Cloning and expression of an actinokinase gene from a thermophilic *Streptomyces* in *Escherichia coli*

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Received 30 August 1999; revised 5 January 2001

A thermophilic *Streptomyces megasporus* strain SD5, could secrete a new fibrinolytic (actinokinase) at 55°C. The gene (*ackS*) encoding actinokinase was isolated from the chromosomal DNA of *S. megasporus* SD5 and cloned in different hosts and vectors. The expression was obtained in *E. coli* JM109 using *Cla*I linearized pBR322 as vector (pSR500). The recombinant *E. coli* containing pSR 500 expressed active actinokinase but the expression was low and the recombinant was unstable in liquid culture. Deletion analysis revealed that removal of *Bam*HI-*Sal*I fragment from downstream and *Cla*I-*EcoR*I from upstream enhanced the stability and expression of *ackS* in both solid and liquid media. For over expression, the *ackS* gene was cloned in *E. coli* C600 using *Bam*HI linearized pTI-7. This seemed to be the most suitable host vector system. The recombinant and native form of actinokinase exhibited similar characteristics. Actinokinase was the first thrombolytic enzyme from a thermophile to be cloned and over expressed in a mesophilic heterologous expression system.

Recent interest in fibrinolytic enzymes has been enhanced due to therapeutic value of the enzymes for recanalization of the occluded blood vessels in myocardial infarction. There are different fibrinolytic enzymes from prokaryotes available in the market. The prokaryotic thermolabile fibrinolytic enzymes, streptokinase and staphylokinase activate plasminogen by forming a stoichiometric complex where as urokinase, a protein available from human urine and foetal kidney is fibrin specific and proteolytic. Thermophiles became important since the pioneering studies of thermal environments by Brock and with the use of Taq polymerase. Actinokinase, a fibrinolytic enzyme from *S. megasporus* strain SD5, belonged to EC.3.4.21.23 and showed some unique features similar to urokinase such as caseinolytic and fibrinolytic activities, molecular weight of 35 kDa and N-terminal dependent activity. Many thermophilic isolates produce extracellular serine proteases. Out of all proteases, the fibrinolytic enzymes, received more attention due to its effect on myocardial infarction for removing the thrombus in the occluded blood vessels. Because of the significance of fibrinolytic enzymes in the treatment of thrombosis in man, microbial sources of such enzymes are being investigated. We have isolated a thermophilic *Streptomyces* strain from a hot water spring. The strain exhibited fibrinolytic activity in the broth. We are interested to study the gene encoding fibrinolytic activity. Although *Streptomyces* genes have been expressed in *E. coli* as recombinant molecules, there were few reports of transformation of *E. coli* using a hybrid plasmid carrying a gene from a thermophile but with low and poor expression. In this paper we have reported the isolation of the gene (*ackS*) encoding actinokinase, a fibrinolytic enzyme from a thermophilic *S. megasporus* strain SD5 and its cloning and over expression in *E. coli*.

**Materials and Methods**

All restriction and modifying enzymes, agarose and ethidium bromide were obtained from Sigma Chemical Co, USA and Genie, India. The chromogenic substrates were purchased from Roche Diagnostics, Mannheim. Other chemical used were of analytical grade.

**Bacterial Strain-Streptomyces megasporus**—SD5 was isolated from a hot spring, situated in the Western Coast of India and identified as described earlier. *E. coli* JM109, DH5α, DH 1, HB101, pUC 18 were gifts from Professor K. Dharmalingam, School of Biotechnology, Madurai, India and *E. coli* C 600 and the vector pT7-7 were gifted by Dr. Stan Tabor, Harvard Medical School, Boston.

**Qualitative and quantitative assay of activity of fibrinolytic enzyme**—Fibrinolytic activity was qualitatively assayed using fibrin plate and quantitatively by lysis of chromogenic substrates using the method.

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of Cleason et al. The reaction mixture consisted of 1 mL of 10 mM sodium phosphate buffer, pH 8.0 containing 0.2 mM chromozyme U and 100 μl enzyme solution and was incubated for 30 min at 37°C. The reaction was stopped with 500 μl of TCA solution (0.11 mol⁻¹ TCA, Sodium acetate, 0.22 mol⁻¹). Increase of colour intensity of 4-nitranilide acetate was measured at 405 nm against a reagent blank using Spectra Scan Chemito 2600 spectrophotometer. One unit of fibrinolytic activity was defined as the amount of the enzyme that liberated 1 ng nitranilide mL⁻¹ min⁻¹ under the specified conditions.

Isolation of total DNA and plasmid—Total DNA from S. megasporus SD5 was prepared as per Chater et al. with slight modification. Mycelia were harvested from 10 hr old liquid culture of S. megasporus SD5 were resuspended in Birnboim buffer (2 mM Tris. Cl pH 7.5, 10 mM EDTA with 0.9% w/v glucose, lysozyme 4 mg mL⁻¹). SDS (1% w/v), and pronase (10 mg mL⁻¹) were added and the suspension was incubated at 37°C for 2 hr. After addition of RNase (20 μg mL⁻¹) it was further incubated for 1 hr. NaCl (5 M) and 100% chilled ethanol were added. DNA was spooled, washed with 70 and 100% ethanol, dried and resuspended in 5 mL TE. Further purification was carried out using cesium chloride/ethidium bromide density gradient and Beckman LM 60 ultracentrifuge. Small scale preparation and purification of plasmid from E. coli was carried out as per Birnboim & Dolly.

Cloning and expression of actinokinase gene in E. coli—Standard techniques described by Sambrook et al. were used in all DNA manipulations. Agarose gels 90.7% w/v, were used to separate DNA bands and HindIII digested lambda DNA was used as size marker. Chromosomal DNA of S. megasporus SD5 was digested for completion with Sau 3 AI and size fractionated in the range of 1 to 10 kb by Prep-a-gene method. Hybrid plasmids were prepared by ligating a ~2kb insert from SD5 DNA with different linearized vectors e.g. ClaI linearized pBR322 and Bam HI treated pUC 18 and were used to prepare partial genomic libraries by transforming different strains of E. coli with both the hybrids separately. All Amp 'tet' colonies obtained were replicated on fibrin plates containing ampicillin (50 μg mL⁻¹) and tetracycline (25 μg mL⁻¹). A few colonies with haloes were selected. The hybrid plasmid was prepared using Bam HI linearized pUC 18 and the same ~2 kb fragment from S. megasporus SD5. Under IPTG induction pUC 18-ackS did not grow in liquid culture and grew slowly on plate forming sticky colonies. Stability and expression of the clones were checked by repeated subculturing on both solid and liquid media. Both active and inactive clones from solid medium were picked up and the plasmid from the active and inactive clones were studied. The clone with the hybrid plasmid in pBR 322 exhibited good activity and was designated as pSR 500. Control transformation experiment was carried out with pBR 322 only.

Restriction endonuclease mapping and subcloning—To determine the reason for instability of ackS gene in pSR 500, ackS gene was cloned up stream just opposite to the previous one also and activity of the clone was determined. Plasmid pSR 500 was restricted with Hind III and Bam HI followed by Klenow DNA polymerase treatment, religated and E. coli JM 109 was transformed. Transformants with haloes of different sizes on fibrin Amp tet plates were analysed. pSR 400, with Hind III-Bam HI deletion, exhibited a small halo where as pSR 350, with a larger deletion showed a medium size halo. Then pSR 500 was double digested down stream with Hind III and Sal I and ligated with a phosphorylated Bam HI linker (Promega) followed by complete digestion with Bam HI and religation to produce pSR 320 where tet gene was removed. pSR 320 exhibited the similar activity as pSR 500. Then pSR 320 was digested down stream with Eco RI-Sal I, religated and transformed E. coli JM 109. The clone pSR 310 was selected on the basis of Amp' and halo on fibrin plate and also for stability in liquid medium. pSR 300 was constructed by subcloning ClaI-SalI fragment from pSR 310 and religated to Bam HI linearized and CIP treated pT7-7 so that pT7-7-ackS contained the complete coding region of ackS under the control of T7 promoter. Activity and stability of the clone in E. coli C600 was checked repeatedly and selected for further studies for protein.

Southern hybridization—Southern hybridization was carried as per Southern DNA (5 μg/lane) from S. megasporus SD5 was partially digested with Sau 3 AI and fractionated on agarose gel (1%) followed by transfer to Hybond-N-membrane and probed with randomly primed digoxigenin (Boehringer Mannheim) labelled linear hybrid plasmid pSR 300. Hybridization was carried out at 30%, formamide, 5X, SSC; Dendardt’s solution; 0.1%, SDS; 0.1 mg/mL, BSA and 50 mM phosphate buffer (pH 7.0) at 42°C. The blot was washed with 0.2% SSC containing (0.1%) SDS at 55°C for 40 min and detected by enzyme-
linked immuno-assay using antibody conjugate and subsequent enzyme catalysed colour reaction with 5-bromo-4-chloro-3-indolyl (x phosphate) and nitroblue tetrazolium salt.

Preparation of enzyme from recombinant E. coli C 600 containing pT7-7-ackS—Extracellular protein from recombinant strain was precipitated with ammonium sulphate saturation (80%), suspended in 0.01 M sodium phosphate buffer, pH 8.0, dialysed overnight at 4°C and the crude protein was analysed in non-denaturing PAGE (10%) followed by in situ enzyme activity as per Lundy et al. The enzyme present in periplasm was released by osmotic shock and the enzyme activity was determined after concentrating as above. The cell mass was sonicated and centrifuged and the activity of the supernatant was determined after concentrating as above and was taken as 100% activity. To determine the purity and similarity between the two proteins, immunological studies were carried out in rabbits using recombinant actinokinase as antigen and standard protocols.

Results

Stability of ackS in E. coli strains—Stability of pSR 500 was examined in a large number of E. coli strains of different genotypes. Percentage of colonies with haloes was assessed before and after subculturing (Table 1). It was observed that pSR 500 was moderately stable in JM 109 and C 600 but on subculturing less than 25% colonies showed activity which might be due to insertional inactivation or plasmid re-arrangement.

Restriction endonuclease mapping and subcloning—To determine the possible cause of loss of actinokinase expression DNA from inactive and active clones were analysed. pSR 500 and DNA from actinokinase active colonies produced a ~6 kb band where as inactive colonies yielded a ~7 kb band (Fig. 1). The addition of ~1 kb indicated that an insertion of some undetermined element might be responsible for inactivation. Southern hybridisation with an alkaline phosphatase conjugated probe specific for ackS gene confirmed the presence of ackS in ~7 kb band. The clone containing ackS gene upstream did not exhibit any activity. To determine the plasmid instability, deletion analysis was carried out using pSR 500 as starting plasmid (Fig. 2). When down stream Hind III-Bam H I fragment was deleted in pSR 400 as 90% activity was lost indicating the probable native promoter was located in this region and it was functional in E. coli. The residual activity expressed by pSR400 (a small halo after 46 hr incubation) might be due to the read through promoter activity from vector where as pSR 350, a fortuitous clone, containing a larger deletion than pSR 400 which might be due to the star activity of Bam H I, exhibited only 30% less actinokinase activity that pSR 500. A distant effect of corresponding read through promoter activity from the vector might be responsible for this low level of expression. pSR 320 was constructed by deleting a larger fragment (Hind III-Sal I) from pSR 500 so that whole tet gene was removed making the clone only resistant to ampicillin. Activity of the clone pSR 320 was nearly same as that of pSR 500.pSR 310, with deletion of 3' flanking region in pSR 320, had no adverse effect on actinokinase activity. The level of actinokinase production on

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<th>Strains of E. coli</th>
<th>Percentage of colonies with haloes</th>
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<tr>
<td>JM 103</td>
<td>&lt;15</td>
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<tr>
<td>DH5a</td>
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<tr>
<td>DHI</td>
<td>&lt;10</td>
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<td>HB 101</td>
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<td>C 600</td>
<td>&lt;96</td>
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Table I — Stability of pSR 500 actinokinase expression in different E. coli host strains

Fig. 1—Plasmids from actinokinase positive and negative clones obtained from amp tet fibrin plate. Lane 1—size marker (λ Hind III digest), Lane 2—pBR 322, Lane 3—pSR 500, Lane 4—Plasmid from actinokinase positive clone, Lane 5—Plasmid from actinokinase negative clone, Lane 6—plasmid from active hybridised with a linear DIG labelled ackS probe, prepared from the insert, Lane 7—Plasmid from inactive clone hybridised with the same probe.
fibrin plate from pSR 310 (Fig. 3) was as good as, if not better than pSR 500, however, in liquid culture it was more stable than pSR 500. pSR 300, containing pT7-7-ackS could produce stable transformants in E. coli JM 109 with low level of expression but in C 600, containing pGP 1-2, stable transformants with higher level of expression were obtained, even under non-induced conditions (Fig. 4).

Expression of actinokinase—Growth conditions were found to be very important for optimum expression of ackS in E. coli. An initial slower growth rate at a higher temperature (42°C) and maintenance

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**Fig. 2**—Deletion analysis of pSR 500. For the convenience of presentation the plasmid maps are given in linear form rather than the conventional circular form. The left part of the figure presents the structure of each plasmid while the right panel showed the actinokinase activity detected on a fibrin plate. The name of each plasmid is given in the extreme right column. The top map represented pSR 500 with important restriction enzyme sites. Gene encoding regions: *amp*—β-lactamase gene, *ori*—origin of replication, *tet*—tetracycline resistance gene and *ackS*—actinokinase gene. Details are given in the text.

**Fig. 3**—Haloes exhibited by clones, with different plasmids, on fibrin plate. A—pSR 500, B—pSR 350, C—pSR 310 and D—pSR 300
of a constant but a steady level of antibiotic selection pressure improved overall expression yield. Fresh transformants of E. coli C 600 with pSR300, grown under these specific conditions gave reproducible yield (150-205 mg L\(^{-1}\)) of actinokinase. Extracts of agar plugs from the regions of clearing around the recombinant E. coli C 600 showed fibrinolytic activity, which revealed that the enzyme was being secreted. Absence of β lactamase activity of the culture supernatant as well as in the agar plug also confirmed that the actinokinase had not leaked but was secreted. It was observed that out of total enzyme 41\% was present in periplasm where as 49\% was secreted in the broth. SDS-PAGE of the concentrated protein from the culture supernatant of recombinant E. coli C 600 revealed that the enzyme was a highly purified protein (Fig. 5). The characteristics of the recombinant actinokinase were similar to those the wild type actinokinase. Immunological studies revealed that the antiserum raised against recombinant actinokinase yielded a single line of precipitation with both native and recombinant antigens (Fig. 6) indicating the similarity between the two proteins.

Discussion

Expression of actinokinase from S. megasperus SD5 in E. coli had no detrimental effect on host cells. The degree of stable expression was strain dependent. Significant increase in production of actinokinase by pSR 300 that pSR 310 might be due to shorter distance between promoter and ackS which might had led to an increase in the plasmid copy number. A strong T7 promoter seemed to be suitable for the expression of actinokinase in E. coli without affecting the plasmid stability. The subtle balance of expression and stability proved to be essential for the expression of actinokinase. High level of actinokinase production was compensated by less incubation time. It supported the earlier views\(^{29,30}\) that extracellular enzyme of Gram positive origin could be secreted by E. coli indicating that E. coli could process the signal
peptide in exactly same manner as S. megasporus SD5. Gram-negative and Gram-positive bacteria carrying the same sequence of a gene may provide an ideal opportunity to study similarities and differences between the mechanisms of the protein secretion.

Acknowledgement

Thanks are due to CSIR, New Delhi, for the award of SRF to R.R. Chitte. The authors are grateful to Dr. Stanley Tabor, Harvard Medical School, Boston for supplying the vector pT7-7 alongwith protocol and Prof. K. Dharmalingam, School of Biotechnology, Madurai Kamraj University, Madurai, for supplying pBR 322, pUC 18 and E. coli JM109.

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