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Streptokinase: Cloning, expression, and excretion by *Escherichia coli*

(recombinant DNA/streptococci/plasminogen activator/periplasmic transport)

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ABSTRACT **Genomic DNA from *Streptococcus equisimilis* strain H46A was cloned in *Escherichia coli* by using the bacteriophage λ replacement vector L47 and an *in vitro* packaging system. A casein/plasminogen overlay technique was used to screen the phage bank for recombinants carrying the streptokinase gene (*skc*). The gene was present with a frequency of 1 in 836 recombinants, and 10 independent clones containing *skc* were isolated and physically characterized. One recombinant clone was used to subclone *skc* in *E. coli* plasmid vectors. Plasmid pMF2 [10.4 kilobases (kb)] consisting of pACYC184 with a 6.4-kb H46A DNA fragment in the *Eco*RI site and pMF5 (6.9 kb) carrying a 2.5-kb fragment in the *Pst* I site of pBR322 were among the recombinant plasmids determining streptokinase production in three different *E. coli* host strains. Expression of *skc* was independent of its orientation in either vector, indicating that its own promoter was present and functional in *E. coli*. However, expression in pBR322 was more efficient in one orientation than in the other, suggesting that one or both of the *bla* gene promoters contributed to *skc* expression. Several lines of evidence, including proof obtained by the immunodiffusion technique, established the identity of *E. coli* streptokinase. Testing cell-free culture supernatant fluids, osmotic shock fluids, and sonicates of osmotically shocked cells for streptokinase activity revealed the substance to be present in all three principal locations, indicating that *E. coli* cells were capable of releasing substantial amounts of streptokinase into the culture medium.**

Streptokinases are a well-defined group of proteins exported by many strains of hemolytic streptococci to the growth medium. They interact stoichiometrically with the enzymatically inert plasma plasminogen to yield the active enzyme plasmin. The plasmin so formed then degrades, by limited proteolysis, the fibrin network to form soluble products (1, 2). Although, unlike other plasminogen activators, streptokinases are not proteases, the recently determined amino acid sequence of one streptokinase species revealed homology to the sequences of bovine trypsin and *Streptomyces griseus* proteases, suggesting that it evolved from a serine protease (3).

The role of streptokinases in the pathogenicity of streptococci is unclear. Potentially, these substances may be determinants of virulence that contribute to the invasiveness of the organisms by preventing the formation of fibrin barriers around infectious lesions. Physical and immunological differences, paralleled by differences in substrate specificity, testify to the molecular heterogeneity of streptokinases from different sources (4, 5). Although these proteins are closely related in function, the genetic basis of their heterogeneity is unknown. To achieve a better understanding of the genetic aspects of this important streptococcal product, we have undertaken to clone a streptokinase gene from a group C *Streptococcus* and report here its expression in *Escherichia coli*. Besides providing approaches to studying the molecular and epidemiological relationships between streptokinases, the cloning of this gene should provide alternative organisms for commercial streptokinase production. As a drug, streptokinase has a place in thrombolytic therapy (6).

MATERIALS AND METHODS

Microorganisms and Culture Conditions. The bacterial strains, phages, and plasmids used are listed in Table 1. *S. equisimilis* H46A cells were grown in brain heart infusion broth (Difco) as standing cultures at 37°C. *E. coli* strains were grown in LB medium (16) with added selective agents [ampicillin (Ap), 50–100 μg/ml; tetracycline (Tc), 12.5 μg/ml; chloramphenicol (Cm, 30 μg/ml] if required. ^{r}, Resistant; ^{s}, sensitive. Solid and liquid media for the preparation of λ phage lysates and for titering phage were prepared as described by Davis *et al.* (17).

DNA Preparation. Strain H46A cells were lysed with mutanolysin (Dainippon Pharmaceutical, Osaka, Japan), and chromosomal DNA was treated with phenol and further purified by CsCl density gradient centrifugation (18). The average size of this DNA was 50 kilobases (kb). Plasmid DNA was isolated from cleared *E. coli* lysates obtained by treatment with lysozyme and sodium dodecyl sulfate (19). Two successive centrifugations to equilibrium in CsCl/ethidium bromide gradients were used to purify this DNA. Plasmid screening in *E. coli* was done according to the "mini prep" method of Ish-Horowicz and Burke (20). Bacteriophage λ DNA was prepared from high-titer lysates [about 10^{10} plaque-forming units (pfu)/ml] after potassium acetate/sodium dodecyl sulfate precipitation of denatured proteins, as described by Cameron *et al.* (21).

Cloning Procedures. Twenty replicate 10-μg amounts of H46A DNA were partially digested with *Sau*3A, pooled, extracted with phenol, and fractionated by centrifugation through a 10–40% linear sucrose gradient (19). One microgram of DNA fragments in the 4- to 15-kb range was ligated with 1 μg of λL47 DNA completely digested with *Bam*HI. We used 0.8 μg of this ligated DNA to package it into λ phage heads under conditions specified by the supplier (Amersham) of the λ DNA *in vitro* packaging kit. Recombinant phage carrying the streptokinase gene were used for the extraction of DNA, which was subcloned as explained in *Results*. Transformation of the *E. coli* strains was as described by Dagert and Ehrlich (22).

Detection and Assay of Streptokinase Activity. Phage plaques or bacterial colonies producing streptokinase were routinely detected by taking advantage of the caseinolytic activity of activated plasminogen. Plates with developed

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Abbreviations: Ap, ampicillin; Tc, tetracycline; Cm, chloramphenicol; ^{r}, resistant; ^{s}, sensitive; kb, kilobases; pfu, plaque-forming units.

Table 1. Bacterial strains, phages, and plasmids used

Strain	Properties	Ref. or source
Streptococcus equisimilis		
H46A	Serological group C strain producing streptokinase	1
Escherichia coli		
HB101	F⁻ *hsdS20 recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 thi-1 supE44* λ⁻; cloning host	7
294	Endonuclease I-negative, endonuclease R-negative, thiamin-requiring; cloning host	8
CP78	F⁻, requiring threonine, leucine, histidine, arginine, and thiamin; cloning host	9
WL87	*recBC*; host for propagating recombinant λL47 phage	Amersham
WL95	*metB supE supF hsdR tonA trpR* P2⁺; host for selecting recombinant λL47	Amersham
Phages		
λ*c*I857	Bacteriophage λ with thermolabile *c*I gene product	10
λL47	λ replacement vector	Ref. 11, Amersham
Plasmids		
pBR322	*E. coli* plasmid vector; ampicillin resistant, tetracycline resistant	12, 13
pACYC184	*E. coli* plasmid vector; chloramphenicol resistant, tetracycline resistant	14

Symbols and genetic markers are used in accordance with Bachmann (15).

plaques or colonies were overlayed with 9 ml of 50 mM Tris·HCl, pH 8.1/150 mM NaCl containing 90 mg of agar or agarose, 100 μg of human plasminogen, and 1 ml of skim milk. After incubation for a minimum of 2 hr at 37°C, clear zones around plaques or colonies indicated phage or bacterial clones carrying the streptokinase gene. Streptokinase activity of culture supernatant fluids or cellular fractions was estimated by comparison with dilutions of a standard purified streptokinase solution, using the casein/plasminogen plate technique. The area of the lysis zone surrounding the wells cut into the agarose medium and filled with the samples can be correlated to the amount of streptokinase.

Recovery of Extracellular, Periplasmic, and Cytoplasmic Streptokinase. Extracellular streptokinase was assayed in chloroform-treated culture supernatant fluids obtained by centrifugation of overnight liquid cultures. Periplasmic protein was prepared by the minishock procedure as described by Hazelbauer and Harayama (23). The cytoplasmic protein fraction was obtained by subjecting the osmotically shocked cells to sonication in 0.5 mM $MgCl_2$ at 0°C, using four intermittent 20-sec pulses at maximal output.

Immunodiffusion. Two-dimensional immunodiffusion was performed on 1% agarose plates containing barbital buffer.

Enzymes and Reagents. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Human plasminogen came from Sigma or was a gift of Dieter Gerlach. The latter also prepared standard streptokinase (55,000 units/mg of protein) from H46A and generously provided us with monospecific streptokinase antibody purified by affinity chromatography.

RESULTS

Construction of a Genomic Library of H46A in λL47. *S. equisimilis* strain H46A total cell DNA partially digested with *Sau*3A and λL47 DNA completely cut with *Bam*HI were mixed and ligated. An aliquot of phage resulting from the *in vitro* packaging was plated on strain WL87 to give the total number of pfu and on strain WL95(P2) to determine the proportion of recombinant phage resistant to phage P2-mediated interference (11). The packaging efficiency was 10^5 total pfu/0.4 μg of vector DNA. Of the total phage, a proportion of 20% were particles carrying recombinant genomes. Assuming that the average insert size was about 10 kb (see below) and the size of the *Streptococcus* genome is 2000 kb (24), the number of recombinant phage particles required to have any target DNA sequence represented at a probability of 0.99 is approximately 900 (25). The yield of recombinant phages resulting from ligation and *in vitro* packaging exceeded this figure by a factor of about 20.

Detection of Streptokinase-Producing Plaques. The plasminogen-casein overlay technique was used to detect streptokinase-producing plaques. Of 8360 recombinant phages plated, 10 yielded plaques showing distinct zones of caseinolysis (Fig. 1). There was no background in the assay, indicating that neither *E. coli* nor phage λ produced detectable amounts of proteases that cleave casein under these conditions. The frequency of the streptokinase gene, henceforth referred to as *skc*, in the phage bank agreed well with the theoretical expectations based on the fraction of the H46A genome in a single recombinant phage (0.5%).

Physical Characterization of Recombinant λL47 Clones Carrying *skc*. The 10 streptokinase-positive phages, designated λL47A-K*skc*, were purified and their DNA was characterized by restriction endonuclease analysis using *Hin*dIII, *Eco*RI, and *Bam*HI. The *Hin*dIII digestion patterns relevant to our analysis are shown in Fig. 2. The insert sizes of the 10 clones, of which E and G were identical, were 7–15 kb. The most prominent feature of the *Hin*dIII patterns was the existence of a 1.9-kb fragment common to all 10 clones. A second *Hin*dIII fragment, 2.65 kb in size, was common to 5 clones, including E and G.

Subcloning of *skc* onto *E. coli* Plasmid Vectors. The DNA from clone λL47E*skc* was partially digested with *Hin*dIII and ligated with pBR322 DNA completely cut with the same enzyme. Of 260 Ap^r *E. coli* HB101 transformants obtained

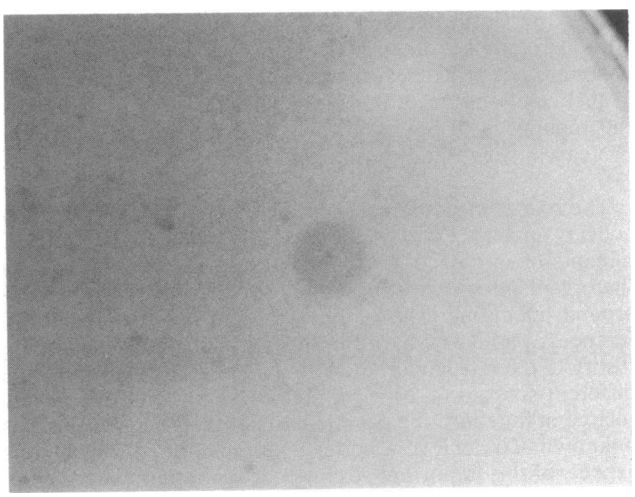

FIG. 1. Detection of recombinant λL47 phage containing the streptokinase gene. Caseinolytic plaque of clone λL47K*skc* plated on WL87 is shown.

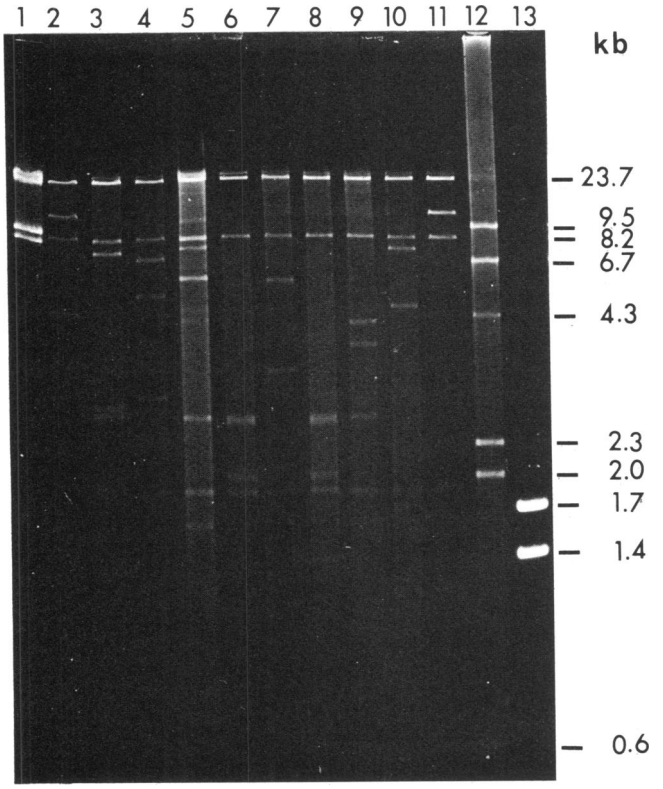

FIG. 2. Analysis of the DNA of phage clones λL47A-K*skc* by *Hin*dIII digestion. Lanes: 1, λL47; 2, λL47A*skc*; 3, λL47B*skc*; 4, λL47C*skc*; 5, λL47D*skc*; 6, λL47E*skc*; 7, λL47F*skc*; 8, λL47G*skc*; 9, λL47H*skc*; 10, λL47I*skc*; 11, λL47K*skc*; 12, λ; 13, pBR322 digested with *Ava* II.

with the ligation mixture, 37 were Tc^s. One of these gave a positive streptokinase reaction. pMF1 (11.8 kb), the plasmid carried by the streptokinase-positive HB101 clone, consisted of pBR322 carrying a 7.4-kb insert in its *Hin*dIII site. A fragment of the same size was identifiable in partial *Hin*dIII digests of λL47E*skc* DNA, indicating that the pBR322 insert did not represent scrambled sequences. The insert consisted of 4 *Hin*dIII fragments (2.65, 2.60, 1.90, and 0.20 kb) which were ordered by partial *Hin*dIII digestion of pMF1 (Fig. 3). As expected, the 1.9- and 2.65-kb fragments were adjacent. Compared to the DNA content of the original λL47E*skc* clone (Fig. 2), pMF1 was devoid of both λ arms and the 2.0-kb *Hin*dIII fragment.

*Eco*RI digestion of pMF1 yielded two fragments (6.4 and 5.4 kb), of which the 6.4-kb fragment consisted of insert DNA containing the complete 1.9- and 2.65-kb *Hin*dIII segments. This fragment was inserted into the *Eco*RI site of pACYC184 to yield, after transformation of HB101 to Tc^r Cm^s, plasmid pMF2 (10.4 kb; Fig. 3). Two HB101(pMF2) clones carrying the *Eco*RI insert in opposite orientations were identified by *Ava* I digestion of pMF2 (size of the *Ava* I fragments in orientation I, 5.3 and 5.1 kb; in orientation II, 6.9 and 3.5 kb) and tested for streptokinase production. The insert was found to determine streptokinase production in either orientation. No detectable difference was noted in the size of the clearing zones produced by colonies containing pMF2I or pMF2II.

Pst I digestion of pMF1 yielded four fragments (6.0, 2.5, 2.2, and 1.1 kb), of which the 2.5-kb fragment containing parts of the 1.9- and 2.65-kb *Hin*dIII fragments (Fig. 3) was ligated with *Pst* I-digested pBR322 DNA and used to transform HB101 to Tc^r Ap^s. *Hin*dIII digestion of the resultant plasmid, pMF5 (6.9 kb; Fig. 3), was used to orient the *Pst* I insert (*Hin*dIII fragment sizes in orientation I, 5.3 and 1.6 kb; in orientation II, 4.4 and 2.5 kb). Plasmid pMF5 specified streptokinase production in either orientation of *skc*. LB agar plates that had developed single colonies of HB101(pMF5I) and HB101(pMF5II) after incubation for 18 hr at 37°C were overlayed with streptokinase assay medium and the diameters of the zones of caseinolysis produced by 100 colonies randomly chosen from each strain were measured after incubation for 8 hr at 37°C. In a representative experiment, the mean (±SD) diameters of the clearing zones were 6.51 ± 0.28 mm (pMF5I) and 7.35 ± 0.30 mm (pMF5II), respectively. This indicated that the orientation of *skc* in the *Pst* I site of pBR322 significantly influenced the expression of the gene ($P > 0.99$). Two repetitions of this experiment yielded similar results.

Identity of the *skc* Gene Product. Several lines of evidence showed unambiguously that we cloned the gene for strepto-

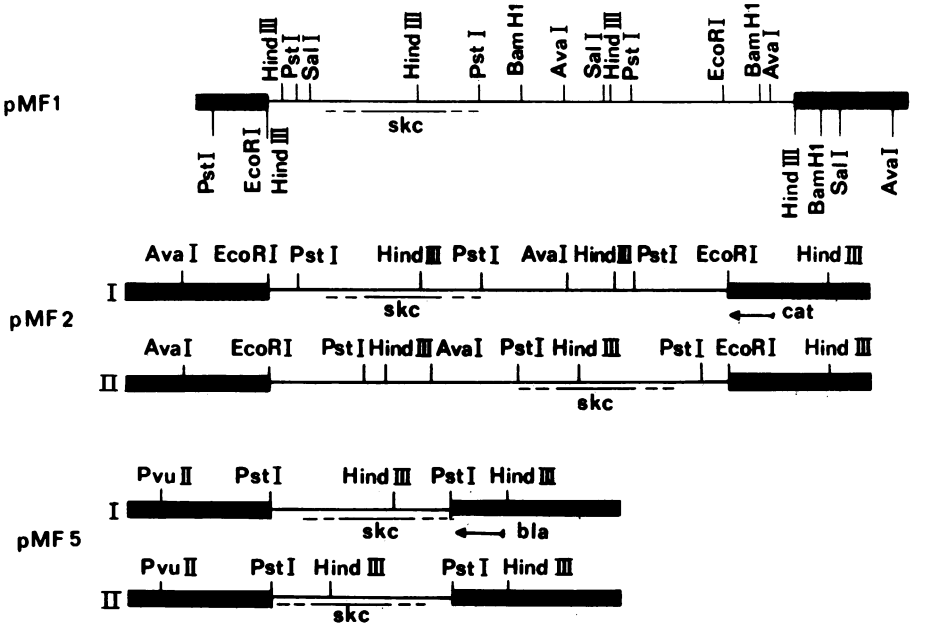

FIG. 3. Restriction maps of pMF1, pMF2, and pMF5. Orientations of the inserts are indicated by a few restriction sites. Heavy lines indicate vector DNA. Arrows indicate the direction of transcription of the *cat* gene in pACYC184 and the *bla* gene in pBR322 (26).

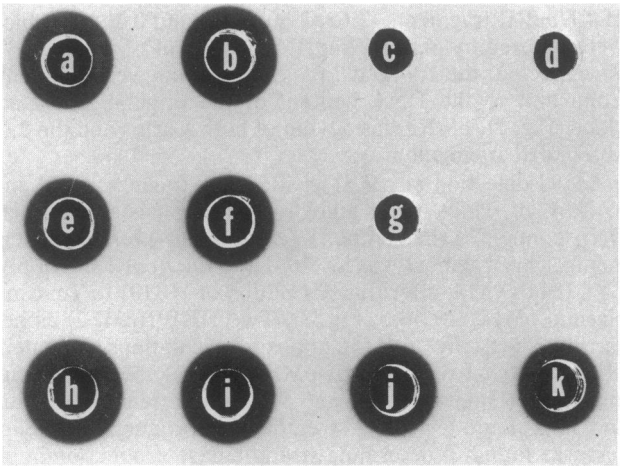

FIG. 4. Neutralization of the caseinolytic activity of *E. coli* streptokinase by monospecific IgG raised against authentic H46A streptokinase. Wells: a, culture supernatant from HB101(pMF1); b, culture supernatant from HB101(pMF1) + nonimmune IgG; c, concentrated culture supernatant from HB101(pMF1) + streptokinase IgG; d, culture supernatant from HB101(pBR322) concentrated (10×) by dialysis against polyethylene glycol; e, H46A streptokinase (5 units); f, H46A streptokinase (5 units) + nonimmune IgG; g, H46A streptokinase (5 units) + streptokinase IgG; h–k, 20, 10, 5, and 2.5 units of purified H46A streptokinase.

kinase rather than a protease gene. (*i*) All 10 primary phage clones produced caseinolytic plaques only in the presence of human plasminogen; (*ii*) diisopropyl fluorophosphate, an inhibitor of serine proteases, did not abolish the activation of plasminogen by any of the recombinant phages upon propagation on WL87 under conditions (10 mM final concentration) in which caseinolysis by trypsin was completely inhibited; (*iii*) replacing human plasminogen by bovine plasminogen in agar plates containing bovine fibrin (provided by Fletcher Taylor) required supernatant fluids from HB101(pMF1) cultures to be mixed with human plasminogen before being capable of mediating the digestion of bovine fibrin (H46A streptokinase does not activate bovine plasminogen); (*iv*) in immunodiffusion tests, concentrated supernatant fluids or cell lysates of HB101(pMF1) cultures and authentic streptokinase preparations from H46A gave a line of identity with monospecific IgG prepared against purified H46A streptokinase. This precipitin band did not develop when similar materials from HB101 cultures containing the insert-free vector were tested. In addition, streptokinase IgG neutralized the caseinolytic activity of supernatant fluids or cell lysates of HB101(pMF1) cultures when added to these preparations before testing on casein/plasminogen plates (Fig. 4).

Synthesis of Streptokinase in *E. coli*. In subcloning the *skc* gene by using plasmid vectors, we did not originally expect the *E. coli* cells to release detectable amounts of streptokinase into the medium. Therefore, we cloned into a λ *c*I857 lysogen of HB101 at 29°C and heat-induced the growing colonies at 42°C (27) before testing for streptokinase production. Subsequently, we found that uninduced HB101 (λ *c*I857, pMF1) and λ-free HB101 colonies transformed with pMF1, -2, or -5 consistently produced pronounced caseinolysis when tested in any state of growth (Fig. 5). Also, two additional *E. coli* strains unrelated to HB101 (294 and CP78) transformed with pMF1 and pMF5, respectively, were caseinolytic. When plasmid-free, none of these strains was caseinolytic under our conditions.

Release of streptokinase into the surrounding medium was observed not only during growth of appropriate *E. coli* strains on solid medium but also during cultivation in LB broth—i.e., cell-free supernatant fluids of saturated overnight cultures were always found to contain streptokinase. In independent 5-ml HB101(pMF1) cultures started from single colonies grown overnight at 37°C to reach about 3×10^9 cells per ml, the approximate amounts released into the medium ranged from 8 to 100 units/ml, depending on the age of the cultures. Fractionation of early stationary phase cells revealed additional streptokinase activity in both cold osmotic shock fluids and sonicates of osmotically shocked cells. In such cultures, typical values for the distribution of the total activity were 17% in the supernatant, 30% in the periplasm, and 52% in the cytoplasm. Cells incubated for a prolonged period after attaining stationary phase (≧8 hr) had no detectable periplasmic activity although still containing streptokinase in the cytoplasm. Qualitatively, corresponding results were obtained with liquid HB101 cultures containing either pMF5I or pMF5II. Quantitatively, the fractions of HB101 cultures containing pMF5 in orientation II of *skc* contained at least 1.5 times more streptokinase than corresponding fractions from HB101(pMF5I) cultures.

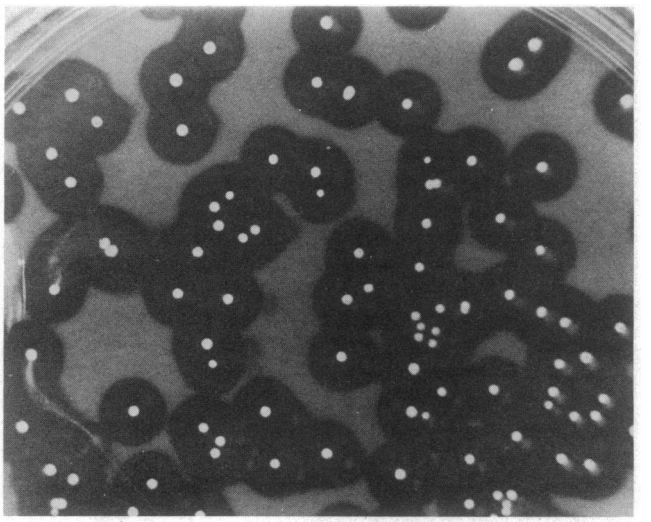

FIG. 5. Caseinolysis mediated by HB101(pMF1) colonies on casein/plasminogen overlay plates.

DISCUSSION

We have identified and cloned in bacteriophage λ and *E. coli* plasmid vectors a segment of *S. equisimilis* strain H46A DNA that codes for streptokinase. In its shortest form, this DNA segment is available as a 2.5-kb insert in the *Pst* I site of pBR322. The *Pst* I fragment is large enough to accommodate the complete coding sequence of mature streptokinase, which consists of 415 amino acid residues (M_r = 47,408) whose sequence has been determined recently (3). The streptokinase gene (*skc*) is likely to contain one *Hin*dIII site but lacks sites for *Ava* I, *Bam*HI, *Eco*RI, and *Pst* I. Assuming that the gene is about 1.3 kb long, the *Sal* I site contained in the *Pst* I fragment is also outside the *skc* gene. This implies that the *Hin*dIII/*Sal* I subfragment (1.5 kb) of the *Pst* I fragment in question contains a minimum of about 400 base pairs of *skc*.

The gene is expressed in *E. coli* when cloned in either λL47 or plasmids pBR322 and pACYC184. Moreover, expression is independent of its orientation in either plasmid, indicating that its own promoter is present and functional in *E. coli*. However, the evidence presented shows that, when cloned in pBR322, *skc* is expressed more efficiently in orientation II (as defined in Fig. 3) than in orientation I. These observations suggest that transcription from one or both of the two *bla* gene promoters in pBR322 (26) proceeds into the *skc* gene region and contributes to its

expression. If this idea is true, note that it defines the orientation of *skc* in the *Pst* I fragment (Fig. 3). The finding that the level of *skc* expression in pMF2 is orientation independent may mean that either the *cat* gene promoter of pACYC184 is weaker than the *skc* promoter or there exists a transcription termination signal(s) in H46A DNA sequences upstream from the *skc* promoter.

Streptokinase produced by *E. coli* has the same substrate specificity as that of the streptococcal donor strain. The identity of the *E. coli* product has also been demonstrated by the immunodiffusion technique. In *Streptococcus*, streptokinase is a secretory protein, suggesting that its immature form is synthesized with a signal sequence at its amino terminus (28). The existence of a signal sequence has also been suggested by Jackson and Tang (3) on the basis of their hypothesis that the streptokinase gene has evolved from a serine protease gene by duplication and fusion, with one copy of the putative signal sequence being conserved in the central part of the processed protein. Clearly, nucleotide sequencing of *skc* is necessary to test the validity of this attractive hypothesis.

In *E. coli* cultures, we find streptokinase activity in all three principal locations. Our data suggest that the extracellular activity is not attributable to leakage of the protein out of dead cells, but we do not know whether or not the exported activity results from correct processing and active secretion. In any event, the phenomenon is conspicuous and quantitatively significant. From a pragmatic point of view, it allows reliable detection of the *skc* gene product in *E. coli* clones without the requirement of fractionating or lysing the cells. The main barrier for the release of the *E. coli* streptokinase seems to be the inner membrane; once it is passed, the periplasmic protein is released upon prolonged incubation. The presence of extracellular and periplasmic streptokinase in cultures of strains carrying *skc* in two different plasmids in either orientation indicates that excretion is not entirely due to signal sequences provided by the attached proximal sequences of the *cat* or *bla* genes of the vectors. Rather, the putative streptokinase-specific signal sequence can be expected to play a crucial role in excretion of the substance by the heterologous host. Assuming that authentic and *E. coli* streptokinase have the same specific activity, the amounts of extracellular streptokinase activity (8–100 units/ml, depending on the age of saturated cultures containing 3×10^9 viable cells per ml) correspond to 0.15–1.82 mg per liter of culture or about 600–7000 streptokinase molecules released per cell.

Of the three drugs available for thrombolytic therapy, the cDNA sequences of urokinase and tissue-type plasminogen activator have been cloned before (29, 30). Another plasminogen activator substance produced by *Staphylococcus aureus*, staphylokinase, has also been cloned and is expressed in *E. coli* (31). The cloning of the streptokinase gene adds to the arsenal of sequences relevant to problems of plasminogen activation and opens up approaches for studying unresolved questions of theoretical and practical significance in the blood clotting field. For the genetics of streptococci, where the study of virulence genes by the recombinant DNA approach is still in its initial stages (26, 32), our work has shown the feasibility of cloning and expressing genes in phage λ vectors. The procedure could be particularly useful for cloning determinants whose products are detrimental to the normal physiology of the cloning host and readily detectable by simple plate assays.

Lewis W. Wannamaker was a source of stimulation, encouragement, and friendship to both authors and we acknowledge his overall contribution to the study of pathogenic streptococci. We thank Dieter Gerlach for suggestions and aid in the final stages of this project. In particular, we are grateful to him for generously providing streptokinase and streptokinase antibody. We also thank Jordan Tang, Charles Esmon, and Fletcher B. Taylor for helpful discussions. Most of this work was done during H.M.'s tenure of a Visiting Scientist Award of the University of Oklahoma Health Sciences Center and was supported by National Institutes of Health Grant AI 9304.

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