity. To demonstrate the antigenic identity of the chymosin part of the tribrid protein, immunodetection was used. As shown in Fig. 4, the 84 kDa band reacted with an antiserum to chymosin in Western blots. In addition to the 84 kDa signal, a second band detected by the chymosin antiserum appeared at a position corresponding to 61 kDa. This band, again presumed to represent a degradation product of the full hybrid protein structure, did not show SK activity. In keeping with

**Table 2.** Open reading frame (ORF) vectors constructed

| Vector | Replicon | Promoter | Signal sequence | Terminus | | Streptokinase (SK) | Target DNA | Host |
|---|---|---|---|---|---|---|---|---|
| | | | | N | C | | | |
| pKM1 | pUC9 | *lacPO* | None | *lacZ* | *skc* | + | None | *Escherichia coli* |
| pKM2 | pUC18 | *lacPO* | None | *lacZ* | *skc* | – | None | *E. coli* |
| pKM3[a] | pUC9 | $P_{speA}$ | *speA* | *speA* | *skc* | – | None | *E. coli* |
| pKM4[a] | pACYC184 | $P_{speA}$ | *speA* | *speA* | *skc* | – | None | *E. coli* |
| pKM5[a] | pACYC184 | $P_{speA}$ | *speA* | *speA* | *skc* | – | None | *E. coli* |
| | + pSM6 | | | | | | | *Streptococcus sanguis*<br>*S. lactis*<br>*Bacillus subtilis*[b] |
| pKMS3 | pUC9 | $P_{speA}$ | *speA* | *speA* | *skc* | + | Prochymosin | *E. coli* |
| pKMS4 | pACYC184 | $P_{speA}$ | *speA* | *speA* | *skc* | + | Prochymosin | *E. coli* |
| pKMS5 | pACYC184 | $P_{speA}$ | *speA* | *speA* | *skc* | + | Prochymosin | *E. coli* |
| | + pLKM6181 | | | | | | | *S. sanguis*<br>*S. lactis*<br>*B. subtilis*[b] |

[a] Identical to, respectively, pKMS3, pKMS4 and pKMS5, except for the absence of the prochymosin target DNA insert
[b] pKM5 and pKMS5 will replicate in *B. subtilis* and, after insertional activation of '*skc*, direct SK synthesis

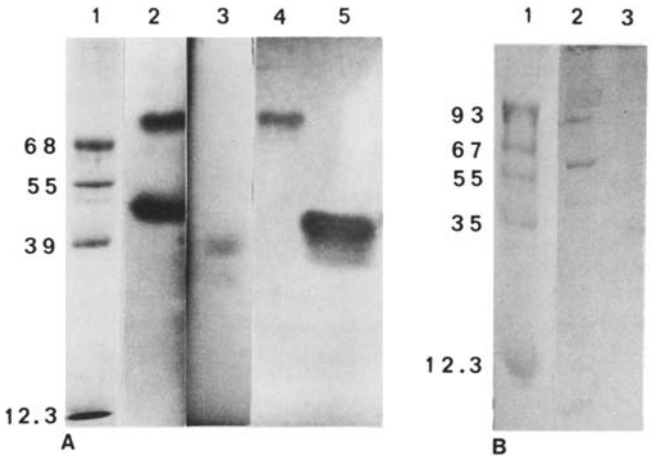


**Fig. 4A, B.** Detection after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (**A**) and Western immunoblotting (**B**) of tribrid streptokinase (SK) specified by pKMS5 in *Escherichia coli*, *Streptococcus sanguis*, and *S. lactis*. **A** Lane 1, marker proteins kDa; 2, SK-active bands obtained from a sonicate of *E. coli* (pKMS5) expressing tribrid SK; 3, SK-active band from the culture filtrate of *S. lactis* (pKMS5) expressing degraded protein; 4, SK-active band from the culture filtrate of *S. sanguis* (pKMS5) expressing tribrid SK; 5, SK-active bands from the culture filtrate of *S. sanguis skc*$^+$ expressing mature SK lacking the N-terminal extension peptide (marker and control). **B** Lane 1, marker proteins (kDa); 2, chymosin-reactive bands from the culture filtrate of *S. sanguis* (pKMS5); 3, absence of chymosin-reactive bands in samples derived from the culture filtrate of *S. sanguis* (pKM5) (control)

what we think corresponds essentially to truncated SK, the 40–46 kDa proteins did not give a chymosin signal in the Western blots (Fig. 4).

## Discussion

We have constructed gene fusion vectors, the key elements of which – replicon, promoter, signal sequence, indicator gene, and transcription terminator – originate from streptococci. This extends the use of the in vitro gene fusion technology for the production of hybrid proteins to this medically and industrially important group of organisms. Hitherto, streptococci were amenable to genetic fusion techniques only through in vivo transcriptional fusion mediated by Tn*917* carrying a *lacZ* gene which seems to be weakly expressed (Weaver and Clewell 1987). The addition to the above elements of a replicon for gram-negative bacteria, as exemplified by pKM5 and pKMS5 (Table 2), allows this system to be used in *E. coli* as well.

The present studies show that the first 13 amino acid residues of mature SK may be substituted with as few as 6 or as many as several hundreds of unrelated amino acids without preventing SK from activating human plasminogen. This is also true for hybrid SKs specified by '*skc* fusions with human plasminogen cDNA coding for a similarly wide range of peptides corresponding to the plasminogen heavy chain (Malke and Ferretti, unpublished). Thus, regardless of the nature of the N-terminal extension peptides, SK continues to be capable of forming functionally active complexes with plasminogen, suggesting that the molecule folds autonomously. The fact that the SK molecule contains no cysteine residues may be germane to this property. In an attempt to suggest a molecular mechanism whereby SK activates plasminogen, Bode and Huber (1976) hypothesized that by analogy with the way trypsinogen is activated, SK intrudes its specific N-terminus into the binding pocket of plasminogen, thus structuring the specificity pocket which results in activity. The present fusion SKs clearly disprove the terminal insertion hypothesis.

One reason for constructing chymosin cDNA-'*skc* fusions was to prove that chymosin is expressed under the *speA* promoter not only in *E. coli* but also in *Streptococcus*. The specific construct (Fig. 2) comprises 90% of the coding sequence for prochymosin which is GC-rich and, therefore, contains a number of codons that are rarely used in the AT-rich streptococci (Malke 1986). By monitoring the SK activity of the fusion protein and demonstrating its antigenic identity, we provide evidence that chymosin is actually expressed and secreted into the streptococcal culture medium. Correct processing of the SPE Type A signal sequence has been demonstrated recently by N-terminal sequence analysis of the mature toxin as expressed in the heterologous *S. sanguis* Challis host (Gerlach et al. 1987). Protein analysis by SDS-PAGE reveals that the chymosin-SK hybrid protein undergoes proteolytic degradation to varying extents in all three organisms. Interestingly, degradation is most pronounced in *S. lactis* MG1363, a strain not producing one major proteinase due to the loss of a 33 MDa lactose-proteinase plasmid (Gasson 1983). Apparently, other proteinases contributing to the breakdown of the hybrid protein render this strain an inappropriate host for the production of certain foreign proteins.

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